Cell Death in Biology and Diseases

Han-Ming Shen
Peter Vandenabeele *Editors*

Necrotic Cell Death

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Xiao-Ming Yin
Zheng Dong

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Han-Ming Shen • Peter Vandenabeele
Editors

Necrotic Cell Death

Editors
Han-Ming Shen
Department of Physiology
Yong Loo Lin School of Medicine
National University of Singapore
Singapore, Singapore

Peter Vandenabeele
Department of Biomedical Molecular Biology (UGent)
Inflammation Research Center (VIB)
VIB/University of Ghent
Zwijnaarde, Belgium

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Series Preface

Cell death, or conversely cell survival, is a major biological phenomenon. Just as with cell proliferation and cell differentiation, cell death is a choice that a cell has to make, sometimes voluntarily, other times accidentally. As such, cell death serves a purpose in the biology of a multicellular organism. The machinery of cell death and that of cell protection are evolutionarily conserved and their elements can even be found in single-celled organisms. The disruption of cell death mechanisms can often cause developmental abnormalities. Factors that can trigger cell death are diverse, and the cell death process is intricately connected with other biological processes. Cell death directly contributes to the pathogenesis of many diseases, including cancer, neurodegenerative diseases, and tissue injury in organ failure.

The study of cell death and cell survival has become a multidisciplinary subject, which requires expertise from all the fields of modern biology. Exploring the role of cell death in disease development and the modulation of cell death for the prevention and treatment of devastating disease demands constant updating of our knowledge through the broadest interactions among all investigators, basic and clinical. The rapid expansion of our knowledge in this field has gone beyond what could be summarized in a single book. Thus, this timely series *Cell Death in Biology and Diseases* summarizes new developments in different areas of cell death research in an elaborate and systemic way. Each volume of this series addresses a particular topic of cell death that either has a broad impact on the field or has an in-depth development in a unique direction. As a whole, this series provides a current and encyclopedic view of cell death.

We would like to sincerely thank the editors of each volume in the series and the authors of each chapter in these volumes for their strong commitment and great effort towards making this mission possible. We are also grateful to our team of

professional Springer editors. They have worked with us diligently and creatively from the initiation and are continuing this on the development and production of each volume of the series. Finally we hope that the readers will enjoy the reading, find the content helpful to their work, and consider this series an invaluable resource.

Indianapolis, IN, USA Xiao-Ming Yin, M.D., Ph.D.
Augusta, GA, USA Zheng Dong, Ph.D.

Book Preface

Cell death is a fundamental aspect of cell biology closely associated with health and disease. So far, various forms of cell death have been recognized and extensively studied. In contrast to apoptosis, well defined as a form of programmed cell death, necrosis used to be considered as accidental (i.e., non-programmed) cell death, usually in response to a severe injury. In the past decade, there is accumulating evidence suggesting that necrosis is also programmed and is controlled by distinctive "death machinery" in response to various stimuli such as cell death ligands, pathogen-associated molecular patterns (such as viral RNA), oxidative stress, and DNA damage. Necroptosis, a particular form of necrotic cell death, has gained particular attention. This form of necrosis is highly regulated by RIP kinases (RIPK); scientists have now developed inhibitors that block these kinases. These inhibitors allow us to explore the role of these kinases in many experimental disease models in inflammation, ischemia-reperfusion damage, degenerative diseases, and infection and hopefully will soon reach the clinic to save lives.

To keep pace with the fast developments in this important research theme, we are pleased to present this book focusing on necrotic cell death as the second volume in the newly launched series *Cell Death in Biology and Diseases*. In this book we attempt to give a comprehensive coverage of programmed necrosis with contributions from the leading experts in this field around the world. The book starts with the history of necrosis research and also immediately jumps to how molecular insights and availability of inhibitors have rapidly led to potentially important therapeutic applications (Chap. 1). The book is divided, like many processes and good things in life, into seven parts. The first part involves a detailed description of the major regulators of necroptosis including the role and regulation of RIPK1 (Chap. 2) and RIPK3 (Chap. 3). The important role of Inhibitors of Apoptosis Proteins (IAPs) and ubiquitylation processes are elaborated as important regulators in the life–death bifurcation of RIPK1 (Chaps. 4 and 5). Caspase 8 is a crucial negative regulator of necroptosis and is paradoxically a pro-apoptotic molecule (Chaps. 6 and 7).

The second part deals with important cellular processes that modulate necrosis such as DNA damage, PARP activation, oxidative stress, and reactive oxygen species (Chaps. 7–9). They may represent different subroutines that result in particular forms of programmed necrosis. The third part of the book examines the intercellular aspects of necrosis and its role in immunity, inflammation, and viral infection (Chap. 10). In the fourth part, the complex relationship between p53, autophagy, autophagic cell death, and necrosis and how this determines cellular fate following stress are described (Chaps. 11–13). The fifth part elaborates on necrosis in model organisms including microbes, yeast, and *C. elegans* (Chaps. 14 and 15). The sixth part deals with the important quest for small molecule inhibitors of necroptosis and their potential implication for a plead of inflammatory, degenerative, and infectious diseases (Chaps. 16 and 17). Finally, in the seventh part, we discuss some methods and techniques for measuring necrosis and discuss the possible pitfalls (Chap. 18).

With the extensive studies on necrosis in the past several years, it is clear that regulated necrosis started as an ugly duckling besides the overwhelming beauty of apoptosis, and it has now become an admired and proud swan in the vast lake of cell death research. It is conceivable that in pathophysiological situations, targeting necrosis may become a dominant paradigm. We hope that this book, as the first one devoted to regulated or programmed necrosis, will become a useful source of reference for the growing number of researchers in this emerging field. We especially thank all the authors and editors for their patience and understanding during the making of this book.

Singapore, Singapore Han-Ming Shen, M.D., Ph.D.
Zwijnaarde, Belgium Peter Vandenabeele, Ph.D.

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Contributors

Tom Vanden Berghe Molecular Signaling and Cell Death Unit, Inflammation Research Center (VIB), Ghent, Belgium

Department of Biomedical Molecular Biology (UGent), University of Ghent, Ghent, Belgium

Konstantin Bogdanov Department of Biological Chemistry, The Weizmann Institute of Science, Rehovot, Israel

Francis Ka-Ming Chan Department of Pathology, Immunology and Microbiology Program, University of Massachusetts Medical School, Worcester, MA, USA

Wanze Chen State Key Laboratory of Cellular Stress Biology, School of Life Sciences, Xiamen University, Xiamen, China

Dana E. Christofferson Department of Cell Biology, Harvard Medical School, Boston, MA, USA

Patrice Codogno French Institute of Health and Medical Research, University Paris-Descartes, Paris, France

Maurice Darding Centre for Cell Death, Cancer, and Inflammation (CCCI), UCL Cancer Institute, University College London, London, UK

Alexei Degterev Department of Biochemistry, Sackler School of Graduate Biomedical Sciences, Tufts University, Boston, MA, USA

Oliver Dittrich-Breiholz Institute of Biochemistry, Medical School Hannover, Hannover, Germany

Yongjun Fan Department of Molecular Genetics & Microbiology, Stony Brook University, Stony Brook, NY, USA

Maria Feoktistova Section for Molecular Dermatology, Department of Dermatology, Venereology, and Allergology, Medical Faculty Mannheim, University of Heidelberg, Mannheim, Germany

Peter Geserick Section for Molecular Dermatology, Department of Dermatology, Venereology, and Allergology, Medical Faculty Mannheim, University of Heidelberg, Mannheim, Germany

Vera Goossens Molecular Signaling and Cell Death Unit, Inflammation Research Center (VIB), Ghent, Belgium

Department of Biomedical Molecular Biology (UGent), University of Ghent, Ghent, Belgium

Sasker Grootjans Molecular Signaling and Cell Death Unit, Inflammation Research Center (VIB), Ghent, Belgium

Department of Biomedical Molecular Biology (UGent), University of Ghent, Ghent, Belgium

Jiahuai Han State Key Laboratory of Cellular Stress Biology, School of Life Sciences, Xiamen University, Xiamen, China

Tae-Bong Kang Department of Biotechnology, College of Biomedical and Health Science, Konkuk University, Chung-Ju, South Korea

Jin-Chul Kim Department of Biological Chemistry, The Weizmann Institute of Science, Rehovot, Israel

You-Sun Kim Department of Biochemistry, Ajou University School of Medicine, Suwon, South Korea

Andrew Kovalenko Department of Biological Chemistry, The Weizmann Institute of Science, Rehovot, Israel

Michael Kracht Rudolf-Buchheim-Institute of Pharmacology, University of Giessen, Giessen, Germany

Stefan Krautwald Clinic for Nephrology and Hypertension, Christian-Albrechts-University, Kiel, Germany

Ulrich Kunzendorf Clinic for Nephrology and Hypertension, Christian-Albrechts-University, Kiel, Germany

Martin Leverkus Section for Molecular Dermatology, Department of Dermatology, Venereology, and Allergology, Medical Faculty Mannheim, University of Heidelberg, Mannheim, Germany

Ying Li Department of Cell Biology, Harvard Medical School, Boston, MA, USA

Yong Lin Molecular Biology and Lung Cancer Program, Lovelace Respiratory Research Institute, Albuquerque, NM, USA

Andreas Linkermann Clinic for Nephrology and Hypertension, Christian-Albrechts-University, Kiel, Germany

Paula Ludovico Life and Health Sciences Research Institute (ICVS), School of Health Sciences, University of Minho, Braga, Portugal

ICVS/3B's – PT Government Associate Laboratory, Braga/Guimarães, Portugal

Colleen R. McNamara Graduate Program in Biochemistry, Sackler School of Graduate Biomedical Sciences, Tufts University, Boston, MA, USA

Ute M. Moll Department of Pathology, Stony Brook University, Stony Brook, NY, USA

Department of Molecular Oncology, University of Göttingen, Göttingen, Germany

Michael J. Morgan Department of Pharmacology, University of Colorado School of Medicine, Anschutz Medical Campus, Aurora, CO, USA

Kenta Moriwaki Department of Pathology, Immunology and Microbiology Program, University of Massachusetts Medical School, Worcester, MA, USA

Vassiliki Nikoletopoulou Institute of Molecular Biology and Biotechnology, Foundation for Research and Technology, Heraklion, Crete, Greece

Andrew Oberst Department of Immunology, University of Washington, Seattle, WA, USA

Akhil Rajput Department of Biological Chemistry, The Weizmann Institute of Science, Rehovot, Israel

Han-Ming Shen Department of Physiology, Yong Loo Lin School of Medicine, National University of Singapore, Singapore, Singapore

John Silke The Walter and Eliza Hall Institute of Medical Research, Parkville, VIC, Australia

Department of Medical Biology, University of Melbourne, Parkville, VIC, Australia

Nozomi Takahashi Molecular Signaling and Cell Death Unit, Inflammation Research Center (VIB), Ghent, Belgium

Department of Biomedical Molecular Biology (UGent), University of Ghent, Ghent, Belgium

Shi-Hao Tan Department of Physiology, Yong Loo Lin School of Medicine, National University of Singapore, Singapore, Republic of Singapore

NUS Graduate School for Integrative Sciences and Engineering, National University of Singapore, Singapore, Republic of Singapore

Nektarios Tavernarakis Institute of Molecular Biology and Biotechnology, Foundation for Research and Technology, Heraklion, Crete, Greece

Beata Toth Department of Biological Chemistry, The Weizmann Institute of Science, Rehovot, Israel

Peter Vandenabeele Molecular Signaling and Cell Death Unit, Inflammation Research Center (VIB), Ghent, Belgium

Department of Biomedical Molecular Biology (UGent), University of Ghent, Ghent, Belgium

David Vaux The Walter and Eliza Hall Institute of Medical Research, Parkville, VIC, Australia

Department of Medical Biology, University of Melbourne, Parkville, VIC, Australia

Henning Walczak Centre for Cell Death, Cancer, and Inflammation (CCCI), UCL Cancer Institute, University College London, London, UK

David Wallach Department of Biological Chemistry, The Weizmann Institute of Science, Rehovot, Israel

Joris Winderickx Functional Biology, University of Leuven, Heverlee, Belgium

Ting Wu State Key Laboratory of Cellular Stress Biology, School of Life Sciences, and Department of Basic Medical Sciences, Medical College, Xiamen University, Xiamen, China

Seung-Hoon Yang Department of Biological Chemistry, The Weizmann Institute of Science, Rehovot, Israel

Junying Yuan Department of Cell Biology, Harvard Medical School, Boston, MA, USA

Katharina Zirngibl Department of Pathology, Stony Brook University, Stony Brook, NY, USA

Wei-Xing Zong Department of Molecular Genetics & Microbiology, Stony Brook University, Stony Brook, NY, USA

Chapter 1
The Potential Role of Necroptosis in Diseases

Andreas Linkermann, Tom Vanden Berghe, Nozomi Takahashi, Ulrich Kunzendorf, Stefan Krautwald, and Peter Vandenabeele

1.1 Necrosis Seen Though the Eye of Pathologists

Until recently, necrosis has mainly been investigated clinically by pathologists. Regarding morphological changes, several macroscopically distinct forms of necrosis regarding whole organs have been described. It is entirely unclear which molecular pathways of regulated necrosis underlie the specific morphological changes associated with necrosis and whether or not they are regulated on a genetic level. The identification of necroptosis, defined by the molecular interplay between RIPK1/RIPK3/MLKL and its negative regulation by caspase-8, FLIP, and inhibitors of apoptosis proteins (IAPs), described in detail in other chapters in this book, and other pathways of regulated necrosis that appear to be virtually distinct on the molecular level, like cyclophilin D-mediated regulated necrosis, have led to the intriguing possibility to specifically target necrosis in various pathologies. For basic researchers, however, there is a lot to learn from the work that has been performed over centuries by pathologists which led to the purely morphologic classification of necrosis. In the eyes of a pathologist, the most common form of necrosis is referred to as coagulative necrosis (Table 1.1).

A. Linkermann (✉) • U. Kunzendorf • S. Krautwald
Clinic for Nephrology and Hypertension, Christian-Albrechts-University, Campus Kiel, Schittenhelmstr. 12, 24105 Kiel, Germany
e-mail: linkermann@nephro.uni-kiel.de

T. Vanden Berghe • N. Takahashi • P. Vandenabeele (✉)
Molecular Signaling and Cell Death Unit, Inflammation Research Center, VIB, 9052 Ghent, Belgium

Department of Biomedical Molecular Biology (UGent), University of Ghent, Technologiepark 927, 9052 Ghent, Belgium
e-mail: Peter.Vandenabeele@irc.vib-ugent.be

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Table 1.1 Pathological aspects of necrotic tissue

	Typical disorders	Typically affected organs	Morphological hallmarks	Involvement of necroptosis
Coagulative necrosis	Solid organ ischemia	Visceral organs apart from pancreas	Dry tissue	Yes
	Dry gangrene	Feet of diabetic patients		Unclear
Colliquative necrosis	Stroke	Brain	Clammy tissue	Unclear
	Pancreatitis	Fat tissue necrosis		Yes
	Wet gangrene	Feet of diabetic patients		Unclear
Fibrinoid necrosis	Atherosclerosis	Large- and medium-sized arteries	Strong eosinophilia in the fibrinoid	Yes
Caseating necrosis	Tuberculosis	Lung	Granuloma and delayed-type hypersensitivity (DTH)	Unclear

1.1.1 Coagulative Necrosis

Coagulative necrosis was first described by C. Weigert in 1880 as the macroscopically visible change in tissues with a dry yellow appearance (Weigert 1880). The hallmarks of this form of necrosis are the diminished moisture content combined with the overall persistence of the organ structure. This type of necrosis is predominantly seen in ischemic tissues, e.g., in the spleen upon infarction, or in other visceral organs like the kidneys. Clearly, RIPK3-dependent necroptosis contributes to this complex picture of organ necrosis (Linkermann et al. 2012b, c). A special form of coagulative necrosis is commonly seen in diabetic patients with combined defects in both micro- and microvasculature, referred to as gangrene (Younes and Ahmad 2006). The clinically important dry aspect of gangrene changes upon superinfection with anaerobic bacteria that can lead to accumulation of lymph and result in wet gangrene that is then regularly referred to as colliquative of liquefactive necrosis (see below). Similar mechanisms as in coagulative necrosis may also occur centrally in rapidly growing solid tumors (Caruso et al. 2012), but these aspects need to be addressed in RIPK3- and MLKL-deficient environments.

1.1.2 Colliquative Necrosis

Colliquative necrosis, also referred to as liquefactive necrosis, results from rapid enzymatic degradation of the necrotic debris following initial tissue swelling. In comparison to coagulative necrosis, colliquative necrosis is predominantly seen in tissues that do not exhibit large amounts of proteins (like the brain) (Estes and Rorke 1986) or organs which actively produce lytic enzymes (like the pancreas)

(Burrell et al. 1980). In addition, colliquative necrosis results from ingestion of NaOH in the esophagus or might rarely result from secondary infections in primarily damaged tissues, like the lung that undergoes hydrolysis upon leukocyte infiltration in so-called secondary-infected pulmonary infarction (Rajagopala et al. 2011; Redline et al. 1985). The most common appearances of colliquative necrosis are myocardial infarction (Baroldi 1975), strokes (Chen et al. 2007), and wet gangrene. In addition, fat tissue necrosis following necrotizing pancreatitis represents a devastating disease which despite intensive care is associated with high mortality rates (Bruennler et al. 2009; Burrell et al. 1980). The mouse model of cerulein-induced pancreatitis (CIP) was among the first in vivo models tested for necroptosis in RIPK3-deficient mice by two groups in 2009, who demonstrated statistically significant protection (He et al. 2009; Zhang et al. 2009). However, we recently applied the RIP1-kinase inhibitor Nec-1 in this model which conversely led to deterioration of the CIP model (Linkermann et al. 2012a). Obviously, Nec-1 protects from several models of acute brain injury (see below) including cerebral IRI, but it remains to be demonstrated that this effect is also seen in RIPK3-deficient mice. Above that, application of necrostatins in larger animal models should be performed before clinical trials are seriously taken into consideration.

1.1.3 Fibrinoid Necrosis

Fibrinoid necrosis is also referred to as collagen necrosis because pathologists recognize an increased eosinophil staining in such areas (Rosenblum 2008). It is generally accepted to interpret these fibrinoid structures as the result of past necrosis with or without inflammation. The most prominent example for fibrinoid necrosis is probably the necrotic center of atherosclerotic plaques to cause arterio- and arteriolosclerosis (Rosenblum 2008), but it also appears in gastric and duodenal ulcers (Aruin et al. 1989; Szabo et al. 2007) and the skin in *necrobiosis lipoidica diabeticorum* (Suarez-Amor et al. 2010). The described subform of fibrinoid necrosis referred to as precipitation fibrinoid is generally and unspecifically associated with the large family of immune complex diseases that surround the collagen and in the entity of collagenosis. It has recently been suggested that RIPK3 might be involved in the pathogenesis of atherosclerotic plaques in a commonly used atherosclerosis model of ApoE-ko mice (Lin et al. 2013a). However, gastrointestinal ulcers, immune complex diseases, and subtypes of collagenosis have not been investigated in the light of necroptosis.

1.1.4 Caseating Necrosis

Caseating necrosis is referred to the debris in the center of granulomas that are typically seen in active and latent mycobacterial infections upon a delayed-type hypersensitivity reaction (Hunter et al. 2007), like tuberculosis. Such granulomas are not restricted to the lungs but are also detected in non-pulmonary manifestations of

tuberculosis like urogenital (Abbara and Davidson 2011) or gastrointestinal tuberculosis (Almadi et al. 2011). Functionally, it is thought that necrosis is required to some extent to defend mycobacteria, but, on the other hand, may also be interpreted as a release mechanism of these intracellular bacteria that is required for spreading, like in disseminated military tuberculosis.

Unlike the interpretation of necrosis over centuries, from our current understanding, all mentioned manifestations of necrosis should be interpreted as potentially regulated and therefore might be therapeutic targets until the opposite is proven. The massive clinical potential of interference with necrotic injury in any case should lead to carefully conducted investigations regarding inhibition of necroptosis (see below in Sect. 3).

1.2 Necroptosis Affects Many Organs in Pathophysiological Settings

The best characterized pathway of regulated necrosis is RIPK3-dependent necroptosis as pointed out in detail in other chapters of this book (Declercq et al. 2009; Han et al. 2011; Kaczmarek et al. 2013). Despite the fact that the dependence of necroptosis on RIPK3 was published in 2007 (Feng et al. 2007) and widely recognized as late as in 2009 (Cho et al. 2009; He et al. 2009; Zhang et al. 2009), diverse organ systems have already been associated with necroptosis in multiple disorders. From 2009, several preclinical in vivo models have employed RIPK3-deficient mice which are thought to lack an overt spontaneous phenotype (Newton et al. 2004). The physiological in vivo relevance of necroptosis was clearly demonstrated as the lethal phenotype of caspase-8-deficient mice which otherwise die in utero at day 10.5 of embryonic development and is reversed with the additional deletion of RIPK3 (Kaiser et al. 2011; Oberst et al. 2011). As demonstrated in Fig. 1.1, the main part of this chapter will provide an overview of the organs affected by necroptosis at the time when the chapter was written. With the rapidly growing number of publications in this field, it is very likely that the number of organs and cell types that can undergo necroptotic cell death will further increase within the next years. As with the published data available today, the affected organs and the model organisms that have been employed to provide the in vivo evidence are summarized in Table 1.2. Ischemia–reperfusion injury (IRI) is of outstanding interest both clinically and in basic science, so we will provide an extra section on IRI before the occurrence of necrosis in different organs will be discussed.

1.2.1 Ischemia–Reperfusion Injury

As early as in 2005, Alexei Degterev et al. published the effects of the RIPK1-inhibitor necrostatin-1 (Nec-1) on middle cerebral artery occlusion (MCAO),

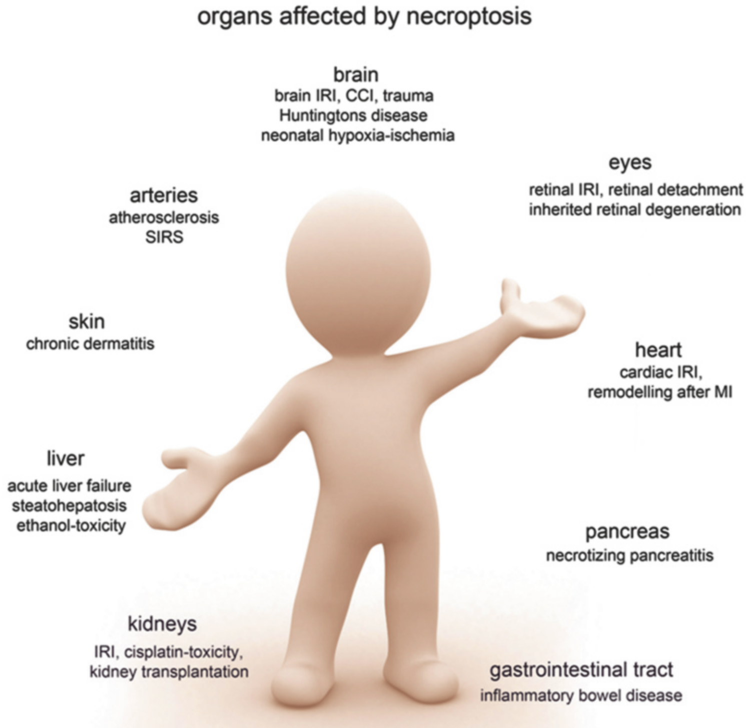

Fig. 1.1 Organs affected by necroptosis. Since 2005, the evidence for the pathophysiological involvement of necroptosis in preclinical disease models has rapidly accumulated. At the time of writing this chapter, necroptosis has already been described to be of importance in brain, eyes, heart, pancreas, gastrointestinal tract, kidneys, liver, and vessels (mainly arteries). It is likely that more disorders are about to be discovered to be affected by necroptosis. *CCI* controlled cortical impact, *IRI* ischemia–reperfusion injury, *MI* myocardial infarction, *SIRS* systemic inflammatory response syndrome

a model of cerebral ischemia, and demonstrated marked protection from stroke (Degterev et al. 2005). Only years later, it became clear that RIPK1 was the therapeutic target (Degterev et al. 2008). Like mice deficient in FADD, caspase-8, or FLIP, RIPK1-deficient mice are not viable, but unlike the FADD-, caspase-8-, and FLIP-deficient mice, RIPK1-deficient mice do not die in utero but perinatally instead (Zhang et al. 2011).

In IRI in mice, hallmarks of necrosis have been detected for long (Hess and Manson 1984), but this was rather interpreted as accidental cell death, and the precise mechanism of the development of the necrotic debris in IRI was neglected, and a causative role for apoptosis was widely accepted. Recently, we directly compared the protective effect of Nec-1 in kidney IRI to the effect of the apoptosis-blocker pan-caspase inhibitor zVAD (Linkermann et al. 2012b), suggesting a predominant role of necroptosis over apoptosis in this setting. In detail, the narrow therapeutic

Table 1.2 Evidence for the pathophysiological in vivo relevance of necroptosis

Disease	In vivo model organism	Citation
Brain		
Brain IRI	C57Bl/6	(Degterev et al. 2005)
Controlled cortical impact	C57Bl/6	(You et al. 2008)
Brain IRI	CD1 mice	(Xu et al. 2010)
Neonatal hypoxia–ischemia	Mice	(Northington et al. 2011)
Huntington's disease	R6/2 mice	(Zhu et al. 2011)
Neonatal hypoxia–ischemia	C57Bl/6	(Chavez-Valdez et al. 2012b)
Traumatic brain injury	CD1 mice	(Wang et al. 2012)
Eye		
Retina IRI	Sprague–Dawley rats	(Rosenbaum et al. 2010)
Retinal detachment	Brown Norway rats, C57Bl/6 and RIPK3-ko mice	(Trichonas et al. 2010)
Retinal detachment	Sprague–Dawley rats	(Dong et al. 2012)
Inherited retinal degeneration	rd10 mice (model of retinal degeneration)	(Murakami et al. 2012b)
GIT		
Inflammatory bowel disease	C57Bl/6, RIPK3-ko mice, conditional FADD-def. mice	(Welz et al. 2011)
Inflammatory bowel disease	C57Bl/6, RIPK3-ko mice, conditional caspase-8-def. mice	(Gunther et al. 2011b)
Heart		
Cardiac IRI	C57Bl/6	(Smith et al. 2007)
Cardiac remodeling after myocardial infarction	C57Bl/6	(Oerlemans et al. 2012b)
Kidney		
Kidney IRI	C57Bl/6	(Linkermann et al. 2012b)
Cisplatin-induced acute kidney injury	C57Bl/6, RIPK3-ko	(Linkermann et al. 2013a)
Kidney transplantation	RIPK3-ko kidney (H-2^{d}) → BALB/c (H-2^{d})	(Lau et al. 2013)
Liver		
Acute liver failure	Conditional caspase-8-ko, RIPK3-ko	(Liedtke et al. 2011b)
Steatohepatitis	C57Bl/6	(von et al. 2012)
Ethanol-induced liver injury	C57Bl/6 and CYP2E1-deficient mice	(Roychowdhury et al. 2012)
Acetaminophen-induced liver necrosis	C57Bl/6	(Ramachandran et al. 2013)
Pancreas		
Necrotizing pancreatitis	C57Bl/6, RIPK3-ko mice	(He et al. 2009)
Necrotizing pancreatitis	C57Bl/6, RIPK3-ko mice	(Zhang et al. 2009)
Skin		
Necrotizing dermatitis	C57Bl/6, RIPK3-ko mice, conditional FADD-def. mice	(Bonnet et al. 2011)

(continued)

Table 1.2 (continued)

Disease	In vivo model organism	Citation
Systemic effects		
SIRS	C57Bl/6, RIPK3-ko mice	(Duprez et al. 2011)
SIRS	C57Bl/6, RIPK3-ko mice	(Linkermann et al. 2012a)
SIRS	C57Bl/6, RIPK3-ko mice	(Linkermann et al. 2013a)
Atherosclerosis	RIPK3-ko, LDLR-ko, RIPK3/LDLR-dko, and ApoE-ko	(Lin et al. 2013a)
Whole body		
Defense against vaccinia-virus infection	C57Bl/6, RIPK3-ko mice	(Cho et al. 2009)

Pathophysiological aspects are separated by affected organ, comparable to Fig. 1.1

window of Nec-1 application was mapped to 15–30 min. before onset of reperfusion, and the protective effect almost completely disappeared after that time point (Linkermann et al. 2012b). Therefore, therapeutic interference with necroptosis in IRI should be applied closely to the onset of reperfusion. This is practical in kidney transplants, and in any transplant model, and to a lesser extent also in myocardial IRI, at least when the coronaries are completely occluded before stenting. We speculate that in most clinical scenarios such as stroke or myocardial infarction (without completely occluded coronary vessels), therapeutically applicable prevention of necroptosis will have limited effects as the death signal will already have passed crucial checkpoints (point of no return) (Linkermann et al. 2012c). Therefore, the most likely situation in which interference with necroptosis will presumably be beneficial are those in which damage can be anticipated rather than to be reversed. Besides solid organ transplantation, these opportunities with high clinical relevance also include cardiac surgery, a very common cause of acute renal failure (Olivero et al. 2012). However, necroptosis is not limited to IRI in brain and kidney but was also demonstrated in the myocardium. The cardioprotective effect of Nec-1 was first discussed in 2007 (Smith et al. 2007). From studies that investigated microRNA-155 which targets RIPK1 (Liu et al. 2011), it was suggested that RIPK1 functioned as an inducer of necrosis of cardiomyocytes, but it was only in 2012 when Oerlemans and colleagues showed a protective effect for Nec-1 in myocardial IRI, focusing on the prevention of adverse cardiac remodeling (Oerlemans et al. 2012a).

It should be noted that the effects of Nec-1 are not entirely understood and may be multiple due defined off-target effects (Degterev et al. 2013; Takahashi et al. 2012; Vandenabeele et al. 2013). Indeed Nec-1 is identical to the indoleamine oxidase (IDO) which is a strong immunomodulator. A more stable form of Nec-1, the 7-chloronated and hydantoin derivate Nec-1s lacks this IDO specificity and may be better used to target RIPK1 without affecting the immune system directly (Degterev et al. 2013; Takahashi et al. 2012; Vandenabeele et al. 2013). Another aspect is that targeting the kinase activity of RIPK1 is different of removing RIPK1, which also has an important platform function in recruiting factors involved in MAPK and NF-κB activation (Vandenabeele et al. 2010). This difference between the kinase

and the platform function of RIPK1 is obvious from the observation that in contrast to caspase-8-deficient mice, RIPK1-deficient mice are not rescued by RIPK3 deficiency (Dillon et al. 2012), suggesting the absence of RIPK1 induces a lethal pathway independent of RIPK3. However, FADD-deficient embryos die early in embryonic development at day 10.5 and are partially rescued by RIPK1 deficiency to die only perinatally (Zhang et al. 2011) and are rescued by RIPK3-deficiency, suggesting a pro-death role of RIPK1 in concert with RIPK3 in early life but a protective role independent of RIPK3 in later life. It is not straightforward to identify the precise role of RIPK1 during embryogenesis and perinatally given these complex genetic interactions. The only way to experimentally study the complex roles of RIPK1 at different stages of life requires conditional RIPK1 kinase dead knock-in mice. Specific inhibitors of RIPK1, like Nec-1, could translate this knowledge to therapeutic strategies. Few other kinase inhibitors have undergone such in-depth specificity analysis (Biton and Ashkenazi 2011; Degterev et al. 2008). Within the entire kinome, only two other kinases are affected by high concentrations of Nec-1 to more than 35 % (Biton and Ashkenazi 2011). Second-generation necrostatins, like 7-chloro-O-Nec-1 (Nec-1s) (see above), might further contribute to our understanding as will RIPK1-kinase dead "knock-in" mice that are currently being generated. In addition, inhibitors of the kinase activity of RIPK3 and inhibitors of MLKL, like necrosulfonamide as the first-in-class compound recently published (Sun et al. 2012), will be developed, with high hopes associated with these interesting compounds in IRI.

1.2.2 Necroptosis and the Gastrointestinal System

The first manifestation of necroptosis that was mentioned in the gastrointestinal tract (GIT) was reported in 2009 when RIPK3 was identified as a crucial necroptosis-associated kinase in the model of cerulein-induced pancreatitis (CIP) in which RIPK3-deficient mice were protected (Zhang et al. 2009; He et al. 2009). This common clinical problem is best known from intensive care units in which hardly anything can be done for these patients apart from applying fluids and pain killers. Therefore, the idea to specifically interfere with necrotizing pancreatitis led to the investigation of Nec-1 in the very same model, but unfortunately, Nec-1 deteriorated rather than protected from CIP (Linkermann et al. 2012a). However, the discrepancy between the Nec-1 data and the RIPK3-ko mice is so far unexplained. One possible explanation might be associated with the short half-life of Nec-1 (Degterev et al. 2013), and second-line derivates of necrostatin such as Nec-1s and newly developed drugs, RIPK3 inhibitors and MLKL inhibitors, have again raised hopes to cure this devastating disorder. However, although likely, the clear identification of MLKL as a mediator of CIP remains to be demonstrated. Apart from the CIP model, Nec-1 was demonstrated to prevent an NO-mediated necrotic-type cell death in pancreatic beta cells, but the role of RIPK1 in this regard remains less clear (Tamura et al. 2011).

In contrast to acute necrotizing pancreatitis, inflammatory bowel disease (IBD) is a more chronic disorder that is understood to be polygenetically inherited.

It remains unclear if the damage associated with the epithelia is triggered by immune cells or if the epithelia themselves are the initiators of the subsequent immune response. The group of Pasparakis first looked at IKK-γ (NEMO) that they specifically deleted from intestinal epithelial cells (IECs) and reported loss of integrity of the gut barrier, an effect that was partly reversed by additional deletion of TNFR1 (Nenci et al. 2007). Following that path the group investigated the role of necroptosis in IBD. Conditionally deleted FADD from IECs, like caspase-8 deficiency (FADD-deficient mice die in utero), initiated necroptosis and the immune response that is associated with any necrotic cell death (Welz et al. 2011). Importantly, it was spontaneous necroptotic cell death that triggered the disease in the presence of standard microbiota because concomitant genetic absence of RIPK3 completely prevented the inflammation in these mice. The second group looked at conditional deletion of caspase-8 in IECs and found, as expected, necroptosis to be activated. These models phenocopy IBD much in the sense of Crohn's disease (Gunther et al. 2011a), and these typical features were prevented on RIPK3-deficient background. However, genome-wide association studies did not identify either FADD or caspase-8 as susceptibility loci for Crohn's disease (Raelson et al. 2007; Hampe et al. 2007). However, the gene of the deubiquitinase A20 (which also deubiquitinylates RIPK1) was demonstrated to be associated with increased risk of developing colitis, and A20 deletion in IECs was associated with partly TNFR1-dependent TUNEL positivity which at that time was interpreted as apoptosis (Vereecke et al. 2010), but effects of RIPK3 or crossing these mice to an RIPK3-deficient background was not yet reported. However, one could wonder whether these sensitized conditions in the absence of FADD, caspase-8, IAPs, or A20 represent pathophysiologically relevant conditions. If yes, one could think of strategies to increase the expression of negative regulators of necroptosis or to downregulate the expression of RIPK3.

Regarding the liver, a major clinical problem is triggered by chronic alcohol abuse that leads to a phenotype that was also described to trigger necrosis in hepatocytes. A recent report has clearly demonstrated the necroptotic pathomechanism in this setting (Roychowdhury et al. 2012). That paper demonstrated RIPK3-ko mice to be protected from steatosis and hepatocyte death and additionally nicely showed the lack of proinflammatory cytokine expression, further underlining the hypothesis that CDAMPs initiate the upcoming immune response (see below). In line with this, the liver-specific deletion of caspase-8 in hepatocytes resulted in severe non-apoptotic liver injury (Liedtke et al. 2011a). Interestingly, subsequent investigations by the same group on these caspase-8-deficient hepatocytes recently revealed accelerated liver regeneration (Freimuth et al. 2013), suggesting a higher cellular turnover within the regular metabolism of these mice.

1.2.3 Neurological Systems Affected by Necroptosis

Apart from IRI, the brain appears to be sensitive to responses mediated by RIPK1 and RIPK3 in a series of other models of brain damage. All of these injury models are closely related to clinical situations and include controlled cortical impact (CCI),

traumatic brain injury, *N*-methyl-D-aspartate (NMDA)-induced excitotoxicity, and glutamate-induced necroptosis in HT-22 cells. In neonatal hypoxia–ischemia models, Nec-1 not only protects from oxidative damage but also prevented the subsequent deteriorating immune cell infiltration (Chavez-Valdez et al. 2012a; Degterev et al. 2005; Li et al. 2008; Northington et al. 2011; You et al. 2008). In addition, it was recently demonstrated that necroptosis also occurs in microglia upon application of caspase inhibitors (Fricker et al. 2013). This mechanism was interpreted as a protective strategy to save neurons, but detailed mechanisms in vivo are lacking, so further investigations are required in this field. It should be pointed out that there is no clear evidence for the induction of necroptosis in primary neurons.

1.2.4 Ophthalmology and Necroptosis

In addition to the abovementioned diseases, necrotic cell death is a hallmark of retinal detachment. It has been demonstrated that in this specialized compartment, both apoptosis and necroptosis are triggered simultaneously (one cell dies by apoptosis, the next one dies by necroptosis) (Trichonas et al. 2010). The same group has now confirmed that cones, but not rods, undergo necroptosis in their model of retinitis pigmentosa (Murakami et al. 2012a). These data support the concept that PCD and RN are not mutually exclusive programs and they may occur in the same organ, following the same stimulus. Additionally, in the exceptional case of retinal ischemic cell death, the death-associated protein DAXX has been suggested to be a critical downstream target of RIPK3 (Lee et al. 2013).

1.2.5 Necroptosis in the Skin

Conditional deletion of FADD from keratinocytes results in chronic inflammation of the skin, and this phenomenon has nicely been demonstrated to be dependent on RIPK3 and partially dependent on TNFR1 (Bonnet et al. 2011). This work implies that the machinery for necroptosis is not limited to mesenchymal and gastrointestinal epithelial tissues but encompasses the epidermal compartment outside the GIT. It remains to be investigated to which extent necroptosis might be involved in other dermatological diseases that are associated with macroscopic appearance of necrosis, like psoriasis, and, in addition, it should be addressed if chronic inflammatory skin diseases, like atopic dermatitis, are kept active by necroptotic cells. As briefly discussed above, also here it should be examined to what extent absence of FADD represents a relevant pathophysiological mechanism in inflammatory skin diseases.

1.2.6 Involvement of Necroptotic Key Molecules in Systemic Disorders

Sepsis is the prototypic systemic disorder, and the complexity of necrotic cell death pathways is highlighted in its pathophysiology, a leading cause of mortality in intensive care patients. Release of TNFα and other proinflammatory cytokines is triggered by LPS on the bacterial surface, which leads to nitric oxide (NO) generation and rapid loss of the peripheral resistance leading to an unstoppable drop in blood pressure eventually resulting in death. We recently demonstrated that RIPK3-deficient mice are protected from lethal TNFα-mediated systemic inflammatory response syndrome (SIRS) and septic shock in a cecal ligation puncture model (CLP) (Linkermann et al. 2012a; Duprez et al. 2011). In the latter paper, absence of RIPK3 prevented CDAMPs release and the sustained cytokine response, which prompted us to postulate that necroptosis triggers immune response through CDAMPs (see below). When it was shown by Cauwels et al. that the pan-caspase inhibitor zVAD, which may have been expected to provide a benefit by blocking TNFα effect, accelerated rather than prevented death (Cauwels et al. 2003), this model attracted attention. Later, when the necroptotic pathway was unraveled in more detail and the central role RIPK3 was identified, these results could be reconciled. However, conflicting results remain in the literature about the therapeutic utility of Nec-1 in this TNF-shock model (Duprez et al. 2011; Linkermann et al. 2012a; Takahashi et al. 2012), which may be explained by various concentrations of Nec-1 (Takahashi et al. 2012; Vandenabeele et al. 2013). More recently, the group of Han reported that neither RIPK3-ko mice nor MLKL-ko mice were protected from CLP (Wu et al. 2013), keeping the debate ongoing.

Another systemic disorder with tremendous clinical impact is atherosclerosis. Mice deficient in low-density lipoprotein (LDL) receptor (LDLR) are known to develop severe central atherosclerosis which involves macrophage-mediated fibrinoid necrosis, as does the phenotype of ApoE-deficient mice. When fed a high-fat "western" diet for 16 weeks (Lin et al. 2013b), those mice lost large parts of this phenotype when on an RIPK3-deficient background. Necrotic affection of vessels therefore likely leads to atherosclerosis and appears to be at least partly mediated by necroptosis (Lin et al. 2013b).

The most important "systemic disorder" to defend in everyday life may be viral infection. The understanding of the physiological relevance of necroptosis is currently best explained by the model that the group of Francis Chan has first described in 2009 (Cho et al. 2009). Therein, the authors took advantage of vaccinia virus, known to express an inhibitor of caspase-8. RIPK3-ko mice were shown to be super-susceptible to vaccinia-virus infections. Whereas most wild-type mice survived this infection, RIPK3-ko mice died rapidly within the first 2 weeks and viral titers markedly increased (Cho et al. 2009). In the special case of murine cytomegalovirus (MCMV), which also expresses a potent caspase inhibitor and thereby might open the gates for necroptotic host defense, it is tempting to speculate that the virus has found a way to adapt to this second-line attack by expressing the viral protein M45

which contains an RHIM domain that interferes with the necroptosome (Upton et al. 2008, 2010). As dangerous as the expression of RIPK3 obviously is, higher organisms apparently take this risk for the sake of defending against viruses, and this model is attracting more and more attention (Kaiser et al. 2013). However, the danger of the necroptotic pathway, as it currently appears, might be limited to pathological conditions (see below), whereas physiological conditions are mostly controlled by apoptosis.

1.2.7 Necroptosis as a Trigger of the Immune System: Aspects of Solid Organ Transplantation

From the point of view of a transplant immunologist, it is unclear how the major clinical problems associated with solid organ transplantation may be interpreted. One particularly challenging entity is antibody-mediated rejection (AMR) which does not only occur rapidly after transplantation but may appear months to years after otherwise successful surgery and initial treatment and may lead to loss of the transplant organ (Kittleson and Kobashigawa 2012). It has been debated for years if IRI which is invariably associated with solid organ transplantation is the trigger of such late immune responses. As we discussed above, IRI is dominated by necrotic cell death and may be not even restricted to necroptosis (Linkermann et al. 2013a). Therefore, understanding cell death-associated molecular patterns (CDAMPs) that are released from cells that die by necrosis gives rise to a novel hypothesis in which CDAMPs trigger adaptive immune responses (Kaczmarek et al. 2013), especially in transplantation medicine. In such a model, one might speculate that local dendritic cells might be the mediators of ischemic/necrotic injury and transfer the initial T-cell signal to B-cells. Indeed, dendritic cells have recently been demonstrated to play a major role in ischemic injury, albeit that paper did not relate the role of DCs to specific necrotic cell death modalities (Li et al. 2012). This model could explain how patients generate donor-specific antibodies that render a second transplantation after AMR and loss of the first transplant a clinically high-risk situation. If future studies could underline these thoughts, prevention of necroptosis might be of interest for all subsequent transplants and, obviously, for the overall outcome of the patient. This hypothesis exemplifies how clinically important and pathophysiologically complex immunogenic cell death by necrotic cells might be. Mechanistically, CDAMPs whether or not in association with cofactors are heavy triggers of the immune system, but unlike expected a decade ago, they are of astonishing specificity. IL-33 and HMGB1 are two such examples that are released by necrotic, but not by apoptotic, cells in which these factors are inactivated by caspases (Kaczmarek et al. 2013; Ladoire et al. 2013). In the case of necroptosis, these events have been worked out in a surgically demanding model of murine RIPK3-deficient kidneys that were transplanted in comparison to wt C57Bl/6 kidneys into Balb/c mice. Whereas 85 % of the transplants were rejected with resultant death of the mice in the wt transplant group, the Balb/c mice that received allogenic RIPK3-ko kidneys

exhibited only 15 % of rejection and death (Lau et al. 2013). Importantly, these results were obtained without any immunosuppression. As tempting as this result is for the initiation of clinical trials with Nec-1 or its derivates, presumably in large-scale, multicenter, randomized prospective trials, it must be considered that (1) RIPK3-ko does not necessarily equal Nec-1 treatment, (2) RIPK3-ko kidneys at least theoretically might be more resistant against adaptive immune responses than wt tissue, and (3) interference with necroptosis might lead to severe side effects in these highly immunosuppressed patients, especially in kidney transplantation which is regularly associated with viral infections. However, the transplant community is about to realize the tremendous potential of interference with necroptosis (Linkermann et al. 2013b), and the quest for the necroptosis-inducing receptor has begun (see below).

1.2.8 Necroptosis in Tumorigenesis, Immune Surveillance, and Immunotherapy

We will only spend a very short description on the possible role of necroptosis in tumorigenesis, immune surveillance, and immunotherapy, as it is not the major topic of this chapter in which we mainly dealt with diseases that can be inhibited pharmacologically, rather than to induce necroptosis in proliferating cells. Necroptosis may play a dual role in tumorigenesis. On the one hand, it could be a tumor suppressor mechanism in the same way as apoptosis prevents tumor formation by eliminating damaged or stressed tumor cells (Kreuzaler and Watson 2012; Hanahan and Weinberg 2011). The regulation of apoptosis and necroptosis shares several common molecular pathways which implies that they may be co-regulated processes. Administration of smac mimetics which block IAP1/2, E3 ubiquityl ligases that keep RIPK1 in the survival modus (Bertrand and Vandenabeele 2011), yet kills cancer cells by apoptosis or necroptosis (Feoktistova et al. 2011; Tenev et al. 2011). Inactivating mutations in the deubiquitylating enzyme CYLD (Trompouki et al. 2003; Kovalenko et al. 2003), a positive regulator of necroptosis (Hitomi et al. 2008a), may protect tumor cells from cell death induction. On the other hand, necroptosis in the necrotic core of tumor could also contribute to tumor growth in a similar way as inflammation-mediated tumorigenesis (Kuraishy et al. 2011). However, this is still a working hypothesis. Finally, therapeutically spoken, necroptosis induction could be an alternative way to induce tumor cell death when the apoptotic machinery is dysfunctional and can be exploited to overcome acquired or therapy-induced apoptosis resistance (Kreuzaler and Watson 2012). First, several actual cancer drugs and radiotherapy often lead to cell death with necrotic morphology, which in several cell lines is shown to be RIPK1 kinase activity dependent (Nehs et al. 2011). Additionally, 5-aminolevulinic acid (5-ALA)-based photodynamic therapy (PDT) in glioblastoma depends on RIPK3 (Coupienne et al. 2011), etoposide-induced killing requires RIPK1 complex formation (Tenev et al. 2011), and obatoclax (GX15-070) in rhabdomyosarcoma cells causes necrosome formation in an Atg5-dependent way (Basit et al. 2013). The remaining question is whether

this induction of necroptosis is a context-dependent phenomenon or whether it may be extended to clinical cancer treatment. Second, disintegration of the plasma membrane during necroptosis results in the release of cell death associated molecular patterns (CDAMPs), evoking immune responses and inflammation (Krysko et al. 2012). As successful anthracycline chemotherapy was demonstrated to depend on immunogenicity of dying cells (Zitvogel et al. 2010), the strong immunogenic properties of necrotic cell death may exert a higher therapeutic potential than apoptotic cell death, but this remains to be experimentally proven.

1.3 The Pharmacological Inhibition of Necroptosis: High Hopes and First Drawbacks

The evolving understanding of the RIPK-family-dependent necroptotic pathway has led to the development of specific inhibitors of necroptosis. The RIPK1 kinase inhibitor necrostatin-1 (Nec-1) has already proven to be of benefit in preclinical models of ischemia–reperfusion injury (IRI) (Degterev et al. 2005, 2008; Hitomi et al. 2008b). However, much of the work provided on necroptosis has been interpreted through inhibitory effects mediated by the RIPK1-inhibitor Nec-1. Hardly any compound has been investigated in this much detail for its kinase specificity (Biton and Ashkenazi 2011; Degterev et al. 2008), and the structure of the Nec-1-bound RIPK1-kinase domain has recently been published (Xie et al. 2013). These data clearly show that most other kinases are ruled out as potential targets for off-target effects. However, it remains unclear if Nec-1 might bind to structures other than kinases during the complex assembly of the necroptosome or elsewhere within cell death induction. In addition, Nec-1 inhibits the potent immunomodulatory enzyme indoleamine 2,3-dioxygenase (IDO) which might explain some side effects (Takahashi et al. 2012). Second-line derivates have been developed and were recently investigated (Takahashi et al. 2012), but until today exclusively in the TNF-shock model (Duprez et al. 2011), but not in sepsis, IRI, or other disorders apart from the brain ischemia model (Degterev et al. 2005). In addition to expanding the specificity of RIPK1 inhibitors, it will certainly be promising to develop RIPK3 inhibitors despite the fear of apoptosis induction (Dondelinger et al. 2013) and RIPK3-mediated effects offside from necroptosis on inflammasome activation, at least in “sensitizing” conditions of caspase-8 inhibition/ablation or IAP inhibition (Kang et al. 2012; Vince et al. 2012). Finally, MLKL might to date be the most promising target of specific necroptosis blockade (Sun et al. 2012; Zhao et al. 2012), but due to the fact that MLKL is a pseudokinase, the precise mechanism of action of such inhibitors, once available, remains to be determined.

At least mice tolerate high serum concentrations of Nec-1 that is rapidly eliminated though free glomerular filtration and excretion via the urine. Therefore, it is to be expected that Nec-1, as many other small molecules, accumulates in acute renal failure or chronic kidney disease, but on the other hand, this allows the most predictable and limited application in patients without renal disease in emergency

situations. However, due to the rapid progression of necroptotic signaling and its execution process, clinically feasible interference to attenuate organ injury may only be possible when necroptosis can be anticipated, such as with ischemia related to surgery as well as solid organ transplantation (Linkermann et al. 2012b, c).

Successful strategies in clinical use often employ specific inhibition of an event-triggering receptor. Whereas in vitro data have often referred to TNFR1 as the primary inducer of necroptosis, this could not be confirmed in vivo in an IRI model (Burne et al. 2001). Another study also showed that TNFR1/2-double-deficient mice are not protected from kidney IRI (Ko et al. 2011). Therefore, the quest for the necroptosis-inducing receptor has started. Promising candidates include other death receptors, Toll-like receptors, and intracellular receptors (Mocarski et al. 2011). Mediators might involve RHIM-domain-containing proteins DAI and TRIF.

1.4 The Predominant Role of Apoptosis in Physiological Conditions Versus the Role of Necroptosis as "Specific for Pathological Conditions"

Until today and to the best of our knowledge, there is no line of evidence for a physiologically occurring necroptotic signal during development and homeostasis in vivo. In line with this thought, RIPK3-deficient mice have been published as early as 2004 by Kim Newton and Vishva Dixit and, albeit carefully investigated, do not exhibit any spontaneous phenotype (Newton et al. 2004). Only upon pathophysiological challenges or upon the removal of the "brakes" on necrosome formation (e.g., ablation of FADD, caspase-8, or IAPs), the development of mice is affected, and pathophysiological signs occur in skin or intestine and probably other organs. It might be speculated that vertebrates employ the presumably more complex program of caspase-dependent apoptosis whenever possible in physiological development and beyond in maintaining organ function by regular cell turnover. The most likely explanation for this hypothesis is the absence of heavy triggers for the immune system in apoptosis that certainly affect organ function and therefore are likely to progress in evolution when available. This hypothesis logically concludes that whenever the apoptotic program is affected in any way, by virally expressed caspase inhibitors, by the simple absence of apoptosis, or in organs that are not expressing a readily available apoptotic machinery, the necroptotic pathway takes over to assure the decision of the cell to die but in such situations needs to accept the disadvantage of severe inflammation triggered by CDMAPs (Kaczmarek et al. 2013). Such situations include most of the disorders that we are dealing with clinically (Fig. 1.1). If necroptosis occurs exclusively under pathologic conditions, we might interpret the presence of necroptosis as a marker for a pathological situation. In this sense, we further might assign necroptosis a "specificity for pathologic conditions." If this thought turns out to be conclusive, the specific interference with necroptosis itself will probably not harm the remaining organism, comparably resulting in "specificity of necroptosis inhibitors for pathologic conditions." In

terms of side effects, obviously, this would be unique in pharmacology and hopefully most favorable for patients. However, today this intriguing hypothesis is purely based on speculation. In cancer therapy, it may be expected that drugs that could sensitize or trigger necroptosis by allosteric activation of RIPK3 or MLKL in cancer cells or tumor-associated endothelium may bypass antiapoptotic mechanisms but also evoke immunogenic cell death. However, in view of the harmful situation of sensitized necroptosis, it will be important to combine pro-necroptotic cancer drugs with a strategy of specific on-target delivery.

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References

Abbara A, Davidson RN (2011) Etiology and management of genitourinary tuberculosis. Nat Rev Urol 8:678–688

Almadi MA, Aljebreen AM, Sanai FM, Marcus V, Almeghaiseeb ES, Ghosh S (2011) New insights into gastrointestinal and hepatic granulomatous disorders. Nat Rev Gastroenterol Hepatol 8:455–466

Aruin LI, Shekhter AB, Milovanova ZP, Gorodinskaia VS (1989) Fibrinoid and fibrinoid necrosis in the morphogenesis of chronic stomach ulcers (electron and light microscopy). Arkh Patol 51:16–23

Baroldi G (1975) Different morphological types of myocardial cell death in man. Recent Adv Stud Cardiac Struct Metab 6:383–397

Basit F, Cristofanon S, Fulda S (2013) Obatoclax (GX15-070) triggers necroptosis by promoting the assembly of the necrosome on autophagosomal membranes. Cell Death Differ 20(9):1161–1173

Bertrand MJ, Vandenabeele P (2011) The ripoptosome: death decision in the cytosol. Mol Cell 43:323–325

Biton S, Ashkenazi A (2011) NEMO and RIP1 control cell fate in response to extensive DNA damage via TNF-alpha feedforward signaling. Cell 145:92–103

Bonnet MC, Preukschat D, Welz PS, Van LG, Ermolaeva MA, Bloch W, Haase I, Pasparakis M (2011) The adaptor protein FADD protects epidermal keratinocytes from necroptosis in vivo and prevents skin inflammation. Immunity 35:572–582

Bruennler T, Hamer OW, Lang S, Gruene S, Wrede CE, Zorger N, Herold T, Siebig S, Rockmann F, Salzberger B, Feuerbach S, Schoelmerich J, Langgartner J (2009) Outcome in a large unselected series of patients with acute pancreatitis. Hepatogastroenterology 56:871–876

Burne MJ, Elghandour A, Haq M, Saba SR, Norman J, Condon T, Bennett F, Rabb H (2001) IL-1 and TNF independent pathways mediate ICAM-1/VCAM-1 up-regulation in ischemia reperfusion injury. J Leukoc Biol 70:192–198
Burrell M, Gold JA, Simeone J, Taylor K, Dobbins J (1980) Liquefactive necrosis of the pancreas: the pancreatic sac. Radiology 135:157–160
Caruso R, Parisi A, Bonanno A, Paparo D, Quattrocchi E, Branca G, Scardigno M, Fedele F (2012) Histologic coagulative tumour necrosis as a prognostic indicator of aggressiveness in renal, lung, thyroid and colorectal carcinomas: a brief review. Oncol Lett 3:16–18
Cauwels A, Janssen B, Waeytens A, Cuvelier C, Brouckaert P (2003) Caspase inhibition causes hyperacute tumor necrosis factor-induced shock via oxidative stress and phospholipase A2. Nat Immunol 4:387–393
Chavez-Valdez R, Martin LJ, Flock DL, Northington FJ (2012) Necrostatin-1 attenuates mitochondrial dysfunction in neurons and astrocytes following neonatal hypoxia-ischemia. Neuroscience 219:192–203
Chen F, Suzuki Y, Nagai N, Jin L, Yu J, Wang H, Marchal G, Ni Y (2007) Rodent stroke induced by photochemical occlusion of proximal middle cerebral artery: evolution monitored with MR imaging and histopathology. Eur J Radiol 63:68–75
Cho YS, Challa S, Moquin D, Genga R, Ray TD, Guildford M, Chan FK (2009) Phosphorylation-driven assembly of the RIP1-RIP3 complex regulates programmed necrosis and virus-induced inflammation. Cell 137:1112–1123
Coupienne I, Fettweis G, Piette J (2011) RIP3 expression induces a death profile change in U2OS osteosarcoma cells after 5-ALA-PDT. Lasers Surg Med 43:557–564
Declercq W, Vanden Berghe T, Vandenabeele P (2009) RIP kinases at the crossroads of cell death and survival. Cell 138:229–232
Degterev A, Hitomi J, Germscheid M, Ch'en IL, Korkina O, Teng X, Abbott D, Cuny GD, Yuan C, Wagner G, Hedrick SM, Gerber SA, Lugovskoy A, Yuan J (2008) Identification of RIP1 kinase as a specific cellular target of necrostatins. Nat Chem Biol 4:313–321
Degterev A, Huang Z, Boyce M, Li Y, Jagtap P, Mizushima N, Cuny GD, Mitchison TJ, Moskowitz MA, Yuan J (2005) Chemical inhibitor of nonapoptotic cell death with therapeutic potential for ischemic brain injury. Nat Chem Biol 1:112–119
Degterev A, Maki JL, Yuan J (2013) Activity and specificity of necrostatin-1, small-molecule inhibitor of RIP1 kinase. Cell Death Differ 20:366
Dillon CP, Oberst A, Weinlich R, Janke LJ, Kang TB, Ben-Moshe T, Mak TW, Wallach D, Green DR (2012) Survival function of the FADD-CASPASE-8-cFLIP(L) complex. Cell Rep 1: 401–407
Dondelinger Y, Aguileta MA, Goossens V, Dubuisson C, Grootjans S, Dejardin E, Vandenabeele P, Bertrand MJM (2013) RIPK3 contributes to TNFR1-mediated RIPK1 kinase-dependent apoptosis in conditions of cIAP1/2 depletion or TAK1 kinase inhibition. Cell Death Diff 20(10):1381–1392
Dong K, Zhu H, Song Z, Gong Y, Wang F, Wang W, Zheng Z, Yu Z, Gu Q, Xu X, Sun X (2012) Necrostatin-1 protects photoreceptors from cell death and improves functional outcome after experimental retinal detachment. Am J Pathol 181:1634–1641
Duprez L, Takahashi N, Van HF, Vandendriessche B, Goossens V, Vanden Berghe T, Declercq W, Libert C, Cauwels A, Vandenabeele P (2011) RIP kinase-dependent necrosis drives lethal systemic inflammatory response syndrome. Immunity 35:908–918
Estes ML, Rorke LB (1986) Liquefactive necrosis in Coxsackie B encephalitis. Arch Pathol Lab Med 110:1090–1092
Feng S, Yang Y, Mei Y, Ma L, Zhu DE, Hoti N, Castanares M, Wu M (2007) Cleavage of RIP3 inactivates its caspase-independent apoptosis pathway by removal of kinase domain. Cell Signal 19:2056–2067
Feoktistova M, Geserick P, Kellert B, Dimitrova DP, Langlais C, Hupe M, Cain K, Macfarlane M, Hacker G, Leverkus M (2011) cIAPs block Ripoptosome formation, a RIP1/caspase-8 containing intracellular cell death complex differentially regulated by cFLIP isoforms. Mol Cell 43:449–463

Freimuth J, Bangen JM, Lambertz D, Hu W, Nevzorova YA, Sonntag R, Gassler N, Riethmacher D, Trautwein C, Liedtke C (2013) Loss of caspase-8 in hepatocytes accelerates the onset of liver regeneration in mice through premature NF-kappaB activation. Hepatology 2013 May 31. doi:10.1002/hep.26538

Fricker M, Vilalta A, Tolkovsky AM, Brown GC (2013) Caspase inhibitors protect neurons by enabling selective necroptosis of inflamed microglia. J Biol Chem 288:9145–9152

Gunther C, Martini E, Wittkopf N, Amann K, Weigmann B, Neumann H, Waldner MJ, Hedrick SM, Tenzer S, Neurath MF, Becker C (2011) Caspase-8 regulates TNF-alpha-induced epithelial necroptosis and terminal ileitis. Nature 477:335–339

Hampe J, Franke A, Rosenstiel P, Till A, Teuber M, Huse K, Albrecht M, Mayr G, De La Vega FM, Briggs J, Gunther S, Prescott NJ, Onnie CM, Hasler R, Sipos B, Folsch UR, Lengauer T, Platzer M, Mathew CG, Krawczak M, Schreiber S (2007) A genome-wide association scan of nonsynonymous SNPs identifies a susceptibility variant for Crohn disease in ATG16L1. Nat Genet 39:207–211

Han J, Zhong CQ, Zhang DW (2011) Programmed necrosis: backup to and competitor with apoptosis in the immune system. Nat Immunol 12:1143–1149

Hanahan D, Weinberg RA (2011) Hallmarks of cancer: the next generation. Cell 144:646–674

He S, Wang L, Miao L, Wang T, Du F, Zhao L, Wang X (2009) Receptor interacting protein kinase-3 determines cellular necrotic response to TNF-alpha. Cell 137:1100–1111

Hess ML, Manson NH (1984) Molecular oxygen: friend and foe. The role of the oxygen free radical system in the calcium paradox, the oxygen paradox and ischemia/reperfusion injury. J Mol Cell Cardiol 16:969–985

Hitomi J, Christofferson DE, Ng A, Yao J, Degterev A, Xavier RJ, Yuan J (2008) Identification of a molecular signaling network that regulates a cellular necrotic cell death pathway. Cell 135:1311–1323

Hunter RL, Jagannath C, Actor JK (2007) Pathology of postprimary tuberculosis in humans and mice: contradiction of long-held beliefs. Tuberculosis (Edinb) 87:267–278

Kaczmarek A, Vandenabeele P, Krysko DV (2013) Necroptosis: the release of damage-associated molecular patterns and its physiological relevance. Immunity 38:209–223

Kaiser WJ, Upton JW, Long AB, Livingston-Rosanoff D, Daley-Bauer LP, Hakem R, Caspary T, Mocarski ES (2011) RIP3 mediates the embryonic lethality of caspase-8-deficient mice. Nature 471:368–372

Kaiser WJ, Upton JW, Mocarski ES (2013) Viral modulation of programmed necrosis. Curr Opin Virol 3(3):296–306

Kang TB, Yang SH, Toth B, Kovalenko A, Wallach D (2012) Caspase-8 blocks kinase RIPK3-mediated activation of the NLRP3 inflammasome. Immunity 38(1):27–40

Kittleson MM, Kobashigawa JA (2012) Antibody-mediated rejection. Curr Opin Organ Transplant 17:551–557

Ko GJ, Jang HR, Huang Y, Womer KL, Liu M, Higbee E, Xiao Z, Yagita H, Racusen L, Hamad AR, Rabb H (2011) Blocking Fas ligand on leukocytes attenuates kidney ischemia-reperfusion injury. J Am Soc Nephrol 22(4):732–742

Kovalenko A, Chable-Bessia C, Cantarella G, Israel A, Wallach D, Courtois G (2003) The tumour suppressor CYLD negatively regulates NF-kappaB signalling by deubiquitination. Nature 424:801–805

Kreuzaler P, Watson CJ (2012) Killing a cancer: what are the alternatives? Nat Rev Cancer 12:411–424

Krysko DV, Garg AD, Kaczmarek A, Krysko O, Agostinis P, Vandenabeele P (2012) Immunogenic cell death and DAMPs in cancer therapy. Nat Rev Cancer 12:860–875

Kuraishy A, Karin M, Grivennikov SI (2011) Tumor promotion via injury- and death-induced inflammation. Immunity 35:467–477

Ladoire S, Hannani D, Vetizou M, Locher C, Aymeric L, Apetoh L, Kepp O, Kroemer G, Ghiringhelli F, Zitvogel L (2013) Cell-death-associated molecular patterns as determinants of cancer immunogenicity. Antioxid Redox Signal. Epub ahead of print

Lau A, Wang S, Jiang J, Haig A, Pavlowsky A, Linkermann A, Zhang ZX, Jevnikar AM (2013) RIPK3 mediated necroptosis promotes donor kidney inflammatory injury and reduces allograft survival. Am J Transplant 13(11):2805–2818

Lee YS, Dayma Y, Park MY, Kim KI, Yoo SE, Kim E (2013) Daxx is a key downstream component of receptor interacting protein kinase 3 mediating retinal ischemic cell death. FEBS Lett 587:266–271

Li L, Huang L, Ye H, Song SP, Bajwa A, Lee SJ, Moser EK, Jaworska K, Kinsey GR, Day YJ, Linden J, Lobo PI, Rosin DL, Okusa MD (2012) Dendritic cells tolerized with adenosine A(2) AR agonist attenuate acute kidney injury. J Clin Invest 122:3931–3942

Li Y, Yang X, Ma C, Qiao J, Zhang C (2008) Necroptosis contributes to the NMDA-induced excitotoxicity in rat's cultured cortical neurons. Neurosci Lett 447:120–123

Liedtke C, Bangen JM, Freimuth J, Beraza N, Lambertz D, Cubero FJ, Hatting M, Karlmark KR, Streetz KL, Krombach GA, Tacke F, Gassler N, Riethmacher D, Trautwein C (2011) Loss of caspase-8 protects mice against inflammation-related hepatocarcinogenesis but induces non-apoptotic liver injury. Gastroenterology 141:2176–2187

Lin J, Li H, Yang M, Ren J, Huang Z, Han F, Huang J, Ma J, Zhang D, Zhang Z, Wu J, Huang D, Qiao M, Jin G, Wu Q, Huang Y, Du J, Han J (2013) A role of RIP3-mediated macrophage necrosis in atherosclerosis development. Cell Rep 3:200–210

Linkermann A, Brasen JH, Darding M, Jin MK, Sanz AB, Heller JO, De ZF, Weinlich R, Ortiz A, Walczak H, Weinberg JM, Green DR, Kunzendorf U, Krautwald S (2013a) Two independent pathways of regulated necrosis mediate ischemia-reperfusion injury. Proc Natl Acad Sci U S A 110:12024–12029

Linkermann A, Brasen JH, De ZF, Weinlich R, Schwendener RA, Green DR, Kunzendorf U, Krautwald S (2012a) Dichotomy between RIP1- and RIP3-mediated necroptosis in tumor necrosis factor alpha-induced shock. Mol Med 18:577–586

Linkermann A, Brasen JH, Himmerkus N, Liu S, Huber TB, Kunzendorf U, Krautwald S (2012b) Rip1 (receptor-interacting protein kinase 1) mediates necroptosis and contributes to renal ischemia/reperfusion injury. Kidney Int 81:751–761

Linkermann A, De ZF, Weinberg J, Kunzendorf U, Krautwald S (2012c) Programmed necrosis in acute kidney injury. Nephrol Dial Transplant 27:3412–3419

Linkermann A, Hackl MJ, Kunzendorf U, Walczak H, Krautwald S, Jevnikar AM (2013b) Necroptosis in immunity and ischemia-reperfusion injury. Am J Transplant 13(11): 2797–2804

Liu J, van MA, Vrijsen K, Zhao J, Gao L, Metz CH, Goumans MJ, Doevendans PA, Sluijter JP (2011) MicroRNA-155 prevents necrotic cell death in human cardiomyocyte progenitor cells via targeting RIP1. J Cell Mol Med 15:1474–1482

Mocarski ES, Upton JW, Kaiser WJ (2011) Viral infection and the evolution of caspase 8-regulated apoptotic and necrotic death pathways. Nat Rev Immunol 12(2):79–88

Murakami Y, Matsumoto H, Roh M, Suzuki J, Hisatomi T, Ikeda Y, Miller JW, Vavvas DG (2012) Receptor interacting protein kinase mediates necrotic cone but not rod cell death in a mouse model of inherited degeneration. Proc Natl Acad Sci U S A 109:14598–14603

Nehs MA, Lin CI, Kozono DE, Whang EE, Cho NL, Zhu K, Moalem J, Moore FD Jr, Ruan DT (2011) Necroptosis is a novel mechanism of radiation-induced cell death in anaplastic thyroid and adrenocortical cancers. Surgery 150:1032–1039

Nenci A, Becker C, Wullaert A, Gareus R, Van LG, Danese S, Huth M, Nikolaev A, Neufert C, Madison B, Gumucio D, Neurath MF, Pasparakis M (2007) Epithelial NEMO links innate immunity to chronic intestinal inflammation. Nature 446:557–561

Newton K, Sun X, Dixit VM (2004) Kinase RIP3 is dispensable for normal NF-kappa Bs, signaling by the B-cell and T-cell receptors, tumor necrosis factor receptor 1, and Toll-like receptors 2 and 4. Mol Cell Biol 24:1464–1469

Northington FJ, Chavez-Valdez R, Graham EM, Razdan S, Gauda EB, Martin LJ (2011) Necrostatin decreases oxidative damage, inflammation, and injury after neonatal HI. J Cereb Blood Flow Metab 31:178–189

Oberst A, Dillon CP, Weinlich R, McCormick LL, Fitzgerald P, Pop C, Hakem R, Salvesen GS, Green DR (2011) Catalytic activity of the caspase-8-FLIP(L) complex inhibits RIPK3-dependent necrosis. Nature 471:363–367

Oerlemans MI, Liu J, Arslan F, den OK, van Middelaar BJ, Doevendans PA, Sluijter JP (2012a) Inhibition of RIP1-dependent necrosis prevents adverse cardiac remodeling after myocardial ischemia-reperfusion in vivo. Basic Res Cardiol 107:270

Oerlemans MI, Liu J, Arslan F, Den OK, Van Middelaar BJ, Doevendans PA, Sluijter JP (2012b) Inhibition of RIP1-dependent necrosis prevents adverse cardiac remodeling after myocardial ischemia-reperfusion in vivo. Basic Res Cardiol 107:270

Olivero JJ, Olivero JJ, Nguyen PT, Kagan A (2012) Acute kidney injury after cardiovascular surgery: an overview. Methodist Debakey Cardiovasc J 8:31–36

Raelson JV, Little RD, Ruether A, Fournier H, Paquin B, Van EP, Bradley WE, Croteau P, Nguyen-Huu Q, Segal J, Debrus S, Allard R, Rosenstiel P, Franke A, Jacobs G, Nikolaus S, Vidal JM, Szego P, Laplante N, Clark HF, Paulussen RJ, Hooper JW, Keith TP, Belouchi A, Schreiber S (2007) Genome-wide association study for Crohn's disease in the Quebec Founder Population identifies multiple validated disease loci. Proc Natl Acad Sci U S A 104:14747–14752

Ramachandran A, McGill MR, Xie Y, Ni HM, Ding WX, Jaeschke H (2013) Receptor interacting protein kinase 3 is a critical early mediator of acetaminophen-induced hepatocyte necrosis in mice. Hepatology 58(6):2099–2108. doi:10.1002/hep.26547. Epub 2013 Oct 11

Rajagopala S, Devaraj U, D'Souza G (2011) Infected cavitating pulmonary infarction. Respir Care 56:707–709

Redline S, Tomashefski JF Jr, Altose MD (1985) Cavitating lung infarction after bland pulmonary thromboembolism in patients with the adult respiratory distress syndrome. Thorax 40:915–919

Rosenbaum DM, Degterev A, David J, Rosenbaum PS, Roth S, Grotta JC, Cuny GD, Yuan J, Savitz SI (2010) Necroptosis, a novel form of caspase-independent cell death, contributes to neuronal damage in a retinal ischemia-reperfusion injury model. J Neurosci Res 88:1569–1576

Rosenblum WI (2008) Fibrinoid necrosis of small brain arteries and arterioles and miliary aneurysms as causes of hypertensive hemorrhage: a critical reappraisal. Acta Neuropathol 116:361–369

Roychowdhury S, McMullen MR, Pisano SG, Liu X, Nagy LE (2012) Absence of receptor interacting protein kinase 3 prevents ethanol-induced liver injury. Hepatology 57(5):1773–1783

Smith CC, Davidson SM, Lim SY, Simpkin JC, Hothersall JS, Yellon DM (2007) Necrostatin: a potentially novel cardioprotective agent? Cardiovasc Drugs Ther 21:227–233

Suarez-Amor O, Perez-Bustillo A, Ruiz-Gonzalez I, Rodriguez-Prieto MA (2010) Necrobiosis lipoidica therapy with biologicals: an ulcerated case responding to etanercept and a review of the literature. Dermatology 221:117–121

Sun L, Wang H, Wang Z, He S, Chen S, Liao D, Wang L, Yan J, Liu W, Lei X, Wang X (2012) Mixed lineage kinase domain-like protein mediates necrosis signaling downstream of RIP3 kinase. Cell 148:213–227

Szabo S, Deng X, Khomenko T, Chen L, Tolstanova G, Osapay K, Sandor Z, Xiong X (2007) New molecular mechanisms of duodenal ulceration. Ann N Y Acad Sci 1113:238–255

Takahashi N, Duprez L, Grootjans S, Cauwels A, Nerinckx W, Duhadaway JB, Goossens V, Roelandt R, Van HF, Libert C, Declercq W, Callewaert N, Prendergast GC, Degterev A, Yuan J, Vandenabeele P (2012) Necrostatin-1 analogues: critical issues on the specificity, activity and in vivo use in experimental disease models. Cell Death Dis 3:e437

Tamura Y, Chiba Y, Tanioka T, Shimizu N, Shinozaki S, Yamada M, Kaneki K, Mori S, Araki A, Ito H, Kaneki M (2011) NO donor induces Nec-1-inhibitable, but RIP1-independent, necrotic cell death in pancreatic beta-cells. FEBS Lett 585:3058–3064

Tenev T, Bianchi K, Darding M, Broemer M, Langlais C, Wallberg F, Zachariou A, Lopez J, Macfarlane M, Cain K, Meier P (2011) The Ripoptosome, a signaling platform that assembles in response to genotoxic stress and loss of IAPs. Mol Cell 43:432–448

Trichonas G, Murakami Y, Thanos A, Morizane Y, Kayama M, Debouck CM, Hisatomi T, Miller JW, Vavvas DG (2010) Receptor interacting protein kinases mediate retinal detachment-induced photoreceptor necrosis and compensate for inhibition of apoptosis. Proc Natl Acad Sci U S A 107(50):21695–21700

Trompouki E, Hatzivassiliou E, Tsichritzis T, Farmer H, Ashworth A, Mosialos G (2003) CYLD is a deubiquitinating enzyme that negatively regulates NF-kappaB activation by TNFR family members. Nature 424:793–796

Upton JW, Kaiser WJ, Mocarski ES (2008) Cytomegalovirus M45 cell death suppression requires receptor-interacting protein (RIP) homotypic interaction motif (RHIM)-dependent interaction with RIP1. J Biol Chem 283:16966–16970

Upton JW, Kaiser WJ, Mocarski ES (2010) Virus inhibition of RIP3-dependent necrosis. Cell Host Microbe 7:302–313

Vandenabeele P, Galluzzi L, Vanden Berghe T, Kroemer G (2010) Molecular mechanisms of necroptosis: an ordered cellular explosion. Nat Rev Mol Cell Biol 11:700–714

Vandenabeele P, Grootjans S, Callewaert N, Takahashi N (2013) Necrostatin-1 blocks both RIPK1 and IDO: consequences for the study of cell death in experimental disease models. Cell Death Differ 20:185–187

Vereecke L, Sze M, Mc GC, Rogiers B, Chu Y, Schmidt-Supprian M, Pasparakis M, Beyaert R, Van LG (2010) Enterocyte-specific A20 deficiency sensitizes to tumor necrosis factor-induced toxicity and experimental colitis. J Exp Med 207:1513–1523

Vince JE, Wong WW, Gentle I, Lawlor KE, Allam R, O'Reilly L, Mason K, Gross O, Ma S, Guarda G, Anderton H, Castillo R, Hacker G, Silke J, Tschopp J (2012) Inhibitor of apoptosis proteins limit RIP3 kinase-dependent interleukin-1 activation. Immunity 36:215–227

von MC, Matias N, Fernandez A, Fucho R, Conde de la Rosa L, Martinez-Chantar ML, Mato JM, Machida K, Tsukamoto H, Murphy MP, Mansouri A, Kaplowitz N, Garcia-Ruiz C, Fernandez-Checa JC (2012) Mitochondrial GSH determines the toxic or therapeutic potential of superoxide scavenging in steatohepatitis. J Hepatol 57:852–859

Wang YQ, Wang L, Zhang MY, Wang T, Bao HJ, Liu WL, Dai DK, Zhang L, Chang P, Dong WW, Chen XP, Tao LY (2012) Necrostatin-1 suppresses autophagy and apoptosis in mice traumatic brain injury model. Neurochem Res 37:1849–1858

Welz PS, Wullaert A, Vlantis K, Kondylis V, Fernandez-Majada V, Ermolaeva M, Kirsch P, Sterner-Kock A, Van LG, Pasparakis M (2011) FADD prevents RIP3-mediated epithelial cell necrosis and chronic intestinal inflammation. Nature 477(7364):330–334

Weigert C (1880) Über die pathologischen Gerinnungsvorgänge. Arch Pathol Anat Physiol Klein Med 79:87–123

Wu J, Huang Z, Ren J, Zhang Z, He P, Li Y, Ma J, Chen W, Zhang Y, Zhou X, Yang Z, Wu SQ, Chen L, Han J (2013) Mlkl knockout mice demonstrate the indispensable role of Mlkl in necroptosis. Cell Res 23(8):994–1006

Xie T, Peng W, Liu Y, Yan C, Maki J, Degterev A, Yuan J, Shi Y (2013) Structural basis of RIP1 inhibition by necrostatins. Structure 21:493–499

Xu X, Chua KW, Chua CC, Liu CF, Hamdy RC, Chua BH (2010) Synergistic protective effects of humanin and necrostatin-1 on hypoxia and ischemia/reperfusion injury. Brain Res 1355: 189–194

You Z, Savitz SI, Yang J, Degterev A, Yuan J, Cuny GD, Moskowitz MA, Whalen MJ (2008) Necrostatin-1 reduces histopathology and improves functional outcome after controlled cortical impact in mice. J Cereb Blood Flow Metab 28:1564–1573

Younes NA, Ahmad AT (2006) Diabetic foot disease. Endocr Pract 12:583–592

Zhang DW, Shao J, Lin J, Zhang N, Lu BJ, Lin SC, Dong MQ, Han J (2009) RIP3, an energy metabolism regulator that switches TNF-induced cell death from apoptosis to necrosis. Science 325:332–336

Zhang H, Zhou X, McQuade T, Li J, Chan FK, Zhang J (2011) Functional complementation between FADD and RIP1 in embryos and lymphocytes. Nature 471:373–376

Zhao J, Jitkaew S, Cai Z, Choksi S, Li Q, Luo J, Liu ZG (2012) Mixed lineage kinase domain-like is a key receptor interacting protein 3 downstream component of TNF-induced necrosis. Proc Natl Acad Sci U S A 109:5322–5327

Zhu S, Zhang Y, Bai G, Li H (2011) Necrostatin-1 ameliorates symptoms in R6/2 transgenic mouse model of Huntington's disease. Cell Death Dis 2:e115

Zitvogel L, Kepp O, Kroemer G (2010) Decoding cell death signals in inflammation and immunity. Cell 140:798–804

Chapter 2
RIP1-Mediated Signaling Pathways in Cell Survival and Death Control

Yong Lin

2.1 Introduction

RIP1 was initially identified through a yeast two-hybrid screening as a Fas-interacting protein and an adaptor protein in the TNF receptor 1 (TNFR1) signaling complex (Stanger et al. 1995; Hsu et al. 1996). The human *rip1* gene is located on chromosome 6 and encodes a protein of 671 amino acids (aa) with a predicted molecular weight of 76 kDa (Hsu et al. 1996). In the 300 aa N-terminus resides a serine/threonine kinase domain (KD), while the C-terminal 112 aa contains a death domain (DD). The DD is homologous to the DD in the intracellular regions of Fas, TNFR1 TRAILR1 (DR4), and TRAILR2 (DR5). Because RIP1 can bind to these death receptors, it is thus called a death domain kinase. The DD can also bind TRADD and FADD in the TNFR1 signaling complex. Between the KD and DD is an intermediate domain (ID) that harbors a RIP homotypic interaction motif (RHIM) (Fig. 2.1). Since the discovery of RIP1, six other RIP-like proteins (RIP2-7) with serine/threonine kinase domain have been found which constitute the RIP family (Meylan and Tschopp 2005). It is noteworthy that other RIP family members cannot compensate RIP1deficiency in cells, indicating a unique cellular role for RIP1.

While RIP1 is a critical adaptor protein for TNFR1-mediated signaling to NF-κB activation, researches have determined RIP1 functions in diverse cell signaling pathways for either cell survival or death. These include death receptor (Fas, TNFR1, DR4, DR5, etc.)-mediated activation of MAPK (JNK, ERK, and p38) (Lin et al. 2000; Festjens et al. 2007), apoptosis and necrosis; Toll-like receptor (TLR)-3- and (TLR)-4-mediated activation of NF-κB and MAPK (Han et al. 2004; Meylan et al. 2004; Kaiser and Offermann 2005), apoptosis and necrosis; and genotoxic

Y. Lin, M.D., Ph.D. (✉)
Molecular Biology and Lung Cancer Program, Lovelace Respiratory Research Institute, 2425 Ridgecrest DR SE, Albuquerque, NM 87108, USA
e-mail: ylin@lrri.org

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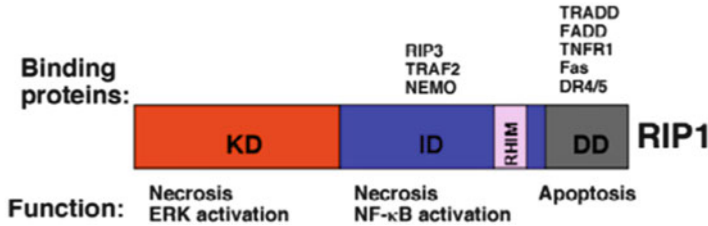

Fig. 2.1 Domain structure of RIP1. The kinase domain (KD), intermediate domain (ID), and the death domain (DD) are *highlighted*. RHIM, RIP homotypic interaction motif. The known interaction proteins and functions of each domain are *listed*

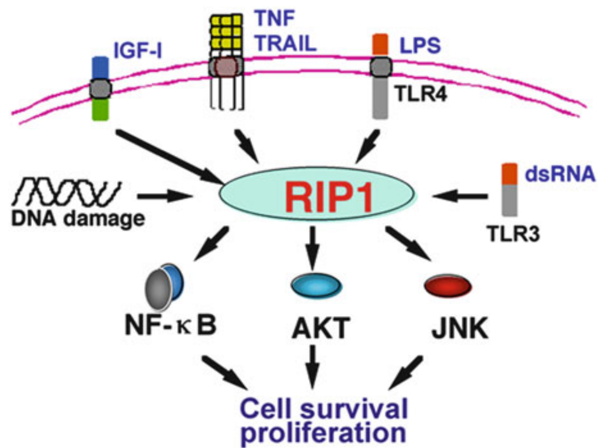

Fig. 2.2 RIP1-mediated cell survival pathways. Activation of receptors such as IGF-IR, TNFR1, DR4/5 (for TRAIL), TLR3 (for dsRNA), or TLR4 (for LPS) and cellular stresses such as DNA damage activate cell survival pathways (NF-κB, Akt, and JNK) depending on cellular context. See text for details

stress-induced activation of NF-κB, apoptosis and necrosis. Therefore, RIP1 is placed at a unique position to relay signals activated by diverse stimuli to different pathways (Fig. 2.2). It is apparent that RIP1 is a key player in regulating cells' fate, survival, or death, in response to different stimulations (Meylan and Tschopp 2005; Festjens et al. 2007; O'Donnell and Ting 2011; Zhang et al. 2011).

2.2 RIP in Cell Survival Signaling

2.2.1 RIP1 in Death Receptor-Mediated Survival Signaling

2.2.1.1 RIP1 in Death Receptor-Mediated NF-κB Activation

The most well-studied NF-κB activation pathway involving RIP1 is that mediated by TNFR1 (Karin and Gallagher 2009) (Fig. 2.3). Ligation of TNFα to its receptor results in the trimerization of TNFR1, which recruits TRADD to form a platform

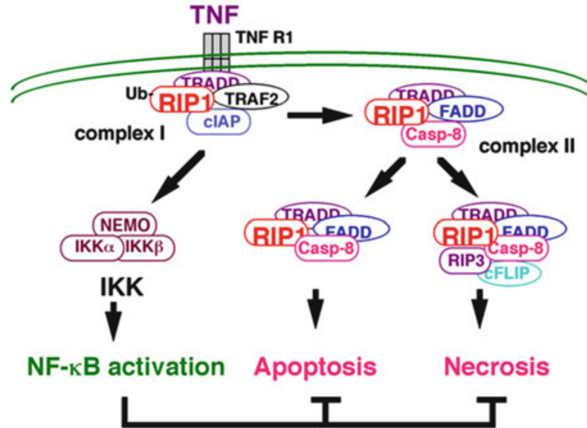

Fig. 2.3 RIP1 in TNF**α**-induced cell survival or death signaling. TNF**α** binding to TNFR1 induces formation of complex I containing TRADD, RIP1, TRAF2, and cIAP1/2. Ubiquitination of RIP1 by cIAP1/2 leads to IKK activation to turn on the NF-κB activation pathway. Internalization of complex I, deubiquitination of RIP1, and recruitment of FADD and caspas-8 result in formation of complex II. When caspase-8 is sufficiently activated, complex II mediates apoptosis. In contrast, if caspase-8 is suppressed by c-FLIP, complex II-mediates RIP3-dependent necrosis. See text for details

for assembling a so-called complex I that consists of RIP1 and TNF receptor-associated factor 2 (TRAF2) in the lipid rafts on the plasma membrane (Hsu et al. 1996; Micheau and Tschopp 2003). RIP1 is then Lys63 polyubiquitinated on its Lys377 within minutes (Ea et al. 2006; Skaug et al. 2009). The Lys63 polyubiquitin chain serves as a platform for binding of NEMO in the IκB kinase (IKK) complex (Wu et al. 2006a). Then IKK is activated by phosphorylation mediated by TAK1 or MEKK3 (Devin et al. 2000; Yang et al. 2001). The adaptor proteins, TAB2 and TAB3, bind to the Lys63 polyubiquitin chain to recruit TAK1 to the complex for IKK activation (Kanayama et al. 2004; Skaug et al. 2009). The activated IKK in turn phosphorylates the inhibitors of NF-κB (IκB), which retains NF-κB in the cytoplasm, to trigger their rapid polyubiquitination followed by degradation in the 26S proteasome. This process allows NF-κB to migrate to the nucleus and bind to the promoters of its target genes. Several of NF-κB's target genes such as c-IAP1, c-IAP2, XIAP, and c-FLIP are found to have anti-apoptotic properties (Karin et al. 2004). Induction of the antioxidant manganese superoxide dismutase (MnSOD) by NF-κB is also suggested to be anti-apoptotic (Kamata et al. 2005). Therefore, the TNFR1-mediated NF-κB activation is generally believed to be for survival through anti-apoptosis. Interestingly, the cIAPs are E3 ubiquitin ligases that execute RIP1 Lys63 polyubiquitination, which may be a positive feedback loop for NF-κB activation (Skaug et al. 2009; Xu et al. 2009). In contrast, A20 and CYLD, both NF-κB targets, remove the Lys63 polyubiquitin chains from RIP1 and promote binding of Lys48-linked ubiquitin chain to RIP1, resulting in proteasomal degradation of RIP1 (Shembade et al. 2008; Skaug et al. 2009). In addition, RIP1 is cleaved at Asp324

by caspase-8 at the early stage of TNFR1 signaling, which shuts off the NF-κB activation pathway and promotes apoptosis (Lin et al. 1999). Thus, RIP1 serves as an important checkpoint for TNFR1-mediated NF-κB activation, and regulation of RIP1 underlies one of the mechanisms for accurate induction of NF-κB activity in terms of extent and duration (Festjens et al. 2007; O'Donnell and Ting 2011).

NF-κB is also activated by other death receptors through a similar mechanism involving RIP1, although the extent is generally weak. Hsp90 binds and stabilizes RIP1, consequently facilitating TNF- or TRAIL-induced NF-κB activation (Lewis et al. 2000; Wang et al. 2006). While NF-κB activation protects cancer cells from TNF- or TRAIL-induced apoptosis, blocking NF-κB sensitizes TNF- or TRAIL-induced cytotoxicity in cancer cells (Wang et al. 2006; Ju et al. 2007; Bai et al. 2009, 2011; Lin et al. 2010).

2.2.1.2 RIP1 in Death Receptor-Mediated MAP Kinase Activation

Residing in complex I during TNFR1 signaling, RIP1 also contributes to activation of MAP kinases (JNK, ERK, and p38) (Devin et al. 2003). The activation of MAPKs also requires TRAF2 and involves sequential activation of the MAPKKK/MAPKK/MAPK cascade. The transient JNK activation appears to promote survival; however, sustained JNK activation leads to cell death (Lin and Dibling 2002; Ventura et al. 2004). Interestingly, NF-κB suppresses sustained JNK activation to maintain cell survival. How the signaling for NF-κB and JNK activation is balanced at RIP1 is still elusive. The role of ERK and p38 in TNFR1-induced cell death is not well understood. Because ERK activation requires the kinase activity of RIP1 and this activity is important for necrosis, it remains to be determined if ERK activation is involved in TNF-induced necrosis. Transient activation of JNK by TRAIL is partially dependent on RIP1 (Lin et al. 2000). In these settings, JNK functions as a cell survival signal to protect cells from death therefore could be a target for sensitizing anticancer chemotherapy (Lin et al. 2000; Wang et al. 2006; Bai et al. 2011).

2.2.2 RIP1 in Genotoxic Stress-Mediated NF-κB Activation

The involvement of RIP1 in DNA damage-induced NF-κB activation was first seen in RIP1−/− mouse embryonic fibroblasts (MEF). DNA topoisomerase inhibitors such as adriamycin and etoposide and ionizing radiation (IR), which cause double-strand DNA breaks (DSB), stimulated NF-κB in wild type but not RIP−/− MEF cells (Hur et al. 2003). Further studies revealed that when genomic DNA is insulted, distinct protein complexes are formed containing different isoforms of p53-induced protein with a death domain (PIDD) for mediating apoptosis, DNA repair, or NF-κB activation (Janssens and Tschopp 2006; Wu et al. 2006b). One complex, caspase-2 PIDDosome, consisting of PIDD, RIP-associated CH1/ECD3-homologous protein with death domain (RAIDD), and procaspase 2, initiates apoptosis in a mitochondria-dependent manner (Janssens et al. 2005; Janssens and Tschopp 2006). The complex

called NEMO PIDDosome, which contains PIDD, RIP1, and the NEMO/IKKγ subunit of IKK, is responsible for NF-κB activation. While PIDD and RIP1 interact directly through their DD, the interaction between NEMO and PIDD and activation of NEMO are mediated by RIP1, indicating the importance of RIP1 in genotoxic stress-induced NF-κB activation (Huang et al. 2003; Janssens et al. 2005; Wu et al. 2006b). Upon the induction of genotoxic stress, two parallel signaling pathways are independently activated for starting the NF-κB activation pathway. The first pathway promotes the nuclear translocation of PIDD followed by recruitment of RIP1 and NEMO to form a complex, where NEMO is rapidly sumoylated by protein inhibitor of activated STAT (PIAS) (Mabb et al. 2006). The second pathway activates the ATM kinase through phosphorylation. The two pathways merge at the point that the sumoylated NEMO and the active ATM kinase meet together. Then ATM phosphorylates NEMO to promote its ubiquitination. Activated NEMO is exported to the cytoplasm where it forms a complex with IKKα and IKKβ, resulting in an active IKK that phosphorylates IκB to trigger its degradation, thereby the downstream cascade for NF-κB activation is activated. The RIP1/NEMO and RAIDD/caspase-2 pathways are mutually exclusive, suggesting that the interaction between RIP1 and PIDD is solely for cell survival to counteract apoptosis mediated by the RAIDD/caspase-2 complex (Tinel et al. 2007; Janssens and Tinel 2012). Additionally, during DNA damage with DSB, RIP1 also interacts with arrest-defective 1 protein (ARD1). ARD1 migrates to the nucleus where the acetyltransferase activity of ARD1 is important for NF-κB activation (Park et al. 2012). It remains to be determined if RIP1 regulates ARD1 nuclear translocation and its acetyltransferase activity during genotoxic stress response.

Although NF-κB is well known as a transcriptional activator, it may function as a transcription repressor in certain circumstances as when cells are responding to DNA damage (Campbell et al. 2004). This may be through ARF-mediated and ATR- and CHK1-dependent phosphorylation of RelA at The505 or through deficiency in Ser536 phosphorylation and acetylation (Ho et al. 2005; Rocha et al. 2005). It is likely that the activation of NF-κB target genes and the cellular outcome in response to DNA damage-induced NF-κB activation are dependent on the cellular context (Wang et al. 2002; Janssens and Tschopp 2006). Accordingly, a pro-apoptosis role of NF-κB has been proposed (Campbell et al. 2004). This may partly be due to differences in cellular context, such as the genetic status of p53 and the current redox status, and the activity of other signaling pathways (Ganapathi et al. 2002; Wang et al. 2002; Lee et al. 2003; Janssens and Tschopp 2006; Chen et al. 2008). Thus, the role of RIP1-mediated NF-κB activation during DNA damage, particularly in cancer cells during chemo- or radiotherapy, requires careful evaluation.

2.2.3 RIP1 in TLR3- and TLR4-Mediated Cell Survival Signaling

TLR are pathogen-associated molecular pattern (PAMP) recognition receptors sensing a wide range of pathogens including bacteria, viruses, fungi, and protozoa.

TLRs are also involved in a wide range of pathophysiological responses such as that in immunity and cancer (Barton and Kagan 2009; Klein Klouwenberg et al. 2009). In the absence of RIP1, TLR3-mediated NF-κB activation, but not the JNK or interferon-β, was abolished. Therefore, TLR 3-induced NF-κB activation is dependent on RIP1 (Meylan et al. 2004). When the cognate ligands bind to TLR3 and TLR4, which are analogous to the dependence of TRADD in binding to the TNFR1 signaling complex, RIP1 is recruited to the receptor mediated by TIR-related adaptor protein inducing INF (TRIF) through the RIP RHIM motif. RIP1 is then phosphorylated followed by polyubiquitination. As an E3 ubiquitin ligase, TRAF6 is suggested to be responsible for RIP1 polyubiquitination (Festjens et al. 2007). Another E3 ubiquitin ligase, Peli1, which was found to bind to and ubiquitinate RIP1 for IKK activation induced by TLR3 and TLR4, suggesting that Peli1 is a ubiquitin ligase for RIP1 in transmission of TRIF-dependent TLR signals (Chang et al. 2009). The E3 ubiquitin ligase Triad3A is also suggested to ubiquitinate RIP1 for TLR signaling (Fearns et al. 2006). The modified RIP1 recruits IKK activating proteins to form a complex consisting of TRIF, TRAF6, RIP1, TAK1, TAB1, and TAB2, which mediates activation of IKKβ and eventually NF-κB (Cusson-Hermance et al. 2005; Festjens et al. 2007). The Bruton's tyrosine kinase (BTK) directly phosphorylates TLR3, leading to formation of the downstream TRIF/RIP1/TBK1 complex (Lee et al. 2012). Whether BTK also modifies TRIF and RIP1 for the signaling needs to be further determined. Interestingly, the RIP family member RIP2 is also involved in TLR3- and TLR4-mediated signaling (Kobayashi et al. 2002). Because TLR3 and TLR4 can induce both survival and death in cells, it remains to be determined if there is functional interaction between RIP1 and RIP2 in modulating cellular outcomes of signaling in these receptors. In addition, recent studies reveal that, despite TRIF, TRADD is also involved in TLR3-mediated RIP1 ubiquitination and NF-κB activation in bone marrow macrophages (Ermolaeva et al. 2008; Pobezinskaya et al. 2008). Thus, it is of interest to determine if TRIF and TRADD contribute to determination of cellular fate during TLR3 signaling.

The evidence showing RIP1 is involved in TLR4-mediated signaling to phosphatidylinositol 3 kinase (PI3K)/Akt activation was from RIP1 (−/−) mouse splenocytes that failed to proliferate and undergo isotype switching in response to LPS. These cells had impaired Akt phosphorylation and increased apoptosis, suggesting that RIP1 is essential for cell survival after TLR4 signaling through mediating the PI3K/Akt pathway (Vivarelli et al. 2004). How RIP1 mediates LPS-/TLR4-induced Akt activation remains to be elucidated.

2.2.4 RIP1 in Other Cell Survival Pathways

RIP1 is also reported to contribute to other cell survival/proliferation pathways. For example, RIP1 was found in the signaling complex of insulin-like growth factor 1 receptor (IGF-1R) for JNK activation, which contributes to cell proliferation (Lin et al. 2006). Also, it was suggested that RIP1 is involved in epidermal growth

factor receptor (EGFR)-mediated signaling (Habib et al. 2001). In addition, RIP1 is overexpressed in glioblastoma. In glioblastoma cells, RIP1 activates PI3K-Akt through dual mechanisms: activates PI3K-Akt by interrupting the mTOR negative feedback loop through negatively regulating mTOR transcription via a NF-κB-dependent pathway and downregulates cellular PTEN levels independent of NF-κB activation. Furthermore, RIP1 suppresses p27 (Kip1) expression to facilitate cell proliferation through the PI3K-/Akt-forkhead pathway (Park et al. 2008, 2009). All these pathways need more attention to their roles in death/survival regulation in different cell types.

2.3 RIP1 in Cell Death Signaling

Although RIP1 possesses a DD and artificial overexpression of RIP1 causes apoptotic cell death that can be rescued by co-expression of the viral caspase-8 inhibiting protein CrmA, in early researches RIP1 was found not to be required for death receptor-mediated cell death under the conditions of transcriptional or translational inhibition (Ting et al. 1996; Kelliher et al. 1998; Lin et al. 1999, 2000; Festjens et al. 2007). Thus, for a long time, RIP1 was not thought to be a death mediator. However, later studies clearly demonstrate that RIP1 actively contributes to cell death, in both apoptosis and necroptosis.

2.3.1 RIP1 in Mediating Apoptosis

2.3.1.1 RIP1 in Death Receptor-Mediated Apoptosis

During TNFα-induced signaling, the TNFR1 complex I that contains TRADD, TRAF2, RIP1, cIAP1, and cIAP2 is internalized and converted into complex II with recruitment of FADD and caspase-8. Complex II mediates signaling to caspase-8 activation and subsequent activation of executor caspases to initiate apoptosis (Fig. 2.3). It has been puzzling that although RIP1 resides in complex II, it appeared not to be involved in apoptosis signaling (Ting et al. 1996; Kelliher et al. 1998). These findings were made in experiments that used RIP1 knockout mouse embryonic fibroblasts (MEF) or RIP1 mutated leukemia cell line Jurkat with addition of TNF or TRAIL in combination with transcription inhibitor or translation inhibitor to block gene expression (Ting et al. 1996; Kelliher et al. 1998; Lin et al. 1999, 2000; Festjens et al. 2007). A later research using stable short-hairpin RNA (shRNA) knockdown (KD) in human tumor cells and immunoprecipitation demonstrated competitive binding of RIP1and TRADD to TNFR1. While FADD is necessary for FasL- or TRAIL- but not TNF-induced apoptosis, RIP1 is required for TNF-induced apoptosis. Furthermore, RIP1 KD abrogated complex II formation after TNF exposure. These observations, although adding more complexity to the roles of death

receptor signaling, suggest that RIP1 contributes to apoptosis induced by TNFR1 in certain tumor cells (Jin and El-Deiry 2006).

Compelling evidence showing RIP1 is involved in TNFR1-mediated apoptosis is that different subtypes of apoptosis are induced by TNFα. With a comparison of apoptosis induced with TNFα combined with cycloheximide that inhibits protein synthesis or second mitochondria-derived activator of caspases (Smac) mimic that targets cIAP1 and cIAP2 for degradation, two distinct caspase-8 activation-mediated apoptosis pathways were identified (Wang et al. 2008). The first well-studied pathway is negatively regulated by the endogenous caspase-8 inhibitor c-FLIP. Cycloheximide eliminates c-FLIP rapidly to promote caspase-8 activation. The second pathway is uncovered with Smac mimetic, which triggers autodegradation of cIAP1 and cIAP2, resulting in the release of RIP1 from complex I to form a caspase-8-activating complex consisting of RIP1, FADD, and caspase-8. While Lys63 polyubiquitination of RIP1 is critical for NF-κB activation, deubiquitination of RIP1 by CYLD is crucial for RIP1/FADD/caspase-8 complex formation and caspase-8 activation (Wang et al. 2008). Thus, it is clear that RIP1 contributes to TNFR1-mediated apoptosis under the condition of cIAP1/2 suppression or CYLD activation. The recently identified CLIP-170-related 59 kDa protein (CLIPR-59) is involved in the formation of complex II and downregulation of TNFα-induced ubiquitination of RIP1 through binding to CYLD, resulting in the formation of complex II and thus promoting caspase-8 activation and apoptosis (Fujikura et al. 2012).

In addition to TNFR1 signaling to apoptosis, other non-death receptor members of the TNFR superfamily also utilize RIP1 for apoptosis. CD40, a cytokine with a prominent role in antitumor immune response, induces apoptosis in cancer cells when its survival signals are blocked. Apoptosis is initiated within a cytosolic death-inducing signaling complex containing RIP1, which is required for CD40 ligand-induced caspase-8 activation and tumor cell killing. Degradation of cIAP1/2 amplifies, whereas inhibition of CYLD reduces the CD40-mediated cytotoxic effect through impacting the ubiquitination on RIP1 (Knox et al. 2011). TNF-like weak inducer of apoptosis (TWEAK, TNFSF12, CD255) induces apoptosis in certain cancer cells via autocrine TNFα. During TWEAK-induced apoptosis, a RIP1-FADD-caspse-8 complex is assembled. Knockdown of RIP1 by siRNA prevented TWEAK-induced association of FADD and caspase-8, suggesting a crucial role of RIP1 in the proapoptotic activity of TWEAK in cancer cells (Ikner and Ashkenazi 2011). A synergy in inducing apoptosis in pediatric acute lymphoblastic leukemia (ALL) occurs with combination of inhibitors of IAPs and various anticancer drugs such as AraC, gemcitabine, doxorubicin, etoposide, vincristine, and Taxol that depends on the formation of a RIP1/FADD/caspase-8 complex via an autocrine/paracrine loop of TNFα. RIP1 is essential for the formation of this complex and subsequent activation of caspase-8 and caspase-3, mitochondrial perturbations, and apoptosis. These findings substantiate the role of RIP1 in cancer therapy that involves death receptor-mediated apoptosis activation with IAP inhibitors and conventional chemotherapy (Loder et al. 2012). Similarly, the Smac mimetic BV6 sensitizes the first-line chemotherapeutic agent in the treatment of glioblastoma temozolomide (TMZ) through apoptosis activation mediated by a RIP1/caspase-8/

FADD complex. Knockdown of RIP1 significantly reduces BV6- and TMZ-induced caspase-8 activation and apoptosis, substantiating that RIP1 is necessary for apoptosis induction and antitumor activity of this therapy regimen (Wagner et al. 2012).

2.3.1.2 RIP1 in DNA Damage-Induced Apoptosis

When DNA damage occurs, PIDDosome is formed for either NF-κB activation-mediated cell survival or caspase-2 activation-mediated apoptosis. While the RIP1 and NEMO containing PIDDosome negatively regulates DNA damage-induced apoptosis through NF-κB activation (Tinel et al. 2007), recent reports show that RIP1 plays a role in facilitating apoptosis when cells acquire DNA damage. Upon excessive DNA damage, ATM is activated to stimulate cytokine secretion, which alerts neighbor cells and induces apoptosis to eliminate the afflicted cell. Extensive DNA lesions stimulate two sequential NF-κB activation phases that induce TNFα-TNFR1 feedforward signaling and drive RIP1 phosphorylation-mediated JNK3 activation, resulting in FADD-mediated pro-apoptotic caspase-8 activation. Thus, in the context of excessive DNA damage, RIP1 kinase participates in TNFα autocrine-mediated apoptosis (Biton and Ashkenazi 2011). Additionally, RIP1-mediated JNK activation has been suggested to be one of the critical components involved in mediating DNA damage-induced and p53-independent cell death (Hur et al. 2006).

A more recent study shed lights on the mechanism of RIP1 in DNA damage-induced apoptotic cell death. Upon genotoxic stress, a large protein complex about 2 MDa called the Ripoptosome is formed to serve as a cell death-inducing platform that can stimulate caspase-8-mediated apoptosis as well as caspase-independent necrosis. Containing RIP1, FADD, and caspase-8, this complex is assembled in response to genotoxic stress-induced depletion of the IAPs (XIAP, cIAP1, and cIAP2). Ripoptosome formation is independent of either death receptors or mitochondria but requires RIP1's kinase activity. The formation and activity of the Ripoptosome are negatively regulated by IAPs. Mechanistically, IAPs serve as a brake for Ripoptosome through mediating RIP1 ubiquitination to keep caspase-8 inactive. These observations shed light on fundamental mechanisms by which RIP1 contributes to chemotherapeutic-induced apoptosis in cancer cells (Tenev et al. 2011). c-FLIP$_L$ prevents, while c-FLIP$_S$ promotes Ripoptosome formation. When cIAPs are absent, caspase activity controlled by c-FLIP isoforms in the Ripoptosome functions as determinants for a cell's fate: RIP3-dependent necroptosis or caspase-dependent apoptosis. While RIP1 is the core component of the complex and the Ripoptosome critically influences the outcome of genotoxic stress, the differential quality of cell death mediated by the Ripoptosome may cause important pathophysiological consequences (Feoktistova et al. 2011).

It should be noted that although RIP1 mediates a cell death pathway in response to DNA damage, it also transduces cell survival signals such as NF-κB. When RIP1 expression is suppressed by gene knockout in MEFs or knockdown in cancer cells, DNA damage-induced cytotoxicity is significantly increased (Yang et al. 2011;

Wang et al. 2014), suggesting that cell survival signaling is predominant in RIP1-mediated genotoxic stress signaling and other cell death pathways independent of RIP1 are sufficient to kill the cells.

2.3.1.3 RIP1 in TLR3- and TLR4-Mediated Apoptotic Death

TLR activation by viral infection can result in apoptosis that is dependent on RIP1, FADD, and caspase. Interestingly, contrasted to TNFR1 signaling, RIP1 functions upstream of FADD in TLR3- and TLR4-induced apoptosis (Ruckdeschel et al. 2004). TRIF physically interacts with the RHIM motif in RIP1. RIP1 recruits FADD and caspase-8 that are essential for apoptosis (Kaiser and Offermann 2005). Engagement of TLR3 by dsRNA in lung cancer cells induces the formation of an atypical caspase-8-containing complex that is devoid of death receptors of the TNFR superfamily. The recruitment of caspase-8 to TLR3 is dependent on RIP1-mediated recruitment of FADD. The TLR3/RIP1/caspase-8 complex is negatively modulated by RIP1 ubiquitination by a ubiquitin ligase complex containing cIAP2-TRAF2-TRADD. These observations uncover the molecular mechanisms underlying TLR3-induced apoptosis (Estornes et al. 2012). Viruses encode proteins suppressing TLR-mediated apoptosis. The murine cytomegalovirus M45 protein directly interacts with RIP 1 and RIP3 via RHIM to suppress cell death. The interaction between M45 and RIP1 underlies the cell tropism role of M45 in preventing premature death of endothelial cells during murine cytomegalovirus infection. Thus, suppressing RIP1 provides a direct cell type-dependent replication benefit to the virus (Mack et al. 2008; Upton et al. 2008). Ribonucleotide reductase R1 subunits of herpes simplex virus type 1 protect cells against TLR3-induced apoptosis by interacting with RIP1 and caspase-8. Collectively, RIP1 is the molecular target for certain viruses to impair the host defense apoptotic mechanism prompted by dsRNA (Dufour et al. 2011).

2.3.2 RIP1 in Programmed Necrosis (Necroptosis)

Necrosis has long been regarded as an uncontrolled, passive, and accidental process where cells experience extreme physicochemical stress conditions. Only in the last decade is it becoming clear that necrotic cell death is an active programmed cellular event and the term necroptosis was coined. Necrotic cell death may be an important cell death mode that is both pathologically and physiologically relevant (Festjens et al. 2006; Vandenabeele et al. 2010). It appears that necrotic cell death is not simply a result of one well-described signaling cascade but is the consequence of extensive cross talk between several biochemical and molecular events at different cellular levels (Festjens et al. 2006; Vandenabeele et al. 2010). Necrotic cell death initiates proinflammatory signaling by actively releasing inflammatory cytokines and releasing cellular contents that can stimulate inflammatory responses.

Necrosis is capable of killing tumor cells that have developed strategies to evade apoptosis. Thus, detailed knowledge of necrosis may be exploited in cancer therapeutic strategies (Festjens et al. 2006).

2.3.2.1 RIP1 in TNFα-Induced Necrosis

Although it was noticed that TNFα induces necrotic cell death long ago, uncovering the first piece of the puzzle of the underlying mechanism was not made until the discovery that RIP1 plays a key role in this pathway. In primary T cells, TNFα-, TRAIL-, or FasL-induced caspase-independent necrotic death is absent when FADD or RIP1 is deficient. In contrast to RIP1's role in NF-κB activation, RIP1 kinase activity is required for necrotic death signaling (Holler et al. 2000). With use of RIP1 knockout MEF cells, it was determined that RIP1-mediated cellular ROS, mainly superoxide, accumulation is crucial for TNF-induced nonapoptotic cell death (Lin et al. 2004). RIP1 is essential for Nox1 recruitment to form a signaling complex for activation of Nox1 which plays a key role in TNF-induced necrotic cell death (Kim et al. 2007).

A genome-wide siRNA screen revealed that another member of the RIP kinase family, RIP3, is required for mediating RIP1-dependent necrosis. Upon induction of necrosis, RIP3 is recruited to RIP1 to form a necrosis-inducing complex and the kinase activity of RIP3 is essential for necrosis execution (He et al. 2009). RIP3 regulates necrosis-specific RIP1 phosphorylation. The phosphorylation of RIP1 and RIP3 stabilizes their association within the pronecrotic complex, activates pronecrotic kinase activity, and triggers downstream ROS production. Furthermore, the pronecrotic RIP1–RIP3 complex is induced during vaccinia virus infection, resulting in tissue necrosis, inflammation, and viral replication suppression (Cho et al. 2009). By activating key enzymes of metabolic pathways, RIP3 regulates TNF-induced ROS production and necrosis, substantiating that modulation of energy metabolism in response to death stimuli has an important role in the choice between apoptosis and necrosis (Zhang et al. 2009). With the RIP3 kinase inhibitor necrosulfonamide, the mixed lineage kinase domain-like protein (MLKL) was identified as the RIP3 interacting target. RIP3 phosphorylates MLKL at the threonine 357 and serine 358 residues for executing necrosis, implicating MLKL as a key mediator of necrosis signaling downstream of the kinase RIP3 (Sun et al. 2012). An independent study with screening a kinase/phosphatase shRNA library also identified MLKL as a key RIP3 downstream component of TNFα-induced necrosis. MLKL functions downstream of RIP1 and RIP3 and is recruited to the necrosome through its interaction with RIP3 for the generation of ROS and the late-phase activation of JNK during TNF-induced necrosis (Zhao et al. 2012). In the RIP1- and RIP3-containing protein complexes resides the mitochondrial protein phosphatase PGAM5. Both two splice variants, PGAM5L (long form) and PGAM5S (short form), are involved in necrosis signaling through ROS production in mitochondria. Upon necrosis induction, PGAM5S binds to the mitochondrial fission factor Drp1 to activate its GTPase activity through dephosphorylation of Drp1, resulting in mitochondrial

fragmentation, an early and obligatory step for necrosis execution (Wang et al. 2012). These observations establish a pathway consisting of RIP1, RIP3, MLKL, and PGAM5 for mitochondria-mediated necrosis in response to TNFα and other stimulations.

Necroptosis could be a target for overcoming cancer's chemoresistance (Kreuzaler and Watson 2012; He et al. 2013). For example, in addition to sensitizing apoptosis-proficient cells to TNFα-mediated and caspase-dependent apoptosis, Smac mimetic primes apoptosis-resistant cells lacking FADD or caspase-8 to TNFα-induced, RIP1-dependent, and caspase-independent necroptosis, highlighting the importance of therapeutic exploitation of necroptosis as an alternative cell death program to overcome chemoresistance (Laukens et al. 2011). Through suppressing RIP1 kinase activity, cIAP1 protects cells from TNFα-induced necrosis by preventing RIP1-/RIP3-dependent ROS production, indicating that cIAPs are key in regulating necrosis and thus appear to be a main target for sensitizing cancer cells to necrosis (Vanlangenakker et al. 2011b). By inhibiting RIP1 recruitment to the death receptor signaling complex, PKC activation suppresses the death receptor-mediated necrotic cell death pathway (Byun et al. 2006).

2.3.2.2 RIP1 in ROS- and PARP-Mediated Necrotic Cell Death

ROS are the main players for propagation and execution of necrotic cell death through directly or indirectly provoking protein, lipid, and DNA damages, culminating in disruption of organelle and cell integrity (Festjens et al. 2006). Oxidative stress and ROS elicit and modulate necrotic cell death. RIP1−/− MEF cells are resistant to ROS-induced cell death. Upon H_2O_2 exposure, RIP1 and TRAF2 form a complex in lipid rafts, which is independent of TNFR1. RIP1 and TRAF2 mediate ROS-induced cell death through JNK activation (Shen et al. 2004). JNK1 subsequently phosphorylates the key DNA repair protein poly(ADP-ribose) polymerase-1 (PARP-1), resulting in sustained activation of PARP-1 (Zhang et al. 2007). Activated PARP1 catalyzes NAD+ into nicotinamide and poly-ADP ribose, resulting in depletion of NAD+ and cellular energy failure that leads to necrotic cell death (Festjens et al. 2006). However, with using the DNA alkylating agent *N*-methyl-*N*′-nitro-N-nitrosoguanidine, a potent PARP-1 activator, JNK was shown to be required for PARP-1-induced mitochondrial dysfunction and subsequent cell death. In this necrosis model, RIP1 is upstream of JNK but downstream of PARP-1 (Xu et al. 2006a). Thus, although RIP1 is clearly involved in ROS- and PARP1-mediated necrosis, the defined mechanisms need further study.

2.3.2.3 RIP1 in Necrotic Cell Death Induced by Other Stimulations

RIP1 is involved in necrosis induced by other cellular stresses. For example, following TLR4 ligation by LPS, when NF-κB and caspase-8 are suppressed, cells undergo necrosis depending on RIP1 (Ma et al. 2005). The interaction between viral proteins and RIP1 prevents necrotic cell death during infection (Mack et al. 2008;

Upton et al. 2008). Thus, RIP1 is the molecular target for certain viruses to modulate necrotic cell death (Dufour et al. 2011).

5-Aminolevulinic acid (5-ALA) for glioblastoma therapy mainly activates a necrotic type of cell death depending on a pronecrotic complex containing RIP3 and RIP1 that mediates singlet oxygen production. Interestingly, the pronecrotic complex is devoid of caspase-8 and FADD, two proteins usually part of the necrosome or Ripoptosome, suggesting different complexes consisting of RIP1 or RIP3 are formed for necrosis under different conditions (Coupienne et al. 2011). Heme leaked from hemolysis or myonecrosis has proinflammatory and cytotoxic effects partly through TLR4-dependent production of TNFα and subsequent necrosis that requires RIP1- and RIP3-mediated ROS production (Fortes et al. 2012). Thus, RIP1 functions as a central player in programmed necrosis. However, TCR-induced necroptosis does not require RIP3 (Osborn et al. 2010). In contrast, RIP1-independent but RIP3-mediated necroptosis in the context of TNFα signaling in particular conditions was also reported (Vanlangenakker et al. 2011a). Therefore, necrosis signaling may be more complex and the role of RIP1 in this context needs further study.

2.3.3 RIP1 in Autophagic Cell Death

Autophagy is a cellular process for degradation and recycling of long-lived proteins and organelles, which is important for cell survival under nutrient starvation conditions and for housekeeping through removal of exhausted, redundant, and unwanted cellular components. However, in certain circumstances autophagy leads to cell death (Todde et al. 2009; Mizushima and Komatsu 2011). LPS induces autophagy in macrophages through a pathway regulated by TRIF-dependent and MyD88-independent TLR4 signaling. RIP1 is downstream of TRIF and MyD88 for inducing autophagy, which contributes to caspase-independent macrophage necrotic cell death (Xu et al. 2006b, 2008). In TRAIL-induced cytoprotective autophagy, RIP1 and TRAF2 mediate JNK activation to blunt apoptosis in cancer cells. Thus, suppression of this RIP1-involved pathway could be utilized for sensitizing cancer cells to therapy with TRAIL (He et al. 2012). On the other hand, in acute lymphoblastic leukemia (ALL), RIP1 is not involved in induction of autophagy but is required for autophagy-mediated necroptosis (Bonapace et al. 2010). These studies reveal a role for RIP1 in autophagic cell death.

2.4 Convergence and Interplay Between RIP1-Mediated Cell Survival and Death Pathways

While RIP1 is involved in both cell survival and death signaling, strict and accurate regulations must be installed to maintain tissue homeostasis and for response to physiological and pathological stimuli. For example, in TNFα-induced signaling to

NF-κB, apoptosis, and necroptosis, multiple shared proteins residing in the TNFR1 complex I and II are involved (Vanlangenakker et al. 2011a). There are two levels of regulation during TNFR1 signaling. The first one is the decision to proceed to survival or death, mainly through regulation of NF-κB. Two cell death checkpoints following TNF stimulation may be involved: an early transcription-independent checkpoint where NEMO restrains RIP1 from activating the caspase cascade, followed by a later checkpoint dependent on NF-κB-mediated transcription of pro-survival genes (Legarda-Addison et al. 2009). Rapid activating expression of NF-κB target anti-apoptosis factors cIAPs that activate RIP1 through ubiquitination and c-FLIP that suppresses caspase-8 is critical for cell survival (Bertrand et al. 2008). In contrast, shutting off cell survival signaling shifts cells' fate to death. In this regard, cleavage of RIP1 by caspase-8 at early time points plays an important role, which blocks NF-κB and enhances apoptosis (Lin et al. 1999). Also, deubiquitination of RIP1 through suppressing cIAPs and activating CYLD shifts RIP1-mediated signaling to death. The second-level regulation is for the modes of cell death. The suppression of caspase-8 by c-FLIP plays a pivotal role for ensuring RIP1-mediated necrosis (Arslan and Scheidereit 2011). Cleavage of RIP1 may also help to suppress necrosis to ensure apoptosis (Sato et al. 2008). In addition, competitive binding of RIP1 and TRADD to TNFR1 may also play a role in determining cells' fate by modulating NF-κB, apoptosis, and nonapoptotic death signals (Zheng et al. 2006).

Analogous to TNFR1 complex II, the main determinant in the Ripoptosome for the mode of cell death is likely the activity of caspase-8. In this regard, c-FLIP plays an important role (Feoktistova et al. 2011). Other mechanisms such as modulation of FADD may exist. In response to Taxol, the mitotic kinase Aurora A and the polo-like kinase Plk1 cooperatively phosphorylate FADD to enhance recruitment of caspase-8 for apoptosis, while dissociation of RIP1 from FADD for necrotic cell death (Jang et al. 2011). Certainly, more defined mechanisms for cell death control need further studies.

2.5 Summary and Perspective

Current research places RIP1 at an important position in mediating cell signaling to cell survival or death. Because cell survival and death control is vital for a variety of cellular functions as well as in disease pathophysiology, further research on RIP1 biology will undoubtedly contribute to elucidation of the mechanisms of pathogenesis in important diseases such as cancer. RIP1 is overexpressed in a portion of human caners without induction of apoptosis as seen in in vitro RIP1 overexpression experiments. Understanding the mechanisms by which the RIP1-mediated apoptosis pathway is attenuated in cancer cells would help to elucidate the role of RIP1 in carcinogenesis and develop new anticancer therapy. Realizing the complexity of RIP1 signaling, one should keep in mind that the role of RIP1 in cell survival and death regulation might be cell context- and stimulus-specific (Wang et al. 2002; Janssens and Tschopp 2006). For example, in some circumstances, RIP1 is

dispensable for TNFR1-mediated NF-κB activation (Wong et al. 2010). With tremendous efforts devoted to researches on cell survival and death signaling involving RIP1, it would be expected that approaches targeting RIP1-mediated pathways will be developed and applied clinically in the near future.

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References

Arslan SC, Scheidereit C (2011) The prevalence of TNFalpha-induced necrosis over apoptosis is determined by TAK1-RIP1 interplay. PLoS One 6:e26069

Bai L, Chen W, Wang X, Ju W, Xu S, Lin Y (2009) Attenuating Smac mimetic compound 3-induced NF-kappaB activation by luteolin leads to synergistic cytotoxicity in cancer cells. J Cell Biochem 108:1125–1131

Bai L, Xu S, Chen W, Li Z, Wang X, Tang H, Lin Y (2011) Blocking NF-kappaB and Akt by Hsp90 inhibition sensitizes Smac mimetic compound 3-induced extrinsic apoptosis pathway and results in synergistic cancer cell death. Apoptosis 16:45–54

Barton GM, Kagan JC (2009) A cell biological view of Toll-like receptor function: regulation through compartmentalization. Nat Rev Immunol 9:535–542

Bertrand MJ, Milutinovic S, Dickson KM, Ho WC, Boudreault A, Durkin J, Gillard JW, Jaquith JB, Morris SJ, Barker PA (2008) cIAP1 and cIAP2 facilitate cancer cell survival by functioning as E3 ligases that promote RIP1 ubiquitination. Mol Cell 30:689–700

Biton S, Ashkenazi A (2011) NEMO and RIP1 control cell fate in response to extensive DNA damage via TNF-alpha feedforward signaling. Cell 145:92–103

Bonapace L, Bornhauser BC, Schmitz M, Cario G, Ziegler U, Niggli FK, Schafer BW, Schrappe M, Stanulla M, Bourquin JP (2010) Induction of autophagy-dependent necroptosis is required for childhood acute lymphoblastic leukemia cells to overcome glucocorticoid resistance. J Clin Invest 120:1310–1323

Byun HS, Park KA, Won M, Yang KJ, Shin S, Piao L, Kwak JY, Lee ZW, Park J, Seok JH, Liu ZG, Hur GM (2006) Phorbol 12-myristate 13-acetate protects against tumor necrosis factor (TNF)-induced necrotic cell death by modulating the recruitment of TNF receptor 1-associated death domain and receptor-interacting protein into the TNF receptor 1 signaling complex: Implication for the regulatory role of protein kinase C. Mol Pharmacol 70:1099–1108

Campbell KJ, Rocha S, Perkins ND (2004) Active repression of antiapoptotic gene expression by RelA(p65) NF-kappa B. Mol Cell 13:853–865

Chang M, Jin W, Sun SC (2009) Peli1 facilitates TRIF-dependent Toll-like receptor signaling and proinflammatory cytokine production. Nat Immunol 10:1089–1095

Chen W, Wang X, Bai L, Liang X, Zhuang J, Lin Y (2008) Blockage of NF-kappaB by IKKbeta- or RelA-siRNA rather than the NF-kappaB super-suppressor IkappaBalpha mutant potentiates adriamycin-induced cytotoxicity in lung cancer cells. J Cell Biochem 105:554–561

Cho YS, Challa S, Moquin D, Genga R, Ray TD, Guildford M, Chan FK (2009) Phosphorylation-driven assembly of the RIP1-RIP3 complex regulates programmed necrosis and virus-induced inflammation. Cell 137:1112–1123

Coupienne I, Fettweis G, Rubio N, Agostinis P, Piette J (2011) 5-ALA-PDT induces RIP3-dependent necrosis in glioblastoma. Photochem Photobiol Sci 10:1868–1878

Cusson-Hermance N, Khurana S, Lee TH, Fitzgerald KA, Kelliher MA (2005) Rip1 mediates the Trif-dependent toll-like receptor 3- and 4-induced NF-{kappa}B activation but does not contribute to interferon regulatory factor 3 activation. J Biol Chem 280:36560–36566

Devin A, Cook A, Lin Y, Rodriguez Y, Kelliher M, Liu Z (2000) The distinct roles of TRAF2 and RIP in IKK activation by TNF-R1: TRAF2 recruits IKK to TNF-R1 while RIP mediates IKK activation. Immunity 12:419–429

Devin A, Lin Y, Liu ZG (2003) The role of the death-domain kinase RIP in tumour-necrosis-factor-induced activation of mitogen-activated protein kinases. EMBO Rep 4:623–627

Dufour F, Bertrand L, Pearson A, Grandvaux N, Langelier Y (2011) The ribonucleotide reductase R1 subunits of herpes simplex virus 1 and 2 protect cells against poly(I. C)-induced apoptosis. J Virol 85:8689–8701

Ea CK, Deng L, Xia ZP, Pineda G, Chen ZJ (2006) Activation of IKK by TNFalpha requires site-specific ubiquitination of RIP1 and polyubiquitin binding by NEMO. Mol Cell 22:245–257

Ermolaeva MA, Michallet MC, Papadopoulou N, Utermohlen O, Kranidioti K, Kollias G, Tschopp J, Pasparakis M (2008) Function of TRADD in tumor necrosis factor receptor 1 signaling and in TRIF-dependent inflammatory responses. Nat Immunol 9:1037–1046

Estornes Y, Toscano F, Virard F, Jacquemin G, Pierrot A, Vanbervliet B, Bonnin M, Lalaoui N, Mercier-Gouy P, Pacheco Y, Salaun B, Renno T, Micheau O, Lebecque S (2012) dsRNA induces apoptosis through an atypical death complex associating TLR3 to caspase-8. Cell Death Differ 19(9):1482–1494

Fearns C, Pan Q, Mathison JC, Chuang TH (2006) Triad3A regulates ubiquitination and proteasomal degradation of RIP1 following disruption of Hsp90 binding. J Biol Chem 281: 34592–34600

Feoktistova M, Geserick P, Kellert B, Dimitrova DP, Langlais C, Hupe M, Cain K, MacFarlane M, Hacker G, Leverkus M (2011) cIAPs block Ripoptosome formation, a RIP1/caspase-8 containing intracellular cell death complex differentially regulated by cFLIP isoforms. Mol Cell 43:449–463

Festjens N, Vanden Berghe T, Cornelis S, Vandenabeele P (2007) RIP1, a kinase on the crossroads of a cell's decision to live or die. Cell Death Differ 14:400–410

Festjens N, Vanden Berghe T, Vandenabeele P (2006) Necrosis, a well-orchestrated form of cell demise: signalling cascades, important mediators and concomitant immune response. Biochim Biophys Acta 1757:1371–1387

Fortes GB, Alves LS, de Oliveira R, Dutra FF, Rodrigues D, Fernandez PL, Souto-Padron T, De Rosa MJ, Kelliher M, Golenbock D, Chan FK, Bozza MT (2012) Heme induces programmed necrosis on macrophages through autocrine TNF and ROS production. Blood 119:2368–2375

Fujikura D, Ito M, Chiba S, Harada T, Perez F, Reed JC, Uede T, Miyazaki T (2012) CLIPR-59 regulates TNF-alpha-induced apoptosis by controlling ubiquitination of RIP1. Cell Death Dis 3:e264

Ganapathi R, Vaziri SA, Tabata M, Takigawa N, Grabowski DR, Bukowski RM, Ganapathi MK (2002) Inhibition of NF-kappaB and proteasome activity in tumors: can we improve the therapeutic potential of topoisomerase I and topoisomerase II poisons. Curr Pharm Des 8:1945–1958

Habib AA, Chatterjee S, Park SK, Ratan RR, Lefebvre S, Vartanian T (2001) The epidermal growth factor receptor engages receptor interacting protein and nuclear factor-kappa B (NF-kappa B)-inducing kinase to activate NF-kappa B. Identification of a novel receptor-tyrosine kinase signalosome. J Biol Chem 276:8865–8874

Han KJ, Su X, Xu LG, Bin LH, Zhang J, Shu HB (2004) Mechanisms of the TRIF-induced interferon-stimulated response element and NF-kappaB activation and apoptosis pathways. J Biol Chem 279:15652–15661

He W, Wang Q, Xu J, Xu X, Padilla MT, Ren G, Gou X, Lin Y (2012) Attenuation of TNFSF10/ TRAIL-induced apoptosis by an autophagic survival pathway involving TRAF2- and RIPK1/ RIP1-mediated MAPK8/JNK activation. Autophagy 8:1811–1821

He S, Wang L, Miao L, Wang T, Du F, Zhao L, Wang X (2009) Receptor interacting protein kinase-3 determines cellular necrotic response to TNF-alpha. Cell 137:1100–1111

He W, Wang Q, Srinivasan B, Xu J, Padilla MT, Li Z, Wang X, Liu Y, Gou X, Shen HM, Xing C, Lin Y (2013) A JNK-mediated autophagy pathway that triggers c-IAP degradation and necroptosis for anticancer chemotherapy. Oncogene. Jul 13. [Epub ahead of print]

Ho WC, Dickson KM, Barker PA (2005) Nuclear factor-kappaB induced by doxorubicin is deficient in phosphorylation and acetylation and represses nuclear factor-kappaB-dependent transcription in cancer cells. Cancer Res 65:4273–4281

Holler N, Zaru R, Micheau O, Thome M, Attinger A, Valitutti S, Bodmer JL, Schneider P, Seed B, Tschopp J (2000) Fas triggers an alternative, caspase-8-independent cell death pathway using the kinase RIP as effector molecule. Nat Immunol 1:489–495

Hsu H, Huang J, Shu HB, Baichwal V, Goeddel DV (1996) TNF-dependent recruitment of the protein kinase RIP to the TNF receptor-1 signaling complex. Immunity 4:387–396

Huang TT, Wuerzberger-Davis SM, Wu ZH, Miyamoto S (2003) Sequential modification of NEMO/IKKgamma by SUMO-1 and ubiquitin mediates NF-kappaB activation by genotoxic stress. Cell 115:565–576

Hur GM, Kim YS, Won M, Choksi S, Liu ZG (2006) The death domain kinase RIP has an important role in DNA damage-induced, p53-independent cell death. J Biol Chem 281:25011–25017

Hur GM, Lewis J, Yang Q, Lin Y, Nakano H, Nedospasov S, Liu ZG (2003) The death domain kinase RIP has an essential role in DNA damage-induced NF-kappa B activation. Genes Dev 17:873–882

Ikner A, Ashkenazi A (2011) TWEAK induces apoptosis through a death-signaling complex comprising receptor-interacting protein 1 (RIP1), Fas-associated death domain (FADD), and caspase-8. J Biol Chem 286:21546–21554

Jang MS, Lee SJ, Kang NS, Kim E (2011) Cooperative phosphorylation of FADD by Aur-A and Plk1 in response to taxol triggers both apoptotic and necrotic cell death. Cancer Res 71:7207–7215

Janssens S, Tinel A (2012) The PIDDosome, DNA-damage-induced apoptosis and beyond. Cell Death Differ 19:13–20

Janssens S, Tinel A, Lippens S, Tschopp J (2005) PIDD mediates NF-kappaB activation in response to DNA damage. Cell 123:1079–1092

Janssens S, Tschopp J (2006) Signals from within: the DNA-damage-induced NF-kappaB response. Cell Death Differ 13:773–784

Jin Z, El-Deiry WS (2006) Distinct signaling pathways in TRAIL-versus tumor necrosis factor-induced apoptosis. Mol Cell Biol 26:8136–8148

Ju W, Wang X, Shi H, Chen W, Belinsky SA, Lin Y (2007) A critical role of luteolin-induced reactive oxygen species in blockage of tumor necrosis factor-activated nuclear factor-kappaB pathway and sensitization of apoptosis in lung cancer cells. Mol Pharmacol 71:1381–1388

Kaiser WJ, Offermann MK (2005) Apoptosis induced by the toll-like receptor adaptor TRIF is dependent on its receptor interacting protein homotypic interaction motif. J Immunol 174:4942–4952

Kamata H, Honda S, Maeda S, Chang L, Hirata H, Karin M (2005) Reactive oxygen species promote TNFalpha-induced death and sustained JNK activation by inhibiting MAP kinase phosphatases. Cell 120:649–661

Kanayama A, Seth RB, Sun L, Ea CK, Hong M, Shaito A, Chiu YH, Deng L, Chen ZJ (2004) TAB2 and TAB3 activate the NF-kappaB pathway through binding to polyubiquitin chains. Mol Cell 15:535–548

Karin M, Gallagher E (2009) TNFR signaling: ubiquitin-conjugated TRAFfic signals control stop-and-go for MAPK signaling complexes. Immunol Rev 228:225–240

Karin M, Yamamoto Y, Wang QM (2004) The IKK NF-kappa B system: a treasure trove for drug development. Nat Rev Drug Discov 3:17–26

Kelliher MA, Grimm S, Ishida Y, Kuo F, Stanger BZ, Leder P (1998) The death domain kinase RIP mediates the TNF-induced NF-kappaB signal. Immunity 8:297–303

Kim YS, Morgan MJ, Choksi S, Liu ZG (2007) TNF-induced activation of the Nox1 NADPH oxidase and its role in the induction of necrotic cell death. Mol Cell 26:675–687

Klein Klouwenberg P, Tan L, Werkman W, van Bleek GM, Coenjaerts F (2009) The role of Toll-like receptors in regulating the immune response against respiratory syncytial virus. Crit Rev Immunol 29:531–550

Knox PG, Davies CC, Ioannou M, Eliopoulos AG (2011) The death domain kinase RIP1 links the immunoregulatory CD40 receptor to apoptotic signaling in carcinomas. J Cell Biol 192:391–399

Kobayashi K, Inohara N, Hernandez LD, Galan JE, Nunez G, Janeway CA, Medzhitov R, Flavell RA (2002) RICK/Rip2/CARDIAK mediates signalling for receptors of the innate and adaptive immune systems. Nature 416:194–199

Kreuzaler P, Watson CJ (2012) Killing a cancer: what are the alternatives? Nat Rev Cancer 12(6):411–424

Laukens B, Jennewein C, Schenk B, Vanlangenakker N, Schier A, Cristofanon S, Zobel K, Deshayes K, Vucic D, Jeremias I, Bertrand MJ, Vandenabeele P, Fulda S (2011) Smac mimetic bypasses apoptosis resistance in FADD- or caspase-8-deficient cells by priming for tumor necrosis factor alpha-induced necroptosis. Neoplasia 13:971–979

Lee CT, Seol JY, Lee SY, Park KH, Han SJ, Yoo CG, Han SK, Shim YS, Kim YW (2003) The effect of adenovirus-IkappaBalpha transduction on the chemosensitivity of lung cancer cell line with resistance to cis-diamminedichloroplatinum(II)(cisplatin) and doxorubicin(adriamycin). Lung Cancer 41:199–206

Lee KG, Xu S, Kang ZH, Huo J, Huang M, Liu D, Takeuchi O, Akira S, Lam KP (2012) Bruton's tyrosine kinase phosphorylates Toll-like receptor 3 to initiate antiviral response. Proc Natl Acad Sci U S A 109:5791–5796

Legarda-Addison D, Hase H, O'Donnell MA, Ting AT (2009) NEMO/IKKgamma regulates an early NF-kappaB-independent cell-death checkpoint during TNF signaling. Cell Death Differ 16:1279–1288

Lewis J, Devin A, Miller A, Lin Y, Rodriguez Y, Neckers L, Liu ZG (2000) Disruption of hsp90 function results in degradation of the death domain kinase, receptor-interacting protein (RIP), and blockage of tumor necrosis factor-induced nuclear factor-kappaB activation. J Biol Chem 275:10519–10526

Lin A, Dibling B (2002) The true face of JNK activation in apoptosis. Aging Cell 1:112–116

Lin Y, Bai L, Chen W, Xu S (2010) The NF-kappaB activation pathways, emerging molecular targets for cancer prevention and therapy. Expert Opin Ther Targets 14:45–55

Lin Y, Choksi S, Shen HM, Yang QF, Hur GM, Kim YS, Tran JH, Nedospasov SA, Liu ZG (2004) Tumor necrosis factor-induced nonapoptotic cell death requires receptor-interacting protein-mediated cellular reactive oxygen species accumulation. J Biol Chem 279:10822–10828

Lin Y, Devin A, Cook A, Keane MM, Kelliher M, Lipkowitz S, Liu ZG (2000) The death domain kinase RIP is essential for TRAIL (Apo2L)-induced activation of IkappaB kinase and c-Jun N-terminal kinase. Mol Cell Biol 20:6638–6645

Lin Y, Devin A, Rodriguez Y, Liu ZG (1999) Cleavage of the death domain kinase RIP by caspase-8 prompts TNF-induced apoptosis. Genes Dev 13:2514–2526

Lin Y, Yang Q, Wang X, Liu ZG (2006) The essential role of the death domain kinase receptor-interacting protein in insulin growth factor-I-induced c-Jun N-terminal kinase activation. J Biol Chem 281:23525–23532

Loder S, Fakler M, Schoeneberger H, Cristofanon S, Leibacher J, Vanlangenakker N, Bertrand MJ, Vandenabeele P, Jeremias I, Debatin KM, Fulda S (2012) RIP1 is required for IAP inhibitor-mediated sensitization of childhood acute leukemia cells to chemotherapy-induced apoptosis. Leukemia 26:1020–1029

Ma Y, Temkin V, Liu H, Pope RM (2005) NF-kappaB protects macrophages from lipopolysaccharide-induced cell death: the role of caspase 8 and receptor-interacting protein. J Biol Chem 280:41827–41834

Mabb AM, Wuerzberger-Davis SM, Miyamoto S (2006) PIASy mediates NEMO sumoylation and NF-kappaB activation in response to genotoxic stress. Nat Cell Biol 8:986–993

Mack C, Sickmann A, Lembo D, Brune W (2008) Inhibition of proinflammatory and innate immune signaling pathways by a cytomegalovirus RIP1-interacting protein. Proc Natl Acad Sci U S A 105:3094–3099

Meylan E, Burns K, Hofmann K, Blancheteau V, Martinon F, Kelliher M, Tschopp J (2004) RIP1 is an essential mediator of Toll-like receptor 3-induced NF-kappa B activation. Nat Immunol 5:503–507

Meylan E, Tschopp J (2005) The RIP kinases: crucial integrators of cellular stress. Trends Biochem Sci 30:151–159

Micheau O, Tschopp J (2003) Induction of TNF receptor I-mediated apoptosis via two sequential signaling complexes. Cell 114:181–190

Mizushima N, Komatsu M (2011) Autophagy: renovation of cells and tissues. Cell 147:728–741

O'Donnell MA, Ting AT (2011) RIP1 comes back to life as a cell death regulator in TNFR1 signaling. FEBS J 278:877–887

Osborn SL, Diehl G, Han SJ, Xue L, Kurd N, Hsieh K, Cado D, Robey EA, Winoto A (2010) Fas-associated death domain (FADD) is a negative regulator of T-cell receptor-mediated necroptosis. Proc Natl Acad Sci U S A 107:13034–13039

Park J, Kanayama A, Yamamoto K, Miyamoto Y (2012) ARD1 binding to RIP1 mediates doxorubicin-induced NF-kappaB activation. Biochem Biophys Res Commun 422(2): 291–297

Park S, Ramnarain DB, Hatanpaa KJ, Mickey BE, Saha D, Paulmurugan R, Madden CJ, Wright PS, Bhai S, Ali MA, Puttaparthi K, Hu W, Elliott JL, Stuve O, Habib AA (2008) The death domain-containing kinase RIP1 regulates p27(Kip1) levels through the PI3K-Akt-forkhead pathway. EMBO Rep 9:766–773

Park S, Zhao D, Hatanpaa KJ, Mickey BE, Saha D, Boothman DA, Story MD, Wong ET, Burma S, Georgescu MM, Rangnekar VM, Chauncey SS, Habib AA (2009) RIP1 activates PI3K-Akt via a dual mechanism involving NF-kappaB-mediated inhibition of the mTOR-S6K-IRS1 negative feedback loop and down-regulation of PTEN. Cancer Res 69:4107–4111

Pobezinskaya YL, Kim YS, Choksi S, Morgan MJ, Li T, Liu C, Liu Z (2008) The function of TRADD in signaling through tumor necrosis factor receptor 1 and TRIF-dependent Toll-like receptors. Nat Immunol 9:1047–1054

Rocha S, Garrett MD, Campbell KJ, Schumm K, Perkins ND (2005) Regulation of NF-kappaB and p53 through activation of ATR and Chk1 by the ARF tumour suppressor. EMBO J 24:1157–1169

Ruckdeschel K, Pfaffinger G, Haase R, Sing A, Weighardt H, Hacker G, Holzmann B, Heesemann J (2004) Signaling of apoptosis through TLRs critically involves toll/IL-1 receptor domain-containing adapter inducing IFN-beta, but not MyD88, in bacteria-infected murine macrophages. J Immunol 173:3320–3328

Sato T, Machida T, Takahashi S, Murase K, Kawano Y, Hayashi T, Iyama S, Takada K, Kuribayashi K, Sato Y, Kobune M, Takimoto R, Matsunaga T, Kato J, Niitsu Y (2008) Apoptosis supercedes necrosis in mitochondrial DNA-depleted Jurkat cells by cleavage of receptor-interacting protein and inhibition of lysosomal cathepsin. J Immunol 181:197–207

Shembade N, Harhaj NS, Parvatiyar K, Copeland NG, Jenkins NA, Matesic LE, Harhaj EW (2008) The E3 ligase Itch negatively regulates inflammatory signaling pathways by controlling the function of the ubiquitin-editing enzyme A20. Nat Immunol 9:254–262

Shen HM, Lin Y, Choksi S, Tran J, Jin T, Chang L, Karin M, Zhang J, Liu ZG (2004) Essential roles of receptor-interacting protein and TRAF2 in oxidative stress-induced cell death. Mol Cell Biol 24:5914–5922

Skaug B, Jiang X, Chen ZJ (2009) The role of ubiquitin in NF-kappaB regulatory pathways. Annu Rev Biochem 78:769–796

Stanger BZ, Leder P, Lee TH, Kim E, Seed B (1995) RIP: a novel protein containing a death domain that interacts with Fas/APO-1 (CD95) in yeast and causes cell death. Cell 81: 513–523

Sun L, Wang H, Wang Z, He S, Chen S, Liao D, Wang L, Yan J, Liu W, Lei X, Wang X (2012) Mixed lineage kinase domain-like protein mediates necrosis signaling downstream of RIP3 kinase. Cell 148:213–227

Tenev T, Bianchi K, Darding M, Broemer M, Langlais C, Wallberg F, Zachariou A, Lopez J, MacFarlane M, Cain K, Meier P (2011) The Ripoptosome, a signaling platform that assembles in response to genotoxic stress and loss of IAPs. Mol Cell 43:432–448

Tinel A, Janssens S, Lippens S, Cuenin S, Logette E, Jaccard B, Quadroni M, Tschopp J (2007) Autoproteolysis of PIDD marks the bifurcation between pro-death caspase-2 and pro-survival NF-kappaB pathway. EMBO J 26:197–208

Ting AT, Pimentel-Muinos FX, Seed B (1996) RIP mediates tumor necrosis factor receptor 1 activation of NF-kappaB but not Fas/APO-1-initiated apoptosis. EMBO J 15:6189–6196
Todde V, Veenhuis M, van der Klei IJ (2009) Autophagy: principles and significance in health and disease. Biochim Biophys Acta 1792:3–13
Upton JW, Kaiser WJ, Mocarski ES (2008) Cytomegalovirus M45 cell death suppression requires receptor-interacting protein (RIP) homotypic interaction motif (RHIM)-dependent interaction with RIP1. J Biol Chem 283:16966–16970
Vandenabeele P, Galluzzi L, Vanden Berghe T, Kroemer G (2010) Molecular mechanisms of necroptosis: an ordered cellular explosion. Nat Rev Mol Cell Biol 11:700–714
Vanlangenakker N, Bertrand MJ, Bogaert P, Vandenabeele P, Vanden Berghe T (2011a) TNF-induced necroptosis in L929 cells is tightly regulated by multiple TNFR1 complex I and II members. Cell Death Dis 2:e230
Vanlangenakker N, Vanden Berghe T, Bogaert P, Laukens B, Zobel K, Deshayes K, Vucic D, Fulda S, Vandenabeele P, Bertrand MJ (2011b) cIAP1 and TAK1 protect cells from TNF-induced necrosis by preventing RIP1/RIP3-dependent reactive oxygen species production. Cell Death Differ 18:656–665
Ventura JJ, Cogswell P, Flavell RA, Baldwin AS Jr, Davis RJ (2004) JNK potentiates TNF-stimulated necrosis by increasing the production of cytotoxic reactive oxygen species. Genes Dev 18:2905–2915
Vivarelli MS, McDonald D, Miller M, Cusson N, Kelliher M, Geha RS (2004) RIP links TLR4 to Akt and is essential for cell survival in response to LPS stimulation. J Exp Med 200(3): 399–404
Wagner L, Marschall V, Karl S, Cristofanon S, Zobel K, Deshayes K, Vucic D, Debatin KM, Fulda S (2012) Smac mimetic sensitizes glioblastoma cells to Temozolomide-induced apoptosis in a RIP1- and NF-kappaB-dependent manner. Oncogene 32:988–997
Wang L, Du F, Wang X (2008) TNF-alpha induces two distinct caspase-8 activation pathways. Cell 133:693–703
Wang Q, Chen W, Bai L, Chen W, Padilla MT, AS Lin, Shi S, Wang X. Lin Y (2014) Receptor-interacting protein 1 increases chemoresistance by maintaining inhibitor of apoptosis protein levels and reducing reactive oxygen species through a microRNA-146a-mediated catalase pathway. J Biol Chem. Jan 14 [Epub ahead of print]
Wang S, Kotamraju S, Konorev E, Kalivendi S, Joseph J, Kalyanaraman B (2002) Activation of nuclear factor-kappaB during doxorubicin-induced apoptosis in endothelial cells and myocytes is pro-apoptotic: the role of hydrogen peroxide. Biochem J 367:729–740
Wang X, Ju W, Renouard J, Aden J, Belinsky SA, Lin Y (2006) 17-allylamino-17-demethoxygeldanamycin synergistically potentiates tumor necrosis factor-induced lung cancer cell death by blocking the nuclear factor-kappaB pathway. Cancer Res 66:1089–1095
Wang Z, Jiang H, Chen S, Du F, Wang X (2012) The mitochondrial phosphatase PGAM5 functions at the convergence point of multiple necrotic death pathways. Cell 148:228–243
Wong WW, Gentle IE, Nachbur U, Anderton H, Vaux DL, Silke J (2010) RIPK1 is not essential for TNFR1-induced activation of NF-kappaB. Cell Death Differ 17:482–487
Wu CJ, Conze DB, Li T, Srinivasula SM, Ashwell JD (2006a) Sensing of Lys 63-linked polyubiquitination by NEMO is a key event in NF-kappaB activation [corrected]. Nat Cell Biol 8:398–406
Wu ZH, Shi Y, Tibbetts RS, Miyamoto S (2006b) Molecular linkage between the kinase ATM and NF-kappaB signaling in response to genotoxic stimuli. Science 311:1141–1146
Xu M, Skaug B, Zeng W, Chen ZJ (2009) A ubiquitin replacement strategy in human cells reveals distinct mechanisms of IKK activation by TNFalpha and IL-1beta. Mol Cell 36:302–314
Xu Y, Huang S, Liu ZG, Han J (2006a) Poly(ADP-ribose) polymerase-1 signaling to mitochondria in necrotic cell death requires RIP1/TRAF2-mediated JNK1 activation. J Biol Chem 281: 8788–8795
Xu Y, Kim SO, Li Y, Han J (2006b) Autophagy contributes to caspase-independent macrophage cell death. J Biol Chem 281:19179–19187

Xu Y, Liu XD, Gong X, Eissa NT (2008) Signaling pathway of autophagy associated with innate immunity. Autophagy 4:110–112

Yang J, Lin Y, Guo Z, Cheng J, Huang J, Deng L, Liao W, Chen Z, Liu Z, Su B (2001) The essential role of MEKK3 in TNF-induced NF-kappaB activation. Nat Immunol 2:620–624

Yang Y, Xia F, Hermance N, Mabb A, Simonson S, Morrissey S, Gandhi P, Munson M, Miyamoto S, Kelliher MA (2011) A cytosolic ATM/NEMO/RIP1 complex recruits TAK1 to mediate the NF-kappaB and p38 mitogen-activated protein kinase (MAPK)/MAPK-activated protein 2 responses to DNA damage. Mol Cell Biol 31:2774–2786

Zhang DW, Shao J, Lin J, Zhang N, Lu BJ, Lin SC, Dong MQ, Han J (2009) RIP3, an energy metabolism regulator that switches TNF-induced cell death from apoptosis to necrosis. Science 325:332–336

Zhang J, Zhang H, Li J, Rosenberg S, Zhang EC, Zhou X, Qin F, Farabaugh M (2011) RIP1-mediated regulation of lymphocyte survival and death responses. Immunol Res 51:227–236

Zhang S, Lin Y, Kim YS, Hande MP, Liu ZG, Shen HM (2007) c-Jun N-terminal kinase mediates hydrogen peroxide-induced cell death via sustained poly(ADP-ribose) polymerase-1 activation. Cell Death Differ 14:1001–1010

Zhao J, Jitkaew S, Cai Z, Choksi S, Li Q, Luo J, Liu ZG (2012) Mixed lineage kinase domain-like is a key receptor interacting protein 3 downstream component of TNF-induced necrosis. Proc Natl Acad Sci U S A 109:5322–5327

Zheng L, Bidere N, Staudt D, Cubre A, Orenstein J, Chan FK, Lenardo M (2006) Competitive control of independent programs of tumor necrosis factor receptor-induced cell death by TRADD and RIP1. Mol Cell Biol 26:3505–3513

Chapter 3
Role of RIP3 in Necrotic Cell Death

Ting Wu, Wanze Chen, and Jiahuai Han

3.1 New Face of Necrosis

Necrotic cell death is originally defined by its morphological characteristics: an increase in cell volume, swelling of the cytoplasmic organelle, rupture of the plasma membrane, and leakage of cell contents. Necrotic cell death often triggers inflammation and immune responses (Kroemer et al. 2009). For a long time, necrosis had been considered a purely accidental and passive cell death and had been neglected as a process lacking physiological relevance. But this "passive" cell death was questioned in 1988 (Laster et al. 1988) when tumor necrosis factor (TNF) was found to cause both apoptosis and necrosis, depending on the cell types. With the discovery that a serine-threonine kinase RIP1 is required for death receptor Fas (CD95)-triggered necrosis of Jurkat cells (Holler et al. 2000) in 2000, more and more molecules were found to be involved in this type of cell death. Another landmark in understanding this process is the discovery of RIP3 (Cho et al. 2009; He et al. 2009; Zhang et al. 2009), which determines whether a cell will go into apoptosis or necrosis. In addition to the RIP1-/RIP3-dependent necrosis termed necroptosis, some other types of necrosis were found to be highly regulated, such as poly-ADP ribose polymerase (PARP) activation-induced necrosis in ischemia-reperfusion

T. Wu
State Key Laboratory of Cellular Stress Biology, School of Life Sciences,
and Department of Basic Medical Sciences, Medical College, Xiamen University,
Xiamen, China

W. Chen • J. Han (✉)
State Key Laboratory of Cellular Stress Biology, School of Life Sciences,
Xiamen University, Xiamen, China
e-mail: jhan@xmu.edu.cn

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injuries (Eliasson et al. 1997), mitochondrial permeability transition pore complex-mediated necrosis in oxidative stress (Vaseva et al. 2012), and lysosomal membrane permeabilization-mediated cell death induced by lysosomotropic compounds (Boya and Kroemer 2008). It is not clear whether these necrosis pathways are independent of each other or share a common "core network." Among different types of necrosis, RIP1-/RIP3-dependent necroptosis is the most studied. Here, we summarize the current knowledge of the molecular mechanism of and the pathophysiology relevance of necroptosis.

3.2 Molecular Mechanism of Necroptosis

3.2.1 Stimuli for Necroptosis

Tumor necrosis factor-induced cellular necrosis is a model system for the study of necroptosis. TNF induces necroptosis in some cell lines, such as L929 cells. TNF can induce necroptosis in a number of cell lines and primary cells when caspase-8 is inhibited by chemical inhibitor benzyloxycarbonyl-Val-Ala- Asp (OMe)-fluoromethylketone(zVAD)or gene deletion. Cellular inhibitor of apoptosis protein (cIAP) antagonists, such as smac-mimic, can markedly enhance this phenomenon. The other death receptors like Fas, tumor necrosis factor-related apoptosis-inducing ligand receptor (TRAIL-R), and T cell receptor (TCR) can induce necroptosis in ways quite similar to TNF (Ch'en et al. 2011), whereas pathogens can trigger cell necroptosis in different ways. The gram-negative bacterium *Shigella flexneri* can induce necroptosis in monocyte-derived macrophages (Koterski et al. 2005) and neutrophils (Francois et al. 2000). Lipopolysaccharide (LPS), a component of the cell walls of gram-negative bacteria, induces macrophage necroptosis when caspase-8 activity is inhibited (Zhang et al. 2009). Double-stranded viral RNA induces necroptosis through toll-like receptor 3 (TLR3) in macrophages in the presence of zVAD (He et al. 2011). Inhibition of caspases can be mediated by viral proteins since many viruses encode natural caspase inhibitors. Infection by such viruses, including the vaccinia virus or cowpox virus, leads to necroptosis of T cells and pig kidney cells, respectively (Ray and Pickup 1996; Cho et al. 2009). Recent research indicates that the DNA sensor, named DNA-dependent activator of IFN regulatory factors (DAI), is the trigger of necroptosis induced by murine cytomegalovirus (MCMV) (Upton et al. 2012). Chemicals such as nitric oxide and etoposide can target mitochondrial respiratory complex I subunit NADH dehydrogenase (ubiquinone) 1 beta subcomplex 8 (NDUFB8) and DNA, respectively, to induce RIP1-/RIP3-dependent necroptosis (Davis et al. 2010; Tenev et al. 2011). Necroptosis needs to be tightly regulated during development since overactivation of necroptosis in caspase-8 and Fas-associated death domain protein (FADD) knockout mice undergo mass necroptosis and the embryos die in the E10–E13 stages (Kaiser et al. 2011; Zhang et al. 2011b).

3.2.2 RIP1/RIP3 Complex Is Essential for Necroptosis

At present, our knowledge of necroptosis is mostly from the research of TNF-induced necrosis. Ligand binding of TNF causes a conformational change in TNF receptor 1(TNFR1) on the cell membrane that recruits TNF receptor-associated death domain (TRADD), which, in turn, recruits RIP1, a cellular inhibitor of apoptosis protein 1 (cIAP1), cIAP2, TNF receptor-associated factor 2 (TRAF2), and TRAF5 (Vandenabeele et al. 2010). It leads to the formation of membrane-associated complex I. In this complex, TRAF2 stabilizes cIAP1 and cIAP2 by preventing their autoubiquitination and recruits them to the cytoplasmic domain of the TNFR1 (Rothe et al. 1995; Shu et al. 1996; Csomos et al. 2009; Vince et al. 2009). RIP1 is K63-polyubiquitinated at K377 by cIAP1 and cIAP2 (Ea et al. 2006; Bertrand et al. 2008) and acts as a scaffold for the assembly of the transforming growth factor-β-activated kinase-1 (TAK1)–TAK-1 binding protein 2 and 3 (TAB2/3) complex, which leads to the activation of the transcription factor nuclear factor κB (NF-κB). K63-polyubiquitination of RIP1, but not RIP1's kinase activity, is crucial for NF-κB activation. When K63-ubiquitination of RIP1 is removed by deubiquitinase cylindromatosis (CYLD) or A20 (Micheau and Tschopp 2003; Schneider-Brachert et al. 2004), then, the plasma membrane-associated complex I is internalized to the cytosol to form complex II (also called cytosolic death-inducing signaling complex (DISC)) (Micheau and Tschopp 2003; Schneider-Brachert et al. 2004).

When the cell does not express RIP3, complex II contains RIP1, caspase-8, and the adaptors TRADD and FADD. Although its kinase activity is not required, RIP1 participates in the activation of caspase-8 and subsequent apoptosis (Bertrand et al. 2008). Other events, such as deletion of c-flice-like inhibitory protein (cFLIP), the cellular form of the caspase-8 inhibitor FLIP, also activate caspase-8 (Wang et al. 2008). Thus, RIP1 may not be indispensable for TNF-induced apoptosis. Indeed, *Ripk1*$^{-/-}$ mouse embryonic fibroblasts (MEFs) still undergo caspase-dependent apoptosis after TNF treatment (Zhang et al. 2011a).

When the cell expresses RIP3, RIP3 incorporates into complex II, called complex IIb or necrosome. Caspase-8 can inhibit necrosis initiation by cleavage of RIP1 and RIP3 (Feng et al. 2007; Cho et al. 2009). However, inhibition of caspase-8 by caspase inhibitors or deletion of either FADD or caspase-8 blocks the cleavage of RIP1 and RIP3 and strengthens the formation of the necrosome and, thus, initiates necroptosis upon TNF stimulation (Vercammen et al. 1998; Holler et al. 2000).

RIP1 and RIP3 belong to the receptor-interacting serine-threonine kinase family. RIP1 contains an N terminal kinase domain, a RIP homotypic interaction motif (RHIM), and a C terminal death domain, while RIP3 lacks the death domain (Zhang et al. 2010). Both RIP1 and RIP3 kinase domains are essential for necroptosis signaling (Holler et al. 2000; Cho et al. 2009; He et al. 2009; Zhang et al. 2009). The kinase activity of RIP1 is required for assembling the RIP1/RIP3 complex since necrostatin-1, an inhibitor of the kinase activity of RIP1, prevents the interaction between RIP1 and RIP3 (Degterev et al. 2008; He et al. 2009). RIP1 is reported to autophosphorylate the serine 161, and the serine 161 autophosphorylation

positively contributes to RIP1 kinase activity and necroptosis (Degterev et al. 2008). The function of RIP1 kinase activity needs to be further investigated, while the role of RIP3 kinase activity is relatively clear. RIP3 autophosphorylates its serine 227; this phosphorylation is not required for RIP1/RIP3 complex assembly, but for the interaction of RIP3 with MLKL, a newly identified necrosome component (Sun et al. 2012).

RIP1 and RIP3 bind to each other through their RHIM domain (Sun et al. 2002; Meylan and Tschopp 2005). Point mutation of the RHIM domain in either protein disrupts the interaction between RIP1 and RIP3 and prevents necroptosis (Sun et al. 2002; Cho et al. 2009; Zhang et al. 2009). Interestingly, recent research indicated that upon TNF stimulation, RIP1/RIP3 complex form a functional amyloid aggregation (Li et al. 2012). The amyloid core consists of the RHIM domain of RIP1 and RIP3, while the kinase domain might flank the core to transduce necroptosis signaling (Li et al. 2012).

3.2.3 Downstream Signaling of RIP3

3.2.3.1 RIP3 Promotes Energy Metabolism

In the early 1990s, TNF-induced necrosis had been linked with energy metabolism. By using inhibitors, TNF-induced necroptosis was shown to depend on ROS (Goossens et al. 1995, 1999; Fiers et al. 1999; Festjens et al. 2007). Mitochondria-derived ROS is believed to be responsible for the ultrastructural changes that occur in the mitochondria and endoplasmic reticulum during cell death (Schulze-Osthoff et al. 1992; Festjens et al. 2006b). Although the role of ROS production in TNF-induced necroptosis has been demonstrated by many laboratories with multiple cell lines, exceptions were reported in studies using the HT29 cell line, indicating that non-ROS mechanisms can also initiate necroptosis (Festjens et al. 2006a; He et al. 2009).

TNF-induced mitochondrial ROS should result when electrons escape from the respiration chain, thus relating to alteration of energy metabolism. RIP3 appears to act upstream of ROS production since RIP3 deficiency in NIH3T3, L929, and primary peritoneal macrophages blocked ROS production and necrosis triggered by different death stimuli (Zhang et al. 2009). By means of liquid chromatography-tandem mass spectrometry analysis, several cellular metabolic enzymes were identified as interacting with RIP3. Further experiments show that RIP3 can directly interact with and enhance the enzymatic activity of liver PYGL, GLUL, and mitochondrial matrix-localized GLUD1 (Zhang et al. 2009). PYGL catalyzes the rate-limiting degradation of glycogen to glucose-1-phosphate, which is subsequently converted into glucose-6-phosphate, directly fueling glycolysis (Bouche et al. 2004). Glycogen accumulation was observed in *RIP3*$^{-/-}$ in a sepsis model, supporting the role of RIP3 in utilizing glycogen (by Sam McNeal, “RIPK1 is necessary for survival during sepsis,” a comment published online at the homepage of Immunity, 2012).

GLUL catalyzes the reaction of glutamate and ammonia to form membrane-permeable glutamine, which stimulates glutathione production, gluconeogenesis, and lipogenesis. Glutamine can transfer into the mitochondria to function as an energy substrate. GLUD1 is found in the mitochondrial matrix and converts glutamate to α-ketoglutarate, which then feeds into the tricarboxylic acid cycle (TCA) (Mates et al. 2009). Consistent with this, an early study had reported a link between glutaminolysis and the generation of cytotoxic ROS during TNF-induced necrosis (Goossens et al. 1996). Activation of these three enzymes all can enhance oxidative phosphorylation and, thus, ROS production. Knockdown of PYGL, GLUL, or GLUD1 partially inhibits TNF- plus zVAD-induced ROS production and necroptosis in NIH3T3 cells, suggesting the contribution of increased energy metabolism in necroptosis (Zhang et al. 2009).

Membrane-associated NADPH oxidase has also been implicated in TNF-induced ROS production (Kim et al. 2007). It was reported that TNF stimulation induces association between the regulatory NADPH oxidase organizer 1 (NOXO1) subunit with RIP1, TRADD, and Rac, which initiates ROS production by the cell membrane-associated NADPH oxidase NOX1 (Kim et al. 2007). In addition, calcium mobilization, activation of phospholipase A2, lipoxygenases, acid sphingomyelinases, and lysosomal destabilization were also reported to contribute to TNF-induced necroptosis (Vandenabeele et al. 2010). But the relationship between RIP3 and these events has yet to be established.

3.2.3.2 MLKL-PGAM5 Pathway in TNF-Induced Necroptosis

Recently, MLKL and PGAM5 were identified as parts of the necrosis signaling machinery downstream of RIP1 and RIP3 activation and are RIP3 substrates (Sun et al. 2012; Wang et al. 2012).

MLKL was found to be involved in necroptosis by searching RIP3 interacting protein with coimmunoprecipitation and by looking for the target of necrosulfonamide (NSA), a small molecule inhibitor of programmed necrosis screened out from a chemical library. NSA inhibits necrosis by covalently binding to Cys86 on the MLKL protein. Since Cys86 is not conserved in mice, NSA has human specificity. Upon necrosis induction, MLKL is recruited to serine 227 phosphorylated RIP3 and is subsequently phosphorylated by RIP3 at the threonine 357 and serine 358 residues. The phosphorylation of these two sites in human MLKL is critical for necroptosis since mutation of these phosphorylation sites on MLKL abrogates the ability of RIP3 and MLKL to signal for necrosis. MLKL was also identified as required for necroptosis shortly after by another group in screening a siRNA library. The data on whether MLKL possesses enzymatic activity were controversial in different reports (Sun et al. 2012; Zhao et al. 2012); however, MLKL's role as an adaptor for bringing the RIP1/RIP3 necrosome complex into proximity to other RIP3 substrates is clear.

PGAM5 is another substrate of RIP3 found by the abovementioned approaches. PGAM5 has two splice variants, PGAM5L (long form) and PGAM5S (small form).

Both splice variants of PGAM5 are required for necrosis execution but seem to have different functions in necrosis. It is likely that PGAM5L tethers the RIP1-RIP3-MLKL necrosome to PGAM5S on the mitochondrial membrane. The activated PGAM5 then dephosphorylates Drp1 and activates it. Drp1 is a GTPase that is essential for mitochondrial fission. It was proposed in a model that mitochondrial fission is required for necroptosis.

3.2.4 The Interplay Between Necroptosis and Apoptosis

The regulation of RIP3-dependent necroptosis tightly links to that of apoptosis. Both types of cell death can be triggered by the same stimulus, for example, TNF, LPS, polyinosinic-polycytidylic acid (PolyI.C), or a virus, depending on the cell status. The adaptor, TRADD, is essential for both TNF-induced necroptosis and apoptosis, since TRADD-deficient cells are both resistant to apoptosis and necroptosis induced by TNF (Ermolaeva et al. 2008). In some situations, necrosis seemed to be a backup of apoptosis when the apoptosis pathway was impaired. For example, vaccine virus encodes caspase inhibitors to prevent apoptosis in the host cell so that they can have a niche for virus production (Upton et al. 2012). But infected cells still can undergo a death program through a necroptotic program (Ray and Pickup 1996; Cho et al. 2009). Since inhibition of caspase-8 by a chemical inhibitor or genetic deletion is a precondition for many necroptoses to occur (Kalai et al. 2002), it is revealed that the apoptotic pathway may inhibit the process of necroptosis. This assumption was supported by in vivo data showing RIP3 deficiency-rescued embryonic lethality of caspase-8 and FADD knockout (Kaiser et al. 2011; Zhang et al. 2011b). Further studies indicated that cleavages of RIP1 and RIP3 by caspase-8 are the underlying mechanisms of the necroptosis inhibition (Feng et al. 2007; He et al. 2009). On the other hand, converting apoptosis to necrosis by RIP3 upregulation has been observed in vitro and in some pathological conditions (Zhang et al. 2009; Zorde-Khvalevsky et al. 2009; Li et al. 2010; Trichonas et al. 2010; Gunther et al. 2011). How RIP3 upregulation prevents apoptosis is still unknown. The competition and interconversion between apoptosis and necroptosis may play an important role in pathological processes.

3.3 The Physiological and Pathological Role of RIP3-Mediated Necrosis

Necroptosis is involved in a variety of physiological and pathological processes, including development and tissue homeostasis and the pathogenesis of some diseases, such as ischemic injury, neurodegeneration, and viral infection, thereby representing an attractive topic of study for the avoidance of unwarranted cell death.

The importance of necroptotic cell death in development has been revealed by several recent works. RIP3 knockout was found to rescue the developmental defects of caspase-8 knockout mice (Oberst et al. 2011). This indicates that the RIP3-mediated necrosis is the underlying mechanism of the cardiac, vascular, and hematopoietic defects that occur during the development of caspase-8 knockout mice (Kaiser et al. 2011; Oberst et al. 2011). Similar results were obtained when *FADD*$^{-/-}$ mice were crossed onto *RIP1*$^{-/-}$ background, indicating that the inhibition of necroptosis occurs in the necrosome (Zhang et al. 2011b). In addition, necroptosis of TCR-activated caspase-8 null T cells also can be completely rescued by RIP3 deletion (Kaiser et al. 2011).

Apoptosis is an antiviral mechanism so many viruses encode caspase inhibitors to suppress infection-induced apoptosis of host cells (Galluzzi et al. 2010; Lamkanfi and Dixit 2010). Necrosis appears to have antiviral functions too, since viruses such as vaccinia virus, herpes virus, and cowpox virus are able to suppress host cell apoptosis, but the host cells still can die in necrosis (Ray and Pickup 1996; Cho et al. 2009). *Rip3*$^{-/-}$ mice are more susceptible to vaccinia virus infection than their wild-type counterparts (Cho et al. 2009). Interestingly, some viruses, such as MCMV, encode a necrosis inhibitor (Lamkanfi and Dixit 2010), which suggests that anti-necrosis mechanisms have already evolved to overcome host control. Upon MCMV infection, DNA sensor DAI interacts with RIP3 through the RHIM domain to initiate necroptosis. The MCMV-encoded viral inhibitor of RIP activation (vIRA) targets the DAI-RIP3 complex to inhibit necroptosis (Upton et al. 2012).

In a mouse model of acute pancreatitis, induction of the RIP3 protein is believed to be responsible for cellular necrosis in the pancreas (He et al. 2009). Upregulation of RIP3 protein levels was also reported in some other disease-related conditions, including hepatocytes showing steatohepatitis (Zorde-Khvalevsky et al. 2009), photoreceptors after retinal detachment (Trichonas et al. 2010), and vascular smooth muscle cells undergoing arterial injury (Li et al. 2010). High expression of RIP3 in Paneth cells was detected in the terminal ileum from patients with Crohn's disease (Gunther et al. 2011), suggesting association of RIP3 with the pathogenesis of this disease. RIP3 induction during the course of disease development probably controls the switch from apoptosis to necrosis in vivo. Although RIP3 does not play an important role in inflammatory cytokine production, RIP3 deficiency or RIP1 kinase inhibition protects mice from mortality due to TNF-induced systemic inflammatory response syndrome (SIRS) (Duprez et al. 2011), suggesting a role played by necroptosis in SIRS. RIP3-mediated necrotic death may exacerbate damage-induced inflammation, thereby aggravating the injury in some pathological conditions.

3.4 Perspective

Programmed necrosis or necroptosis has been demonstrated to be an important, intrinsic cellular death program. It plays a role in a variety of biological processes and has functions ranging from development to pathological conditions.

Although significant progress has been made in elucidating the molecular mechanisms of necroptosis, the necroptotic pathway is still largely unknown. It is unclear how RIP3 switches the apoptotic pathway to the necroptotic pathway. Although mitochondria ROS and mitochondria fission were proposed to be part of the execution mechanism of necroptosis, exceptions have been observed. Whether there is a common execution mechanism for programmed necrosis is still an unsolved question.

Mitochondria have been implicated to play an essential role in both apoptosis and necrosis (Kroemer et al. 1998). Bcl-X_L, an anti-apoptosis member of the Bcl-2 family, also inhibits TNF-induced necroptosis (Karsan et al. 1996). Mitochondria permeability pore opening, a trigger of apoptosis, is believed to cause necrosis as well (Kim et al. 2003). Cyclophilin D is a component of the permeability pore, and its deletion was reported to block necrosis, but not apoptosis (Zamzami et al. 2005). It is unknown at present whether cyclophilin D deletion will affect TNF-induced necroptosis. The mitochondrial event(s) in the process of necroptosis still need to be further explored.

The study of the clinical implication of necroptosis is still hampered by lack of in vivo biomarkers. In addition to phosphorylation of RIP3, a recent study on MLKL suggests that MLKL phosphorylation could also be a marker of necrosis initiation. Examining a large number of patients' samples is urgently needed to determine the role of necroptosis in human disease. We speculate that inhibition of necroptosis can be one of the effective cytotoxic or cytoprotective therapies in the future.

References

Bertrand MJ, Milutinovic S, Dickson KM, Ho WC, Boudreault A, Durkin J, Gillard JW, Jaquith JB, Morris SJ, Barker PA (2008) cIAP1 and cIAP2 facilitate cancer cell survival by functioning as E3 ligases that promote RIP1 ubiquitination. Mol Cell 30:689–700

Bouche C, Serdy S, Kahn CR, Goldfine AB (2004) The cellular fate of glucose and its relevance in type 2 diabetes. Endocr Rev 25:807–830

Boya P, Kroemer G (2008) Lysosomal membrane permeabilization in cell death. Oncogene 27: 6434–6451

Ch'en IL, Tsau JS, Molkentin JD, Komatsu M, Hedrick SM (2011) Mechanisms of necroptosis in T cells. J Exp Med 208:633–641

Cho YS, Challa S, Moquin D, Genga R, Ray TD, Guildford M, Chan FK (2009) Phosphorylation-driven assembly of the RIP1-RIP3 complex regulates programmed necrosis and virus-induced inflammation. Cell 137:1112–1123

Csomos RA, Brady GF, Duckett CS (2009) Enhanced cytoprotective effects of the inhibitor of apoptosis protein cellular IAP1 through stabilization with TRAF2. J Biol Chem 284:20531–20539

Davis CW, Hawkins BJ, Ramasamy S, Irrinki KM, Cameron BA, Islam K, Daswani VP, Doonan PJ, Manevich Y, Madesh M (2010) Nitration of the mitochondrial complex I subunit NDUFB8 elicits RIP1- and RIP3-mediated necrosis. Free Radic Biol Med 48:306–317

Degterev A, Hitomi J, Germscheid M, Ch'en IL, Korkina O, Teng X, Abbott D, Cuny GD, Yuan C, Wagner G, Hedrick SM, Gerber SA, Lugovskoy A, Yuan J (2008) Identification of RIP1 kinase as a specific cellular target of necrostatins. Nat Chem Biol 4:313–321

Duprez L, Takahashi N, Van Hauwermeiren F, Vandendriessche B, Goossens V, Vanden Berghe T, Declercq W, Libert C, Cauwels A, Vandenabeele P (2011) RIP kinase-dependent necrosis drives lethal systemic inflammatory response syndrome. Immunity 35:908–918

Ea CK, Deng L, Xia ZP, Pineda G, Chen ZJ (2006) Activation of IKK by TNFalpha requires site-specific ubiquitination of RIP1 and polyubiquitin binding by NEMO. Mol Cell 22:245–257

Eliasson MJ, Sampei K, Mandir AS, Hurn PD, Traystman RJ, Bao J, Pieper A, Wang ZQ, Dawson TM, Snyder SH, Dawson VL (1997) Poly(ADP-ribose) polymerase gene disruption renders mice resistant to cerebral ischemia. Nat Med 3:1089–1095

Ermolaeva MA, Michallet MC, Papadopoulou N, Utermohlen O, Kranidioti K, Kollias G, Tschopp J, Pasparakis M (2008) Function of TRADD in tumor necrosis factor receptor 1 signaling and in TRIF-dependent inflammatory responses. Nat Immunol 9:1037–1046

Feng S, Yang Y, Mei Y, Ma L, Zhu DE, Hoti N, Castanares M, Wu M (2007) Cleavage of RIP3 inactivates its caspase-independent apoptosis pathway by removal of kinase domain. Cell Signal 19:2056–2067

Festjens N, Kalai M, Smet J, Meeus A, Van Coster R, Saelens X, Vandenabeele P (2006a) Butylated hydroxyanisole is more than a reactive oxygen species scavenger. Cell Death Differ 13:166–169

Festjens N, Vanden Berghe T, Cornelis S, Vandenabeele P (2007) RIP1, a kinase on the crossroads of a cell's decision to live or die. Cell Death Differ 14:400–410

Festjens N, Vanden Berghe T, Vandenabeele P (2006b) Necrosis, a well-orchestrated form of cell demise: signalling cascades, important mediators and concomitant immune response. Biochim Biophys Acta 1757:1371–1387

Fiers W, Beyaert R, Declercq W, Vandenabeele P (1999) More than one way to die: apoptosis, necrosis and reactive oxygen damage. Oncogene 18:7719–7730

Francois M, Le Cabec V, Dupont MA, Sansonetti PJ, Maridonneau-Parini I (2000) Induction of necrosis in human neutrophils by Shigella flexneri requires type III secretion, IpaB and IpaC invasins, and actin polymerization. Infect Immun 68:1289–1296

Galluzzi L, Kepp O, Morselli E, Vitale I, Senovilla L, Pinti M, Zitvogel L, Kroemer G (2010) Viral strategies for the evasion of immunogenic cell death. J Intern Med 267:526–542

Goossens V, Grooten J, De Vos K, Fiers W (1995) Direct evidence for tumor necrosis factor-induced mitochondrial reactive oxygen intermediates and their involvement in cytotoxicity. Proc Natl Acad Sci U S A 92:8115–8119

Goossens V, Grooten J, Fiers W (1996) The oxidative metabolism of glutamine. A modulator of reactive oxygen intermediate-mediated cytotoxicity of tumor necrosis factor in L929 fibrosarcoma cells. J Biol Chem 271:192–196

Goossens V, Stange G, Moens K, Pipeleers D, Grooten J (1999) Regulation of tumor necrosis factor-induced, mitochondria- and reactive oxygen species-dependent cell death by the electron flux through the electron transport chain complex I. Antioxid Redox Signal 1:285–295

Gunther C, Martini E, Wittkopf N, Amann K, Weigmann B, Neumann H, Waldner MJ, Hedrick SM, Tenzer S, Neurath MF, Becker C (2011) Caspase-8 regulates TNF-alpha-induced epithelial necroptosis and terminal ileitis. Nature 477:335–339

He S, Liang Y, Shao F, Wang X (2011) Toll-like receptors activate programmed necrosis in macrophages through a receptor-interacting kinase-3-mediated pathway. Proc Natl Acad Sci U S A 108:20054–20059

He S, Wang L, Miao L, Wang T, Du F, Zhao L, Wang X (2009) Receptor interacting protein kinase-3 determines cellular necrotic response to TNF-alpha. Cell 137:1100–1111

Holler N, Zaru R, Micheau O, Thome M, Attinger A, Valitutti S, Bodmer JL, Schneider P, Seed B, Tschopp J (2000) Fas triggers an alternative, caspase-8-independent cell death pathway using the kinase RIP as effector molecule. Nat Immunol 1:489–495

Kaiser WJ, Upton JW, Long AB, Livingston-Rosanoff D, Daley-Bauer LP, Hakem R, Caspary T, Mocarski ES (2011) RIP3 mediates the embryonic lethality of caspase-8-deficient mice. Nature 471:368–372

Kalai M, Van Loo G, Vanden Berghe T, Meeus A, Burm W, Saelens X, Vandenabeele P (2002) Tipping the balance between necrosis and apoptosis in human and murine cells treated with interferon and dsRNA. Cell Death Differ 9:981–994

Karsan A, Yee E, Harlan JM (1996) Endothelial cell death induced by tumor necrosis factor-alpha is inhibited by the Bcl-2 family member, A1. J Biol Chem 271:27201–27204

Kim JS, He L, Lemasters JJ (2003) Mitochondrial permeability transition: a common pathway to necrosis and apoptosis. Biochem Biophys Res Commun 304:463–470

Kim YS, Morgan MJ, Choksi S, Liu ZG (2007) TNF-induced activation of the Nox1 NADPH oxidase and its role in the induction of necrotic cell death. Mol Cell 26:675–687

Koterski JF, Nahvi M, Venkatesan MM, Haimovich B (2005) Virulent *Shigella flexneri* causes damage to mitochondria and triggers necrosis in infected human monocyte-derived macrophages. Infect Immun 73:504–513

Kroemer G, Dallaporta B, Resche-Rigon M (1998) The mitochondrial death/life regulator in apoptosis and necrosis. Annu Rev Physiol 60:619–642

Kroemer G, Galluzzi L, Vandenabeele P, Abrams J, Alnemri ES, Baehrecke EH, Blagosklonny MV, El-Deiry WS, Golstein P, Green DR, Hengartner M, Knight RA, Kumar S, Lipton SA, Malorni W, Nunez G, Peter ME, Tschopp J, Yuan J, Piacentini M, Zhivotovsky B, Melino G (2009) Classification of cell death: recommendations of the nomenclature committee on cell death 2009. Cell Death Differ 16:3–11

Lamkanfi M, Dixit VM (2010) Manipulation of host cell death pathways during microbial infections. Cell Host Microbe 8:44–54

Laster SM, Wood JG, Gooding LR (1988) Tumor necrosis factor can induce both apoptic and necrotic forms of cell lysis. J Immunol 141:2629–2634

Li J, McQuade T, Siemer AB, Napetschnig J, Moriwaki K, Hsiao YS, Damko E, Moquin D, Walz T, McDermott A, Chan FK, Wu H (2012) The RIP1/RIP3 necrosome forms a functional amyloid signaling complex required for programmed necrosis. Cell 150:339–350

Li Q, Li G, Lan X, Zheng M, Chen KH, Cao CM, Xiao RP (2010) Receptor interacting protein 3 suppresses vascular smooth muscle cell growth by inhibition of the phosphoinositide 3-kinase-Akt axis. J Biol Chem 285:9535–9544

Mates JM, Segura JA, Campos-Sandoval JA, Lobo C, Alonso L, Alonso FJ, Marquez J (2009) Glutamine homeostasis and mitochondrial dynamics. Int J Biochem Cell Biol 41:2051–2061

Meylan E, Tschopp J (2005) The RIP kinases: crucial integrators of cellular stress. Trends Biochem Sci 30:151–159

Micheau O, Tschopp J (2003) Induction of TNF receptor I-mediated apoptosis via two sequential signaling complexes. Cell 114:181–190

Oberst A, Dillon CP, Weinlich R, McCormick LL, Fitzgerald P, Pop C, Hakem R, Salvesen GS, Green DR (2011) Catalytic activity of the caspase-8-FLIP(L) complex inhibits RIPK3-dependent necrosis. Nature 471:363–367

Ray CA, Pickup DJ (1996) The mode of death of pig kidney cells infected with cowpox virus is governed by the expression of the crmA gene. Virology 217:384–391

Rothe M, Pan MG, Henzel WJ, Ayres TM, Goeddel DV (1995) The TNFR2-TRAF signaling complex contains two novel proteins related to baculoviral inhibitor of apoptosis proteins. Cell 83:1243–1252

Schneider-Brachert W, Tchikov V, Neumeyer J, Jakob M, Winoto-Morbach S, Held-Feindt J, Heinrich M, Merkel O, Ehrenschwender M, Adam D, Mentlein R, Kabelitz D, Schutze S (2004) Compartmentalization of TNF receptor 1 signaling: internalized TNF receptosomes as death signaling vesicles. Immunity 21:415–428

Schulze-Osthoff K, Bakker AC, Vanhaesebroeck B, Beyaert R, Jacob WA, Fiers W (1992) Cytotoxic activity of tumor necrosis factor is mediated by early damage of mitochondrial functions. Evidence for the involvement of mitochondrial radical generation. J Biol Chem 267:5317–5323

Shu HB, Takeuchi M, Goeddel DV (1996) The tumor necrosis factor receptor 2 signal transducers TRAF2 and c-IAP1 are components of the tumor necrosis factor receptor 1 signaling complex. Proc Natl Acad Sci U S A 93:13973–13978

Sun L, Wang H, Wang Z, He S, Chen S, Liao D, Wang L, Yan J, Liu W, Lei X, Wang X (2012) Mixed lineage kinase domain-like protein mediates necrosis signaling downstream of RIP3 kinase. Cell 148:213–227

Sun X, Yin J, Starovasnik MA, Fairbrother WJ, Dixit VM (2002) Identification of a novel homotypic interaction motif required for the phosphorylation of receptor-interacting protein (RIP) by RIP3. J Biol Chem 277:9505–9511
Tenev T, Bianchi K, Darding M, Broemer M, Langlais C, Wallberg F, Zachariou A, Lopez J, MacFarlane M, Cain K, Meier P (2011) The Ripoptosome, a signaling platform that assembles in response to genotoxic stress and loss of IAPs. Mol Cell 43:432–448
Trichonas G, Murakami Y, Thanos A, Morizane Y, Kayama M, Debouck CM, Hisatomi T, Miller JW, Vavvas DG (2010) Receptor interacting protein kinases mediate retinal detachment-induced photoreceptor necrosis and compensate for inhibition of apoptosis. Proc Natl Acad Sci U S A 107:21695–21700
Upton JW, Kaiser WJ, Mocarski ES (2012) DAI/ZBP1/DLM-1 complexes with RIP3 to mediate virus-induced programmed necrosis that is targeted by murine cytomegalovirus vIRA. Cell Host Microbe 11:290–297
Vandenabeele P, Declercq W, Van Herreweghe F, Vanden Berghe T (2010) The role of the kinases RIP1 and RIP3 in TNF-induced necrosis. Sci Signal 3:re4
Vaseva AV, Marchenko ND, Ji K, Tsirka SE, Holzmann S, Moll UM (2012) p53 Opens the mitochondrial permeability transition pore to trigger necrosis. Cell 149:1536–1548
Vercammen D, Beyaert R, Denecker G, Goossens V, Van Loo G, Declercq W, Grooten J, Fiers W, Vandenabeele P (1998) Inhibition of caspases increases the sensitivity of L929 cells to necrosis mediated by tumor necrosis factor. J Exp Med 187:1477–1485
Vince JE, Pantaki D, Feltham R, Mace PD, Cordier SM, Schmukle AC, Davidson AJ, Callus BA, Wong WW, Gentle IE, Carter H, Lee EF, Walczak H, Day CL, Vaux DL, Silke J (2009) TRAF2 must bind to cellular inhibitors of apoptosis for tumor necrosis factor (tnf) to efficiently activate nf-{kappa}b and to prevent tnf-induced apoptosis. J Biol Chem 284:35906–35915
Wang L, Du F, Wang X (2008) TNF-alpha induces two distinct caspase-8 activation pathways. Cell 133:693–703
Wang Z, Jiang H, Chen S, Du F, Wang X (2012) The mitochondrial phosphatase PGAM5 functions at the convergence point of multiple necrotic death pathways. Cell 148:228–243
Zamzami N, Larochette N, Kroemer G (2005) Mitochondrial permeability transition in apoptosis and necrosis. Cell Death Differ 12(Suppl 2):1478–1480
Zhang D, Lin J, Han J (2010) Receptor-interacting protein (RIP) kinase family. Cell Mol Immunol 7:243–249
Zhang DW, Shao J, Lin J, Zhang N, Lu BJ, Lin SC, Dong MQ, Han J (2009) RIP3, an energy metabolism regulator that switches TNF-induced cell death from apoptosis to necrosis. Science 325:332–336
Zhang DW, Zheng M, Zhao J, Li YY, Huang Z, Li Z, Han J (2011a) Multiple death pathways in TNF-treated fibroblasts: RIP3- and RIP1-dependent and independent routes. Cell Res 21: 368–371
Zhang H, Zhou X, McQuade T, Li J, Chan FK, Zhang J (2011b) Functional complementation between FADD and RIP1 in embryos and lymphocytes. Nature 471:373–376
Zhao J, Jitkaew S, Cai Z, Choksi S, Li Q, Luo J, Liu ZG (2012) Mixed lineage kinase domain-like is a key receptor interacting protein 3 downstream component of TNF-induced necrosis. Proc Natl Acad Sci U S A 109:5322–5327
Zorde-Khvalevsky E, Abramovitch R, Barash H, Spivak-Pohis I, Rivkin L, Rachmilewitz J, Galun E, Giladi H (2009) Toll-like receptor 3 signaling attenuates liver regeneration. Hepatology 50:198–206

Chapter 4
IAPs and Necroptotic Cell Death

John Silke and David Vaux

4.1 Technological Advance

Important discoveries are often preceded by technological advances. The discovery of the role of IAPs in regulating necroptosis was due in large part to the development of small molecule IAP antagonist compounds, also known as "smac-mimetics", which upon binding trigger the rapid auto-ubiquitylation and degradation of cIAPs (Gaither et al. 2007; Varfolomeev et al. 2007; Vince et al. 2007). Equally important was the development of the specific RIPK1 kinase inhibitors, most notably nec-1, frequently referred to as necrostatin (Degterev et al. 2005, 2008; Takahashi et al. 2012; Vandenabeele et al. 2013), and potent, broad-spectrum caspase inhibitors such as Q-VD-Oph (Caserta et al. 2003). These molecular tools made it feasible to survey large numbers of cell types and conditions to reveal a novel, caspase- and Bax/Bak-independent mechanism by which cells can kill themselves: necroptosis. The challenges now are to determine all the components of the necroptotic mechanism, to elucidate its physiological and pathophysiological roles and to develop further drugs that can promote or inhibit necroptosis to treat disease.

4.2 What Is Necroptosis? Maxwell's Silver Hammer

Necroptosis is the third mechanism for cell suicide to be identified in metazoans. The first, based on genetic analysis of *C. elegans*, showed that developmentally programmed cell death during development required a cysteine protease, CED-3, that

J. Silke (✉) • D. Vaux
The Walter and Eliza Hall Institute of Medical Research, Parkville, VIC 3052, Australia

Department of Medical Biology, University of Melbourne, Parkville, VIC 3050, Australia
e-mail: silke@wehi.edu.au; vaux@wehi.edu.au

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was the archetype of a family of proteases now known as caspases. Caspase-dependent cell death also occurs in mammalian cells and can be defined as a form of cell death that can be inhibited by a caspase inhibitor. Cells undergoing caspase-dependent cell death typically display a classic "apoptotic" morphology by forming membrane blebs and cleaving their DNA following activation of caspase-dependent DNAse (CAD). The internucleosomally cleaved DNA forms a characteristic "ladder" upon electrophoresis. The necroptotic pathway of cell death appears to be held in check by caspase, particularly caspase-8, activity. If therefore caspase-8 activity is inhibited (perhaps by a virus), the necroptosis pathway can come into play, and to paraphrase the words of the Beatles' song, "make sure that it is dead".

The other well-characterised form of cell death depends on the Bcl-2 family members Bax and Bak and also exhibits an apoptotic morphology, because in this case cytochrome c released from the mitochondria (via Bax/Bak) triggers activation of Apaf-1, caspase-9 and caspase-3. However, in the case of Bax/Bak-dependent apoptosis, caspase inhibitors do not allow the treated cells to permanently cheat death, because loss of cytochrome c causes the cells to die from lack of ATP, even if caspases are inhibited.

Like caspase-dependent apoptosis and Bax/Bak-dependent apoptosis, necroptosis is a form of cell suicide because the mechanism for the cell's demise is encoded by the host's genes, and the function of those gene products is, amongst other functions, to cause the cell to die. Necroptosis can be functionally distinguished from the other two forms of cell death as cell death is not prevented by broad-spectrum caspase inhibitors, or deletion of Bax and Bak, or over-expression of anti-apoptotic Bcl-2 family members. In some, but not all cases, necroptosis can be inhibited by treatment with necrostatin.

Of course, cells in which necroptosis and Bax-/Bak- and caspase-dependent apoptosis are blocked are still mortal: although they cannot commit suicide by these mechanisms, they can still be killed if a vital metabolic pathway is inhibited. Cells that are killed (e.g. by a toxin, anoxia, starvation or physical injury such as heating) may exhibit an apoptotic morphology, because stress often induces activation of an apoptotic mechanism, or the morphology known as "necrosis", in which there is cell swelling and early loss of plasma membrane integrity. The neologism "necroptosis" was coined to indicate a genetically programmed cell death that is caspase and Bax/Bak independent and which typically induces cellular changes that resemble those of cells undergoing necrosis.

The history of necroptosis research is presented elsewhere in this volume, and while it is indisputable that smac-mimetics and necrostatins drove necroptosis research forward, as William Gibson wrote, "The future is already here; it's just not very evenly distributed". Thus groundbreaking studies were made without these reagents. What all these early studies have in common was that they were all investigating caspase-independent cell death induced by the extrinsic apoptosis pathway (Vercammen et al. 1998a, b; Holler et al. 2000) and this pathway features heavily in subsequent studies. The extrinsic apoptosis pathway is initiated by ligands of the TNF superfamily (TNFSF) binding to receptors of the same superfamily (Schneider 2008).

4.3 Death Receptor Signalling: The Long and Winding Road

Death receptors (DRs) are a subset of the TNF receptor superfamily (TNFRSF) that contain a cytoplasmic death domain (DD). The DD is a conserved intracellular domain that, upon receptor ligation, recruits other intracellular proteins via homotypic DD-DD interaction. Despite the evocative name, there are two important points to note. Firstly, while activation of death receptors can certainly lead to cell death, the name can be misleading because when they signal they do not necessarily kill: for example, cells rarely die when exposed to TNF (Fig. 4.1a), and while Fas ligand and TRAIL often trigger cell death, they do not always do so (Peter et al. 2007; Silke 2011; Wajant and Scheurich 2011). Secondly, ligation of DRs induces formation of signalling complexes that activate MAP kinases and transcription factors such as NF-κB. Signalling is clearly initiated from the plasma membrane where the receptors first interact with their respective ligands, but it is also accepted that these complexes mature to generate cytoplasmic signalling complexes that are no longer associated with the plasma membrane (Lavrik and Krammer 2012; Silke 2011; Schütze et al. 2008; Dickens et al. 2012) (Fig. 4.1). Exactly how these secondary signalling complexes are generated is still unclear, but it appears to occur not only for TNFR1 (Micheau and Tschopp 2003; Schneider-Brachert et al. 2004; Harper et al. 2003) but also for Fas and TRAIL receptors (Lavrik et al. 2008; Geserick et al. 2009; Varfolomeev et al. 2005; Lavrik and Krammer 2012).

The mechanics of Fas and TRAILR signalling have principally been examined in cells where FasL and TRAIL induce cell death (Figs. 4.1b, c and 4.2), but this is not the sole type of physiological signalling from these receptors (Peter et al. 2007; O'Reilly et al. 2009; Varfolomeev et al. 2005). When their ligands bind, the cytosolic DDs of Fas and TRAILR recruit Fas-associated death domain protein (FADD), and FADD in turn recruits caspase-8, forming the so-called death-inducing signalling complex (DISC) and leading to the oligomerisation and activation of this upstream caspase at the plasma membrane (Fig. 4.1a) (Kischkel et al. 1995; Muzio et al. 1996; Chang et al. 2003; Lavrik and Krammer 2012).

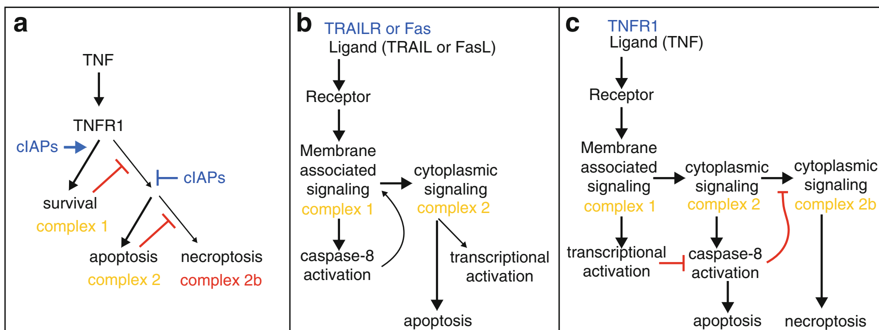

Fig. 4.1 Schematic of key concepts in death receptor signaling

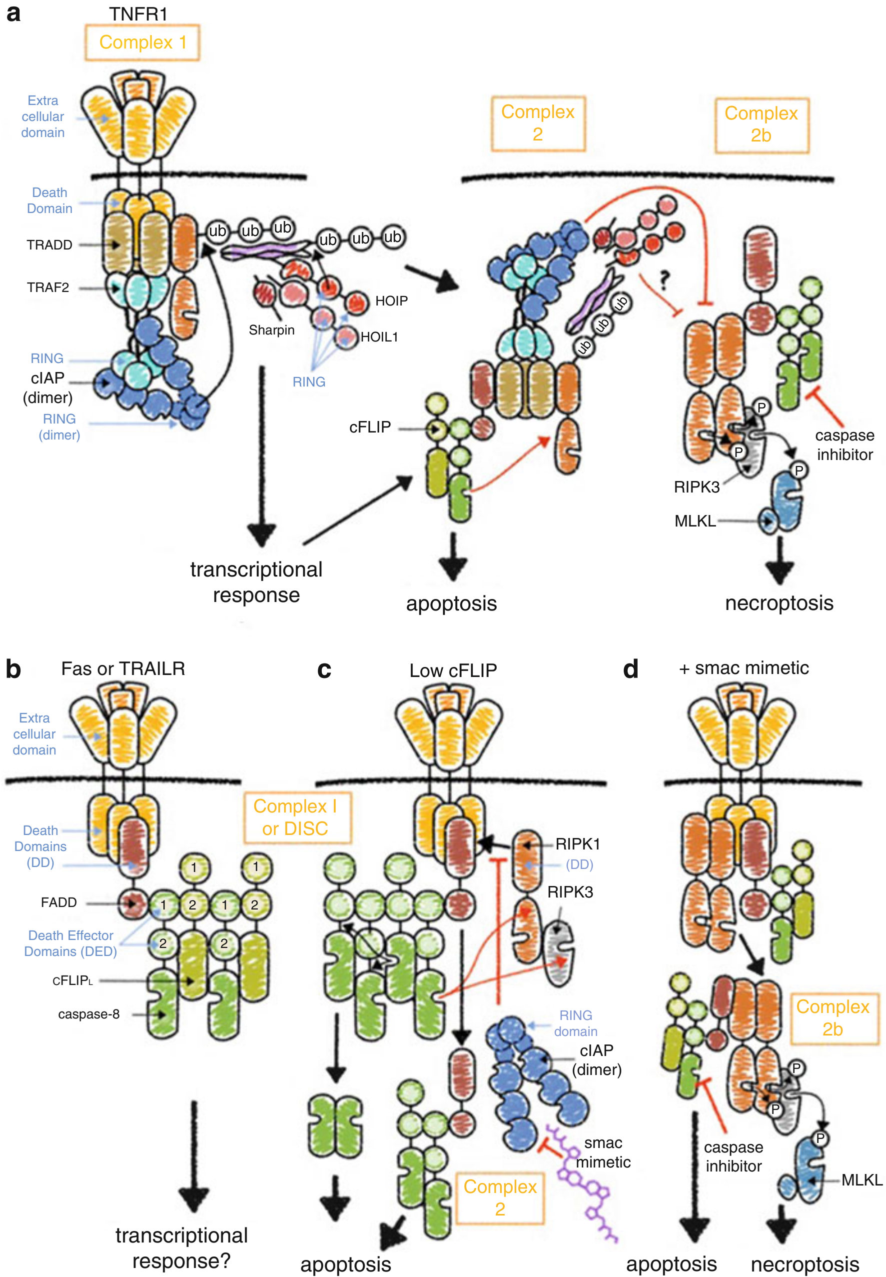

Fig. 4.2 Schematic of molecular components of death receptor signaling platforms

Because addition of TNF alone rarely causes cell death, TNFR1 signalling has been more thoroughly characterised with regard to the transcriptional response than for the death response. In fact, in most normal and transformed cell types, TNF only induces cell death if the transcriptional signalling pathway is blocked (Fig. 4.1a). Some of the additional complexity of TNFR1 signalling, compared to Fas or TRAILR, might be due to the adaptor protein TRADD, which binds to the DDs of TNFR1 and recruits TRAF2 together with cIAP1 and cIAP2. However it should be noted that TRADD is apparently not required in all cell types for TNF signalling (see Silke (2011) and Wajant and Scheurich (2011) for discussion).

In addition to TRADD and FADD, another DD-bearing adaptor protein recruited to DR signalling complexes is RIPK1. This protein sits at the core of the bipotential transcriptional and cell death signalling pathways emanating from DRs, and because the amount and activity of RIPK1 are regulated by IAPs and caspase- 8, we should introduce the IAPs.

4.4 IAPs

Inhibitor of apoptosis proteins were originally identified in baculoviruses, where they prevent apoptosis of their host cell, thereby gaining time for viral replication (Crook et al. 1993). IAPs are defined by the presence of one or more baculovirus IAP repeat (BIR) domain(s), a ~70 residue large zinc-binding domain that mediates protein–protein interactions (Birnbaum et al. 1994; Hinds et al. 1999; LaCasse et al. 1998; Vaux and Silke 2005). The mammalian cellular IAPs cIAP1 and cIAP2, which bear three BIR domains and a carboxy-terminal RING domain, were identified because of their similarity to viral IAPs and by virtue of their indirect binding to TNFR2 via the adaptor protein TRAF2 (Rothe et al. 1994, 1995; Liston et al. 1996; Uren et al. 1996).

XIAP, which is the other major IAP with a role in inhibiting cell death, was identified by its similarity to the baculoviral IAPs (Duckett et al. 1996; Liston et al. 1996; Uren et al. 1996), and like the cIAPs, it bears three BIRs and a RING domain, but unlike the cIAPs, it does not bind to TRAF1 or TRAF2 (Uren et al. 1996). Because XIAP also differs from cIAP1 and cIAP2 in its ability to directly inhibit caspase-3 (Deveraux et al. 1997) and caspase-9 (Deveraux et al. 1998), it is not thought to play a major role in regulating signalling by TNF receptor family members.

4.5 Smac/Diablo, Other IAP Antagonists and Small Molecule Smac-Mimetics

The IAP-binding protein Smac/Diablo was identified by Verhagen et al. by co-immunoprecipitation with XIAP (Verhagen et al. 2000) and by Du et al. (2000) by its ability to promote caspase activation by cytochrome c and Apaf-1. Other IAP antagonist proteins were soon identified (Verhagen et al. 2001, 2007; Hegde et al. 2001;

Martins et al. 2001), and all shared a common motif, four amino acids at a processed N-terminal end that interact with the BIRs of XIAP (Silke et al. 2000; Wu et al. 2000). The ability of this motif to displace active caspases (Srinivasula et al. 2001; Ekert et al. 2001) attracted the interest of several pharmaceutical companies (Garber 2005), who developed "smac-mimetic" compounds that not only relieved inhibition of caspase-3 by XIAP in vitro but also caused the death of certain cancer cell lines. Because XIAP is the only mammalian IAP that can directly inhibit caspase activity, and XIAP does not bind to TRAFs, and smac-mimetics were designed to bind to XIAP, the ability of smac-mimetics to regulate DR-induced cell death was not immediately apparent.

However, smac-mimetics also promote loss of cIAP1 within minutes of being added to cells (Gaither et al. 2007; Varfolomeev et al. 2007; Vince et al. 2007; Darding et al. 2011; Bertrand et al. 2008; Wang et al. 2008), and this discovery provided some rationale for their effects on DR signalling. For smac-mimetics to promote proteasomal degradation of their IAP targets, they have to bind to the BIR2 or BIR3 of the IAP, the IAP RING must be intact, and the RING has to be able to bind to an E2 ubiquitin-conjugating enzyme (Varfolomeev et al. 2007; Vince et al. 2007; Feltham et al. 2011; Dueber et al. 2011; Blankenship et al. 2009). Some smac-mimetics promote XIAP degradation, but the mechanism has not been as well characterised as for cIAPs.

Smac-mimetics, and particularly the bivalent compounds, are therefore useful tools that promote rapid cIAP1/cIAP2 auto-ubiquitylation and degradation (Gaither et al. 2007; Varfolomeev et al. 2007; Vince et al. 2007) and bind to XIAP predominantly in the BIR3 and therefore may inhibit some (but not all) XIAP interactions. However not all smac-mimetics are equal, and each may affect each major IAP distinctly (Ndubaku et al. 2009; Darding et al. 2011). Furthermore, some on-target effects of these compounds mean that their effects on cells are not equivalent to cIAP1 and cIAP2 knockout. For example, smac-mimetic-induced loss of cIAP1 leads to activation of the NF-κB-activating kinase NIK1 (Varfolomeev et al. 2007; Vince et al. 2007). Because cIAP2 is rapidly induced by NF-κB and cIAP2 homodimers are relatively resistant to smac-mimetic-induced degradation (Darding et al. 2011; Feltham et al. 2011), smac-mimetic treatment can result in increased levels of cIAP2.

Because smac-mimetics are a diverse set of chemicals, they will have distinct off-target effects. Similarly, while genetic experiments allow the contribution of each individual IAP to be disentangled, there are limitations to what can be learnt especially when they result in embryonic lethality. On the other hand, findings that are consistent with both smac-mimetic experiments as well as with genetically mutated cells are very likely to be reliable.

4.6 IAPs and Death Receptors: Come Together

Early indications that IAPs might be important in regulating DR signalling included a pivotal study showing that smac or smac peptides could dramatically sensitise glioma, neuroblastoma and melanoma cells to killing by TRAIL (Fulda et al. 2002).

Similarly, a small molecule smac-mimetic sensitised a glioblastoma cell line to TRAIL- and TNF-induced cell death (Li et al. 2004). Although this particular smac-mimetic was designed to antagonise XIAP, it nevertheless appeared to sensitise cells to DR-induced cell death. As described above it was not at all clear at the time why, until it was subsequently shown that the same compound, like other smac-mimetics (Varfolomeev et al. 2007; Vince et al. 2007), promoted degradation of cIAP1 and cIAP2 by the proteasome (Wang et al. 2008).

Because most cells do not die when exposed to TNF alone, but die when it is added together with an inhibitor of translation or transcription (such as actinomycin D or cycloheximide, respectively), it has become accepted that transcription of a labile cell death inhibitor(s) is required to stop TNF from triggering cell death (Wajant and Scheurich 2011). The finding that cell lines derived from p65/RelA NF-κB deleted mice are killed by TNF alone (Beg and Baltimore 1996) and that the early embryonic lethality of p65 knockout mice was prevented by crossing to TNF knockout mice (Doi et al. 1999) was consistent with this notion and suggested that wild-type cells do not die in response to TNF because it activates canonical (p65/RelA) NF-κB which drives production of one or more cell death inhibitory proteins. Consistent with this model, expression of a super-repressor of IκBα that cannot be degraded and which thereby prevents NF-κB activation also sensitises cells to TNF-induced cell death (Van Antwerp et al. 1996).

The finding that cIAP1-gene-deleted or cIAP1-gene-depleted but not XIAP-gene-deleted cell lines was killed by TNF alone, just like p65/RelA mutant cells, suggested that either cIAP1 was required for TNF to efficiently activate NF-κB or that cIAP1 was the cell death inhibitor induced by NF-κB. cFLIP is the best candidate for the NF-κB-induced inhibitor of cell death (Micheau et al. 2001), and experiments studying TNF-induced degradation of IκBα and nuclear localisation of p65/RelA in wild-type and cIAP-deficient cell lines (Varfolomeev et al. 2008; Mahoney et al. 2008; Feltham et al. 2010; Moulin et al. 2012) indicate that the former is the case: cIAPs are needed for TNF to efficiently activate canonical NF-κB (Fig. 4.2a).

One explanation for why cIAP-deficient cells might be defective in activating NF-κB relates to the role of RIPK1 in TNF-induced NF-κB. RIPK1 has been reported to play a pivotal role in TNF-induced NF-κB activation (Kelliher et al. 1998), and there is no doubt that RIPK1 becomes rapidly ubiquitylated in response to TNF signalling (see Haas et al. (2009) for particularly stunning examples of TNF-induced RIPK1 ubiquitylation). Findings such as these suggested RIPK1 ubiquitylation was required for TNF-induced NF-κB activation and that K63-linked ubiquitin chains on RIPK1 directly recruited NEMO/IKK1/IKK2 (Fig. 4.2a). As a consequence, IKK2 was activated and phosphorylated IκBα, resulting in translocation of p65/p50 NF-κB into the nucleus (Ea et al. 2006; Wu et al. 2006). This model has been extensively refined (Wajant and Scheurich 2011; Wertz and Dixit 2010; Silke and Brink 2010; Silke 2011) by identifying a new type of M-1-linked ubiquitin chain, “linear ubiquitin” (Kirisako et al. 2006), and a new ubiquitin E3 ligase, LUBAC, that play pivotal roles in TNF-induced activation of NF-κB (Kirisako et al. 2006; Haas et al. 2009; Tokunaga et al. 2009; Gerlach et al. 2011; Tokunaga et al. 2011; Ikeda et al. 2011). But it should also be noted that RIPK1 is not

universally required for TNF-induced NF-κB (Wong et al. 2010; Wajant and Scheurich 2011; Blackwell et al. 2013) and wild-type embryonic hepatocytes do not express RIPK1 and yet activate NF-κB in response to TNF (Wong et al. 2010; Blackwell et al. 2013).

Whatever the precise details, it is clear that in several cell types RIPK1 appears to be a focus of the ubiquitin platform assembling machinery activated upon TNF binding to TNFR1 and that the assembly of ubiquitin chains on components of the TNFR1 signalling complex is essential for activation of NF-κB in response to TNF/TNFR1 signalling. Significantly, one of the most prominent and readily observable effects of depleting cIAPs is a reduction in RIPK1 ubiquitylation (Vince et al. 2007; Varfolomeev et al. 2008; Bertrand et al. 2008; Mahoney et al. 2008; Haas et al. 2009; Feltham et al. 2010; Moulin et al. 2012). Furthermore, cells in which cIAP protein levels are reduced, whether genetically or chemically, do not efficiently activate canonical NF-κB in response to TNF; rather, the cells die (Varfolomeev et al. 2008; Mahoney et al. 2008; Feltham et al. 2010; Moulin et al. 2012). It is only natural therefore to draw the conclusion that cIAP-deficient cells die in response to TNF because they fail to ubiquitylate RIPK1 which leads to a failure to activate NF-κB and a failure to drive production of cFLIP.

Only natural, but not the whole story. When TNF is added, but canonical NF-κB signalling is blocked, cells usually die by caspase-8-mediated apoptosis, presumably because cFLIP fails to be induced (Micheau et al. 2001; Nakajima et al. 2006). Similarly, treatment with smac-mimetics sensitises cells to a caspase-8-dependent death because crmA, a viral inhibitor of caspase-8, is able to inhibit smac-mimetic-induced death in some types of cells (Vince et al. 2007). However cell death induced by TNF/CHX can be distinguished from cell death induced by TNF and smac-mimetic, because TNF-/CHX-induced death is increased by RIPK1 deficiency whereas TNF-/smac-mimetic-induced death is prevented by loss of RIPK1 (Wang et al. 2008; Wong et al. 2010).

Therefore increased sensitivity of smac-mimetic-treated cells to TNF cannot be as simple as a loss of NF-κB signalling. One of the first experiments to hint at what this added complication might be was an increase in RIPK1 accumulation at the cytoplasmic tail of TNFR1 following addition of TNF to either smac-mimetic-treated or cIAP1 knockout cells (Vince et al. 2007). With this fact in mind, we discuss the other data that lead to a proposed model to explain this added complication.

4.7 cIAPs and RIPK1: Regulating the Regulators

Consistent with the reduction in RIPK1 ubiquitylation in cIAP-deficient cells, it has been known for almost 10 years that cIAPs are able to ubiquitylate RIP kinases (Park et al. 2004; Bertrand et al. 2008, 2011; Dynek et al. 2010) and this may be the K48-type linkage that promotes proteasomal degradation, or the K63 type that promotes NF-κB activation, or some other type (Blankenship et al. 2009; Dynek et al. 2010; Bertrand et al. 2011). We have proposed therefore that in the absence of

cIAPs, RIPK1 in complex 1 is not ubiquitylated, so its level increases abnormally (Geserick et al. 2009) (Fig. 4.2a, Complex 2). This indicates that at least some of the ubiquitylation on RIPK1 is K48 linked, thereby promoting proteasomal degradation. However, there are other potential scenarios, and it is difficult experimentally to address this because the proportion of RIPK1 recruited to receptors appears to be a minor part of the total pool. In the case of the Fas and TRAILR DRs, when ligand (FasL or TRAIL) is added together with smac-mimetic, the high levels of RIPK1 that accumulate in complex 1 cause increased activation of caspase-8 (Geserick et al. 2009; Wang et al. 2008). This is possibly because of the extra DD supplied by RIPK1 that could promote increased recruitment and activation of caspase-8. Smac-mimetic treatment also increases the formation of a cytoplasmic caspase-8-containing complex (Fig. 4.2a, d, complex 2; Geserick et al. 2009). This model passes one test in that it predicts that in smac-mimetic plus TNF-, FasL- or TRAIL-induced cell death, loss of RIPK1 would prevent death because high levels of RIPK1 are required for caspase-8 activation.

A variation on this model is that recruitment of RIPK1 into the DISC is inhibited when it is ubiquitylated by cIAP. The experiments described by Geserick et al. could not distinguish whether cIAPs limit RIPK1 recruitment into the DISC or whether they limit the accumulation of RIPK1 within the DISC by K48 ubiquitylating RIPK1, leading to its proteasomal degradation.

On the other hand, in cells treated with TNF and low-dose cycloheximide, the only part of the TNFR1 signalling pathway that is affected is production of the NF-κB-responsive cell death inhibitor (most likely, cFLIP). In the absence of RIPK1, low-dose cycloheximide would reduce NF-κB activation even further, such that RIPK1 knockout cells are more sensitive to cycloheximide + TNF-induced cell death.

It has been shown that knock-down of CYLD, a deubiquitinating enzyme, decreases smac-mimetic + TNF-induced apoptosis (Wang et al. 2008; Vanlangenakker et al. 2010). This could mean that the absolute levels of RIPK1 might not determine whether complex 1 progresses to complex 2 (as suggested above) but that RIPK1 might have to be deubiquitylated for complex 2 to form. However, the interpretation of these data in this way is difficult to reconcile with the fact that levels of ubiquitylated RIPK1 in complex 1 are already dramatically reduced in cIAP-defective cells (Geserick et al. 2009; Haas et al. 2009; Moulin et al. 2012; Gerlach et al. 2011) and that the degree of RIPK1 ubiquitylation appears to be identical for RIPK1 associated with complex 1 and complex 2 (Geserick et al. 2009). If the deubiquitylation model for transition from complex 1 to complex 2 is correct, one prediction is that CYLD knock-down should also inhibit TNF + cycloheximide-induced death; however, this test has not, to our knowledge, been reported.

4.8 Cell Death Without Complex 1: The Ripoptosome

As we have discussed, it is an axiom in DR signalling that formation of complex 1 is followed by formation of complex 2. Consistently, when treating cells with one of the death ligands together with a smac-mimetic, it has not been possible to

decisively demonstrate complex 2 formation without complex 1 (Geserick et al. 2009). However, when cell death was induced by combining smac-mimetics with a non-DR stimulus, such as double-stranded RNA, which activates Toll-like receptor 3 (TLR3), a complex 2 was formed without DR signalling (Feoktistova et al. 2011; Tenev et al. 2011). These results suggest two possibilities: either cIAPs are involved in preventing recruitment and activation of RIPK1 to DRs in the absence of ligand or cIAPs prevent the formation of complex 2 and that normally DR ligands overcome this inhibition. Both Feoktistova and Tenev et al. examined recruitment of RIPK1 to TNFR1 in smac-mimetic-treated cells and did not observe recruitment in the absence of a DR ligand, indicating that the first model is less likely to be true, although they did not examine all DR signalling platforms. As with FasL and smac-mimetic treatment, Feoktistova et al. showed that $cFLIP_L$ inhibited complex 2 formation (Fig. 4.2e), while $cFLIP_s$ promoted it (Fig. 4.2f). Because this complex 2 contained RIPK1, it was referred to as the "ripoptosome" (Fig. 4.2g).

Thus a common mechanism is suggested for DR and non-death receptor induced cell death that is dependent upon RIPK1 and caspase-8, and the results suggest that DR signalling normally inactivates IAPs in some way to promote the generation of the death-inducing complex.

We have covered the formation of complex 2 in detail because it is integral to understanding the final necroptotic arm of the cell death pathway induced by DRs, which again involves RIPK1 as the cornerstone of the process.

4.9 RIPK1, RIPK3 and MLKL Required for Necroptosis

Our limited recent understanding of necroptosis means that the models explaining it are still, thankfully, very simple and are also described in detail elsewhere in this volume. In essence it has been shown that RIPK1 kinase activity is required for most cases of DR-induced necroptosis and the function of RIPK1 is to phosphorylate and activate RIPK3 (He et al. 2009; Cho et al. 2009; Zhang et al. 2009). RIPK3 knockout mice and cells are relatively normal (Newton et al. 2004) but are resistant to treatments that cause wild-type cells to die by necroptosis (He et al. 2009; Cho et al. 2009). RIPK3 phosphorylates and activates the pseudokinase MLKL (Sun et al. 2012; Zhao et al. 2012; Murphy et al. 2013). How MLKL activation results in necroptotic cell death is still unknown. It has been suggested that PGAM5, a mitochondrial phosphatase, may play a role (Wang et al. 2012), but it does not seem to be universally required (Murphy et al. 2013).

The small molecule necrostatin (Nec-1) was isolated in a screen in U937 cells for inhibitors of cell death induced by TNF together with the caspase inhibitor zVAD.fmk (Degterev et al. 2005). These and later results (He et al. 2009; Cho et al. 2009) indicate that the kinase activity of RIPK1 is required for DR-induced necroptosis, presumably because it phosphorylates and thereby activates RIPK3. Although Nec-1 inhibits the kinase activity of RIPK1, it does so in a very unusual manner whereby it is "caged" in a hydrophobic pocket between the N- and C-lobes of the

kinase domain that disrupts the structure of RIPK1. Therefore it may have effects that are distinct from its ability simply to inhibit the kinase activity of RIPK1. In this regard it is interesting that Nec-1 appears to inhibit recruitment of RIPK1 into complex 2/ripoptosome (Geserick et al. 2009; Tenev et al. 2011; Feoktistova et al. 2011).

Caspase-8 usually plays the executioner role in TNF-, FasL- or TRAIL + smac-mimetic-induced death, but, remarkably, when caspase-8 is blocked by an inhibitor such as CrmA, zVAD or Q-VD-Oph, in some cell types the amount of cell death actually increases. The proposed explanation for this is that normally caspase-8 homodimers or caspase-8/cFLIP heterodimers within complex 2 are able to cleave and inactivate RIPK1 (Lin et al. 1999; Geserick et al. 2009; Feoktistova et al. 2011; Pop et al. 2011), and it has also been shown that RIPK3 can be similarly inactivated by caspase-8 cleavage (Feng et al. 2007). In the absence of caspase-8 proteolytic activity, enough active RIPK1 and RIPK3 accumulate to cause cell death by necroptosis.

Consistent with caspase-8 having a role in limiting complex 2b/ripoptosome formation and hence blocking necroptosis, cFLIP, which structurally resembles a catalytically inactive form of caspase-8, is able to inhibit necroptosis as well as caspase-8-dependent apoptosis. Evidence supporting this idea is the striking similarity between the embryonic lethal phenotypes of mice mutant for caspase-8, FADD and cFLIP and the protection afforded to each by combined deletion of genes for RIPK3 (Oberst et al. 2011; Kaiser et al. 2011; Dillon et al. 2012). However, the role of cFLIP is complex, because $cFLIP_s$' expression sensitises cells to smac-mimetic-induced necroptosis while $cFLIP_L$ inhibits it (Geserick et al. 2009; Tenev et al. 2011; Feoktistova et al. 2011). Because both the short and long forms of cFLIP inhibit activation of caspase-8, the difference cannot simply be due to inhibiting caspase-8 activity, and indeed $cFLIP_L$, in contrast to $cFLIP_s$, inhibits recruitment of RIPK1 into complex 2b/ripoptosome.

The model we favour is that recruitment of RIPK1 into complex 2 is enhanced by smac-mimetics because smac-mimetic-induced depletion of cIAPs prevents RIPK1 from being ubiquitylated. According to this model, a RIPK1 deubiquitylating enzyme would enhance rather than prevent smac-mimetic-induced necroptosis (Wang et al. 2008). Recent work suggests CYLD is required for necroptosis and is one of the targets of caspase-8 in apoptotic cells (O'Donnell et al. 2011). However when complex 2b was analysed, there appeared to be very little difference in RIPK1 recruitment (O'Donnell et al. 2011), and the evidence from the CYLD knockout mice that CYLD is a negative regulator of RIPK1 is weak (Wright et al. 2007). An alternative hypothesis is that the deubiquitylating activity of CYLD on Tak1 is more important in regulating the necroptotic response (Vanlangenakker et al. 2010).

4.10 IAPs and Necroptosis In Vivo

As discussed elsewhere in this review, and in this book, FADD, cFLIP and caspase-8 knockout mice are all embryonic lethal around embryonic day 10 (E10; Yeh et al. 1998; Varfolomeev et al. 1998; Yeh et al. 2000). Remarkably the embryonic

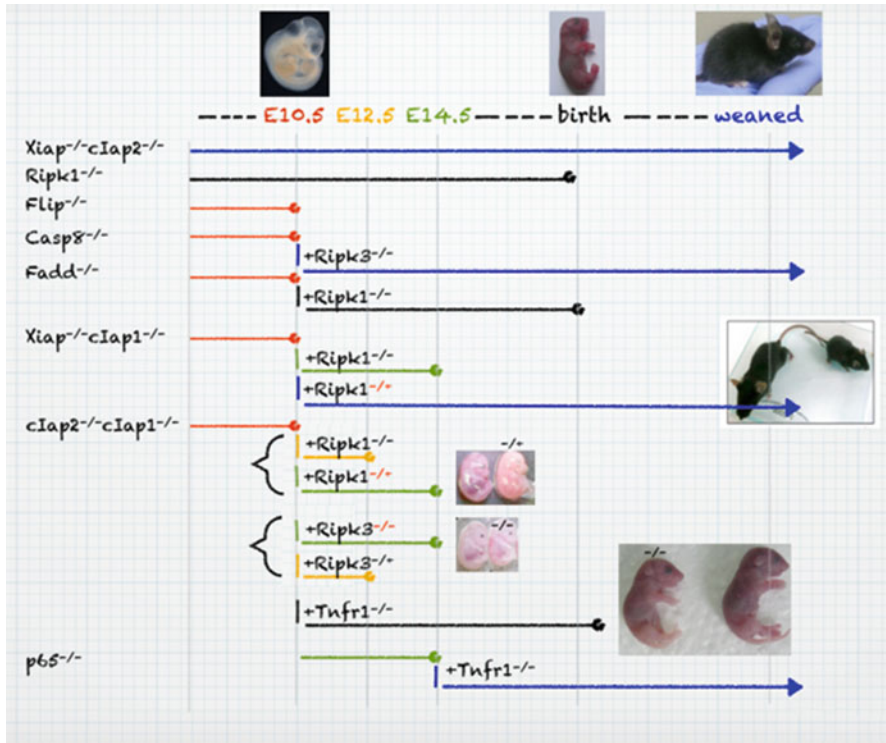

Fig. 4.3 Table of phenotypes of knockout mice relevant to death receptor signaling

lethality of caspase-8 knockout mice is prevented by crossing them to RIPK3 knockout mice (Kaiser et al. 2011; Oberst et al. 2011), while the embryonic lethality of FADD knockout mice is prevented by crossing them to RIPK1 or RIPK3 knockout mice (Dillon et al. 2012; Zhang et al. 2011). These results are sometimes interpreted as genetic evidence that FADD/caspase-8 and cFLIP are engaged in inhibiting a necroptotic pathway that occurs at this stage of embryonic development. However, because of the potential roles that RIPK1 and RIPK3 play in generating inflammatory cytokines, there are also other potential explanations.

Consistent with the ability for cIAPs to regulate components of complex 2 and thereby necroptosis, it was gratifying to find that the cIAP1 cIAP2 double-knockout embryos also die at E10 with a very similar phenotype to the caspase-8 knockouts (Fig. 4.3; Varfolomeev et al. 1998; Kang et al. 2004; Moulin et al. 2012). And again, consistent with the FLIP, FADD and caspase-8 knockouts, the phenotype was suppressed and survival was prolonged to birth when the cIAP double knockouts were combined with deletion of RIPK1 or RIPK3 (Moulin et al. 2012). Most remarkably, the cIAP1/cIAP2 double-knockout embryonic lethality was also prevented by crossing these mice to TNFR1 knockout mice (Moulin et al. 2012). This strongly suggests that the embryonic lethality of FADD, cFLIP and caspase-8 knockout mice

will also be delayed or prevented if these mice are crossed to TNFR1 knockout mice, a result that would be highly counterintuitive because TNFR1 signalling is not required for survival of mice in utero (Kang et al. 2004). However, it is plausible that there is a certain level of TNFR1 signalling that occurs in embryos around day E10 but which is not required for development but that when one of the essential components of complex 2 described above is absent, this provokes an inappropriate response, leading to death of certain cells in the developing placenta and loss of viability of the embryo at E10.

4.11 IAPs, Pyroptosis and the Inflammasome

The role of IAPs in regulating the inflammasome is worth commenting on briefly. The inflammasome, like the apoptosome and the ripoptosome, is a caspase-activating complex, but in this case the core adaptor is ASC, and the caspase is caspase-1 (and sometimes caspase-8 (Sagulenko et al. 2013)). Its role is to activate the cytokine IL-1β and sometimes to cause cell death, which in this case is termed "pyroptosis" (Bergsbaken et al. 2009). This type of cell death typically has an appearance resembling that of necroptosis, including plasma membrane rupture and release of intracellular contents. The fact that when LPS-primed bone marrow-derived macrophages or dendritic cells (BMDMs or BMDCs) were treated with the smac-mimetic compound A, the inflammasome was activated, IL-1β was produced and they died by pyroptosis indicates that IAPs have a role in limiting assembly or activation of the inflammasome (Vince et al. 2012).

IL-1ß levels are usually very low in resting cells, and in order to detect measurable quantities of the pro form of this cytokine, they therefore need to be "primed" with a stimulus, typically a TLR ligand such as LPS (Fig. 4.4). The pro form of IL-1ß is cleaved and secreted, typically in a caspase-1-dependent manner. Vince et al. demonstrated that single cIAP1, cIAP2 or XIAP knockout BMDMs each behaved like wild-type BMDMs, and in order to see significant processing of IL-1ß, cells had to be first primed with LPS to produce pro-IL-1ß and then activated with a classic inflammasome activator such as alum. However in XIAP/cIAP1/cIAP2 triple-knockout BMDMs (somewhat analogous to smac-mimetic-treated cells), all that was required to see processing and secretion of IL-1ß was LPS priming. This dramatic result demonstrates that in the absence of the three IAPs, the inflammasome can form and activate spontaneously and suggests that the inflammasome is already poised to process IL-1ß in these triple-knockout cells. Intriguingly, this work also showed that IAPs not only hold the canonical NLRP3/ASC/caspase-1 inflammasome in check in a RIPK3-dependent manner but also inhibit a RIPK3/caspase-8 ripoptosome-like complex that is also capable of processing pro-IL-1ß into the active and secreted mature form (Vince et al. 2012). Whether this apparent processing of pro-IL-1ß by a ripoptosome is a direct consequence of IAP depletion that occurs in parallel to inflammasome activation, or whether the two pathways are somehow connected sequentially, will be an interesting problem to solve. Certainly it is clear that

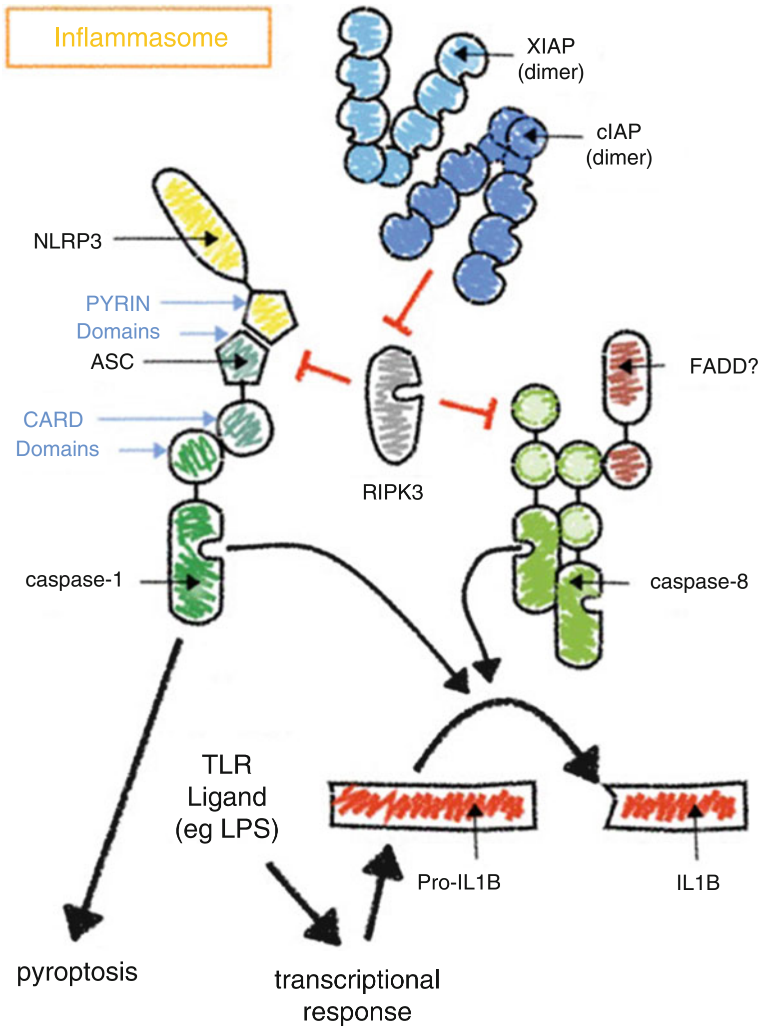

Fig. 4.4 Schematic of inflammasome components

caspase-8 processing of IL-1ß can occur physiologically (Miwa et al. 1998; Bossaller et al. 2012) and that this is not simply a smac-mimetic-induced artefact.

These results, while consistent with the systemic inflammatory phenotype of *Ciap1*$^{LysM\text{-}cre}$*Xiap*$^{-/-}$*Ciap2*$^{-/-}$X knockout mice that lack all three IAPs in their macrophage lineages (unpublished), are however controversial. Labbé et al. showed that cIAP 1 and cIAP 2 and TRAF2 associated with caspase-1 and that cIAP2 weakly ubiquitylated caspase-1. In their hands, BMDMs from cIAP2 mutant mice were defective in their response to a range of different inflammasome-activating stimuli. Furthermore cIAP1 or cIAP2 knockout mice or mice treated with the bivalent

smac-mimetic, BV6, were as defective as caspase-1 knockout mice in their response to inflammasome activators such as alum. Their data therefore are in direct contrast to those of Vince et al. and led these authors to propose that TRAF2, cIAP1 and cIAP2 together are required to ubiquitylate caspase-1 and this ubiquitylation is required for full caspase-1 activation. This model has some parallels with recent work showing that caspase-8 ubiquitylation by a CUL3/RBX ubiquitin E3 was required for full activation of caspase-8 by the death receptors DR4 and DR5 (Jin et al. 2009), but it is unknown how general this mechanism might be.

It is hard to reconcile such different conclusions: Labbé et al. state that IAPs are required to activate the inflammasome, while Vince et al. claim that IAPs are required to inhibit inflammasome activity. It is unlikely that the differences between the two groups are solely due to the different smac-mimetics used because each group also obtained different results with knockout cells and mice. One point worth bearing in mind is that the genotypes of the knockouts used were different. Labbé et al. used knockout mice derived from 129/Sv ES cells, while Vince et al.'s mice were from C57BL/6 ES cells. Embryonic stem cells derived from the 129/Sv mouse strain harbour a caspase-11-inactivating mutation, and mouse knockout lines derived from 129/Sv ES cells obtained by targeting genes closely linked to the caspase-11 locus might also be mutated for caspase-11 (Kayagaki et al. 2011). In the case of closely linked genes, such as caspase-1, this is despite extensive back-crossing of these animals to C57BL/6 mice. The cIAP locus is approximately 2.5 Mbp from the caspase-11 locus, and Kenneth et al. reported that the 129/Sv-generated cIAP1-deficient mice over several backcrosses have still not recombined with the C57BL/6 wild-type caspase-11 (Conze et al. 2005; Kenneth et al. 2012). These are the same strain of cIAP1 knockout mice used by Labbé et al. The cIAP2-deficient mice analysed by Kenneth et al. are also the same strain as those used by Labbé et al. (Conte et al. 2006) and are now carrying functional caspase-11 genes, although precisely when this occurred is unclear (Kenneth et al. 2012). However Labbé et al. observed the same defect in inflammasome activation in both cIAP1 and cIAP2 knockout mice indicating that differences in caspase-11 probably do not explain the differences between their work and that of Vince et al.

4.12 Physiological Role of IAPs: IAPs or Regulators of Cell Death

It is too late to rename the IAPs, but it should be clear from the foregoing that these proteins are not pure and simple apoptosis inhibitors. As discussed elsewhere (Silke and Brink 2010), the recent gene duplication of the cIAPs is probably a reflection of an extremely important function for these proteins in regulating TNFSF signalling and cell death. Their regulation of RIP kinase function and levels by ubiquitylation (Bertrand et al. 2011) is likely to be key to this function, but in this regard much remains to be discovered: How are cIAPs activated to ubiquitylate RIP kinases? Is it a mechanism analogous to smac-mimetic-induced activation? Does XIAP have

any role to play in this regulation? How do cIAPs create the correct ubiquitin chain needed for signalling when they are capable of generating all ubiquitin chain linkage types? How does ubiquitylation of RIPK1 affect complex 2 and 2b formation? What role does cIAP-dependent recruitment of LUBAC play in regulating complex 2 formation? Who knows, maybe we need more technological advances before we find out!

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References

Beg AA, Baltimore D (1996) An essential role for Nf-kappa-B in preventing Tnf-alpha-induced cell death. Science 274:782–784

Bergsbaken T, Fink SL, Cookson BT (2009) Pyroptosis: host cell death and inflammation. Nat Rev Microbiol 7:99–109

Bertrand MJ, Milutinovic S, Dickson KM, Ho WC et al (2008) cIAP1 and cIAP2 facilitate cancer cell survival by functioning as E3 ligases that promote RIP1 ubiquitination. Mol Cell 30:689–700

Bertrand MJ, Lippens S, Staes A, Gilbert B et al (2011) cIAP1/2 are direct E3 ligases conjugating diverse types of ubiquitin chains to receptor interacting proteins kinases 1 to 4 (RIP1-4). PLoS One 6:e22356

Birnbaum MJ, Clem RJ, Miller LK (1994) An apoptosis inhibiting gene from a nuclear polyhedrosis virus encoding a polypeptide with cys/his sequence motif. J Virol 68:2521–2528

Blackwell K, Zhang L, Workman LM, Ting AT et al (2013) Two coordinated mechanisms underlie tumor necrosis factor alpha-induced immediate and delayed IκB kinase activation. Mol Cell Biol 33:1901–1915

Blankenship JW, Varfolomeev E, Goncharov T, Fedorova AV et al (2009) Ubiquitin binding modulates IAP antagonist-stimulated proteasomal degradation of c-IAP1 and c-IAP2. Biochem J 417:149–160

Bossaller L, Chiang PI, Schmidt-Lauber C, Ganesan S et al (2012) Cutting edge: FAS (CD95) mediates noncanonical IL-1β and IL-18 maturation via caspase-8 in an RIP3-independent manner. J Immunol 189:5508–5512

Caserta TM, Smith AN, Gultice AD, Reedy MA, Brown TL (2003) Q-VD-OPh, a broad spectrum caspase inhibitor with potent antiapoptotic properties. Apoptosis 8:345–352

Chang DW, Xing Z, Capacio VL, Peter ME, Yang X (2003) Interdimer processing mechanism of procaspase-8 activation. EMBO J 22:4132–4142

Cho YS, Challa S, Moquin D, Genga R et al (2009) Phosphorylation-driven assembly of the RIP1-RIP3 complex regulates programmed necrosis and virus-induced inflammation. Cell 137:1112–1123

Conte D, Holcik M, Lefebvre CA, Lacasse E et al (2006) Inhibitor of apoptosis protein cIAP2 is essential for lipopolysaccharide-induced macrophage survival. Mol Cell Biol 26:699–708

Conze DB, Albert L, Ferrick DA, Goeddel DV et al (2005) Posttranscriptional downregulation of c-IAP2 by the ubiquitin protein ligase c-IAP1 in vivo. Mol Cell Biol 25:3348–3356

Crook NE, Clem RJ, Miller LK (1993) An apoptosis inhibiting baculovirus gene with a zinc finger like motif. J Virol 67:2168–2174

Darding M, Feltham R, Tenev T, Bianchi K et al (2011) Molecular determinants of Smac mimetic induced degradation of cIAP1 and cIAP2. Cell Death Differ 18:1376–1386

Degterev A, Huang Z, Boyce M, Li Y et al (2005) Chemical inhibitor of nonapoptotic cell death with therapeutic potential for ischemic brain injury. Nat Chem Biol 1:112–119

Degterev A, Hitomi J, Germscheid M, Ch'en IL et al (2008) Identification of RIP1 kinase as a specific cellular target of necrostatins. Nat Chem Biol 4:313–321

Deveraux QL, Takahashi R, Salvesen GS, Reed JC (1997) X-linked IAP is a direct inhibitor of cell-death proteases. Nature 388:300–304

Deveraux QL, Roy N, Stennicke HR, Van Arsdale T et al (1998) IAPs block apoptotic events induced by caspase-8 and cytochrome c by direct inhibition of distinct caspases. EMBO J 17:2215–2223

Dickens LS, Powley IR, Hughes MA, MacFarlane M (2012) The 'complexities' of life and death: death receptor signalling platforms. Exp Cell Res 318:1269–1277

Dillon CP, Oberst A, Weinlich R, Janke LJ et al (2012) Survival function of the FADD-CASPASE-8-cFLIP(L) complex. Cell Rep 1:401–407

Doi TS, Marino MW, Takahashi T, Yoshida T et al (1999) Absence of tumor necrosis factor rescues RelA-deficient mice from embryonic lethality. Proc Natl Acad Sci U S A 96:2994–2999

Du C, Fang M, Li Y, Li L, Wang X (2000) Smac, a mitochondrial protein that promotes cytochrome c-dependent caspase activation by eliminating IAP inhibition. Cell 102:33–42

Duckett CS, Nava VE, Gedrich RW, Clem RJ et al (1996) A conserved family of cellular genes related to the baculovirus iap gene and encoding apoptosis inhibitors. Embo J 15:2685–2694

Dueber EC, Schoeffler AJ, Lingel A, Elliott JM et al (2011) Antagonists induce a conformational change in cIAP1 that promotes autoubiquitination. Science 334:376–380

Dynek JN, Goncharov T, Dueber EC, Fedorova AV et al (2010) c-IAP1 and UbcH5 promote K11-linked polyubiquitination of RIP1 in TNF signalling. EMBO J 29:4198–4209

Ea CK, Deng L, Xia ZP, Pineda G, Chen ZJ (2006) Activation of IKK by TNFalpha requires site-specific ubiquitination of RIP1 and polyubiquitin binding by NEMO. Mol Cell 22:245–257

Ekert PG, Silke J, Hawkins CJ, Verhagen AM, Vaux DL (2001) DIABLO promotes apoptosis by removing MIHA/XIAP from processed caspase 9. J Cell Biol 152:483–490

Feltham R, Moulin M, Vince JE, Mace PD et al (2010) Tumor Necrosis Factor (TNF) Signaling, but Not TWEAK (TNF-like Weak Inducer of Apoptosis)-triggered cIAP1 (Cellular Inhibitor of Apoptosis Protein 1) Degradation, Requires cIAP1 RING Dimerization and E2 Binding. J Biol Chem 285:17525–17536

Feltham R, Bettjeman B, Budhidarmo R, Mace PD et al (2011) Smac mimetics activate the E3 ligase activity of cIAP1 protein by promoting RING domain dimerization. J Biol Chem 286:17015–17028

Feng S, Yang Y, Mei Y, Ma L et al (2007) Cleavage of RIP3 inactivates its caspase-independent apoptosis pathway by removal of kinase domain. Cell Signal 19:2056–2067

Feoktistova M, Geserick P, Kellert B, Dimitrova DP et al (2011) CIAPs block ripoptosome formation, a RIP1/caspase-8 containing intracellular cell death complex differentially regulated by cFLIP isoforms. Mol Cell 43:449–463

Fulda S, Wick W, Weller M, Debatin KM (2002) Smac agonists sensitize for Apo2L/TRAIL- or anticancer drug-induced apoptosis and induce regression of malignant glioma in vivo. Nat Med 8:808–815

Gaither A, Porter D, Yao Y, Borawski J et al (2007) A Smac mimetic rescue screen reveals roles for inhibitor of apoptosis proteins in tumor necrosis factor-alpha signaling. Cancer Res 67: 11493–11498

Garber K (2005) New apoptosis drugs face critical test. Nat Biotechnol 23:409–411

Gerlach B, Cordier SM, Schmukle AC, Emmerich CH et al (2011) Linear ubiquitination prevents inflammation and regulates immune signalling. Nature 471:591–596

Geserick P, Hupe M, Moulin M, Wong WW et al (2009) Cellular IAPs inhibit a cryptic CD95-induced cell death by limiting RIP1 kinase recruitment. J Cell Biol 187:1037–1054

Haas TL, Emmerich CH, Gerlach B, Schmukle AC et al (2009) Recruitment of the linear ubiquitin chain assembly complex stabilizes the TNF-R1 signaling complex and is required for TNF-mediated gene induction. Mol Cell 36:831–844

Harper N, Hughes M, MacFarlane M, Cohen GM (2003) Fas-associated death domain protein and caspase-8 are not recruited to the tumor necrosis factor receptor 1 signaling complex during tumor necrosis factor-induced apoptosis. J Biol Chem 278:25534–25541

He S, Wang L, Miao L, Wang T et al (2009) Receptor interacting protein kinase-3 determines cellular necrotic response to TNF-alpha. Cell 137:1100–1111

Hegde R, Srinivasula SM, Zhang Z, Wassell R et al (2001) Identification of Omi/HtrA2 as a mitochondrial apoptotic serine protease that disrupts inhibitor of apoptosis protein–caspase interaction. J Biol Chem 277:432–438

Hinds MG, Norton RS, Vaux DL, Day CL (1999) Solution structure of a baculoviral inhibitor of apoptosis (IAP) repeat. Nat Struct Biol 6:648–651

Holler N, Zaru R, Micheau O, Thome M et al (2000) Fas triggers an alternative, caspase-8-independent cell death pathway using the kinase RIP as effector molecule. Nat Immunol 1:489–495

Ikeda F, Deribe YL, Skånland SS, Stieglitz B et al (2011) SHARPIN forms a linear ubiquitin ligase complex regulating NF-κB activity and apoptosis. Nature 471:637–641

Jin Z, Li Y, Pitti R, Lawrence D et al (2009) Cullin3-based polyubiquitination and p62-dependent aggregation of caspase-8 mediate extrinsic apoptosis signaling. Cell 137:721–735

Kaiser WJ, Upton JW, Long AB, Livingston-Rosanoff D et al (2011) RIP3 mediates the embryonic lethality of caspase-8-deficient mice. Nature 471:368–372

Kang TB, Ben-Moshe T, Varfolomeev EE, Pewzner-Jung Y et al (2004) Caspase-8 serves both apoptotic and nonapoptotic roles. J Immunol 173:2976–2984

Kayagaki N, Warming S, Lamkanfi M, Vande Walle L et al (2011) Non-canonical inflammasome activation targets caspase-11. Nature 479:117–121

Kelliher MA, Grimm S, Ishida Y, Kuo F et al (1998) The death domain kinase RIP mediates the TNF-induced NF-kappaB signal. Immunity 8:297–303

Kenneth NS, Younger JM, Hughes ED, Marcotte D et al (2012) An inactivating caspase 11 passenger mutation originating from the 129 murine strain in mice targeted for c-IAP1. Biochem J 443:355–359

Kirisako T, Kamei K, Murata S, Kato M et al (2006) A ubiquitin ligase complex assembles linear polyubiquitin chains. EMBO J 25:4877–4887

Kischkel FC, Hellbardt S, Behrmann I, Germer M et al (1995) Cytotoxicity-dependent APO-1 (Fas/CD95)-associated proteins form a death-inducing signaling complex (DISC) with the receptor. Embo Journal 14:5579–5588

LaCasse EC, Baird S, Korneluk RG, MacKenzie AE (1998) The inhibitors of apoptosis (IAPs) and their emerging role in cancer. Oncogene 17:3247–3259

Lavrik IN, Krammer PH (2012) Regulation of CD95/Fas signaling at the DISC. Cell Death Differ 19:36–41

Lavrik IN, Mock T, Golks A, Hoffmann JC et al (2008) CD95 stimulation results in the formation of a novel death effector domain protein-containing complex. J Biol Chem 283:26401–26408

Li L, Thomas RM, Suzuki H, De Brabander JK et al (2004) A small molecule smac mimic potentiates TRAIL- and TNFα-mediated cell death. Science 305:1471–1474

Lin Y, Devin A, Rodriguez Y, Liu ZG (1999) Cleavage of the death domain kinase RIP by caspase-8 prompts TNF-induced apoptosis. Genes Dev 13:2514–2526

Liston P, Roy N, Tamai K, Lefebvre C et al (1996) Suppression of apoptosis in mammalian cells by naip and a related family of iap genes. Nature 379:349–353

Mahoney DJ, Cheung HH, Mrad RL, Plenchette S et al (2008) Both cIAP1 and cIAP2 regulate TNFalpha-mediated NF-kappaB activation. Proc Natl Acad Sci U S A 105:11778–11783

Martins LM, Iaccarino I, Tenev T, Gschmeissner S et al (2001) The serine protease Omi/HtrA2 regulates apoptosis by binding XIAP through a reaper-like motif. J Biol Chem 277:439–444

Micheau O, Tschopp J (2003) Induction of TNF receptor I-mediated apoptosis via two sequential signaling complexes. Cell 114:181–190

Micheau O, Lens S, Gaide O, Alevizopoulos K, Tschopp J (2001) NF-kappaB signals induce the expression of c-FLIP. Mol Cell Biol 21:5299–5305

Miwa K, Asano M, Horai R, Iwakura Y et al (1998) Caspase 1-independent IL-1beta release and inflammation induced by the apoptosis inducer Fas ligand. Nat Med 4:1287–1292

Moulin M, Anderton H, Voss AK, Thomas T et al (2012) IAPs limit activation of RIP kinases by TNF receptor 1 during development. EMBO J 31:1679–1691

Murphy JM, Czabotar PE, Hildebrand JM, Lucet IS et al (2013) The pseudokinase MLKL mediates necroptosis via a molecular switch mechanism. Immunity 39(3):443–453

Muzio M, Chinnaiyan AM, Kischkel FC, O'Rourke K et al (1996) FLICE, a novel FADD-homologous ICE/CED-3-like protease, is recruited to the CD95 (Fas/APO-1) death-inducing signaling complex. Cell 85:817–827

Nakajima A, Komazawa-Sakon S, Takekawa M, Sasazuki T et al (2006) An antiapoptotic protein, c-FLIPL, directly binds to MKK7 and inhibits the JNK pathway. EMBO J 25:5549–5559

Ndubaku C, Varfolomeev E, Wang L, Zobel K et al (2009) Antagonism of c-IAP and XIAP proteins is required for efficient induction of cell death by small-molecule IAP antagonists. ACS Chem Biol 4:557–566

Newton K, Sun X, Dixit VM (2004) Kinase RIP3 is dispensable for normal NF-κB signaling by the B-cell and T-cell receptors, tumor necrosis factor receptor 1 and Toll-like receptors 2 and 4. Mol Cell Biol 24:1464–1469

O'Donnell MA, Perez-Jimenez E, Oberst A, Ng A et al (2011) Caspase 8 inhibits programmed necrosis by processing CYLD. Nat Cell Biol 13:1437–1442

O'Reilly LA, Tai L, Lee L, Kruse EA et al (2009) Membrane-bound Fas ligand only is essential for Fas-induced apoptosis. Nature 461:659–663

Oberst A, Dillon CP, Weinlich R, McCormick LL et al (2011) Catalytic activity of the caspase-8-FLIP(L) complex inhibits RIPK3-dependent necrosis. Nature 471:363–367

Park SM, Yoon JB, Lee TH (2004) Receptor interacting protein is ubiquitinated by cellular inhibitor of apoptosis proteins (c-IAP1 and c-IAP2) in vitro. FEBS Lett 566:151–156

Peter ME, Budd RC, Desbarats J, Hedrick SM et al (2007) The CD95 receptor: apoptosis revisited. Cell 129:447–450

Pop C, Oberst A, Drag M, Van Raam BJ et al (2011) FLIP(L) induces caspase 8 activity in the absence of interdomain caspase 8 cleavage and alters substrate specificity. Biochem J 433: 447–457

Rothe M, Wong SC, Henzel WJ, Goeddel DV (1994) A novel family of putative signal transducers associated with the cytoplasmic domain of the 75 kda tumor necrosis factor receptor. Cell 78:681–692

Rothe M, Pan MG, Henzel WJ, Ayres TM, Goeddel DV (1995) The TNF-R2-TRAF signaling complex contains two novel proteins related to baculoviral-inhibitor of apoptosis proteins. Cell 83:1243–1252

Sagulenko V, Thygesen SJ, Sester DP, Idris A et al (2013) AIM2 and NLRP3 inflammasomes activate both apoptotic and pyroptotic death pathways via ASC. Cell Death Differ 20(9): 1149–1160

Schneider P (2008) Signaling by TNF and related ligands. In: Epstein C, Erickson R, Wynshaw-Boris A (eds.). Inborn errors of development. 2nd edition, Oxford monographs on medical genetics 54, Oxford University Press, Oxford, pp 433–441

Schneider-Brachert W, Tchikov V, Neumeyer J, Jakob M et al (2004) Compartmentalization of TNF receptor 1 signaling: internalized TNF receptosomes as death signaling vesicles. Immunity 21:415–428

Schütze S, Tchikov V, Schneider-Brachert W (2008) Regulation of TNFR1 and CD95 signalling by receptor compartmentalization. Nat Rev Mol Cell Biol 9:655–662

Silke J (2011) The regulation of TNF signalling: what a tangled web we weave. Curr Opin Immunol 23:620–626

Silke J, Brink R (2010) Regulation of TNFRSF and innate immune signalling complexes by TRAFs and cIAPs. Cell Death Differ 17:35–45

Silke J, Verhagen A, Ekert P, Vaux D (2000) Sequence as well as functional similarity for DIABLO/smac and grim, reaper and Hid ? Cell Death Diff 7:1275

Srinivasula SM, Hegde R, Saleh A, Datta P et al (2001) A conserved XIAP-interaction motif in caspase-9 and Smac/DIABLO regulates caspase activity and apoptosis. Nature 410:112–116
Sun L, Wang H, Wang Z, He S et al (2012) Mixed lineage kinase domain-like protein mediates necrosis signaling downstream of RIP3 kinase. Cell 148:213–227
Takahashi N, Duprez L, Grootjans S, Cauwels A et al (2012) Necrostatin-1 analogues: critical issues on the specificity, activity and in vivo use in experimental disease models. Cell Death Dis 3:e437
Tenev T, Bianchi K, Darding M, Broemer M et al (2011) The Ripoptosome, a signaling platform that assembles in response to genotoxic stress and loss of IAPs. Mol Cell 43:432–448
Tokunaga F, Sakata SI, Saeki Y, Satomi Y et al (2009) Involvement of linear polyubiquitylation of NEMO in NF-kappaB activation. Nat Cell Biol 11:123–132
Tokunaga F, Nakagawa T, Nakahara M, Saeki Y et al (2011) SHARPIN is a component of the NF-κB-activating linear ubiquitin chain assembly complex. Nature 471:633–636
Uren AG, Pakusch M, Hawkins CJ, Puls KL, Vaux DL (1996) Cloning and expression of apoptosis inhibitory protein homologs that function to inhibit apoptosis and/or bind TRAFs. Proc Natl Acad Sci U S A 93:4974–4978
Van Antwerp DJ, Martin SJ, Kafri T, Green DR, Verma IM (1996) Suppression of TNFα-induced apoptosis by NF-κB. Science 274:787–789
Vandenabeele P, Grootjans S, Callewaert N, Takahashi N (2013) Necrostatin-1 blocks both RIPK1 and IDO: consequences for the study of cell death in experimental disease models. Cell Death Differ 20:185–187
Vanlangenakker N, Vanden Berghe T, Bogaert P, Laukens B et al (2010) cIAP1 and TAK1 protect cells from TNF-induced necrosis by preventing RIP1/RIP3-dependent reactive oxygen species production. Cell Death Differ 18:656–665
Varfolomeev EE, Schuchmann M, Luria V, Chiannilkulchai N et al (1998) Targeted disruption of the mouse Caspase 8 gene ablates cell death induction by the TNF receptors, Fas/Apo1, and DR3 and is lethal prenatally. Immunity 9:267–276
Varfolomeev E, Maecker H, Sharp D, Lawrence D et al (2005) Molecular determinants of kinase pathway activation by Apo2 ligand/tumor necrosis factor-related apoptosis-inducing ligand. J Biol Chem 280:40599–40608
Varfolomeev E, Blankenship JW, Wayson SM, Fedorova AV et al (2007) IAP antagonists induce autoubiquitination of c-IAPs, NF-kappaB activation, and TNFalpha-dependent apoptosis. Cell 131:669–681
Varfolomeev E, Goncharov T, Fedorova AV, Dynek JN et al (2008) c-IAP1 and c-IAP2 are critical mediators of tumor necrosis factor alpha (TNFalpha)-induced NF-kappaB activation. J Biol Chem 283:24295–24299
Vaux DL, Silke J (2005) IAPs, RINGs and ubiquitylation. Nat Rev Mol Cell Biol 6:287–297
Vercammen D, Beyaert R, Denecker G, Goossens V et al (1998a) Inhibition of caspases increases the sensitivity of L929 cells to necrosis mediated by tumor necrosis factor. J Exp Med 187:1477–1485
Vercammen D, Brouckaert G, Denecker G, Van de Craen M et al (1998b) Dual signaling of the Fas receptor: initiation of both apoptotic and necrotic cell death pathways. J Exp Med 188: 919–930
Verhagen AM, Ekert PG, Pakusch M, Silke J et al (2000) Identification of DIABLO, a mammalian protein that promotes apoptosis by binding to and antagonizing IAP proteins. Cell 102:42–53
Verhagen AM, Silke J, Ekert PG, Pakusch M et al (2001) HtrA2 Promotes cell death through its serine protease activity and its ability to antagonize inhibitor of apoptosis proteins. J Biol Chem 277:445–454
Verhagen AM, Kratina TK, Hawkins CJ, Silke J et al (2007) Identification of mammalian mitochondrial proteins that interact with IAPs via N-terminal IAP binding motifs. Cell Death Diff 14:348–357
Vince JE, Wong WW, Khan N, Feltham R et al (2007) IAP antagonists target cIAP1 to induce TNFalpha-dependent apoptosis. Cell 131:682–693

Vince JE, Wong WW, Gentle I, Lawlor KE et al (2012) Inhibitor of apoptosis proteins limit RIP3 kinase-dependent interleukin-1 activation. Immunity 36:215–227
Wajant H, Scheurich P (2011) TNFR1-induced activation of the classical NF-κB pathway. FEBS J 278:862–876
Wang L, Du F, Wang X (2008) TNF-alpha induces two distinct caspase-8 activation pathways. Cell 133:693–703
Wang Z, Jiang H, Chen S, Du F, Wang X (2012) The mitochondrial phosphatase PGAM5 functions at the convergence point of multiple necrotic death pathways. Cell 148:228–243
Wertz IE, Dixit VM (2010) Regulation of death receptor signaling by the ubiquitin system. Cell Death Differ 17:14–24
Wong WW, Gentle IE, Nachbur U, Anderton H et al (2010) RIPK1 is not essential for TNFR1-induced activation of NF-kappaB. Cell Death Differ 17:482–487
Wright A, Reiley WW, Chang M, Jin W et al (2007) Regulation of early wave of germ cell apoptosis and spermatogenesis by deubiquitinating enzyme CYLD. Dev Cell 13:705–716
Wu G, Chai JJ, Suber TL, Wu JW et al (2000) Structural basis of IAP recognition by Smac/DIABLO. Nature 408:1008–1012
Wu CJ, Conze DB, Li T, Srinivasula SM, Ashwell JD (2006) Sensing of Lys 63-linked polyubiquitination by NEMO is a key event in NF-kappaB activation [corrected]. Nat Cell Biol 8:398–406
Yeh WC, Delapompa JL, Mccurrach ME, Shu HB et al (1998) Fadd—essential for embryo development and signaling from some, but not all, inducers of apoptosis. Science 279:1954–1958
Yeh WC, Itie A, Elia AJ, Ng M et al (2000) Requirement for Casper (c-FLIP) in regulation of death receptor-induced apoptosis and embryonic development. Immunity 12:633–642
Zhang DW, Shao J, Lin J, Zhang N et al (2009) RIP3, an energy metabolism regulator that switches TNF-induced cell death from apoptosis to necrosis. Science 325:332–336
Zhang H, Zhou X, McQuade T, Li J et al (2011) Functional complementation between FADD and RIP1 in embryos and lymphocytes. Nature 471:373–376
Zhao J, Jitkaew S, Cai Z, Choksi S et al (2012) Mixed lineage kinase domain-like is a key receptor interacting protein 3 downstream component of TNF-induced necrosis. Proc Natl Acad Sci U S A 109:5322–5327

Chapter 5
Regulation of Death Receptor-Induced Necroptosis by Ubiquitination

Maurice Darding and Henning Walczak

5.1 Introduction

Programmed cell death is critically important for the development and survival of multicellular organisms. Hence, deregulation of cell death processes lies at the root of many human diseases including neurodegenerative disorders, immunodeficiencies, autoimmune diseases and cancer. Apoptosis, the best studied form of programmed cell death, relies on signalling pathways leading to the activation of caspases, an evolutionarily conserved family of cysteine proteases. During apoptosis, caspases cleave a large number of substrates in the cell, facilitating efficient removal of the cellular corpse. In contrast, necrosis was long regarded as a form of accidental and unregulated cell death caused by physical stresses. Necrotic cell death occurs independently from caspases and lacks the morphological features that characterise apoptotic cells. However, there is a growing body of evidence that necrotic cell death can also occur in a regulated, programmed manner (Vandenabeele et al. 2010). This mode of cell death is referred to as programmed necrosis or necroptosis. Whereas apoptosis is generally thought of as an anti-inflammatory form of cell death, through the release of damage-associated molecular patterns (DAMPs), necrosis is thought to result in inflammation.

The first findings that indicated that necrosis can occur as a regulated process came from research on members of the tumour necrosis factor (TNF) superfamily (TNFSF) of cytokines. TNF, the first identified member of this family, is a pleiotropic cytokine involved in inflammatory responses, immunity and cell death (Balkwill 2009; Walczak 2011). TNFSF cytokines bind to and cross-link their cognate

M. Darding • H. Walczak (✉)
Centre for Cell Death, Cancer, and Inflammation (CCCI), UCL Cancer Institute,
University College London, London WC1E 6BT, UK
e-mail: h.walczak@ucl.ac.uk

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receptors, the members of the TNF-receptor superfamily (TNF-RSF). These single-pass membrane-spanning receptors are characterised by the presence of cysteine-rich domains in their extracellular portion. Death receptors (DR) constitute a subset of the TNF-RSF and are characterised by the presence of an intracellular death domain (DD). The DR subfamily includes TNF-R1, TRAMP (DR3), CD95 (Fas/APO-1), TRAIL-R1 (DR4) and TRAIL-R2 (KILLER/TRICK/DR5/Apo2) as bona fide members and DR6 as a less well-characterised member (Walczak 2013). DRs are able to mediate programmed cell death by apoptosis following ligation by their respective ligands, at least under certain conditions. The ligands of the bona fide members are TNF, TL1A, CD95L (FasL/APO-1L) and TNF-related apoptosis inducing ligand (TRAIL, also known as Apo2L), respectively. The signalling mechanisms induced by TNF, CD95L and TRAIL are best characterised. Whereas TNF can mediate its effects through binding to two different receptors, TNF-R1 and TNF-R2, of which only TNF-R1 contains a DD (Gray et al. 1984; Loetscher et al. 1990; Pennica et al. 1984; Schall et al. 1990; Smith et al. 1990), CD95L can induce cell death via CD95 (Itoh et al. 1991; Oehm et al. 1992) or be inhibited by the soluble receptor DcR3 (Pitti et al. 1998). TRAIL can induce apoptosis through binding to its DD-containing receptors TRAIL-R1 and TRAIL-R2 (Pan et al. 1997a, b; Screaton et al. 1997; Sheridan et al. 1997; Walczak et al. 1997; Wu et al. 1997). However, TRAIL can also bind to three other receptors, TRAIL-R3, TRAIL-R4 and osteoprotegerin (OPG), but these receptors lack a DD and, hence, do not trigger apoptosis (Walczak and Krammer 2000).

Apart from their ability to activate signalling pathways that result in apoptosis, DRs are also capable of gene activation and induction of pro-inflammatory cytokines. The intracellular signalling pathways involved include the NF-κB pathway and MAP kinase pathways (ERK and JNK pathways). Although the precise mechanisms are currently unclear, the relative balance between the pro-apoptotic and gene activation signals may be determined by several factors, including cell type, strength of ligand-induced signal and presence or absence of certain intracellular proteins that form part of the signalling complexes triggered by the different ligands. When the gene activation pathway dominates over the pro-apoptotic pathway, the consequence would be resistance to ligand-induced cell death.

TNF has long been known to also induce necrosis. In fact, when TNF was first identified, it was shown to selectively induce necrosis in the mouse fibrosarcoma cell line L929 (Carswell et al. 1975). Intriguingly, inhibition of caspases, the central executioners of apoptotic cell death, was found to exacerbate the necrosis-inducing capacity of TNF, and this observation has been extended to cell death induction by TRAIL and CD95L (Holler et al. 2000; Vercammen et al. 1998a, b). Hence, TNF, TRAIL and CD95L are not only able to induce apoptosis but also necrosis.

So why was the necrosis-inducing capacity of TNF and its siblings virtually ignored by the cell death research community during the past two decades? The reason for this is quite simple: apoptosis took centre stage and pushed necrosis off into a "Schattendasein". During this time there were some scattered reports about TNF-, TRAIL- or CD95L-induced non-apoptotic cell death being necrotic, and receptor-interacting protein 1 (RIP1) was the most likely candidate to be involved in

mediating this (Holler et al. 2000). However, it was not until 2008 that Degterev et al. discovered that TNF-induced necrosis can in fact be prevented pharmacologically, indeed by interfering with the kinase activity of RIP1 (Degterev et al. 2008). This discovery has reignited interest in studying DR-mediated necrosis induction because it suddenly appeared feasible to pharmacologically interfere with this type of cell death, i.e. a cell death that could not only not be inhibited by caspase inhibitors but was even exacerbated by caspase inhibition. Much hope was suddenly placed on specific inhibitors of this newly discovered form of programmed necrosis, also referred to as necroptosis, in regard to the possible treatment of several pathological conditions that involve cell death. Clearly, the feat now is to identify the components of and best pharmacological targets within the necroptosis signalling pathways, as well as the matching pathological conditions in which inhibition of programmed necrosis is likely to be beneficial. It is therefore important to gain a detailed understanding of the processes that lead to the triggering of necrosis, both in physiological situations and under pathological conditions, and the exact biochemical mechanisms of necroptosis induction.

Ubiquitination, i.e. the post-translation modification of target proteins by ubiquitin, is a central theme in DR signalling (Bianchi and Meier 2009; Walczak 2011; Wertz and Dixit 2010). In this chapter, we will focus on the role of ubiquitination and its reversal, de-ubiquitination, in DR-mediated signalling that results in necroptosis. Following an introduction into the ubiquitin system and an overview of DR signalling pathways, we will discuss how signalling towards necroptosis is mediated by DRs, and how ubiquitination and de-ubiquitination events in DR-triggered signalling complexes are currently thought to determine the balance between gene activation, apoptosis or necroptosis as outputs of DR triggering.

5.2 The Ubiquitin System

Ubiquitin is a small 8 kDa protein belonging to the family of ubiquitin-like (Ubl) proteins. Ubiquitination is important in a wide range of intracellular signalling processes and normally results in the covalent attachment of ubiquitin to lysine (K) residues of target proteins (Komander and Rape 2012). Ubiquitination involves the action of a three-step enzymatic cascade involving a ubiquitin-activating enzyme (E1), a ubiquitin-conjugating enzyme (E2) and a ubiquitin ligase (E3). In the first step, ubiquitin is activated in an ATP-dependent manner by forming a thioester bond with a cysteine residue in the E1 enzyme. Next, the ubiquitin is transferred from the E1 to the E2 enzyme, which can bind E3s. Finally, the E3, which recruits specific substrates, mediates the transfer of ubiquitin, usually via an isopeptide bond to the lysine residues of the target protein. Ubiquitin E3s can be divided into HECT- and RING-type E3s. In the case of HECT E3 ligases, ubiquitin is first transferred from the E2 to a catalytic cysteine residue of the HECT domain. This ubiquitin-thioester intermediate subsequently transfers ubiquitin to the substrate that is bound to the HECT E3. In contrast to HECT E3s, RING-domain E3s do not form a

ubiquitin-thioester intermediate and instead stimulate the direct transfer of ubiquitin from E2 to substrate. An exception to this are the RING E3s that harbour RING-in-between-RING domains (RBR) which were recently shown to function like RING/HECT hybrids whereby the first RING domain binds the E2 after which the ubiquitin moiety is first transferred to a cysteine in the second "RING" domain—which therefore acts as a HECT rather than a RING domain—before ubiquitination of the substrate (Stieglitz et al. 2012; Wenzel et al. 2011). The removal of ubiquitin moieties from protein substrates is catalysed by a diverse class of proteases termed de-ubiquitinases (DUBs).

When a single moiety of ubiquitin is covalently attached to a lysine residue of a target protein, this is referred to as mono-ubiquitination. Ubiquitin itself contains seven lysine residues (K6, K11, K27, K29, K33, K48 and K63), and these can in turn be conjugated with another ubiquitin molecule, allowing the formation of ubiquitin chains. Additionally, also the N-terminal methionine (M1) of ubiquitin can be conjugated with another ubiquitin, resulting in M1-linked chains (also called linear chains) (Kirisako et al. 2006; Rieser et al. 2013; Walczak 2011). All lysines and M1 of ubiquitin are present in different positions on the surface of the protein. Therefore, depending on which ubiquitin-contained lysine or whether M1 is used for ubiquitin chain formation, each type of ubiquitin linkage adopts a structurally distinct conformation (Komander 2009). The different ubiquitin linkages are connected to specific downstream signalling processes which are, in turn, translated into distinct cellular functions induced by specific proteins that are termed ubiquitin receptors. Ubiquitin receptors harbour ubiquitin-binding domains (UBDs) that allow them to recognise the different ubiquitin modifications through non-covalent interactions, in many cases in a linkage-type-specific manner (Dikic et al. 2009). Ubiquitination-dependent signalling is terminated by the action of DUBs that specifically hydrolyse ubiquitin chains, sometimes in a linkage-specific manner, and thereby counteract the action of E3 ligases.

5.3 Death Receptor Signalling

Two groups of DRs can be identified: those that recruit the adaptor protein Fas-associated death domain (FADD) (CD95, TRAIL-R1, TRAIL-R2) and those that recruit the adaptor protein TNFR-associated death domain (TRADD) (TNF-R1 and DR3). The primary signalling outcome for the FADD-recruiting receptors is cell death, whilst recruitment of TRADD results in gene activation as the primary signalling output (Fig. 5.1). Binding of TRAIL to TRAIL-R1 and/or TRAIL-R2, and of CD95L to CD95, results in the formation of the death-inducing signalling complex (DISC). First, the adaptor protein FADD is recruited to the intracellular portion of the receptor(s) whose cross-linking was triggered by the respective ligand. This happens through homotypic interaction between the DD of FADD and the DD of TRAIL-R1/R2 or that of CD95. In addition to a DD, FADD also contains a death

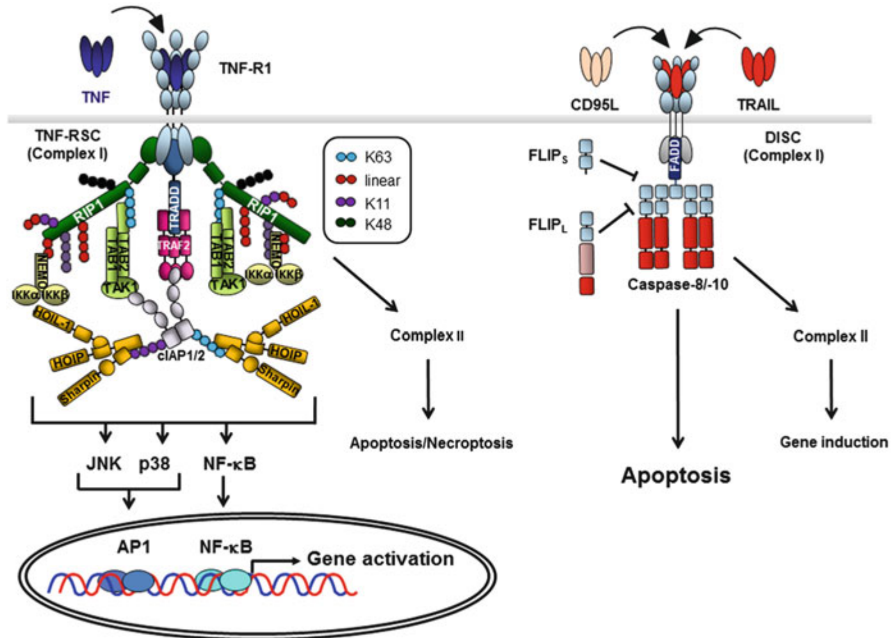

Fig. 5.1 Death receptor signalling. TNF-R1 signalling (*left*) and TRAIL-R1, TRAIL-R2 and CD95 signalling (*right*) are depicted. DRs trigger two main signals. The primary signalling output for TNF-R1 is gene activation via NF-κB and MAPK, whereas CD95, TRAIL-R1 and TRAIL-R2 primarily induce apoptosis. For all DR systems, the protein complex that forms at the plasma membrane and exerts the primary function of the respective receptor is defined as complex-I. Binding of TNF to its receptor TNF-R1 results in the formation of the TNF-R1 signalling complex (TNF-RSC). Cross-linked TNF-R1 recruits RIP1 and the adaptor protein TRADD to the DD of the receptor. Subsequently, TRADD recruits TRAF2, which in turn provides the platform for recruitment of the ubiquitin E3 ligases cIAP1 and cIAP2. cIAP1/2 then place various ubiquitin chains of different linkages on several components of the TNF-RSC. cIAP1/2-mediated ubiquitination of TNF-RSC components allows recruitment of LUBAC. Once recruited, LUBAC places M1-linked (linear) ubiquitin chains on RIP1 and NEMO. The different ubiquitin chains generated by cIAP1/2 and LUBAC in the TNF-RSC enable recruitment of the IKK and TAB/TAK complexes, which leads to downstream gene activation. Binding of TRAIL or CD95L to their cognate receptors leads to the formation of the death-inducing signalling complex (DISC). First, the adaptor protein FADD is recruited by a homotypic interaction of the DD of one FADD molecule with the DDs of three ligand-cross-linked receptors. Subsequently, the pro-forms of caspase-8 and caspase-10 are recruited by interaction of their DED with that of FADD. Oligomerisation of caspase-8/10 in the DISC leads to its activation and induction of apoptosis. FLIP isoforms can also be recruited to the DISC, resulting in inhibition of apoptosis by completely or partially preventing caspase-8 activation. The primary complexes can dissociate from the DD of the respective receptor and recruit additional proteins from the cytosol to form a secondary complex, also called complex-II, which triggers the respective secondary signal. In the case of TNF-R1, this can be the induction of necroptosis or apoptosis (see Fig. 5.2), whereas in the case of CD95 and TRAIL-R1/2, the secondary signal is gene activation

effector domain (DED) through which it recruits the two initiator caspases, caspase-8 and caspase-10. Recruitment of caspase-8/10 to FADD results in their activation at the DISC. Active caspase-8/10 subsequently undergoes a series of autocatalytic events, which separates the pro-domain from the caspase domains, and stabilises caspase-8/10 dimers. Subsequently, fully activated caspase-8 cleaves the downstream effector caspases caspase-3 and caspase-7, which results in apoptotic cell death.

Caspase-8-mediated apoptosis is negatively regulated at the DISC by the caspase-8-like molecule FLICE-like inhibitory protein (FLIP). FLIP can also be recruited to the DISC through binding to the DED of FADD. The initially discovered form of FLIP was a viral protein that was found to inhibit caspase-8 (Thome et al. 1997). Shortly thereafter, the cellular version of this protein was identified (also called cellular FLIP, cFLIP) (Irmler et al. 1997). In human cancer cells, two FLIP isoforms are prominently expressed: the long, 55 kDa ($FLIP_L$), and the short, 26 kDa ($FLIP_S$), isoforms (Budd et al. 2006). $FLIP_L$ resembles caspase-8 in its domain architecture but lacks a catalytic cysteine in the caspase-like domain necessary for enzymatic activity. Hence, $FLIP_L$ represents an inactive pseudo-caspase. $FLIP_S$ is highly homologous to viral FLIP and only encodes the pro-domain, which contains the DED domains necessary for FADD binding. Dimerisation of $FLIP_{L/S}$ with caspase-8 inhibits full caspase-8 activation and apoptosis. However, the $FLIP_S$ and $FLIP_L$ isoforms differentially regulate DR-induced necroptosis, as will be discussed in Sect. 5.4. Caspase-8 activation at the DISC can also be regulated by ubiquitination events. For instance, in DR signalling the E3 ligase Cullin-3 interacts with caspase-8 in the DISC and modifies it with K48- and K63-linked poly-ubiquitin chains. The ubiquitin receptor p62 is then recruited to poly-ubiquitinated caspase-8, which promotes its aggregation and activation (Jin et al. 2009). In addition, K48-linked poly-ubiquitination of caspase-8 was also reported to promote its degradation, thereby shutting off apoptotic signalling (Gonzalvez et al. 2012).

Unlike stimulation of the FADD-recruiting receptors, binding of TNF to TNF-R1 does not usually result in cell death but instead induces transcription of genes important for inflammation and cell survival. The TNF-induced signalling pathway that results in gene activation has been best described for the activation of NF-κB transcription factors. NF-κB family members (p65/RelA, c-REL, REL-B, NF- κB1 and NF-κB2) exist as homo- and heterodimers and regulate genes involved in inflammation and cell survival. NF-κB dimers can shuttle between nucleus and cytoplasm, but under resting conditions they are sequestered in the cytoplasm because they are bound to inhibitor of κB (IκB) proteins. Ligation of TNF-R1 by TNF results in the formation of a signalling complex that is referred to as the TNF-R1 signalling complex (TNF-RSC) or complex-I of TNF-R1 signalling (Micheau and Tschopp 2003) (Fig. 5.1). Both TRADD and RIP1 are independently recruited to the DD of TNF-R1 through their respective DDs. TRADD serves as a platform for the recruitment of TRAF2 and/or TRAF5. Whilst TRAF2 carries a RING domain, the role of TRAF2 as an E3 ligase in TNF signalling is controversial (Alvarez et al. 2010; Yin et al. 2009). Although recruitment of TRAF2 to complex-I is required for downstream signalling and gene activation, the RING of TRAF2 is

reportedly dispensable for TNF-mediated activation of NF-κB (Vince et al. 2009; Zhang et al. 2010). The importance of TRAF2 for TNF signalling can therefore be attributed to the fact that it serves as a platform for recruitment of the RING E3 ligases cIAP1 and cIAP2 to the TNF-RSC through its cIAP interaction motif (CIM) (Vince et al. 2009). The homologous proteins cIAP1 and cIAP2 are members of the inhibitor of apoptosis (IAP) protein family. It appears that they can, at least in part, compensate for the loss of each other. Nevertheless, subtle functional differences seem to exist between cIAP1 and cIAP2 (Darding et al. 2011; Feltham et al. 2011; Gyrd-Hansen et al. 2008) and it will be interesting to dissect this further. Members of the IAP family, such as XIAP in mammals, are classically known to inhibit apoptotic cell death by binding and inhibiting caspases directly. However, cIAPs do not inhibit caspases directly but instead are implicated in ubiquitin-dependent signalling pathways. Structural studies have demonstrated that the TRAF2-cIAP2 complex consists of a TRAF2 trimer binding to one cIAP2 molecule (Mace et al. 2010; Zheng et al. 2010). TRAF5 is also a RING-containing protein, yet it apparently cannot bind cIAPs which leaves its role in TNF signalling less clear than originally thought (Silke 2011). Following recruitment of cIAP1/2 to the TNF-RSC, cIAPs ubiquitinate various components of complex-I, most prominently RIP1 and cIAP1 itself, with K63- and K11-linked poly-ubiquitin chains (Bertrand et al. 2008; Dynek et al. 2010; Gerlach et al. 2011; Varfolomeev et al. 2008). cIAP1-mediated ubiquitination is critically important for TNF signalling as genetic or pharmacological depletion of cIAPs results in loss of ubiquitination of RIP1 and failure to activate NF-κB (Bertrand et al. 2008; Vince et al. 2007). cIAP1/2-generated ubiquitin linkages, placed on components of complex-I, allow the subsequent recruitment of the linear ubiquitin chain assembly complex (LUBAC) (Gerlach et al. 2011; Haas et al. 2009; Ikeda et al. 2011; Tokunaga et al. 2009, 2011). LUBAC is an E3 ligase complex that consists of two RBR proteins, HOIP (RNF31) and HOIL-1 (RBCK1), and one additional factor, SHARPIN (SIPL1). Currently, LUBAC is the only E3 ligase known to generate M1-ubiquitin linkages under physiological conditions. Moreover, LUBAC exclusively generates linear ubiquitin chains. HOIP is the central component of this complex as the formation of linear linkages depends on the enzymatic activity of the RBR of HOIP (Haas et al. 2009; Smit et al. 2012; Tokunaga et al. 2009). LUBAC is recruited to cIAP1/2-generated linkages by a UBD (a NZF2 domain) present in HOIP. Although cIAPs also contain a UBD, its role as a ubiquitin receptor in TNF signalling is not known (Gyrd-Hansen et al. 2008).

The ubiquitin linkages placed on RIP1—and possibly other components of the TNF-RSC—by cIAPs and LUBAC allow for more efficient recruitment and retention of the kinase complexes consisting of TAK1 and TAB2 or TAB3 and of NEMO, IKKα and IKKβ, i.e. the TAK/TAB and IκB kinase (IKK) complexes, respectively. In the native TNF-RSC, RIP1 is simultaneously modified with K11-, K48-, K63- and M1-linked chains (Gerlach et al. 2011). Recruitment of the TAK/TAB and IKK complexes is mediated by the UBDs present in TAB2 and NEMO, respectively. The ubiquitin binding in ABIN and NEMO (UBAN) domain of NEMO is able to bind to K63-, K11- and M1-linked ubiquitin chains, all of which are present in the native TNF-RSC (Dynek et al. 2010; Gerlach et al. 2011; Lo et al. 2009; Rahighi et al. 2009).

However, the affinity of NEMO's UBAN for M1-linked ubiquitins is 100-fold greater than for K63- and K11-linked ubiquitins. The UBD of TAB2, in turn, does not bind M1-linkages but specifically interacts with K63-linked ubiquitins (Komander et al. 2009). In addition to RIP1, LUBAC modifies NEMO with linear ubiquitin chains. In contrast to RIP1, on NEMO exclusively linear ubiquitin chains have been identified when present in the native TNF-RSC, although it is currently unclear what the functional role of this modification is (Gerlach et al. 2011). Following activation, IKKβ phosphorylates IκB which leads to recognition by the Skp1-Cullin-F-box (SCF)β-TRCP E3 ubiquitin ligase complex. SCFβ-TRCP ubiquitinates IκB with K48-linked chains, thereby targeting it for proteasomal degradation. This liberates NF-κB dimers from IκB-imposed inhibition so that they can now translocate to the nucleus and drive expression of NF-κB target genes.

TNF-induced NF-κB signalling is negatively regulated by its gene products. For instance, IκB is rapidly transcriptionally upregulated upon NF-κB activation. In addition, DUBs such as A20 and Cezanne are upregulated by NF-κB and mediate disassembly of complex-I by hydrolysing the ubiquitin modifications present in the complex. Other DUBs such as CYLD and USP21, whose expression does not dependent on NF-κB, are also implicated in negatively regulating TNF signalling. Although the specific roles of the individual DUBs in the disassembly of the TNF-RSC are not clear, together they achieve removal of the different ubiquitin linkages from the various components of the complex. Notably, RIP1 is usually almost completely stripped of its many ubiquitinations. It is likely that different DUBs are recruited to different ubiquitin linkages on different components of the complex, mediated by their own UBDs or UBDs present in their adaptor proteins. For instance, ABINs are adaptor proteins required for the recruitment of A20 to the TNF-RSC. ABINs contain a UBAN domain, allowing them to bind linear ubiquitin chains (Wagner et al. 2008).

Apart from being recruited to specific ubiquitin linkages, different DUBs appear to be specific in cleaving certain ubiquitin-linkage types. For instance, A20 has been reported to cleave K63-linked chains from RIP1, and subsequently targeting it for degradation, thereby terminating TNF signalling (Wertz et al. 2004). CYLD is able to cleave K63- and M1-linked ubiquitin chains whereas Cezanne reportedly specifically hydrolyses K11-linkages (Bremm et al. 2010; Komander et al. 2009). The targeted recruitment of specific DUBs to certain linkages and their preferences for catalysing specific linkage types probably serve to precisely fine-tune the extent and duration of a given signal.

Whilst gene activation is the primary signalling outcome of TNF stimulation, under certain circumstances TNF can also induce cell death. TNF-induced cell death is mediated by a secondary complex that forms approximately 2 h after TNF stimulation (Micheau and Tschopp 2003). This cytoplasmic complex, which derives from complex-I, is frequently referred to as complex-II. Complex-II forms when TRADD dissociates from the receptor and instead binds to FADD in the cytosol. FADD in turn can serve as a platform for the recruitment of caspase-8 and FLIP. When TNF successfully stimulates NF-κB-mediated expression of target genes, formation of complex-II does not lead to apoptosis because anti-apoptotic genes,

such as Bcl-2, cIAP2 and FLIP, are transcriptionally upregulated. Therefore, TNF only kills under conditions where expression of NF-κB target genes is blocked, such as upon genetic deletion of NF-κB, expression of a dominant negative form of IκB or in the presence of the protein synthesis inhibitor cycloheximide. In these cases, FLIP is not upregulated after TNF stimulation, allowing full caspase-8 activation in complex-II and induction of apoptosis. As will be discussed further in the next sections of this chapter, complex-II can also be formed when ubiquitination events in complex-I are perturbed, thereby altering the ubiquitination status of RIP1. Unmodified RIP1 can then bind to FADD and caspase-8 and stimulate their association, forming complex-II (Wang et al. 2008).

5.4 Necroptosis Signalling

As mentioned above, apart from gene activation and apoptosis, stimulation with death ligands can also lead to the induction of necroptosis, in particular in the presence of caspase inhibitors. As necroptosis has only recently been discovered, at present the necroptosis signalling pathway is not well understood but currently under intense investigation. Importantly, whilst RIP1 plays a central role in ubiquitin-mediated NF-κB signalling downstream of TNF-R1, RIP1 was also the first factor found to be required for death-ligand-induced necroptosis in the presence of caspase inhibition (Holler et al. 2000). Subsequently, the kinase activity of RIP1 was found to be the target of necrostatin-1, a chemical inhibitor of necroptosis (Degterev et al. 2008). In addition to the kinase activity of RIP1, TNF- and virus-induced necroptosis is dependent on the RIP1-related kinase RIP3 (Cho et al. 2009; He et al. 2009; Hitomi et al. 2008; Zhang et al. 2009). Whereas RIP1 is involved in the induction of apoptosis, necrosis and gene activation by TNF, RIP3 appears to play a more specific role as a key determinant of necroptosis. RIP3 and RIP1 are similar in structure as they both harbour an N-terminal kinase domain and can associate with each other through their respective C-terminal RIP homotypic interaction motif (RHIM) domains. However, RIP3 lacks the DD through which RIP1 is capable of interacting with other DD-containing proteins such as FADD, TRADD or TNF-R1 itself.

When caspase activity is inhibited, RIP1 in complex-II is now able to recruit RIP3 to form a pro-necroptotic signalling complex frequently referred to as the necrosome. The kinase activity of RIP1 is required for necrosome formation as this can be inhibited by necrostatin-1 (Cho et al. 2009). Both RIP1 and RIP3 are highly phosphorylated during necroptosis, which again can be inhibited by necrostatin-1, suggesting that RIP1 is the upstream kinase responsible for phosphorylation of its target RIP3 (Cho et al. 2009; Degterev et al. 2008). Phosphorylation of RIP3 on S227 is needed for recruitment of the mixed-lineage kinase-like (MLKL) protein, a pseudo-kinase that was recently identified as a crucial factor for necroptotic signalling downstream of RIP1 and RIP3 (Sun et al. 2012; Zhao et al. 2012). MLKL interacts with phosphorylated RIP3 through its C-terminal kinase-like domain

during necroptosis induction. Following recruitment, MLKL is phosphorylated by RIP3 at T357 and S358 (Chen et al. 2013; Sun et al. 2012). MLKL was identified as the target of the small molecule inhibitor of necroptosis necrosulfonamide (NSA) in a chemical library screen (Sun et al. 2012). NSA blocks necroptosis by covalently binding to Cys86 in the N-terminal coiled-coil (CC) domain of MLKL, indicating that this domain is important for MLKL's function. As this residue is not conserved in murine MLKL, NSA is unable to block necroptosis in mouse cells. One concept that has recently been put forward, but awaits confirmation, is that RIP1/RIP3/MLKL-containing necrosomes aggregate into amyloid-like structures, resulting in the manifestation of the physical features of necroptosis (Li et al. 2012). Although several other components have been implicated in the execution of necroptosis, the biochemical mechanism of necroptosis induction is not yet understood and formal genetic proof for the requirement of any factors for the execution of necroptosis, apart from RIP1, RIP3 and MLKL, is currently lacking.

Recent studies in mice deficient for key components of the apoptosis machinery provided insight on the mechanisms that regulate RIP1-/RIP3-dependent necroptosis (Dillon et al. 2012; Kaiser et al. 2011; Oberst et al. 2011; Zhang et al. 2011) (Fig. 5.2). At the same time, they provided an explanation for the puzzling observation that deficiency in caspase-8, FADD or FLIP results in an embryonically lethal phenotype that cannot be attributed to deregulated apoptosis. Since all of these three gene-deficient mice die at the same stage of embryonic development at around E10.5, displaying a phenotype that showed lack of vascularisation of the yolk sac (Sakamaki et al. 2002; Varfolomeev et al. 1998), it could be deduced that caspase-8, FADD and FLIP likely act in concert to prevent the death of a precursor cell of yolk sac vascularisation. Until recently it was, however, unclear whether this hypothesis was correct and, if so, what type of cell death the caspase-8/FLIP/FADD triad would be capable of inhibiting. Strikingly, normal development is completely restored in caspase-8- and FADD-deficient mice when RIP3 is absent (Kaiser et al. 2011; Oberst et al. 2011). In addition, the embryonic lethality of FADD-deficient mice is also rescued by the deletion of RIP1 (Zhang et al. 2011). However, similar to RIP1-deficient mice, these animals die shortly after birth. These studies revealed that, together, FADD, caspase-8 and FLIP antagonise RIP1-/RIP3-dependent necroptosis signalling.

What could be the explanation for these observations on the molecular level? The different FLIP isoforms appear to be the key determinants in deciding cell fate. Whilst $FLIP_S$ completely inhibits caspase-8 activation, counter-intuitively $FLIP_L$ actually appears to promote localised activation of caspase-8 (Boatright et al. 2004; Micheau et al. 2002; Oberst et al. 2011; Pop et al. 2011) (Fig. 5.2). $FLIP_L$-mediated activation of caspase-8 is achieved through dimerisation-induced conformational changes of the $FLIP_L$-caspase-8 heterodimer. Heterodimerisation of $FLIP_L$ and caspase-8 allows cleavage between their respective large and small subunits and, consequently, activation of the caspase-8 portion of the heterodimer. Although $FLIP_L$ thereby imparts catalytic activity to caspase-8, this activity is not sufficient to trigger cell death, because $FLIP_L$ prevents cleavage of the pro-domain of caspase-8. Moreover, it appears that $FLIP_L$-caspase-8 heterodimers display less proteolytic

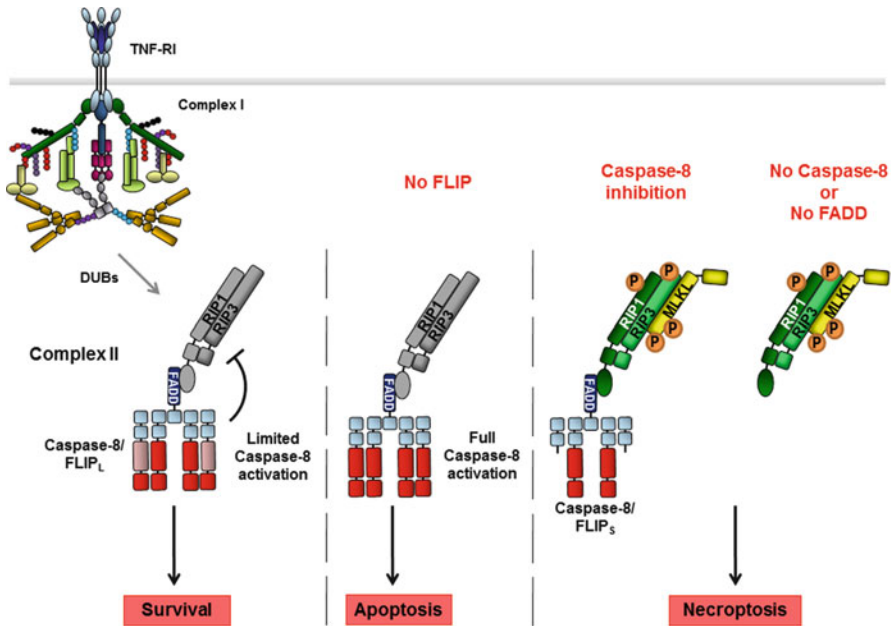

Fig. 5.2 Necroptosis signalling. The different cell death outcomes downstream of TNF-R1 are depicted. Following formation of the TNF-RSC (complex-I), components of this complex, notably TRADD and RIP1, can be released into the cytosol, forming a secondary complex referred to as complex-II. This process is most likely facilitated by the action of DUBs. Complex-II can recruit FADD, caspase-8/10, FLIP isoforms and RIP3, when expressed. The presence of FLIP isoforms in complex-II inhibits full caspase-8 activation and hence apoptosis. The caspase-8/FLIP$_L$ heterodimer also counteracts RIP1 and RIP3 signalling, thereby preventing necroptosis. When FLIP levels are low, for example, when the gene activation signals of complex-I are inhibited, caspase-8 activation is uncontrolled and leads to apoptosis. When caspase-8 activity is completely absent, for instance, by genetic deletion or by the presence of high levels of FLIP$_S$ in complex-II, RIP1 and RIP3 signalling is unchecked. The kinase activities of RIP1 and RIP3 allow their stable oligomerisation and recruitment and phosphorylation of MLKL, resulting in execution of necroptosis by a yet undefined downstream pathway

activity than caspase-8 homodimers on apoptotic substrates such as Bid and caspase-3 (Hughes et al. 2007; Pop et al. 2011). FADD, serving as the scaffold for caspase-8 and FLIP$_L$, is also able to bind RIP1. The current model is that the FADD/caspase-8/FLIP$_L$ complex allows localised caspase-8 activity that results in cleavage of RIP1 and RIP3 which, in turn, probably results in their inactivation (Feng et al. 2007; Oberst et al. 2011). Hence, FLIP$_L$ inhibits both caspase-8-mediated apoptosis and RIP1-/RIP3-dependent necroptosis (Fig. 5.2). In contrast, FLIP$_S$, which completely prevents catalytic activity of caspase-8, cannot inhibit RIP1/3-dependent necroptosis. Since FLIP$_L$ inhibits both apoptosis and necroptosis, in FLIP knockout mice both pathways need to be neutralised in order to rescue the lethal phenotype. Accordingly, embryonic lethality in mice lacking FLIP cannot be rescued by deletion of RIP3 because in the absence of FLIP, apoptotic cell death can occur in an

uncontrolled manner and, indeed, these embryos display apoptotic cell death (Dillon et al. 2012). Consistent with this notion, the deletion of FADD in addition to FLIP and RIP3 resulted in viable FLIP/FADD/RIP3 triple-knockout mice (Dillon et al. 2012). Thus, the components of the apoptotic machinery, FADD, caspase-8 and FLIP have a survival role by means of negatively regulating RIP1 and RIP3, thereby controlling aberrant necroptosis in the developing embryo.

Currently, it is not clear what the physiological roles of necroptosis are. Whereas RIP1-deficient mice die perinatally, both RIP3- and MLKL-deficient mice are healthy and do not display any remarkable developmental defects (Cho et al. 2009; He et al. 2009; Wu et al. 2013). Whilst these knockouts show that necroptosis is not required for normal development, they also provide invaluable means to study the pathophysiological role of necroptosis. Evidence is emerging that necroptosis is likely to play a crucial role in the host defence against viruses and other pathogens. As viruses commonly bear genes expressing apoptosis inhibitors, RIP3-dependent necrosis most likely serves as a second-line defence cell death mechanism to eliminate infected cells when apoptosis is inhibited. Indeed, although RIP3-deficient mice are developmentally normal, they are unable to control viral infections (Cho et al. 2009; Upton et al. 2010). The notion that necroptosis is a crucial component of anti-viral immunity is further supported by the recent finding that certain viruses also express necroptosis inhibitors, such as the cytomegalovirus protein M45/vIRA that binds and inhibits RIP3 (Upton et al. 2010). In addition, it has been suggested that necroptosis of infected cells enhances the inflammatory response since necroptotic cells release pro-inflammatory DAMPs (Weinlich et al. 2011).

5.5 Regulation of Necroptosis by Ubiquitination

As discussed above, ubiquitination events play an important role in TNF signalling. In fact, over the years the ubiquitin system has emerged as a key regulator of cell death and survival signalling. The first indications of the importance of ubiquitination came from studies on apoptotic signalling in *Drosophila melanogaster*. The drosophila IAPs, DIAP1 and DIAP2, limit caspase activation by modifying both initiator and effector caspases with degradative and non-degradative ubiquitin chains (Broemer and Meier 2009). In mammals, recent evidence suggests that IAPs can regulate caspase-8 activation directly and independently from DR signalling. Loss of IAPs, either due to genotoxic stress or pharmacological inhibition, results in spontaneous formation of a RIP1-containing cytoplasmic death-inducing complex dubbed the “Ripoptosome” (Feoktistova et al. 2011; Tenev et al. 2011). Similar to complex-II, the Ripoptosome contains the core components RIP1, caspase-8, FLIP and FADD and can stimulate caspase-8-dependent apoptosis as well as necroptosis when RIP3 is present. An important difference with complex-II is that the Ripoptosome is assembled in the cytosol independently from DR signalling. Most likely, cIAP1, cIAP2 and XIAP negatively regulate the Ripoptosome by targeting its components for ubiquitination and degradation.

Downstream of TNF-R1, the formation of ubiquitin linkages by cIAP1/2 plays an important role in regulating the signalling outcome. First of all, as outlined above, NF-κB activation downstream of TNF-R1 depends on various degradative and non-degradative ubiquitination events. In addition, ubiquitination of RIP1, and possibly other components of the TNF-RSC, by cIAPs and LUBAC, is important to actively protect cells against the lethal effects of TNF. The importance of IAPs in TNF signalling was manifested by the use of Smac mimetics (SMs). SMs are a class of small molecule drugs that mimic the N-terminal IAP-binding motif (IBM) of the mature form of the endogenous IAP antagonist Smac. Originally, these compounds were designed to cause apoptosis of cancer cells by antagonising XIAP and relieving caspases from its inhibition (Li et al. 2004; Schimmer et al. 2004). However, SM compounds exert their strongest effect on cIAP1 and cIAP2 by causing their proteasomal degradation (Petersen et al. 2007; Varfolomeev et al. 2007; Vince et al. 2007). These two cellular IAPs normally mediate the constitutive degradation of NF-κB-inducing kinase (NIK), a kinase that mediates non-canonical activation of NF-κB. Depletion of cIAPs by treatment with SMs results in stabilisation of NIK and spontaneous activation of non-canonical NF-κB signalling. In some cells, SM-mediated activation of NF-κB leads to induction of TNF, resulting in subsequent autocrine stimulation of TNF-R1. In the absence of cIAPs, cells are sensitised to the lethal effects of TNF since components of complex-I, in particular RIP1, are no longer ubiquitinated. The lack of ubiquitination in complex-I leads to the formation of the cytoplasmic complex-II, which is dependent on RIP1 (Petersen et al. 2007; Wang et al. 2008). RIP1 promotes the association between caspase-8 and FADD in complex-II, resulting in caspase-8 activation and induction of apoptosis (Fig. 5.2). When RIP3 is present, however, it can also be recruited to RIP1 in complex-II; when, in addition, caspase-8 is inhibited, the combination of SM treatment and TNF stimulation can result in necroptosis.

Apart from the E3 ligase activity of cIAPs, linear ubiquitination of components of complex-I by LUBAC is also required to protect cells against the lethal effects of TNF. Interestingly, mice carrying a mutation in the LUBAC component SHARPIN, which results in loss of SHARPIN expression, develop chronic proliferative dermatitis (*cpdm*) (Seymour et al. 2007). Whilst gene induction by TNF and other inflammatory ligands is attenuated, *cpdm* cells are sensitised to TNF-induced cell death (Gerlach et al. 2011; Ikeda et al. 2011; Tokunaga et al. 2011). The observation that TNF stimulation results in aberrant death of *cpdm*-derived cells and that this cell death is both apoptotic and necroptotic—and hence possibly inflammatory—inspired the cross between TNF-deficient and *cpdm* mice, with the result that the formation of inflammatory lesions that characterises *cpdm* mice was completely prevented in the absence of TNF (Gerlach et al. 2011). These data strongly support a model according to which TNF-induced cell death, the only TNF signal that was increased in cells derived from SHARPIN-deficient *cpdm* mice, is causative for inflammation in *cpdm* mice. It remains to be shown, however, to which extent TNF-induced necroptosis and/or apoptosis contribute to the *cpdm*-characterising inflammation and whether, together, these two forms of cell death might be required and sufficient for it to occur.

Apart from E3 ligases, DUBs also play an important role in the regulation of DR-mediated necroptosis. CYLD is recruited to the TNF-R1 signalling complex where it targets ubiquitin linkages on RIP1 (Wright et al. 2007). CYLD has been identified in a genome-wide siRNA screen in the mouse cell line L929 to be required for efficient induction of TNF-induced necroptosis (Hitomi et al. 2008). Subsequently, several other studies have confirmed the role of CYLD in necroptosis as well as apoptosis (Bonnet et al. 2011; O'Donnell et al. 2011; Welz et al. 2011). Similar to RIP1 and RIP3, CYLD is also being cleaved by caspase-8 after DR stimulation. This inactivates CYLD and is another mechanism by which caspase-8 suppresses necroptosis (O'Donnell et al. 2011). The finding that CYLD appears to be capable of not only cleaving K63- but also M1-linked ubiquitin chains (Komander et al. 2009) strengthens the notion that linear ubiquitination by LUBAC prevents RIP1 from inducing cell death. Recently, the novel DUB FAM105B/OTULIN/Gumby has been identified as specifically hydrolysing linear ubiquitin linkages, thereby antagonising LUBAC activity (Keusekotten et al. 2013; Rivkin et al. 2013).

Detailed investigations will be required to understand the exact mechanisms by which cIAPs, LUBAC, OTULIN and CYLD, and most likely other E3 ligases and DUBs, regulate the balance between the gene-activatory, apoptotic and necroptotic outcomes of signalling downstream of death receptors. This will not only be important downstream of TNF-R1 but also of TRAIL-R1/2 and CD95, as the necroptotic signalling arm and the role of ubiquitination downstream of these receptors is less well understood. Moreover, the elucidation of hitherto unknown components and regulators of the necroptosis pathway beyond the ubiquitin system will clearly be the focus of future studies. As necroptosis is being reported to be involved in a wider range of pathologies, it will be crucial to completely dissect the signalling pathways that results in necroptosis. Evidently, the understanding of the exact molecular mechanism underlying necroptosis will lead to the identification of novel therapeutic targets and opportunities.

References

Alvarez SE, Harikumar KB, Hait NC, Allegood J, Strub GM, Kim EY, Maceyka M, Jiang H, Luo C, Kordula T et al (2010) Sphingosine-1-phosphate is a missing cofactor for the E3 ubiquitin ligase TRAF2. Nature 465:1084–1088

Balkwill F (2009) Tumour necrosis factor and cancer. Nat Rev Cancer 9:361–371

Bertrand M, Milutinovic S, Dickson K, Ho W, Boudreault A, Durkin J, Gillard J, Jaquith J, Morris S, Barker P (2008) cIAP1 and cIAP2 Facilitate Cancer Cell Survival by Functioning as E3 Ligases that Promote RIP1 Ubiquitination. Mol Cell 30:689–700

Bianchi K, Meier P (2009) A tangled web of ubiquitin chains: breaking news in TNF-R1 signaling. Mol Cell 36:736–742

Boatright KM, Deis C, Denault JB, Sutherlin DP, Salvesen GS (2004) Activation of caspases-8 and −10 by FLIP(L). Biochem J 382:651–657

Bonnet MC, Preukschat D, Welz PS, van Loo G, Ermolaeva MA, Bloch W, Haase I, Pasparakis M (2011) The adaptor protein FADD protects epidermal keratinocytes from necroptosis in vivo and prevents skin inflammation. Immunity 35:572–582

Bremm A, Freund SM, Komander D (2010) Lys11-linked ubiquitin chains adopt compact conformations and are preferentially hydrolyzed by the deubiquitinase Cezanne. Nat Struct Mol Biol 17:939–947

Broemer M, Meier P (2009) Ubiquitin-mediated regulation of apoptosis. Trends Cell Biol 19:130–140

Budd RC, Yeh W-C, Tschopp J (2006) cFLIP regulation of lymphocyte activation and development. Nat Rev Immunol 6:196–204

Carswell EA, Old LJ, Kassel RL, Green S, Fiore N, Williamson B (1975) An endotoxin-induced serum factor that causes necrosis of tumors. Proc Natl Acad Sci U S A 72:3666–3670

Chen W, Zhou Z, Li L, Zhong CQ, Zheng X, Wu X, Zhang Y, Ma H, Huang D, Li W et al (2013) Diverse Sequence Determinants Control Human and Mouse Receptor Interacting Protein 3 (RIP3) and Mixed Lineage Kinase domain-Like (MLKL) Interaction in Necroptotic Signaling. J Biol Chem 288(23):16247–16261

Cho YS, Challa S, Moquin D, Genga R, Ray TD, Guildford M, Chan FK-M (2009) Phosphorylation-driven assembly of the RIP1-RIP3 complex regulates programmed necrosis and virus-induced inflammation. Cell 137:1112–1123

Darding M, Feltham R, Tenev T, Bianchi K, Benetatos C, Silke J, Meier P (2011) Molecular determinants of Smac mimetic induced degradation of cIAP1 and cIAP2. Cell Death Differ 18(8):1376–1386

Degterev A, Hitomi J, Germscheid M, Ch'en IL, Korkina O, Teng X, Abbott D, Cuny GD, Yuan C, Wagner G et al (2008) Identification of RIP1 kinase as a specific cellular target of necrostatins. Nat Chem Biol 4:313–321

Dikic I, Wakatsuki S, Walters KJ (2009) Ubiquitin-binding domains—from structures to functions. Nat Rev Mol Cell Biol 10:659–671

Dillon CP, Oberst A, Weinlich R, Janke LJ, Kang TB, Ben-Moshe T, Mak TW, Wallach D, Green DR (2012) Survival function of the FADD-CASPASE-8-cFLIP(L) complex. Cell Rep 1:401–407

Dynek JN, Goncharov T, Dueber EC, Fedorova AV, Izrael-Tomasevic A, Phu L, Helgason E, Fairbrother WJ, Deshayes K, Kirkpatrick DS et al (2010) c-IAP1 and UbcH5 promote K11-linked polyubiquitination of RIP1 in TNF signalling. EMBO J 29:4198–4209

Feltham R, Bettjeman B, Budhidarmo R, Mace PD, Shirley S, Condon SM, Chunduru SK, McKinlay MA, Vaux DL, Silke J et al (2011) Smac mimetics activate the E3 ligase activity of cIAP1 protein by promoting RING domain dimerization. J Biol Chem 286:17015–17028

Feng S, Yang Y, Mei Y, Ma L, Zhu DE, Hoti N, Castanares M, Wu M (2007) Cleavage of RIP3 inactivates its caspase-independent apoptosis pathway by removal of kinase domain. Cellular Signalling 19:2056–2067

Feoktistova M, Geserick P, Kellert B, Dimitrova DP, Langlais C, Hupe M, Cain K, MacFarlane M, Hacker G, Leverkus M (2011) cIAPs block Ripoptosome formation, a RIP1/caspase-8 containing intracellular cell death complex differentially regulated by cFLIP isoforms. Mol Cell 43: 449–463

Gerlach B, Cordier SM, Schmukle AC, Emmerich CH, Rieser E, Haas TL, Webb AI, Rickard JA, Anderton H, Wong WW et al (2011) Linear ubiquitination prevents inflammation and regulates immune signalling. Nature 471:591–596

Gonzalvez F, Lawrence D, Yang B, Yee S, Pitti R, Marsters S, Pham VC, Stephan JP, Lill J, Ashkenazi A (2012) TRAF2 Sets a threshold for extrinsic apoptosis by tagging caspase-8 with a ubiquitin shutoff timer. Mol Cell 48:888–899

Gray PW, Aggarwal BB, Benton CV, Bringman TS, Henzel WJ, Jarrett JA, Leung DW, Moffat B, Ng P, Svedersky LP et al (1984) Cloning and expression of cDNA for human lymphotoxin, a lymphokine with tumour necrosis activity. Nature 312:721–724

Gyrd-Hansen M, Darding M, Miasari M, Santoro MM, Zender L, Xue W, Tenev T, Fonseca PCAD, Zvelebil M, Bujnicki JM et al (2008) IAPs contain an evolutionarily conserved ubiquitin-binding domain that regulates NF-kappaB as well as cell survival and oncogenesis. Nat Cell Biol 10(11):1309–1317

Haas TL, Emmerich CH, Gerlach B, Schmukle AC, Cordier SM, Rieser E, Feltham R, Vince J, Warnken U, Wenger T et al (2009) Recruitment of the linear ubiquitin chain assembly complex stabilizes the TNF-R1 signaling complex and is required for TNF-mediated gene induction. Mol Cell 36:831–844

He S, Wang L, Miao L, Wang T, Du F, Zhao L, Wang X (2009) Receptor interacting protein kinase-3 determines cellular necrotic response to TNF-alpha. Cell 137:1100–1111

Hitomi J, Christofferson D, Ng A, Yao J, Degterev A, Xavier R, Yuan J (2008) Identification of a molecular signaling network that regulates a cellular necrotic cell death pathway. Cell 135:1311–1323

Holler N, Zaru R, Micheau O, Thome M, Attinger A, Valitutti S, Bodmer JL, Schneider P, Seed B, Tschopp J (2000) Fas triggers an alternative, caspase-8-independent cell death pathway using the kinase RIP as effector molecule. Nat Immunol 1:489–495

Hughes DA, Ginsberg L, Baker R, Goodwin S, Milligan A, Richfield L, Mehta AB (2007) Effective treatment of an elderly patient with Gaucher's disease and Parkinsonism: a case report of 24 months' oral substrate reduction therapy with miglustat. Parkinson Relat Dis 13:365–368

Ikeda F, Deribe YL, Skanland SS, Stieglitz B, Grabbe C, Franz-Wachtel M, van Wijk SJ, Goswami P, Nagy V, Terzic J et al (2011) SHARPIN forms a linear ubiquitin ligase complex regulating NF-kappaB activity and apoptosis. Nature 471:637–641

Irmler M, Thome M, Hahne M, Schneider P, Hofmann K, Steiner V, Bodmer JL, Schroter M, Burns K, Mattmann C et al (1997) Inhibition of death receptor signals by cellular FLIP. Nature 388:190–195

Itoh N, Yonehara S, Ishii A, Yonehara M, Mizushima S, Sameshima M, Hase A, Seto Y, Nagata S (1991) The polypeptide encoded by the cDNA for human cell surface antigen Fas can mediate apoptosis. Cell 66:233–243

Jin Z, Li Y, Pitti R, Lawrence D, Pham VC, Lill JR, Ashkenazi A (2009) Cullin3-based polyubiquitination and p62-dependent aggregation of caspase-8 mediate extrinsic apoptosis signaling. Cell 137:721–735

Kaiser WJ, Upton JW, Long AB, Livingston-Rosanoff D, Daley-Bauer LP, Hakem R, Caspary T, Mocarski ES (2011) RIP3 mediates the embryonic lethality of caspase-8-deficient mice. Nature 471:368–372

Keusekotten K, Elliott PR, Glockner L, Fiil BK, Damgaard RB, Kulathu Y, Wauer T, Hospenthal MK, Gyrd-Hansen M, Krappmann D et al (2013) OTULIN antagonizes LUBAC signaling by specifically hydrolyzing Met1-linked polyubiquitin. Cell 153:1312–1326

Kirisako T, Kamei K, Murata S, Kato M, Fukumoto H, Kanie M, Sano S, Tokunaga F, Tanaka K, Iwai K (2006) A ubiquitin ligase complex assembles linear polyubiquitin chains. EMBO J 25:4877–4887

Komander D (2009) The emerging complexity of protein ubiquitination. Biochem Soc Trans 37:937–953

Komander D, Rape M (2012) The ubiquitin code. Ann Rev Biochem 81:203–229

Komander D, Reyes-Turcu F, Licchesi JD, Odenwaelder P, Wilkinson KD, Barford D (2009) Molecular discrimination of structurally equivalent Lys 63-linked and linear polyubiquitin chains. EMBO Rep 10:466–473

Li J, McQuade T, Siemer AB, Napetschnig J, Moriwaki K, Hsiao YS, Damko E, Moquin D, Walz T, McDermott A et al (2012) The RIP1/RIP3 necrosome forms a functional amyloid signaling complex required for programmed necrosis. Cell 150:339–350

Li L, Thomas RM, Suzuki H, De Brabander JK, Wang X, Harran PG (2004) A small molecule Smac mimic potentiates TRAIL- and TNFalpha-mediated cell death. Science 305:1471–1474

Lo YC, Lin SC, Rospigliosi CC, Conze DB, Wu CJ, Ashwell JD, Eliezer D, Wu H (2009) Structural basis for recognition of diubiquitins by NEMO. Mol Cell 33:602–615

Loetscher H, Pan YC, Lahm HW, Gentz R, Brockhaus M, Tabuchi H, Lesslauer W (1990) Molecular cloning and expression of the human 55 kd tumor necrosis factor receptor. Cell 61:351–359

Mace PD, Smits C, Vaux DL, Silke J, Day CL (2010) Asymmetric recruitment of cIAPs by TRAF2. J Mol Biol 400:8–15

Micheau O, Thome M, Schneider P, Holler N, Tschopp J, Nicholson DW, Briand C, Grutter MG (2002) The long form of FLIP is an activator of caspase-8 at the Fas death-inducing signaling complex. J Biol Chem 277:45162–45171

Micheau O, Tschopp J (2003) Induction of TNF receptor I-mediated apoptosis via two sequential signaling complexes. Cell 114:181–190

O'Donnell MA, Perez-Jimenez E, Oberst A, Ng A, Massoumi R, Xavier R, Green DR, Ting AT (2011) Caspase 8 inhibits programmed necrosis by processing CYLD. Nat Cell Biol 13:1437–1442

Oberst A, Dillon CP, Weinlich R, McCormick LL, Fitzgerald P, Pop C, Hakem R, Salvesen GS, Green DR (2011) Catalytic activity of the caspase-8-FLIP(L) complex inhibits RIPK3-dependent necrosis. Nature 471:363–367

Oehm A, Behrmann I, Falk W, Pawlita M, Maier G, Klas C, Li-Weber M, Richards S, Dhein J, Trauth BC et al (1992) Purification and molecular cloning of the APO-1 cell surface antigen, a member of the tumor necrosis factor/nerve growth factor receptor superfamily. Sequence identity with the Fas antigen. J Biol Chem 267:10709–10715

Pan G, Ni J, Wei YF, Yu G, Gentz R, Dixit VM (1997a) An antagonist decoy receptor and a death domain-containing receptor for TRAIL. Science 277:815–818

Pan G, O'Rourke K, Chinnaiyan AM, Gentz R, Ebner R, Ni J, Dixit VM (1997b) The receptor for the cytotoxic ligand TRAIL. Science 276:111–113

Pennica D, Nedwin GE, Hayflick JS, Seeburg PH, Derynck R, Palladino MA, Kohr WJ, Aggarwal BB, Goeddel DV (1984) Human tumour necrosis factor: precursor structure, expression and homology to lymphotoxin. Nature 312:724–729

Petersen S, Wang L, Yalcin-Chin A, Li L, Peyton M, Minna J, Harran P, Wang X (2007) Autocrine TNFalpha Signaling Renders Human Cancer Cells Susceptible to Smac-Mimetic-Induced Apoptosis. Cancer Cell 12:445–456

Pitti RM, Marsters SA, Lawrence DA, Roy M, Kischkel FC, Dowd P, Huang A, Donahue CJ, Sherwood SW, Baldwin DT et al (1998) Genomic amplification of a decoy receptor for Fas ligand in lung and colon cancer. Nature 396:699–703

Pop C, Oberst A, Drag M, Van Raam BJ, Riedl SJ, Green DR, Salvesen GS (2011) FLIP(L) induces caspase 8 activity in the absence of interdomain caspase 8 cleavage and alters substrate specificity. Biochem J 433:447–457

Rahighi S, Ikeda F, Kawasaki M, Akutsu M, Suzuki N, Kato R, Kensche T, Uejima T, Bloor S, Komander D et al (2009) Specific recognition of linear ubiquitin chains by NEMO is important for NF-kappaB activation. Cell 136:1098–1109

Rieser E, Cordier SM, Walczak H (2013) Linear ubiquitination: a newly discovered regulator of cell signalling. Trends Biochem Sci 38:94–102

Rivkin E, Almeida SM, Ceccarelli DF, Juang YC, MacLean TA, Srikumar T, Huang H, Dunham WH, Fukumura R, Xie G et al (2013) The linear ubiquitin-specific deubiquitinase gumby regulates angiogenesis. Nature 498:318–324

Sakamaki K, Inoue T, Asano M, Sudo K, Kazama H, Sakagami J, Sakata S, Ozaki M, Nakamura S, Toyokuni S et al (2002) Ex vivo whole-embryo culture of caspase-8-deficient embryos normalize their aberrant phenotypes in the developing neural tube and heart. Cell Death Differ 9:1196–1206

Schall TJ, Lewis M, Koller KJ, Lee A, Rice GC, Wong GH, Gatanaga T, Granger GA, Lentz R, Raab H et al (1990) Molecular cloning and expression of a receptor for human tumor necrosis factor. Cell 61:361–370

Schimmer AD, Welsh K, Pinilla C, Wang Z, Krajewska M, Bonneau MJ, Pedersen IM, Kitada S, Scott FL, Bailly-Maitre B et al (2004) Small-molecule antagonists of apoptosis suppressor XIAP exhibit broad antitumor activity. Cancer Cell 5:25–35

Screaton GR, Mongkolsapaya J, Xu XN, Cowper AE, McMichael AJ, Bell JI (1997) TRICK2, a new alternatively spliced receptor that transduces the cytotoxic signal from TRAIL. Curr Biol 7:693–696

Seymour RE, Hasham MG, Cox GA, Shultz LD, Hogenesch H, Roopenian DC, Sundberg JP (2007) Spontaneous mutations in the mouse Sharpin gene result in multiorgan inflammation, immune system dysregulation and dermatitis. Genes Immun 8:416–421

Sheridan JP, Marsters SA, Pitti RM, Gurney A, Skubatch M, Baldwin D, Ramakrishnan L, Gray CL, Baker K, Wood WI et al (1997) Control of TRAIL-induced apoptosis by a family of signaling and decoy receptors. Science 277:818–821

Silke J (2011) The regulation of TNF signalling: what a tangled web we weave. Curr Opin Immunol 23:620–626

Smit JJ, Monteferrario D, Noordermeer SM, van Dijk WJ, van der Reijden BA, Sixma TK (2012) The E3 ligase HOIP specifies linear ubiquitin chain assembly through its RING-IBR-RING domain and the unique LDD extension. EMBO J 31:3833–3844

Smith CA, Davis T, Anderson D, Solam L, Beckmann MP, Jerzy R, Dower SK, Cosman D, Goodwin RG (1990) A receptor for tumor necrosis factor defines an unusual family of cellular and viral proteins. Science 248:1019–1023

Stieglitz B, Morris-Davies AC, Koliopoulos MG, Christodoulou E, Rittinger K (2012) LUBAC synthesizes linear ubiquitin chains via a thioester intermediate. EMBO Rep 13:840–846

Sun L, Wang H, Wang Z, He S, Chen S, Liao D, Wang L, Yan J, Liu W, Lei X et al (2012) Mixed lineage kinase domain-like protein mediates necrosis signaling downstream of RIP3 kinase. Cell 148:213–227

Tenev T, Bianchi K, Darding M, Broemer M, Langlais C, Wallberg F, Zachariou A, Lopez J, MacFarlane M, Cain K et al (2011) The Ripoptosome, a signaling platform that assembles in response to genotoxic stress and loss of IAPs. Mol Cell 43:432–448

Thome M, Schneider P, Hofmann K, Fickenscher H, Meinl E, Neipel F, Mattmann C, Burns K, Bodmer JL, Schroter M et al (1997) Viral FLICE-inhibitory proteins (FLIPs) prevent apoptosis induced by death receptors. Nature 386:517–521

Tokunaga F, Nakagawa T, Nakahara M, Saeki Y, Taniguchi M, Sakata S, Tanaka K, Nakano H, Iwai K (2011) SHARPIN is a component of the NF-kappaB-activating linear ubiquitin chain assembly complex. Nature 471:633–636

Tokunaga F, Sakata S, Saeki Y, Satomi Y, Kirisako T, Kamei K, Nakagawa T, Kato M, Murata S, Yamaoka S et al (2009) Involvement of linear polyubiquitylation of NEMO in NF-kappaB activation. Nat Cell Biol 11(2):123–132

Upton JW, Kaiser WJ, Mocarski ES (2010) Virus inhibition of RIP3-dependent necrosis. Cell Host Microbe 7:302–313

Vandenabeele P, Galluzzi L, Vanden Berghe T, Kroemer G (2010) Molecular mechanisms of necroptosis: an ordered cellular explosion. Nat Rev Mol Cell Biol 11:700–714

Varfolomeev E, Blankenship J, Wayson S, Fedorova A, Kayagaki N, Garg P, Zobel K, Dynek J, Elliott L, Wallweber H et al (2007) IAP antagonists induce autoubiquitination of c-IAPs, NF-kappaB activation, and TNFalpha-dependent apoptosis. Cell 131:669–681

Varfolomeev E, Goncharov T, Fedorova AV, Dynek JN, Zobel K, Deshayes K, Fairbrother WJ, Vucic D (2008) c-IAP1 and c-IAP2 are critical mediators of tumor necrosis factor alpha (TNFalpha)-induced NF-kappaB activation. J Biol Chem 283:24295–24299

Varfolomeev EE, Schuchmann M, Luria V, Chiannilkulchai N, Beckmann JS, Mett IL, Rebrikov D, Brodianski VM, Kemper OC, Kollet O et al (1998) Targeted disruption of the mouse Caspase 8 gene ablates cell death induction by the TNF receptors, Fas/Apo1, and DR3 and is lethal prenatally. Immunity 9:267–276

Vercammen D, Beyaert R, Denecker G, Goossens V, Van Loo G, Declercq W, Grooten J, Fiers W, Vandenabeele P (1998a) Inhibition of caspases increases the sensitivity of L929 cells to necrosis mediated by tumor necrosis factor. J Exp Med 187:1477–1485

Vercammen D, Brouckaert G, Denecker G, Van de Craen M, Declercq W, Fiers W, Vandenabeele P (1998b) Dual signaling of the Fas receptor: initiation of both apoptotic and necrotic cell death pathways. J Exp Med 188:919–930

Vince J, Pantaki D, Feltham R, Mace P, Cordier S, Schmuckle A, Davidson A, Callus B, Wong W, Gentle I et al (2009) TRAF2 must bind to cIAPs for TNF to efficiently activate NF-{kappa}B and to prevent TNF-induced apoptosis. J Biol Chem 284(51):35906–35915

Vince J, Wong W, Khan N, Feltham R, Chau D, Ahmed A, Benetatos C, Chunduru S, Condon S, McKinlay M et al (2007) IAP antagonists target cIAP1 to induce TNFalpha-dependent apoptosis. Cell 131:682–693

Wagner S, Carpentier I, Rogov V, Kreike M, Ikeda F, Lohr F, Wu CJ, Ashwell JD, Dotsch V, Dikic I et al (2008) Ubiquitin binding mediates the NF-kappaB inhibitory potential of ABIN proteins. Oncogene 27:3739–3745

Walczak H (2011) TNF and ubiquitin at the crossroads of gene activation, cell death, inflammation, and cancer. Immunol Rev 244:9–28

Walczak H (2013) Death receptor-ligand systems in cancer, cell death, and inflammation. Cold Spring Harbor Perspect Biol 5:a008698

Walczak H, Degli-Esposti MA, Johnson RS, Smolak PJ, Waugh JY, Boiani N, Timour MS, Gerhart MJ, Schooley KA, Smith CA et al (1997) TRAIL-R2: a novel apoptosis-mediating receptor for TRAIL. EMBO J 16:5386–5397

Walczak H, Krammer PH (2000) The CD95 (APO-1/Fas) and the TRAIL (APO-2 L) apoptosis systems. Exp Cell Res 256:58–66

Wang L, Du F, Wang X (2008) TNF-alpha induces two distinct caspase-8 activation pathways. Cell 133:693–703

Weinlich R, Dillon CP, Green DR (2011) Ripped to death. Trends Cell Biol 21:630–637

Welz PS, Wullaert A, Vlantis K, Kondylis V, Fernandez-Majada V, Ermolaeva M, Kirsch P, Sterner-Kock A, van Loo G, Pasparakis M (2011) FADD prevents RIP3-mediated epithelial cell necrosis and chronic intestinal inflammation. Nature 477:330–334

Wenzel DM, Lissounov A, Brzovic PS, Klevit RE (2011) UBCH7 reactivity profile reveals parkin and HHARI to be RING/HECT hybrids. Nature 474:105–108

Wertz IE, Dixit VM (2010) Regulation of death receptor signaling by the ubiquitin system. Cell Death Differ 17:14–24

Wertz IE, O'Rourke KM, Zhou H, Eby M, Aravind L, Seshagiri S, Wu P, Wiesmann C, Baker R, Boone DL et al (2004) De-ubiquitination and ubiquitin ligase domains of A20 downregulate NF-kappaB signalling. Nature 430:694–699

Wright A, Reiley WW, Chang M, Jin W, Lee AJ, Zhang M, Sun SC (2007) Regulation of early wave of germ cell apoptosis and spermatogenesis by deubiquitinating enzyme CYLD. Dev Cell 13:705–716

Wu GS, Burns TF, McDonald ER 3rd, Jiang W, Meng R, Krantz ID, Kao G, Gan DD, Zhou JY, Muschel R et al (1997) KILLER/DR5 is a DNA damage-inducible p53-regulated death receptor gene. Nat Genet 17:141–143

Wu J, Huang Z, Ren J, Zhang Z, He P, Li Y, Ma J, Chen W, Zhang Y, Zhou X et al (2013) Mlkl knockout mice demonstrate the indispensable role of Mlkl in necroptosis. Cell Res 23:994–1006

Yin Q, Lamothe B, Darnay BG, Wu H (2009) Structural basis for the lack of E2 interaction in the RING domain of TRAF2. Biochemistry 48:10558–10567

Zhang DW, Shao J, Lin J, Zhang N, Lu BJ, Lin SC, Dong MQ, Han J (2009) RIP3, an energy metabolism regulator that switches TNF-induced cell death from apoptosis to necrosis. Science 325:332–336

Zhang H, Zhou X, McQuade T, Li J, Chan FK, Zhang J (2011) Functional complementation between FADD and RIP1 in embryos and lymphocytes. Nature 471:373–376

Zhang L, Blackwell K, Shi Z, Habelhah H (2010) The RING domain of TRAF2 plays an essential role in the inhibition of TNFalpha-induced cell death but not in the activation of NF-kappaB. J Mol Biol 396(3):528–539

Zhao J, Jitkaew S, Cai Z, Choksi S, Li Q, Luo J, Liu ZG (2012) Mixed lineage kinase domain-like is a key receptor interacting protein 3 downstream component of TNF-induced necrosis. Proc Natl Acad Sci U S A 109:5322–5327

Zheng C, Kabaleeswaran V, Wang Y, Cheng G, Wu H (2010) Crystal structures of the TRAF2: cIAP2 and the TRAF1: TRAF2: cIAP2 complexes: affinity, specificity, and regulation. Mol Cell 38:101–113

Chapter 6
Dead if You Do, Dead if You Don't: How Caspase-8 Causes and Prevents Cell Death

Andrew Oberst

6.1 Apoptosis: It All Makes Sense

Apoptosis is a form of programmed cell death defined by the activation of the caspase proteases. Billions of cells are eliminated from the body by apoptosis each day, and apoptosis can be triggered by normal developmental and homeostatic cues, as well as by stresses or pathogen infection. The mechanisms by which apoptosis is initiated and executed have been very well studied and are generally well understood. Since these pathways are exhaustively described elsewhere (Green 2011), I will provide only a brief summary.

6.1.1 The Apoptotic Pathway

The apoptotic signaling cascade is generally divided into two branches, termed the "intrinsic" and the "extrinsic" apoptotic pathways. Intrinsic apoptosis is triggered by cell-intrinsic stresses, such as DNA damage, oncogene upregulation, or ER stress; these stimuli converge on the Bcl-2 protein family, which mediates the permeabilization of the outer mitochondrial membrane. This event leads to release of caspase-activating proteins and activation of caspase-9 in a large protein complex called the "apoptosome."

In contrast, extrinsic apoptosis—upon which we will focus in this chapter—is triggered by engagement of cell surface receptors of the TNF superfamily, collectively called the "death receptors." These include TNF receptor-1 (TNFR1),

A. Oberst (✉)
Department of Immunology, University of Washington,
750 Republican St., Seattle, WA 98109, USA
e-mail: Oberst@uw.edu

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Fas (also called CD95), and the TRAIL receptors DR4 and DR5, also respectively called TRAIL-R1 and TRAIL-R2. Ligation of these receptors has complex and pleiotropic effects, many of which lie beyond the scope of this chapter (Wilson et al. 2009). For our purposes, it is important to understand that one consequence of death receptor ligation is the recruitment and activation of the pro-apoptotic protease caspase-8. Once activated, caspase-8 can directly activate the downstream effector caspases, caspase-3 and caspase-7; however, in most cell types this process is prevented by the IAP proteins (Jost et al. 2009). Thus, caspase-8-dependent apoptosis generally requires an amplification step, which is achieved by the caspase-8-dependent processing and activation of the Bcl-2 family member Bid. Processed Bid promotes mitochondrial permeabilization and thereby engages the intrinsic apoptotic pathway, leading to efficient apoptosis.

6.2 Caspase Inhibitor-Induced Programmed Cell Death: A Challenge to the Apoptotic Paradigm

The pathways described above neatly explain the activation of the caspases and the execution of apoptosis. In the course of working out these pathways, potent, cell-permeable inhibitors of the caspases were developed; these were quite effective at inhibiting cell death in response to many stimuli and in numerous cell types (Garcia-Calvo et al. 1998; McStay et al. 2008). However, it was found that caspase inhibition failed to protect certain cells—notably L929 murine fibroblasts, in which many seminal early observations were made—from TNF-induced cell death (Vercammen et al. 1997, 1998). In fact, addition of the caspase inhibitor zVAD actually sensitized these cells to death, though the death appeared morphologically more similar to necrosis than to apoptosis. The concept of TNF-induced "programmed necrosis" (also called necroptosis; the terms are used interchangeably here) was thereby born; however, the molecular mechanisms that govern this non-apoptotic form of cell death are only partly understood.

6.2.1 The Necroptotic Pathway

Let us now turn to the pathway of necroptosis as we understand it; after examining this signaling pathway, we will return to the apoptotic players described above and consider how these two cell death processes are functionally linked. Early findings implicated receptor-interacting protein kinase-1 (RIPK1) in the process of TNF-induced necroptosis (Holler et al. 2000; Lin et al. 2004); indeed, a major breakthrough was the identification of a small molecule that blocked this form of cell death, called necrostatin-1, and the demonstration that this molecule is an inhibitor of RIPK1 (Degterev et al. 2005, 2008). However, it remained mysterious why the combination of TNF and zVAD led to potent necroptosis only in certain cell lines;

indeed, this observation led to speculation that this "zVAD toxicity" was an artifact restricted to specific cultured cells. This notion was put to rest with the identification of RIPK3 as a downstream target of RIPK1 in the necroptotic pathway (Cho et al. 2009; He et al. 2009; Zhang et al. 2009). RIPK3 is silenced in many commonly used cell lines, but is notably present in L929 and Jurkat cell lines, as well as in many primary cell types; this finding explained why these lines, but not other commonly cultured cells, undergo necroptosis in response to TNF+zVAD treatment. The identification of RIPK3 as a key mediator of necroptosis also confirmed this as a bona fide signaling pathway.

The molecular mechanism by which the TNF receptor activates RIPK1 and RIPK3 is described in greater detail elsewhere (Van Herreweghe et al. 2010; Vandenabeele et al. 2010), including in this book. Briefly, TNFR1 ligation recruits many proteins, including RIPK1 and the cIAPs; this complex, often called "complex I," leads to activation of the NF-kB transcriptional program, which in turn upregulates numerous genes involved in inflammation and cell survival (Ea et al. 2006). (We will return to the importance of this event later.) Subsequently, RIPK1 translocates out of the receptor-associated complex to form additional complexes in the cytosol (Hsu et al. 1996; Wang et al. 2008). The nature of these complexes is a subject of continuing study; for example, it is not clear how—or indeed if—RIPK1 is modified by its recruitment to complex I and how such modifications influence the formation and function of subsequent complexes. However, it is clear that once in the cytosol, RIPK1 can interact with RIPK3, via the RIP homotypic interaction motif (RHIM) shared by both proteins. Structural studies suggest that this interaction involves formation of an amyloid-like oligomeric structure (Li et al. 2012), though how the formation of this oligomer influences kinase activity and signal transduction remains unclear. Downstream of RIPK3 activation, the pathway becomes murky; the pseudokinase MLKL has been identified as a RIPK3 substrate required for RIPK3-dependent necroptosis (Sun et al. 2012), but how this protein—which seems to lack kinase activity—causes rapid cell death remains unclear.

6.3 Meanwhile, in the Apoptotic Pathways, a Genetic Mystery

Having surveyed both apoptosis and necroptosis, let us now consider the intersection of these cell death pathways, as defined by the control of necroptosis by the pro-apoptotic protease caspase-8. The importance of this cross talk was highlighted by genetic studies, which provide a useful jumping-off point.

Apoptosis is generally considered to be a mechanism for removing superfluous or damaged cells from the organism; one would therefore predict that knocking out pro-apoptotic genes would lead to phenotypes associated with an overabundance of cells. In many cases, this is true: for example, knockout or inactivation of the death receptor Fas or its ligand causes accumulation of an aberrant lymphocyte population (Watanabe-Fukunaga et al. 1992; Takahashi et al. 1994; Fisher et al. 1995)

(more on this later), while knockout of pro-apoptotic Bcl-2 family members produces mice that manifest autoimmunity or increased susceptibility to tumors (Lindsten et al. 2000; Bouillet et al. 2002; Rathmell et al. 2002; Pellegrini et al. 2003; Tan et al. 2005). Similarly, knockout of caspase-9 or APAF-1 causes severe neurological phenotypes associated with an overabundance of neurons (Kuida et al. 1998; Yoshida et al. 1998); all these phenotypes are consistent with a failure to eliminate unwanted cells.

However, there are a few exceptions to this paradigm, and they prove interesting. Specifically, knockout of caspase-8, its adapter FADD, or its catalytically inactive paralog $cFLIP_L$ (hereafter called FLIP) leads to embryonic lethality at developmental day 10.5 due to a failure of hematopoiesis (Varfolomeev et al. 1998; Yeh et al. 1998, 2000). Further studies using conditional knockouts demonstrated that lymphocytes lacking caspase-8, FADD, or FLIP failed to proliferate following antigen receptor activation (Zhang et al. 2001; Kang et al. 2004; Zhang and He 2005; He and He 2012). Mysteriously, it was found that these phenotypes are not shared by knockout of the death receptors that lie upstream of caspase-8 in the apoptotic pathway nor by knockout of Bid or the executioner caspases downstream of caspase-8. These observations, first made over 15 years ago, led to extensive speculation regarding possible non-apoptotic roles for caspase-8. Candidates included participation in NF-kB signaling (Hu et al. 2000; Dohrman et al. 2005; Golks et al. 2006), cell motility (Helfer et al. 2006; Senft et al. 2007), inflammation (Ben Moshe et al. 2007; Kovalenko et al. 2009), lymphocyte activation (Su et al. 2005), and TLR signaling (Imtiyaz et al. 2006; Lemmers et al. 2007); however, none of these proposed functions fully explained the phenotypes of the knockout animals. Something else was going on; but what?

6.3.1 *Suppression of Necroptosis by Caspase-8/FLIP: Genetic Evidence*

A key finding came when Ch'en et al. took a closer look at the phenotype of mice lacking caspase-8 only in their T cells (Ch'en et al. 2008). It had been previously reported that T cells from these animals failed to proliferate upon TCR ligation; this finding was explained by a proposed requirement for caspase-8 in the TCR-driven NF-kB transcriptional response, which is required to coordinate the proliferative program in these circumstances (Su et al. 2005). What Ch'en et al. observed, however, was that caspase-8-deficient T cells did in fact proliferate upon antigen receptor stimulation; however, they then rapidly underwent cell death. Importantly, the authors demonstrated that the RIPK1 inhibitor Nec-1—but not caspase inhibitors—could rescue this cell death phenotype. This finding strongly implied that the requirement for caspase-8 in T cell proliferation is defined by the suppression of RIPK1-dependent, caspase-independent cell death. In other words, caspase-8 is needed to prevent necroptosis in activated T cells (Ch'en et al. 2008).

This finding raised an obvious question: if the requirement for caspase-8 in T cells is defined by the suppression of necroptosis, what about the requirement for caspase-8 in normal embryonic development? This question is not easily addressed using a chemical inhibitor of RIPK1, and the RIPK1 knockout is itself perinatally lethal (Kelliher et al. 1998); however, the studies demonstrating a fundamental role for RIPK3 in necroptosis had recently been published, and the RIPK3 knockout had no overt phenotype (Newton et al. 2004). Based on these findings, I set out to cross the caspase-8 knockout animals to RIPK3-deficient mice during my postdoctoral work in the laboratory of Dr. Douglas Green; Dr. William Kaiser at Emory, thinking along similar lines, was carrying out similar crosses concurrently.

What we found, to our surprise, was that caspase-8::RIPK3 double-knockout (DKO) animals were born at Mendelian frequencies and were overtly healthy (Kaiser et al. 2011; Oberst et al. 2011). Consistent with elimination of the extrinsic pathway of apoptosis by caspase-8 ablation, these DKO animals were completely resistant to injection of an activating anti-Fas antibody, a treatment that causes massive liver apoptosis and rapid lethality in wild-type animals. Interestingly, these animals also developed a progressive accumulation of an aberrant lymphocyte population identical to that observed in Fas- or FasL-deficient mice (Watanabe-Fukunaga et al. 1992; Takahashi et al. 1994). The origin of these cells—which are positive for B220 and CD3, but lack CD4 and CD8 expression—remains poorly understood, but subsequent studies found that conditional deletion of caspase-8 in T cells using CD4-driven Cre led to emergence of this cell population only if RIPK3 was also absent (Ch'en et al. 2011). Together, these findings indicate that this lymphocyte population is eliminated by Fas-dependent cell death, presumably dying by apoptosis in RIPK3-deficient animals and necroptosis in mice conditionally lacking caspase-8 in T cells; when either the Fas receptor signal or *both* RIPK3 and caspase-8 are absent, this population is allowed to accumulate.

The unexpected viability of the caspase-8::RIPK3 DKO animals also allowed us to test the putative role of caspase-8 in other processes, such as proliferation and cell motility. The striking finding from these animals was, in fact, the lack of striking findings; T cells from DKO animals (prior to accumulation of the aberrant lymphocyte population discussed above) expanded and contracted normally in response to a bacterial superantigen and exhibited normal NF-kB activation, and with the exception of the progressive lymphoaccumulation, the DKO animals were normal, fertile, and viable. It is tempting to interpret the overall normality of the DKO animals as demonstrating that caspase-8 does not participate in NF-kB activation, cell motility, inflammation, or other non-apoptotic processes. However, all we can truly say is that any function of caspase-8 in these processes is either (1) not required for normal development and survival or (2) defined by regulation of RIPK3 and therefore fails to manifest in mice lacking both caspase-8 and RIPK3. Indeed, ongoing studies are uncovering roles for RIPK1 and RIPK3 that are independent of the induction of necroptosis (Kang et al. 2012; Vince et al. 2012), and the regulation of these processes by caspase-8 may point to broader functions for this signaling axis.

6.4 Caspase-8 in Cell Survival: A Killer Turns Over a New Leaf

The rescue of the early embryonic lethality of caspase-8 knockout mice by ablation of RIPK3 indicates that caspase-8 is required during normal development to suppress necroptosis. Furthermore, tissue-specific deletion of caspase-8 shows that this requirement for suppression of RIPK3 extends to several other tissues. Caspase-8 is therefore a key pro-survival protein, because it is needed to prevent necroptosis. However, this idea raises another conundrum: as you recall from Part I of this chapter, caspase-8 is a key effector of extrinsic apoptosis. How, then, can caspase-8 be brought to bear to limit necroptosis without killing cells by apoptosis? In other words, how do you activate this pro-death enzyme to promote survival? To answer this question, we must look more closely at the mechanism of caspase-8 activation.

6.4.1 The Molecular Mechanism of Caspase-8 Activation

Caspases are cysteine proteases that cleave substrates at aspartic acid residues (Pop and Salvesen 2009). Caspases are present as inactive zymogens in the cytosol of healthy cells, where they can be activated by a variety of stimuli. The caspases can be broadly divided into "initiators" and "executioners." Initiators—such as caspase-8—possess long N-terminal domains, called "prodomains," that mediate their interactions with regulatory and activating proteins. Executioners, such as caspase-3 and caspase-7, lack these domains. These differences give rise to distinct modes of activation between initiator and executioner caspases: initiator caspases are activated by recruitment and dimerization of monomeric proenzymes. In the case of caspase-8, prodomain-driven recruitment to receptor- or RIPK1-associated complexes by the adapter protein FADD leads to dimerization of two molecules of procaspase-8 (Boatright et al. 2003) (Fig. 6.1). Following this dimerization—and importantly for our discussion of the regulation of necroptosis—caspase-8 undergoes key autoproteolytic cleavage events, which allows rearrangement of the caspase-8 catalytic domain and stabilization of the active caspase-8 enzyme (Pop et al. 2007; Oberst et al. 2010). Executioner caspases, on the other hand, are present in healthy cells as inactive dimers and are activated by interdomain cleavage by initiator caspases. Thus, initiator caspases are activated by recruitment to specific complexes and induced dimerization of inactive proenzymes, and these can then activate executioner caspases by cleaving them (Fig. 6.1).

So, what does this have to do with the selective activation of caspase-8 to suppress necroptosis? A significant clue to how this process might work was provided by the generation of a BAC transgenic mouse expressing a version of caspase-8 with a mutation in the autoproteolytic processing site within the catalytic domain (Kang et al. 2008). As mentioned, this cleavage event normally stabilizes the active homodimeric enzyme, and processing at this site is required for formation of a fully

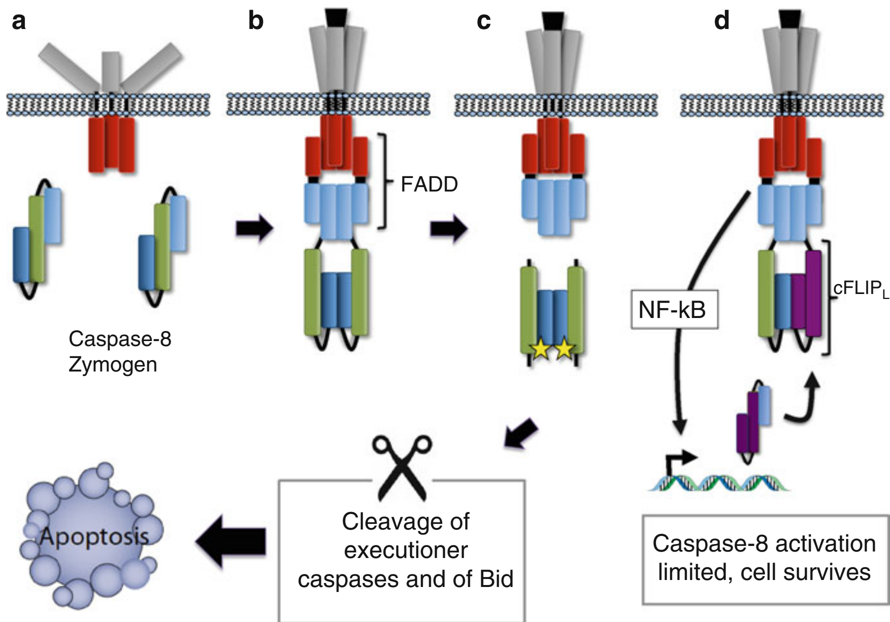

Fig. 6.1 Molecular mechanism of caspase-8 activation. (**a**) Caspase-8 is present in the cytosol of healthy cells as an inactive, monomeric zymogen. (**b**) Upon receptor ligation (depicted here, as occurs with FasL and TRAIL signaling) or formation of cytosolic caspase-8-activating complexes (as occurs with TNF, not shown here), recruitment of the adapter protein FADD leads to dimerization of caspase-8 zymogens. (**c**) Dimerization causes interdomain autoproteolytic cleavage events, which stabilize the mature caspase-8 enzyme and allow full proteolytic activity (stars). Activated caspase-8 can then cleave substrates and trigger apoptosis. (**d**) When FLIP is present, it can substitute for one half of the caspase-8 homodimer. This caspase-8/FLIP heterodimeric species allows cell survival by limiting caspase-8 activation

active, mature caspase-8 enzyme. It was therefore striking that the transgenic mouse expressing non-cleavable caspase-8 was found to survive normally—that is, it did not manifest embryonic lethality—but that cells and tissues from this mouse were resistant to caspase-8-dependent apoptosis. This finding indicated that non-cleavable caspase-8 was able to carry out the survival functions of caspase-8, but not the pro-apoptotic functions. But, if the autoproteolytic processing of caspase-8 is required for its activation (Oberst et al. 2010), how could the non-cleavable form have any activity at all?

6.4.2 Things Get FLIPping Complicated

In order to understand how caspase-8 can be activated to suppress necroptosis without killing cells, we need to add the protein FLIP to the picture. FLIP stands for "FLICE-like inhibitory protein," FLICE being the former name of caspase-8.

As this name indicates, FLIP bears significant sequence and structural homology to caspase-8, but importantly lacks a catalytic cysteine. This property allows it to substitute for one half of the caspase-8 homodimer, thereby forming a FLIP-caspase-8 heterodimer instead (Fig. 6.1). Also as its name implies, FLIP was initially described as an inhibitor of caspase-8 (Irmler et al. 1997), and formation of this heterodimer was thought to block caspase-8 activation.

In fact, the effect of FLIP on caspase-8 is more complicated than that. FLIP is clearly able to prevent caspase-8-dependent apoptosis during DR stimulation, as evidenced by the fact that knockdown or knockout of FLIP greatly sensitizes cells to death receptor-induced apoptosis (Yeh et al. 2000; Kreuz et al. 2001; Micheau et al. 2001). Indeed, FLIP is a potent transcriptional target of NF-kB, and NF-kB activation is an early event in TNFR1 and TLR signaling; in most cellular settings, TNFR1 and TLR ligation does not induce cell death, and this is largely attributable to FLIP upregulation blocking caspase-8.

However, careful analysis of the heterodimeric FLIP-caspase-8 complex indicated that this enzyme species does have catalytic activity (Chang et al. 2002; Micheau et al. 2002; Boatright et al. 2004). And this is where a key piece of the puzzle arrives: heterodimerization with FLIP is able to impart catalytic activity to non-cleavable caspase-8 (Pop et al. 2011). Thus, non-cleavable caspase-8, which is able to suppress necroptosis but not induce apoptosis, can be activated by heterodimerization with FLIP but not by homodimerization. This finding strongly implies that it is the caspase-8/FLIP heterodimer, rather than the caspase-8/caspase-8 homodimer, that is responsible for suppression of necroptosis. Indeed, this idea is now supported by extensive experimental evidence (Geserick et al. 2009; Feoktistova et al. 2011; Oberst et al. 2011) and provides a neat explanation for the pro-survival engagement of caspase-8 (Fig. 6.2).

6.4.3 Summing Up the Mechanism: How Caspase-8 Is Engaged to Suppress Necroptosis

In the preceding discussion of the history and discovery of these mechanisms, details may have impeded a clear understanding of how we think this pathway works. So here's a direct explanation, using signaling through TNFR1 as an example:

Ligation of TNFR1 by its cognate ligand, TNF, leads to formation of a signaling complex at the cytoplasmic tail of the receptor. This complex contains RIPK1, among several other proteins, and formation of this complex causes the degradation of the inhibitory IkB proteins and thereby activates the NF-kB transcriptional program. This, in turn, leads to transcriptional upregulation of FLIP. Meanwhile, RIPK1 leaves the receptor-associated complex and forms a second complex in the cytosol. This complex leads to the recruitment of RIPK3 but also to the recruitment of the caspase-8 adapter protein FADD, which interacts with the C-terminal death

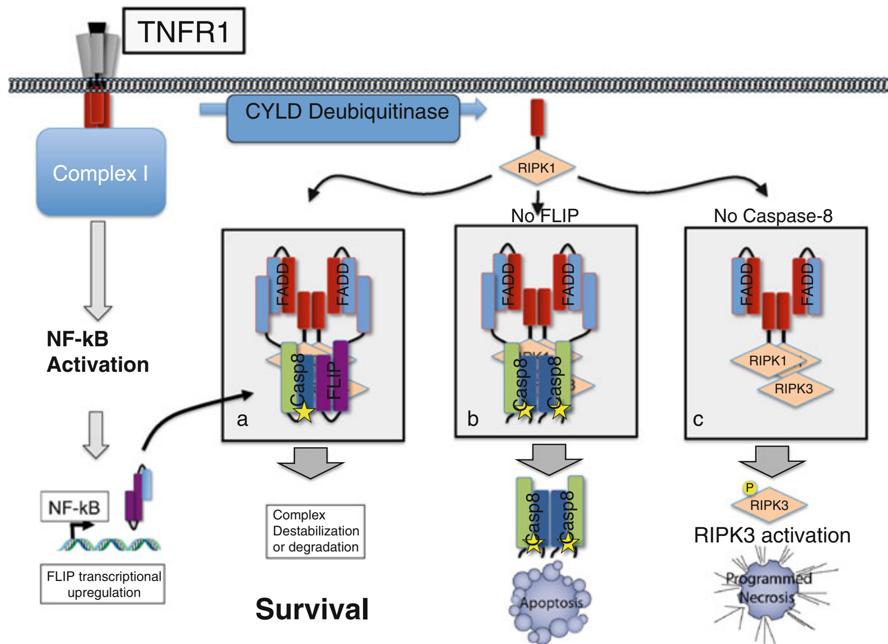

Fig. 6.2 TNF-induced complexes and their effects. TNFR1 ligation leads to NF-kB activation. Thereafter, RIPK1 translocates from the receptor-proximal complex into the cytosol, through the action of the deubiquitinase CYLD. In the cytosol, RIPK1 can interact with RIPK3, caspase-8, and FLIP; the relative abundance and activity of these proteins determine cell fate. (**a**) If both caspase-8 and FLIP are present, RIPK1/RIPK3 signaling is inhibited, and the cell survives. (**b**) If FLIP is absent, caspase-8 activation proceeds unchecked, resulting in apoptosis. (**c**) If caspase-8 is absent, RIPK3 signaling dominates, leading to RIPK3-dependent necroptosis

domain of RIPK1. FADD, in turn, recruits both caspase-8 and FLIP. They form a heterodimeric enzyme complex, which suppresses RIPK3 activation and thereby prevents necroptosis. However, heterodimerization with FLIP also limits caspase-8 activation and thereby does not trigger apoptosis (Fig. 6.2).

An important feature of this proposed mechanism is that TNFR1 signaling can result in cell survival, apoptosis, or necroptosis, depending on the relative abundance of the enzymes involved. If caspase-8, FLIP, and FADD are all present, cell death is prevented. However, if caspase-8 or FADD is absent, RIPK3 activation proceeds unchecked, leading to necroptosis. If FLIP is absent, caspase-8 activation is uncontrolled, and apoptosis ensues. (Note: this last statement is not strictly true, as we will discuss in Sect. 6.6).

While complicated, this model received genetic backing from recent work by Dillon et al. (Dillon et al. 2012). This study used FADD and FLIP knockout mice, both of which die at E10.5, apparently due to the same hematopoietic defect observed in caspase-8 knockouts. This work found that while FADD knockout mice are rescued by knockout of RIPK3 (analogous to caspase-8 knockouts), FLIP

knockout mice are not. Analysis of FLIP::RIPK3 double-knockout embryos revealed that these pups die due to massive apoptosis in hematopoietic precursors, as revealed by cleaved caspase-3 staining; by contrast, while FADD knockout animals die at the same time, they do not display hallmarks of apoptosis. These observations are consistent with the idea that FADD knockouts die due to unchecked RIPK3 and necroptosis, while FLIP::RIPK3 DKO mice die due to unchecked caspase-8 activation. Interestingly, Dillon et al. showed that FADD::FLIP::RIPK3 triple knockout mice survived to adulthood; thus, the apoptotic phenotype of the FLIP::RIPK3 double knockout is rescued by also ablating FADD. In addition to being informative, this study turns a neat trick: it crosses two embryonically lethal genetic strains (FADD knockout crossed to FLIP::RIPK3 double knockout) and emerges with a viable mouse.

6.5 How Does Caspase-8/FLIP Block Necroptosis?

As discussed, extensive evidence points to the suppression of necroptosis by the caspase-8/FLIP complex as a key component of survival signaling. But how does this really work? Caspase-8 is a protease; what is it cutting to block necroptosis, and how is FLIP involved in this process? The answer to this question remains unclear, and several mechanisms have been proposed to account for this phenomenon.

One caspase-8 substrate proposed to account for the suppression of necroptosis is CYLD (O'Donnell et al. 2011). CYLD is a deubiquitinase, and its activity is associated with the translocation of RIPK1 from the receptor-associated complex I to the RIPK3-activating cytosolic complex. O'Donnell et al. showed that CYLD is processed by caspase-8 during normal TNF signaling and that this processing leads to proteasomal degradation of CYLD. This inhibition of CYLD would prevent RIPK1 deubiquitination and thereby prevent formation of a cytosolic RIPK1 complex capable of inducing necroptosis. However, it remains unclear exactly how and where this processing occurs; caspase-8 forms a cytosolic complex with RIPK1 and RIPK3, but it is not clear that CYLD is also present in this complex.

More directly, both RIPK1 and RIPK3 have been shown to contain caspase cleavage sites (Lin et al. 1999; Feng et al. 2007). In particular, careful analysis of cytosolic RIPK1 complexes has demonstrated the presence of a cleaved RIPK1 fragment when caspase-8 and FLIP are present (Feoktistova et al. 2011; Tenev et al. 2011), and reconstitution with non-cleavable RIPK1 mutants has been shown to eliminate the protective effects of caspase-8/FLIP in multiple systems (Lu et al. 2011; Rajput et al. 2011). Furthermore, in some cases these studies have proposed that RIPK1 cleavage may produce an inhibitory fragment of RIPK1, which is capable of acting in a dominant-negative manner to prevent RIPK1/RIPK3 complex formation (Rajput et al. 2011).

6.5.1 The IAPs Get in on the Act

Lurking in the wings of this discussion are the IAP proteins. The IAPs are ubiquitin ligases, and several reports indicate that they can ubiquitinate and promote the degradation of components of the necroptosis-inducing RIPK1/RIPK3 complex (Feoktistova et al. 2011; Tenev et al. 2011). The IAPs can thereby prevent necroptosis, a fact that has been demonstrated in multiple systems (Geserick et al. 2009; Vince et al. 2012). However, it remains unclear how the IAPs interact with caspase-8 and FLIP to promote this function. These observations are further clouded by the fact that the IAPs participate in NF-kB signaling by TNFR1 complexes; inhibition or knockout of the IAPs thereby prevents FLIP upregulation while also preventing degradation of cytosolic RIPK1 complexes. Thus, while the cIAP1/cIAP2 double-knockout animals die at E10.5 due to the same defects observed in caspase-8, FADD, or FLIP knockouts, this phenotype is only slightly ameliorated by RIPK1 or RIPK3 knockout (Moulin et al. 2012). This may be due to unrestrained activation of caspase-8; consistent with this idea, TNFR1 knockout rescues cIAP1/cIAP2 double-knockout mice to a greater degree.

The action of the IAP ubiquitin ligases may help to answer another persistent question regarding the suppression of necroptosis by caspase-8/FLIP: how does FLIP prevent caspase-8-dependent apoptosis? That this is the case is clear from knockout and knockdown studies; however, these observations are not supported by biochemical studies. Specifically, the caspase-8/FLIP heterodimer has similar substrate specificities as the caspase-8/caspase-8 homodimer, and artificially forming caspase-8/FLIP heterodimers in the cytosol using inducible dimerization systems leads to potent apoptosis (Pop et al. 2011). Thus, there is nothing inherent to the caspase-8/FLIP enzyme complex that prevents apoptosis. This is where other components of the RIPK1 complex may play a role; for example, the presence of FLIP may allow the recognition and ubiquitination of the complex by the IAPs, or the activity or the RIP kinases themselves may affect caspase-8 activity and thereby limit apoptosis. A full understanding of the interactions of these proteins remains a challenge for future studies.

6.6 But Why Is It Wired This Way?

To this point, we have discussed genetic and biochemical data that prove that the apoptotic enzyme caspase-8 is required to suppress RIPK3-dependent necroptosis. These data are both compelling and complicated, and the mechanisms discussed here represent the culmination of dozens of studies. However, in considering these we risk losing sight of a bigger question: why are the cell death systems set up this way? That is, why is caspase-8, a key mediator of one cell death pathway, also required to prevent another? Why has this arrangement been favored by evolution, and what are its implications? We cannot yet answer these questions, but we can speculate.

Most studies of necroptosis use chemical inhibitors of the caspases and IAPs or genetic systems in which these proteins are ablated. Of course, neither of these conditions regularly occur naturally, which begs the question: when is necroptosis engaged under physiological conditions? One possible answer lies in the observation that many viruses encode caspase inhibitors. The subversion of cell death pathways by viruses is covered elsewhere in this book, but it is sufficient to note that viral infection can promote TNF signaling, as well as activating TLR3. These stimuli, in combination with caspase inhibition, could be sufficient to trigger physiological necroptosis; in this hypothetical scenario necroptosis would be a "backup" mechanism to overcome viral inhibition of apoptosis. The idea that necroptosis is a physiological response to viral infection is attractive; it has been demonstrated that RIPK3 knockout mice are hypersusceptible to MCMV and vaccinia infection (Cho et al. 2009; Upton et al. 2010), and MCMV encodes a specific RIPK3 inhibitor (Upton et al. 2010), a sure sign that this pathway is relevant to viral infection.

However, while this is undoubtedly the case, this may not fully explain the links between caspase-8 and necroptosis. CMV infection induces RIPK3 activation via DAI (Upton et al. 2012), and neither caspase-8 nor RIPK1 seems to alter this phenomenon; that is, caspase-8 inhibition is dispensable for necroptosis in this setting. Furthermore, our analysis of one viral caspase inhibitor, the poxvirus serpin CrmA, showed that this protein preferentially inhibits the pro-apoptotic caspase-8/caspase-8 homodimer while leaving the activity of the anti-necroptotic caspase-8/FLIP heterodimer intact (Oberst et al. 2011). This finding explains the previously puzzling result that CrmA transgenic T cells proliferate normally upon activation, while caspase-8 knockouts do not (Smith et al. 1996). It may also demonstrate a refined approach to caspase-8 inhibition by the virus, blocking apoptosis without triggering necroptosis.

If viral caspase inhibitors are not sufficient to explain the induction of necroptosis under physiological conditions, what alternatives do we have? One interesting possibility is that it is not the inhibition of caspase-8 that allows physiological necroptosis, but rather the absence of FLIP. In considering this possibility, we must return to a statement made back in Sect. 6.4 that when FLIP levels are low, caspase-8 is unrestrained and apoptosis ensues. As I mentioned, this is not strictly true. Interestingly, FLIP not only is required to prevent caspase-8 activation but is in fact necessary for the suppression of necroptosis per se. Thus, when FLIP is absent, caspase-8 is unrestrained, but so is RIPK3; the protective link between caspase-8 and necroptosis is effectively broken, and both apoptosis and necroptosis can proceed unchecked. While this is highly speculative, it may be that in many cases necroptosis "wins" this race to the death (Fig. 6.3).

What are the physiological implications of this scenario? Several bacteria express inhibitors of NF-kB or of general protein translation. Furthermore, PKR-eIF2α-dependent inhibition of general protein synthesis is a common feature of viral infection. Inhibition of protein synthesis is also a component of various stress response pathways, including ER stress and DNA damage. Thus, in all these scenarios, de novo FLIP expression would be prevented, and necroptosis may thereby ensue in response to TNF or TLR signaling (Fig. 6.3).

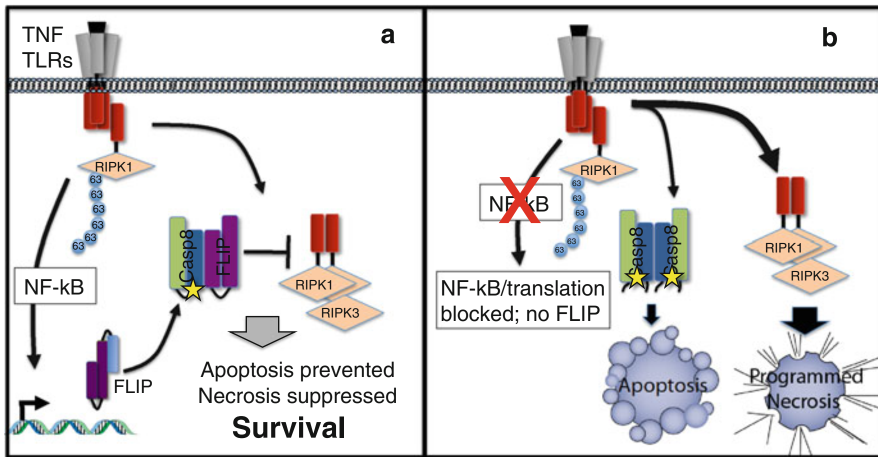

Fig. 6.3 A speculative scenario for physiological necroptosis. (**a**) In healthy cells, TNF-dependent NF-kB activation leads to formation of the caspase-8/FLIP heterodimer, which suppresses RIPK3-dependent necroptosis while also preventing apoptosis. The cell survives, and NF-kB-dependent inflammatory signaling is the result. (**b**) If NF-kB signaling or *de novo* protein synthesis is prevented by pathogen-encoded effectors or cellular stress, FLIP is not upregulated. In this case, the suppressive link between caspase-8 and RIPK3 is broken, and both apoptosis and necroptosis may occur. In such a scenario, necroptosis may be kinetically favored in some cells

But again, why would such an arrangement be evolutionarily favorable? One possible answer lies in the presumed inflammatory nature of necroptosis. TNF and TLR signaling is normally pro-inflammatory, not pro-death; these signals tell cells to produce cytokines and thereby propagate inflammatory signals and coordinate immune responses. In the situations outlined above—bacterial or viral infection or severe cellular stress—cells would be unable to heed these signals, because protein synthesis would be blocked. It may therefore be that in these instances, necroptosis has evolved as an emergency fail-safe means of propagating inflammatory signals. If a cell is unable to respond to the normal cues due to an infection or other crisis, it may be advantageous for that cell to die in a manner that is itself inflammatory, thereby alerting surrounding cells to the danger and promoting the recruitment of immune cells to the site of death. This idea may provide a more general explanation for the induction of necroptosis under physiological conditions. (Or it might be *totally* wrong (Fig. 6.3)).

6.7 Concluding Remarks

The recent years have seen an explosion in interest in the phenomenon of necroptosis, but many fundamental questions remain to be answered. Most notably, the definition of necroptosis has led to a reevaluation of the field of cell death research in

general. Because necroptosis is both genetically programmed and (presumably) inflammatory, its addition to the cell death lexicon means that we must now consider not only *whether* a cell dies in response to a given stimulus but *how* that cell dies and what the effects of that death are for surrounding tissues and global immune responses. Many of the topics discussed in this chapter contain significant speculation, and finding definitive answers to them remains a challenge for future studies. For those interested in these questions, the coming years are certain to prove exciting.

References

Ben Moshe T, Barash H, Kang TB, Kim JC, Kovalenko A, Gross E, Schuchmann M, Abramovitch R, Galun E, Wallach D (2007) Role of caspase-8 in hepatocyte response to infection and injury in mice. Hepatology 45:1014–1024

Boatright KM, Deis C, Denault JB, Sutherlin DP, Salvesen GS (2004) Activation of caspases-8 and −10 by FLIP(L). Biochem J 382:651–657

Boatright KM, Renatus M, Scott FL, Sperandio S, Shin H, Pedersen IM, Ricci JE, Edris WA, Sutherlin DP, Green DR, Salvesen GS (2003) A unified model for apical caspase activation. Mol Cell 11:529–541

Bouillet P, Purton JF, Godfrey DI, Zhang LC, Coultas L, Puthalakath H, Pellegrini M, Cory S, Adams JM, Strasser A (2002) BH3-only Bcl-2 family member Bim is required for apoptosis of autoreactive thymocytes. Nature 415:922–926

Ch'en IL, Beisner DR, Degterev A, Lynch C, Yuan J, Hoffmann A, Hedrick SM (2008) Antigen-mediated T cell expansion regulated by parallel pathways of death. Proc Natl Acad Sci U S A 105:17463–17468

Ch'en IL, Tsau JS, Molkentin JD, Komatsu M, Hedrick SM (2011) Mechanisms of necroptosis in T cells. J Exp Med 208:633–641

Chang DW, Xing Z, Pan Y, Algeciras-Schimnich A, Barnhart BC, Yaish-Ohad S, Peter ME, Yang X (2002) c-FLIP(L) is a dual function regulator for caspase-8 activation and CD95-mediated apoptosis. Embo J 21:3704–3714

Cho YS, Challa S, Moquin D, Genga R, Ray TD, Guildford M, Chan FK (2009) Phosphorylation-driven assembly of the RIP1-RIP3 complex regulates programmed necrosis and virus-induced inflammation. Cell 137:1112–1123

Degterev A, Hitomi J, Germscheid M, Ch'en IL, Korkina O, Teng X, Abbott D, Cuny GD, Yuan C, Wagner G, Hedrick SM, Gerber SA, Lugovskoy A, Yuan J (2008) Identification of RIP1 kinase as a specific cellular target of necrostatins. Nat Chem Biol 4:313–321

Degterev A, Huang Z, Boyce M, Li Y, Jagtap P, Mizushima N, Cuny GD, Mitchison TJ, Moskowitz MA, Yuan J (2005) Chemical inhibitor of nonapoptotic cell death with therapeutic potential for ischemic brain injury. Nat Chem Biol 1:112–119

Dillon CP, Oberst A, Weinlich R, Janke LJ, Kang TB, Ben-Moshe T, Mak TW, Wallach D, Green DR (2012) Survival function of the FADD-CASPASE-8-cFLIP(L) complex. Cell Rep 1:401–407

Dohrman A, Kataoka T, Cuenin S, Russell JQ, Tschopp J, Budd RC (2005) Cellular FLIP (long form) regulates CD8+ T cell activation through caspase-8-dependent NF-kappa B activation. J Immunol 174:5270–5278

Ea CK, Deng L, Xia ZP, Pineda G, Chen ZJ (2006) Activation of IKK by TNFalpha requires site-specific ubiquitination of RIP1 and polyubiquitin binding by NEMO. Mol Cell 22:245–257

Feng S, Yang Y, Mei Y, Ma L, Zhu DE, Hoti N, Castanares M, Wu M (2007) Cleavage of RIP3 inactivates its caspase-independent apoptosis pathway by removal of kinase domain. Cell Signal 19:2056–2067

Feoktistova M, Geserick P, Kellert B, Dimitrova DP, Langlais C, Hupe M, Cain K, Macfarlane M, Hacker G, Leverkus M (2011) cIAPs block ripoptosome formation, a RIP1/caspase-8 containing intracellular cell death complex differentially regulated by cFLIP isoforms. Mol Cell 43(3):449–463

Fisher GH, Rosenberg FJ, Straus SE, Dale JK, Middleton LA, Lin AY, Strober W, Lenardo MJ, Puck JM (1995) Dominant interfering Fas gene mutations impair apoptosis in a human autoimmune lymphoproliferative syndrome. Cell 81:935–946

Garcia-Calvo M, Peterson EP, Leiting B, Ruel R, Nicholson DW, Thornberry NA (1998) Inhibition of human caspases by peptide-based and macromolecular inhibitors. J Biol Chem 273:32608–32613

Geserick P, Hupe M, Moulin M, Wong WW, Feoktistova M, Kellert B, Gollnick H, Silke J, Leverkus M (2009) Cellular IAPs inhibit a cryptic CD95-induced cell death by limiting RIP1 kinase recruitment. J Cell Biol 187:1037–1054

Golks A, Brenner D, Krammer PH, Lavrik IN (2006) The c-FLIP-NH2 terminus (p22-FLIP) induces NF-kappaB activation. J Exp Med 203:1295–1305

Green DR (2011) Means to an end : apoptosis and other cell death mechanisms. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY

He MX, He YW (2012) A role for c-FLIP(L) in the regulation of apoptosis, autophagy, and necroptosis in T lymphocytes. Cell Death Differ 20(2):188–197

He S, Wang L, Miao L, Wang T, Du F, Zhao L, Wang X (2009) Receptor interacting protein kinase-3 determines cellular necrotic response to TNF-alpha. Cell 137:1100–1111

Helfer B, Boswell BC, Finlay D, Cipres A, Vuori K, Bong Kang T, Wallach D, Dorfleutner A, Lahti JM, Flynn DC, Frisch SM (2006) Caspase-8 promotes cell motility and calpain activity under nonapoptotic conditions. Cancer Res 66:4273–4278

Holler N, Zaru R, Micheau O, Thome M, Attinger A, Valitutti S, Bodmer JL, Schneider P, Seed B, Tschopp J (2000) Fas triggers an alternative, caspase-8-independent cell death pathway using the kinase RIP as effector molecule. Nat Immunol 1:489–495

Hsu H, Shu HB, Pan MG, Goeddel DV (1996) TRADD-TRAF2 and TRADD-FADD interactions define two distinct TNF receptor 1 signal transduction pathways. Cell 84:299–308

Hu WH, Johnson H, Shu HB (2000) Activation of NF-kappaB by FADD, Casper, and caspase-8. J Biol Chem 275:10838–10844

Imtiyaz HZ, Rosenberg S, Zhang Y, Rahman ZS, Hou YJ, Manser T, Zhang J (2006) The Fas-associated death domain protein is required in apoptosis and TLR-induced proliferative responses in B cells. J Immunol 176:6852–6861

Irmler M, Thome M, Hahne M, Schneider P, Hofmann K, Steiner V, Bodmer JL, Schroter M, Burns K, Mattmann C, Rimoldi D, French LE, Tschopp J (1997) Inhibition of death receptor signals by cellular FLIP. Nature 388:190–195

Jost PJ, Grabow S, Gray D, McKenzie MD, Nachbur U, Huang DC, Bouillet P, Thomas HE, Borner C, Silke J, Strasser A, Kaufmann T (2009) XIAP discriminates between type I and type II FAS-induced apoptosis. Nature 460:1035–1039

Kaiser WJ, Upton JW, Long AB, Livingston-Rosanoff D, Daley-Bauer LP, Hakem R, Caspary T, Mocarski ES (2011) RIP3 mediates the embryonic lethality of caspase-8-deficient mice. Nature 471:368–372

Kang TB, Ben-Moshe T, Varfolomeev EE, Pewzner-Jung Y, Yogev N, Jurewicz A, Waisman A, Brenner O, Haffner R, Gustafsson E, Ramakrishnan P, Lapidot T, Wallach D (2004) Caspase-8 serves both apoptotic and nonapoptotic roles. J Immunol 173:2976–2984

Kang TB, Oh GS, Scandella E, Bolinger B, Ludewig B, Kovalenko A, Wallach D (2008) Mutation of a self-processing site in caspase-8 compromises its apoptotic but not its nonapoptotic functions in bacterial artificial chromosome-transgenic mice. J Immunol 181:2522–2532

Kang TB, Yang SH, Toth B, Kovalenko A, Wallach D (2012) Caspase-8 blocks kinase RIPK3-mediated activation of the NLRP3 inflammasome. Immunity 38(1):27–40

Kelliher MA, Grimm S, Ishida Y, Kuo F, Stanger BZ, Leder P (1998) The death domain kinase RIP mediates the TNF-induced NF-kappaB signal. Immunity 8:297–303

Kovalenko A, Kim JC, Kang TB, Rajput A, Bogdanov K, Dittrich-Breiholz O, Kracht M, Brenner O, Wallach D (2009) Caspase-8 deficiency in epidermal keratinocytes triggers an inflammatory skin disease. J Exp Med 206:2161–2177

Kreuz S, Siegmund D, Scheurich P, Wajant H (2001) NF-kappaB inducers upregulate cFLIP, a cycloheximide-sensitive inhibitor of death receptor signaling. Mol Cell Biol 21:3964–3973

Kuida K, Haydar TF, Kuan CY, Gu Y, Taya C, Karasuyama H, Su MS, Rakic P, Flavell RA (1998) Reduced apoptosis and cytochrome c-mediated caspase activation in mice lacking caspase 9. Cell 94:325–337

Lemmers B, Salmena L, Bidere N, Su H, Matysiak-Zablocki E, Murakami K, Ohashi PS, Jurisicova A, Lenardo M, Hakem R, Hakem A (2007) Essential role for caspase-8 in Toll-like receptors and NFkappaB signaling. J Biol Chem 282:7416–7423

Li J, McQuade T, Siemer AB, Napetschnig J, Moriwaki K, Hsiao YS, Damko E, Moquin D, Walz T, McDermott A, Chan FK, Wu H (2012) The RIP1/RIP3 necrosome forms a functional amyloid signaling complex required for programmed necrosis. Cell 150:339–350

Lin Y, Choksi S, Shen HM, Yang QF, Hur GM, Kim YS, Tran JH, Nedospasov SA, Liu ZG (2004) Tumor necrosis factor-induced nonapoptotic cell death requires receptor-interacting protein-mediated cellular reactive oxygen species accumulation. J Biol Chem 279:10822–10828

Lin Y, Devin A, Rodriguez Y, Liu ZG (1999) Cleavage of the death domain kinase RIP by caspase-8 prompts TNF-induced apoptosis. Genes Dev 13:2514–2526

Lindsten T, Ross AJ, King A, Zong WX, Rathmell JC, Shiels HA, Ulrich E, Waymire KG, Mahar P, Frauwirth K, Chen Y, Wei M, Eng VM, Adelman DM, Simon MC, Ma A, Golden JA, Evan G, Korsmeyer SJ, MacGregor GR, Thompson CB (2000) The combined functions of proapoptotic Bcl-2 family members bak and bax are essential for normal development of multiple tissues. Mol Cell 6:1389–1399

Lu JV, Weist BM, van Raam BJ, Marro BS, Nguyen LV, Srinivas P, Bell BD, Luhrs KA, Lane TE, Salvesen GS, Walsh CM (2011) Complementary roles of Fas-associated death domain (FADD) and receptor interacting protein kinase-3 (RIPK3) in T-cell homeostasis and antiviral immunity. Proc Natl Acad Sci U S A 108(37):15312–15317

McStay GP, Salvesen GS, Green DR (2008) Overlapping cleavage motif selectivity of caspases: implications for analysis of apoptotic pathways. Cell Death Differ 15:322–331

Micheau O, Lens S, Gaide O, Alevizopoulos K, Tschopp J (2001) NF-kappaB signals induce the expression of c-FLIP. Mol Cell Biol 21:5299–5305

Micheau O, Thome M, Schneider P, Holler N, Tschopp J, Nicholson DW, Briand C, Grutter MG (2002) The long form of FLIP is an activator of caspase-8 at the Fas death-inducing signaling complex. J Biol Chem 277:45162–45171

Moulin M, Anderton H, Voss AK, Thomas T, Wong WW, Bankovacki A, Feltham R, Chau D, Cook WD, Silke J, Vaux DL (2012) IAPs limit activation of RIP kinases by TNF receptor 1 during development. Embo J 31:1679–1691

Newton K, Sun X, Dixit VM (2004) Kinase RIP3 is dispensable for normal NF-kappa Bs, signaling by the B-cell and T-cell receptors, tumor necrosis factor receptor 1, and Toll-like receptors 2 and 4. Mol Cell Biol 24:1464–1469

O'Donnell MA, Perez-Jimenez E, Oberst A, Ng A, Massoumi R, Xavier R, Green DR, Ting AT (2011) Caspase 8 inhibits programmed necrosis by processing CYLD. Nat Cell Biol 13: 1437–1442

Oberst A, Dillon CP, Weinlich R, McCormick LL, Fitzgerald P, Pop C, Hakem R, Salvesen GS, Green DR (2011) Catalytic activity of the caspase-8-FLIP(L) complex inhibits RIPK3-dependent necrosis. Nature 471:363–367

Oberst A, Pop C, Tremblay AG, Blais V, Denault JB, Salvesen GS, Green DR (2010) Inducible dimerization and inducible cleavage reveal a requirement for both processes in caspase-8 activation. J Biol Chem 285:16632–16642

Pellegrini M, Belz G, Bouillet P, Strasser A (2003) Shutdown of an acute T cell immune response to viral infection is mediated by the proapoptotic Bcl-2 homology 3-only protein Bim. Proc Natl Acad Sci U S A 100:14175–14180

Pop C, Fitzgerald P, Green DR, Salvesen GS (2007) Role of proteolysis in caspase-8 activation and stabilization. Biochemistry 46:4398–4407

Pop C, Oberst A, Drag M, Van Raam BJ, Riedl SJ, Green DR, Salvesen GS (2011) FLIP(L) induces caspase 8 activity in the absence of interdomain caspase 8 cleavage and alters substrate specificity. Biochem J 433:447–457

Pop C, Salvesen GS (2009) Human caspases: activation, specificity, and regulation. J Biol Chem 284:21777–21781

Rajput A, Kovalenko A, Bogdanov K, Yang SH, Kang TB, Kim JC, Du J, Wallach D (2011) RIG-I RNA helicase activation of IRF3 transcription factor is negatively regulated by caspase-8-mediated cleavage of the RIP1 protein. Immunity 34:340–351

Rathmell JC, Lindsten T, Zong WX, Cinalli RM, Thompson CB (2002) Deficiency in Bak and Bax perturbs thymic selection and lymphoid homeostasis. Nat Immunol 3:932–939

Senft J, Helfer B, Frisch SM (2007) Caspase-8 interacts with the p85 subunit of phosphatidylinositol 3-kinase to regulate cell adhesion and motility. Cancer Res 67:11505–11509

Smith KG, Strasser A, Vaux DL (1996) CrmA expression in T lymphocytes of transgenic mice inhibits CD95 (Fas/APO-1)-transduced apoptosis, but does not cause lymphadenopathy or autoimmune disease. Embo J 15:5167–5176

Su H, Bidere N, Zheng L, Cubre A, Sakai K, Dale J, Salmena L, Hakem R, Straus S, Lenardo M (2005) Requirement for caspase-8 in NF-kappaB activation by antigen receptor. Science 307:1465–1468

Sun L, Wang H, Wang Z, He S, Chen S, Liao D, Wang L, Yan J, Liu W, Lei X, Wang X (2012) Mixed lineage kinase domain-like protein mediates necrosis signaling downstream of RIP3 kinase. Cell 148:213–227

Takahashi T, Tanaka M, Brannan CI, Jenkins NA, Copeland NG, Suda T, Nagata S (1994) Generalized lymphoproliferative disease in mice, caused by a point mutation in the Fas ligand. Cell 76:969–976

Tan TT, Degenhardt K, Nelson DA, Beaudoin B, Nieves-Neira W, Bouillet P, Villunger A, Adams JM, White E (2005) Key roles of BIM-driven apoptosis in epithelial tumors and rational chemotherapy. Cancer Cell 7:227–238

Tenev T, Bianchi K, Darding M, Broemer M, Langlais C, Wallberg F, Zachariou A, Lopez J, Macfarlane M, Cain K, Meier P (2011) The ripoptosome, a signaling platform that assembles in response to genotoxic stress and loss of IAPs. Mol cell 43(3):432–448

Upton JW, Kaiser WJ, Mocarski ES (2010) Virus inhibition of RIP3-dependent necrosis. Cell Host Microbe 7:302–313

Upton JW, Kaiser WJ, Mocarski ES (2012) DAI/ZBP1/DLM-1 complexes with RIP3 to mediate virus-induced programmed necrosis that is targeted by murine cytomegalovirus vIRA. Cell Host Microbe 11:290–297

Van Herreweghe F, Festjens N, Declercq W, Vandenabeele P (2010) Tumor necrosis factor-mediated cell death: to break or to burst, that's the question. Cell Mol Life Sci 67:1567–1579

Vandenabeele P, Galluzzi L, Vanden Berghe T, Kroemer G (2010) Molecular mechanisms of necroptosis: an ordered cellular explosion. Nat Rev Mol Cell Biol 11:700–714

Varfolomeev EE, Schuchmann M, Luria V, Chiannilkulchai N, Beckmann JS, Mett IL, Rebrikov D, Brodianski VM, Kemper OC, Kollet O, Lapidot T, Soffer D, Sobe T, Avraham KB, Goncharov T, Holtmann H, Lonai P, Wallach D (1998) Targeted disruption of the mouse Caspase 8 gene ablates cell death induction by the TNF receptors, Fas/Apo1, and DR3 and is lethal prenatally. Immunity 9:267–276

Vercammen D, Beyaert R, Denecker G, Goossens V, Van Loo G, Declercq W, Grooten J, Fiers W, Vandenabeele P (1998) Inhibition of caspases increases the sensitivity of L929 cells to necrosis mediated by tumor necrosis factor. J Exp Med 187:1477–1485

Vercammen D, Vandenabeele P, Beyaert R, Declercq W, Fiers W (1997) Tumour necrosis factor-induced necrosis versus anti-Fas-induced apoptosis in L929 cells. Cytokine 9:801–808

Vince JE, Wong WW, Gentle I, Lawlor KE, Allam R, O'Reilly L, Mason K, Gross O, Ma S, Guarda G, Anderton H, Castillo R, Hacker G, Silke J, Tschopp J (2012) Inhibitor of apoptosis proteins limit RIP3 kinase-dependent interleukin-1 activation. Immunity 36:215–227

Wang L, Du F, Wang X (2008) TNF-alpha induces two distinct caspase-8 activation pathways. Cell 133:693–703

Watanabe-Fukunaga R, Brannan CI, Copeland NG, Jenkins NA, Nagata S (1992) Lymphoproliferation disorder in mice explained by defects in Fas antigen that mediates apoptosis. Nature 356:314–317

Wilson NS, Dixit V, Ashkenazi A (2009) Death receptor signal transducers: nodes of coordination in immune signaling networks. Nat Immunol 10:348–355

Yeh WC, Itie A, Elia AJ, Ng M, Shu HB, Wakeham A, Mirtsos C, Suzuki N, Bonnard M, Goeddel DV, Mak TW (2000) Requirement for Casper (c-FLIP) in regulation of death receptor-induced apoptosis and embryonic development. Immunity 12:633–642

Yeh WC, Pompa JL, McCurrach ME, Shu HB, Elia AJ, Shahinian A, Ng M, Wakeham A, Khoo W, Mitchell K, El-Deiry WS, Lowe SW, Goeddel DV, Mak TW (1998) FADD: essential for embryo development and signaling from some, but not all, inducers of apoptosis. Science 279:1954–1958

Yoshida H, Kong YY, Yoshida R, Elia AJ, Hakem A, Hakem R, Penninger JM, Mak TW (1998) Apaf1 is required for mitochondrial pathways of apoptosis and brain development. Cell 94:739–750

Zhang DW, Shao J, Lin J, Zhang N, Lu BJ, Lin SC, Dong MQ, Han J (2009) RIP3, an energy metabolism regulator that switches TNF-induced cell death from apoptosis to necrosis. Science 325:332–336

Zhang J, Kabra NH, Cado D, Kang C, Winoto A (2001) FADD-deficient T cells exhibit a disaccord in regulation of the cell cycle machinery. J Biol Chem 276:29815–29818

Zhang N, He YW (2005) An essential role for c-FLIP in the efficient development of mature T lymphocytes. J Exp Med 202:395–404

Chapter 7
The In Vivo Significance of Necroptosis: Lessons from Exploration of Caspase-8 Function

David Wallach, Tae-Bong Kang, Akhil Rajput, Seung-Hoon Yang, Jin-Chul Kim, Beata Toth, Konstantin Bogdanov, Oliver Dittrich-Breiholz, Michael Kracht, and Andrew Kovalenko

7.1 Growing Knowledge of Necrotic Cell Death in Inflammation and Causal Interrelationships Between Necrotic Cell Death and Inflammation: A Brief Historical Perspective

Cell death in tissues damaged as a result of inflammation was noticed almost as early as the detection of the leukocyte mobilization events that underlie inflammation. However, definition of the death process was initially so vague that today we cannot tell exactly what was observed in those early studies. Furthermore, we are not even sure what the terms for death used in those studies actually referred to (see Majno et al. (1960) for a discussion of such terms). The clear definition of apoptosis in 1972 (Kerr et al. 1972) opened the way to exploration of the relevance of this form of death to inflammation. Some evidence for apoptotic cell death was reported well before that time, but the concept had not been clearly defined (Clarke and Clarke 1996). The post-1972 studies disclosed that apoptosis serves an

D. Wallach (✉) • A. Rajput • S.-H. Yang • J.-C. Kim • B. Toth • K. Bogdanov • A. Kovalenko
Department of Biological Chemistry, The Weizmann Institute of Science,
76100 Rehovot, Israel
e-mail: d.wallach@weizmann.ac.il

T.-B. Kang
Department of Biotechnology, College of Biomedical and Health Science,
Konkuk University, Chung-Ju 380-701, South Korea

O. Dittrich-Breiholz
Institute of Biochemistry, Medical School Hannover, Hannover 30625, Germany

M. Kracht
Rudolf-Buchheim-Institute of Pharmacology, University of Giessen,
Frankfurter Strasse 107, 35392 Giessen, Germany

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important role in the eventual elimination of leukocytes that mediate various manifestations of inflammation and, as a consequence, in the resolution of this process (e.g., Haslett et al. 1994). Identification of molecular changes specific to apoptotic cell death, and of signaling mechanisms that induce it, contributed to more accurate recognition of the occurrence of apoptosis in inflammatory processes and provided evidence for its induction in these processes by specific ligands.

In contrast, studies of the occurrence of non-apoptotic cell death in inflammation yielded perplexing data on its role in the process. Accumulating evidence suggested that other cellular changes occurring in inflammation, particularly vascular blockage and activation of granulocytes, precede such death and are the cause of it (Menkin 1948; Henson and Johnston 1987). Yet there was also evidence suggesting that non-apoptotic death plays causal role in inflammation. Of particular note were the studies of Valy Menkin, who suggested that many of the manifestations of inflammation are mediated by molecules generated by injured cells (Menkin 1948), and, much later, the work of Polly Matzinger attributing crucial roles to "danger signals" that emanate from injured tissues both in adaptive and in innate immunity (Matzinger 1994). Accumulating knowledge about cellular components that indeed act as "danger signals" (danger-associated molecular patterns—DAMPs) supports Menkin's and Matzinger's suggestions, though understanding of the exact functional role of individual DAMPs in inflammation is still limited (Rock and Kono 2008). More recently it was shown that necrotic cell death can be induced by binding of specific agonists such as the cytokine TNF or bacterial endotoxin (LPS) to specific receptors and by subsequent activation of a distinct group of signaling molecules (reviewed in Vandenabeele et al. 2010). These findings provided further support for the idea that necrosis might serve to promote inflammation and possibly some other physiological roles as well.

7.2 Roles of Caspase-8 in Induction of Apoptotic Cell Death and in Necroptosis

Caspase-8 was initially discovered as the proximal enzyme in the induction of apoptotic cell death by receptors of the TNF family (Boldin et al. 1996; Muzio et al. 1996). It mediates this effect by cleaving and hence activating executioner caspases such as caspase-3 and also via proteolytic processing of the Bcl-2 family member BID which, once cleaved, initiates proapoptotic changes in the mitochondria (Wallach et al. 1999).

Receptors of the TNF family that induce apoptotic cell death can also, in some cells and certain situations, induce necrotic cell death. Two protein kinases, RIPK1 and RIPK3, were recently found to initiate the signaling for necrotic cell death by these and other inducers (Holler et al. 2000; Degterev et al. 2008; Cho et al. 2009; He et al. 2009; Zhang et al. 2009). While serving a crucial function in triggering apoptotic cell-death induction, caspase-8 also plays a major role in restricting the

RIPK1-/RIPK3-mediated induction of necrotic cell death. It is believed to serve this latter role by cleaving the activated RIPK1 and RIPK3 molecules and also by cleaving the deubiquitinase CYLD, a tumor suppressor that regulates the initiation of necroptosis (Green et al. 2011; O'Donnell et al. 2011).

7.3 Caspase-8 Deficiency In Vivo Stimulates Inflammation

Consistent with the crucial role of caspase-8 in the induction of apoptotic cell death through the extrinsic cell-death pathway, knockout of this enzyme in mice was found to block activation of this pathway (Varfolomeev et al. 1998). However, the main phenotypic changes so far observed in mice as a result of such knockout reflect other functional roles of this enzyme, distinct from the induction of apoptotic death. One of the most obviously apparent of these changes is an increased propensity for inflammation or even its spontaneous emergence. We first observed this functional consequence when studying the phenotype of mice whose hepatocytes were deficient in caspase-8. Under normal conditions this deficiency seemed to have no effect on the mice. However, partial hepatectomy in these mice triggered chronic liver inflammation characterized by increased accumulation of leukocytes, phosphorylation of STAT-3, and hypertrophy of the liver (Fig. 7.1, Ben Moshe et al. 2007). A much more severe inflammatory process was found to develop spontaneously in the mouse skin when *caspase-8* was deleted in the basal cells of the epidermis (Fig. 7.2, Kovalenko et al. 2009). This acute inflammation first became evident

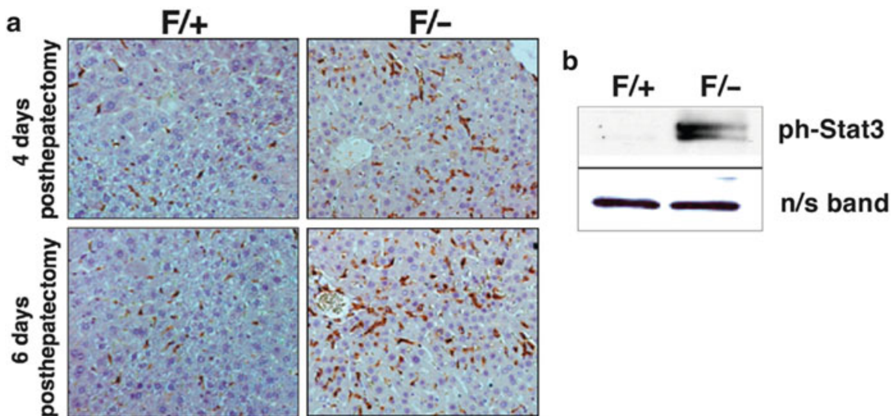

Fig. 7.1 Caspase-8 deficiency in hepatocytes prompts inflammation of the liver after partial hepatectomy. Comparison of the effects of partial hepatectomy on wild-type mice (F/+) and mice with caspase-8-deficient hepatocytes (F/−). (**a**) Staining with the anti-F4/80 antibody for accumulation of leukocytes in the liver 4 and 6 days after partial hepatectomy. Magnification: 400×. (**b**) Western blot analysis of STAT-3 phosphorylation in the liver 14 days after partial hepatectomy. From Ben Moshe et al. (2007)

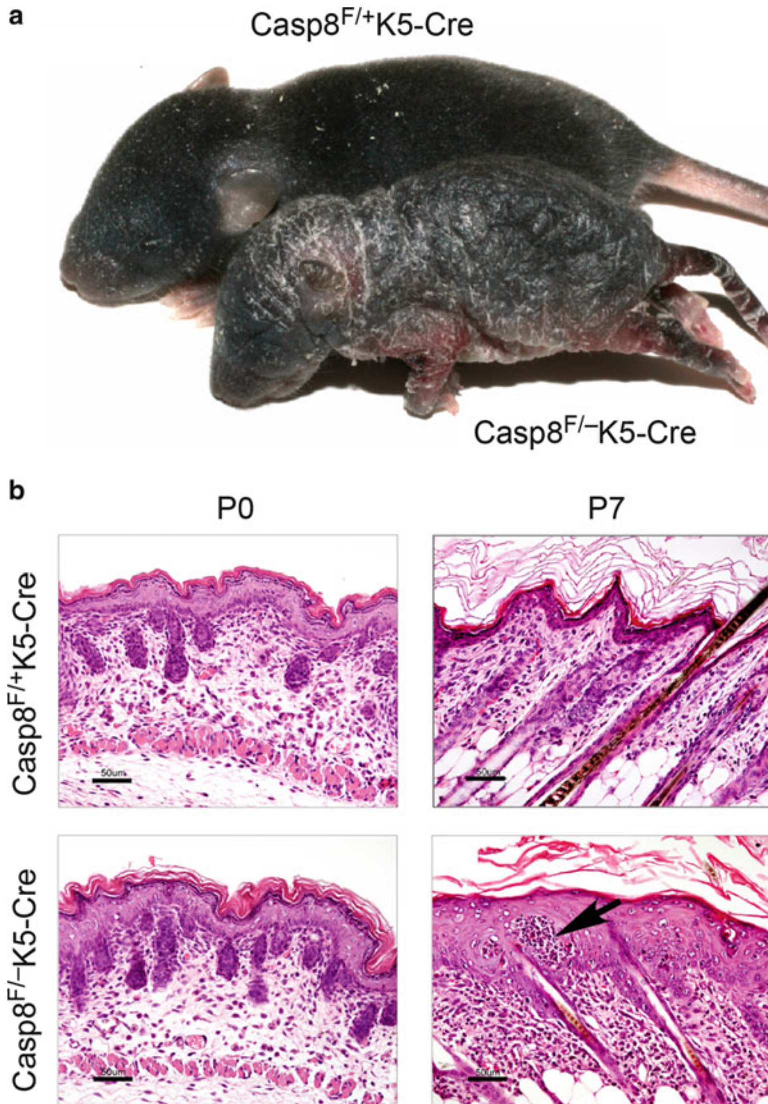

Fig. 7.2 Caspase-8 deficiency in the basal layer of the epidermis prompts acute spontaneous skin inflammation. (**a**) General appearance of mice that express caspase-8 (*Casp-8*$^{F/+}$*K5-Cre*) or do not express it (*Casp-8*$^{F/-}$*K5-Cre*) in the basal cells of the epidermis at P7 (7 days after birth). (**b**) Microscopy of the skin. At P0, the skin of the *Casp-8*$^{F/-}$*K5-Cre* mouse is indistinguishable in appearance from the skin of the *Casp-8*$^{F/+}$*K5-Cre* mouse. At P7 there is marked epidermal hyperplasia and widespread dermal inflammatory cellular infiltration in the *Casp8*$^{F/-}$*K5-Cre* mouse. *Arrows* in (**b**) point to intraepidermal eosinophilic pustules. H&E; bars, 50 µm. From Kovalenko et al. (2009)

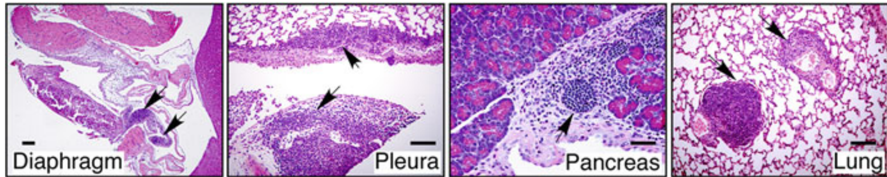

Fig. 7.3 Ubiquitous expression of an enzymatically inactive caspase-8 allele induces inflammation in various organs. Tissue specimens of mice with one wild-type and one enzymatically inactive *caspase-8* allele demonstrate multifocal cellular inflammatory infiltration in diaphragmatic parietal pleura, lung visceral pleura, and interstitium of pancreas and lung (*arrows*). Scale bar, 100 μm. From Kovalenko et al. (2009)

about 3 days after birth and rapidly strengthened, with death occurring a few days later. Deletion of *caspase-8* in enterocytes was similarly shown to trigger chronic inflammation of the intestine (Gunther et al. 2011).

Ubiquitous deletion of *caspase-8* is fatal in utero (Varfolomeev et al. 1998). Therefore, to obtain a broad view of the function of this enzyme, we had to find a way to impose partial blockage of its activity throughout the body. Mice expressing subnormal amounts of caspase-8 owing to deletion of one of its two alleles did not display any evident abnormality. However, mice that also expressed an enzymatically inactive allele of *caspase-8* developed chronic inflammation in the skin, as well as in a variety of internal organs, apparently as a result of interference of the mutant enzyme with the function of the enzyme expressed by the wild-type allele (Fig. 7.3, Kovalenko et al. 2009). This finding further demonstrated that caspase-8 acts to restrict inflammation in various tissues.

7.4 Several Different Functions of Caspase-8 May Contribute to Restriction of Inflammation

The suspected contribution of necroptosis to inflammation is currently the focus of much research interest. It is therefore important to stress that although caspase-8 deficiency can result in enhanced necroptosis in cultured cells treated with certain inflammation-related ligands and that if this process occurs in vivo it may indeed promote inflammation, the inhibition of necroptosis induction is by no means the only mechanism by which caspase-8 restricts the initiation of inflammation. Therefore, ablation of this particular effect is not the only factor that can account for the inflammation observed in mice when caspase-8 function is obliterated (Fig. 7.4). For one thing, apoptotic cells block activation of the innate immune response in other cells (Voll et al. 1997; Fadok et al. 1998). Therefore, the function that occasioned the discovery of caspase-8, namely, its initiation of the extrinsic apoptotic cell-death pathway, can contribute to restriction of inflammation through the effects of cells that have died apoptotically as a result of caspase-8 activation ("1" in Fig. 7.4). Even when cell death does not occur, activation of executioner caspases

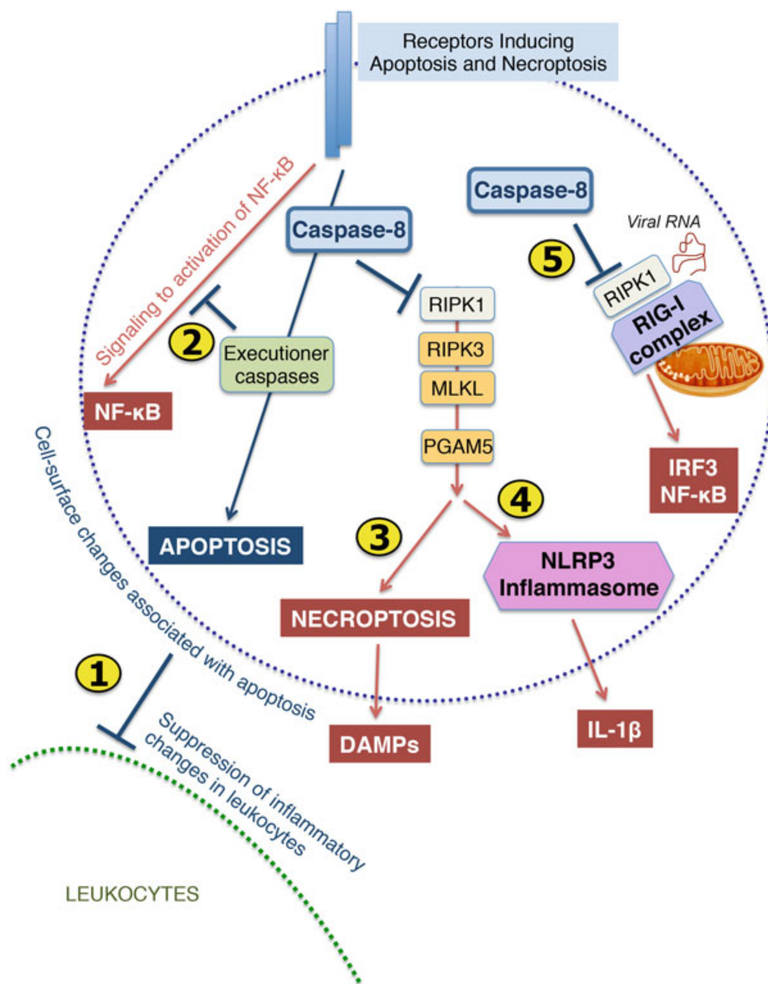

Fig. 7.4 Signaling mechanisms participating in various anti-inflammatory effects of caspase-8. See text for further explanation

by the extrinsic cell-death pathway may suppress signaling for inflammation (such as that mediated by the NF-κB transcription factors) by cleaving proteins that participate in these signaling pathways (e.g., cf. Levkau et al. 1999; Lin et al. 1999; Henkler et al. 2003; Frelin et al. 2008, “2” in Fig. 7.4).

The extrinsic cell-death signaling pathway and signaling for necroptosis branch out from a common starting point, namely, a signaling complex whose assembly is dictated by receptors such as the TNF receptor or certain Toll-like receptors (TLRs) that can initiate both effects. Downstream of the ramification point the necroptotic pathway leads to activation of several proteins, including RIPK3, MLKL, and PGAM5 (Cho et al. 2009; He et al. 2009; Zhang et al. 2009; Sun et al. 2012;

Wang et al. 2012; Zhao et al. 2012), which do not participate in the extrinsic cell-death pathway (“3” in Fig. 7.4). However, these further downstream events are still not distinctive of necroptosis. Persuasive evidence indicates that the kinase function of RIPK1 serves several functions other than induction of necrotic cell death (e.g., Devin et al. 2003; Biton and Ashkenazi 2011; Lukens et al. 2013). Moreover, we recently found that activation of RIPK3, MLML, and PGAM5 in a way that depends on the kinase function of RIPK1 can also lead to activation of the NLRP3 inflammasome, a complex that mediates generation of inflammatory cytokines such as interleukin (IL)-1β, and that this function is elicited independently of the induction of cell death (Fig. 7.5 (Kang et al. 2013) and “4” in Fig. 7.4). It might well turn out that this pathway leads to various additional kinds of functional changes. As with the induction of necroptosis, triggering of these various other changes is likely to be suppressed by caspase-8 and thus enhanced in its absence.

Moreover, caspase-8 also participates in signaling complexes other than those triggered by the cell-surface receptors that activate the extrinsic cell-death pathway, and its effects in the latter complexes might also result in suppression of inflammation. An example of this function is the role served by caspase-8 in controlling the response to cytoplasmic nucleic acids. RIG-I, a complex that contains both caspase-8 and RIPK1, associates with the mitochondria and peroxisomes, senses viral and cellular cytoplasmic nucleic acids, and mediates antiviral responses through signaling for activation of IRF3 and NF-κB. Binding of caspase-8 with this complex and the resulting caspase-8-induced cleavage of RIPK1 restrict the activation of IRF3 by cytoplasmic nucleic acids (Fig. 7.6 (Rajput et al. 2011) and “5” in Fig. 7.4).

There are also functions of caspase-8 that might yield the opposite consequence—enhancement of inflammation or even its triggering. It was shown, for example, that once caspase-8 is activated, either by the extrinsic cell-death pathway or in some other way, it can cleave pro-IL1β molecules and thus generate active molecules of this cytokine (Bossaller et al. 2012; Gringhuis et al. 2012; Vince et al. 2012). However, since the present discussion concerns the relative contribution of necroptosis to enhancement of inflammation, we have restricted it to those functions of caspase-8 whose arrest can result in such enhancement.

7.5 Which of the Various Anti-inflammatory Effects of Caspase-8 Contribute to the Inflammatory Processes Initiated by Its Genetic Ablation?

When we first discerned inflammation in mice as a result of *caspase-8* deletion, the only known signaling activity of this enzyme was the extrinsic cell-death pathway. It therefore initially seemed possible that the observed inflammation had resulted from deficient killing of certain cells through the extrinsic cell-death pathway or from impairment of some other consequences of that pathway’s activation. These possibilities, however, were excluded by a subsequent study in which we applied

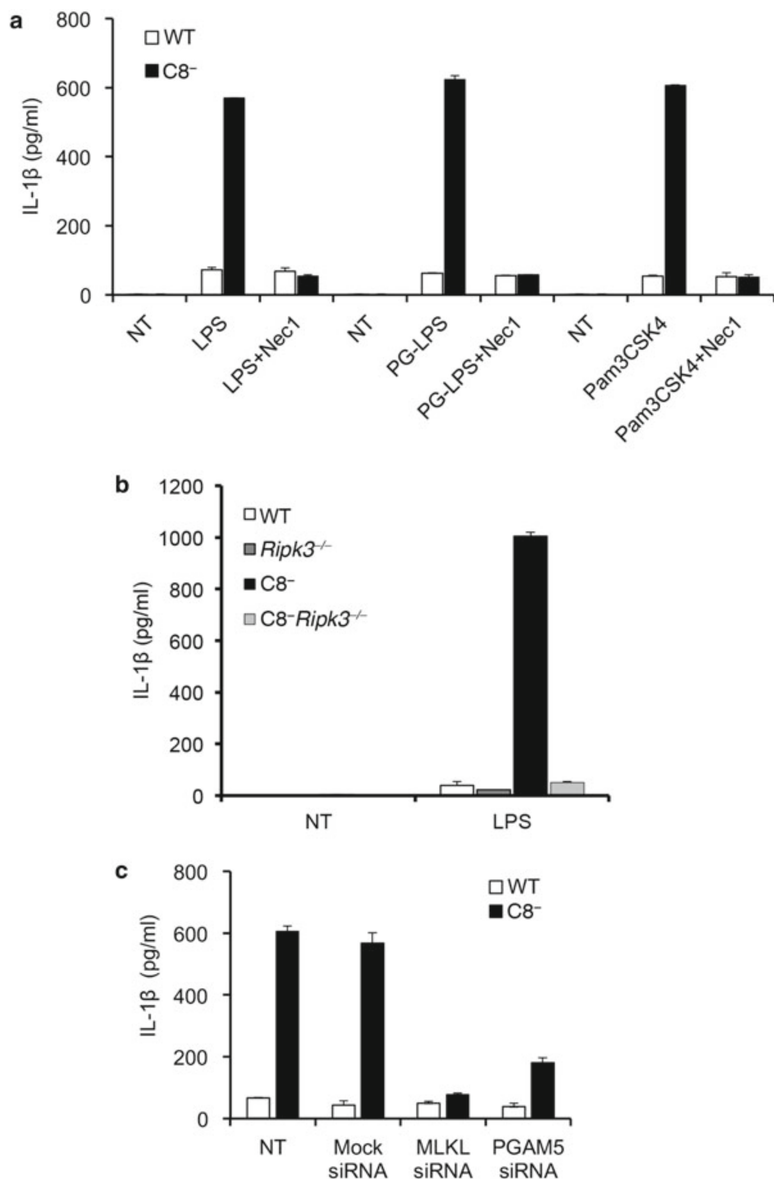

Fig. 7.5 Caspase-8 deficiency in dendritic cells facilitates activation of the NLRP3 inflammasome. (**a**) Secretion of IL-1β by wild-type (WT) and caspase-8-deficient ($C8^-$) dendritic cells in response to treatment for 6 h with the indicated TLR ligands (which induce IL-1β through activation of the NLRP3 inflammasome) and the effect of added necrostatin (Nec1), an inhibitor of the kinase function of RIPK1. (**b**) Secretion of IL-1β by dendritic cells derived from the indicated mouse strains in response to treatment with LPS. (**c**) Effects of knockdown of MLKL or PGAM5 in WT and $C8^-$ dendritic cells on secretion of IL-1β in response to their treatment for 3 h with LPS. From Kang et al. (2013)

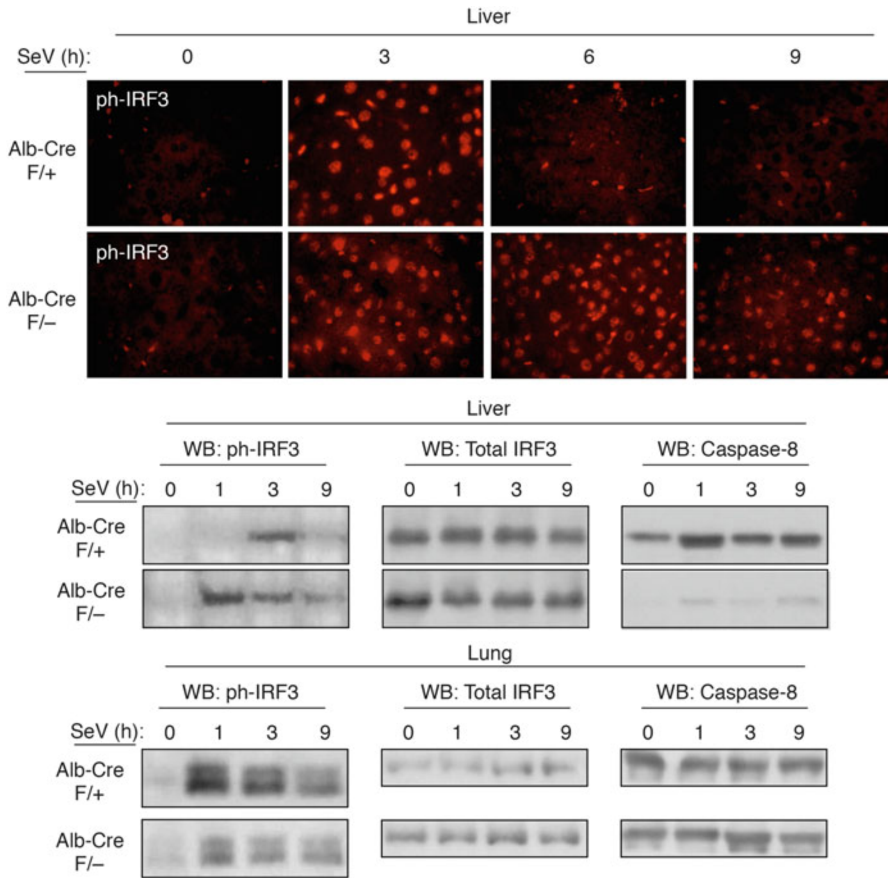

Fig. 7.6 Enhanced viral RNA-induced activation of IRF3 in caspase-8-deficient cells. Effect of conditional deletion of *caspase-8* in hepatocytes on IRF3 activation by Sendai virus (SeV) infection in vivo. *Top* and *middle*, immunohistochemical analyses of IRF3 phosphorylation and translocation to the nuclei and immunoblot analyses of IRF3 phosphorylation in the livers of control mice (Alb-Cre F/+) and of mice with hepatocyte-specific deletion of *Casp8* (Alb-Cre F/−) at various times after SeV injection. *Bottom*, immunoblot analysis of IRF3 phosphorylation in the lungs of the same mice. Immunostained liver sections were inspected and photographed under a Nikon ECLIPSE E600 fluorescence microscope at a magnification of 100×. From Rajput et al. (2011)

bacterial artificial chromosome (BAC) transgenesis to explore structure/function relationships for the various activities of caspase-8 in vivo. That study revealed that activation of the extrinsic cell-death pathway depends on self-processing of caspase-8. However, mutational ablation of such self-processing did not trigger inflammation in the mice (Kang et al. 2008), implying that those caspase-8 function(s) whose ablation upon caspase-8 deletion results in inflammation do not depend on activation of the extrinsic cell-death pathway, but involve a molecular form of caspase-8 distinct from the form that mediates this pathway.

Of the various inflammatory processes that we and others have found to be inflicted by caspase-8 deficiency in mice, my laboratory chose to focus on the skin inflammation imposed by deletion of *caspase-8* at the basal layer of the epidermis. This inflammatory process and similar inflammation resulting from epidermal-specific deletion of FADD (an adapter protein to which caspase-8 binds) have been studied in some other laboratories as well (Li et al. 2010; Bonnet et al. 2011). Our main findings (some of which do not fully concur with the findings from those other laboratories) were as follows:

1. While the first signs of skin pathology and histological evidence of inflammation could be discerned only about 3 days after birth, detailed analyses of gene expression revealed that activation of inflammatory genes in the skin occurs well before that, as early as 2 days before birth (Fig. 7.7 and data not shown). Various signaling enzymes that contribute to inflammation were likewise found to be activated very early. Among the earliest observed were activation of IRF3 and of the kinase TBK1 that phosphorylates it (Kovalenko et al. 2009).
2. Skin inflammation as a result of caspase-8 deficiency in keratinocytes also occurs, albeit to a lesser extent, on a TNF null background. It also occurs in mice deficient in IL-1α and IL-1β and in either of the two main adapter proteins (MyD88 and TRIFF) activated by the TLRs, suggesting that this inflammation does not depend on triggering of the TNF or IL1 receptors or of TLRs (Kovalenko et al. 2009).
3. In situ hybridization with probes corresponding to some of the main inflammatory genes upregulated in the inflamed skin revealed that whereas deletion of *caspase-8* occurs at the basal layer of the epidermis, activation of these inflammatory genes mainly occurs several cell layers further upwards, in the granular layer of the epidermis (Fig. 7.8, Kovalenko et al. 2009).
4. Our histological analysis of the skin at the time of its extensive inflammation revealed some dead keratinocytes in the epidermis along with various other histological aberrations and accumulation of leukocytes in this tissue. Prior to these massive histological changes, however, death of cells in the epidermis was rare. Despite detailed histological analyses, we could not discern any increase in the frequency of cell death at that time in the caspase-8-deficient epidermis compared to that in the epidermis of normal mice, nor could we see a consistent increase in the number of TUNEL-positive cells in the knockout skin.
5. In agreement with the observations of others (Kaiser et al. 2011), we found that inflammation of the skin resulting from caspase-8 deficiency in the epidermis did not occur on a RIPK3 null background, implying a crucial role of RIPK3 in its induction.

Fig. 7.7 (continued) For each mouse, mRNA expression of the indicated four genes was analyzed by real-time PCR. Samples from pairs of *Casp-8$^{F/-}$K5-Cre* and *Casp-8$^{F/+}$K5-Cre* mice were tested simultaneously; these pairs are depicted at identical horizontal positions. (In the case of Isg15, four additional D − 1 WT samples were analyzed.) Shown are the −Δ cycle threshold (Ct) values obtained by subtraction of the Ct value of each PCR product from the Ct value of the housekeeping gene *Hprt1*. *Red arrows* point to values for two *Casp-8$^{F/-}$K5-Cre* mice that showed significant upregulation of Isg15 expression before birth. From Kovalenko et al. (2009)

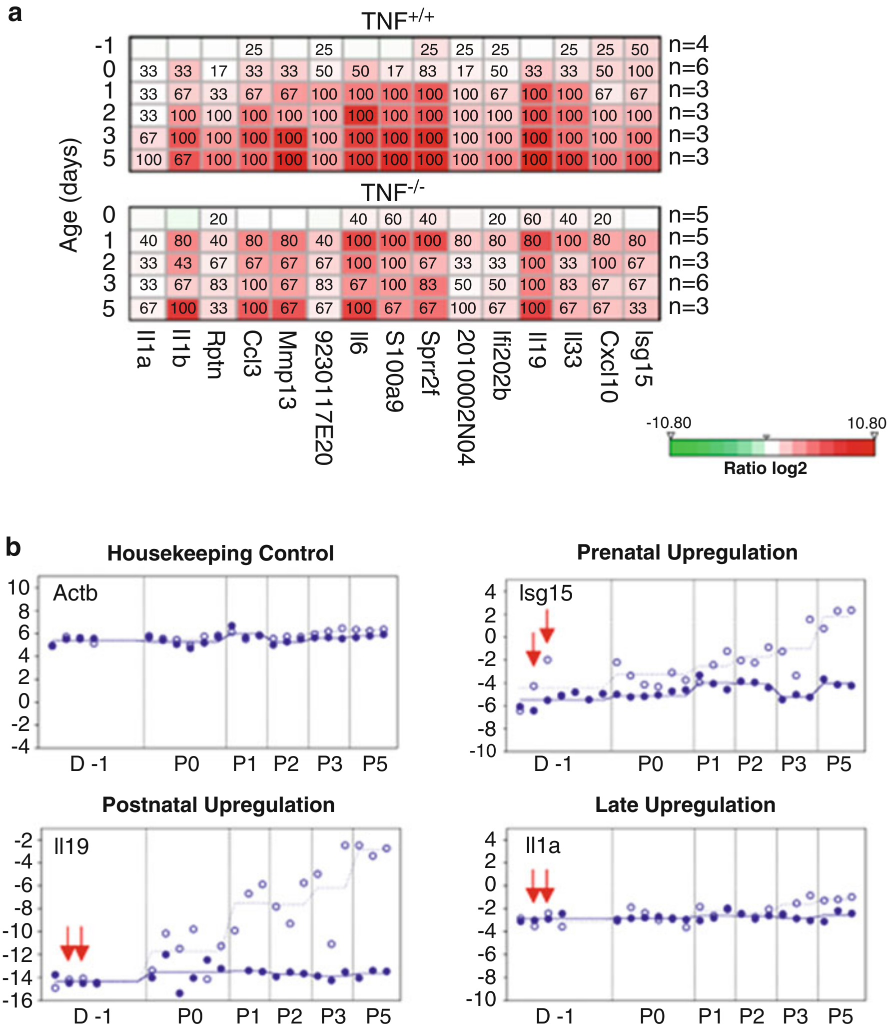

Fig. 7.7 Caspase-8 deficiency in the basal cells of the epidermis already facilitates expression of inflammatory genes in the skin before birth. Kinetic analysis of gene expression changes in the *caspase-8*-null epidermis and assessment of their dependence on TNF. (**a**) RNA isolated from the epidermal tissues of 22 pairs of *Casp-8*$^{F/+}$*K5-Cre* and *Casp-8* $^{F/-}$*K5-Cre* mice (from eight litters) and from the epidermal tissues of 22 pairs of *TNF*$^{-/-}$*Casp-8*$^{F/+}$*K5-Cre*/*TNF*$^{-/-}$*Casp-8*$^{F/-}$*K5-Cre* mice (from seven litters) was used for real-time RT-PCR determination of the expression of 15 selected genes representative of most of the functional groups of genes found to be upregulated in the skin as a result of *caspase-8* deletion in the basal cells of the epidermis. For heat-map visualization, log2-transformed expression ratios were ordered according to increasing consistency of changes in mRNA expression in the TNF+/+ mice (horizontal axis). Percentages of *Casp-8*$^{F/-}$*K5-Cre* mice at a given age with a significantly upregulated transcript relative to its mean expression in all *Casp-8*$^{F/+}$*K5-Cre* age-matched mice are recorded in the heat-map boxes. *n*, number of experimental/control mice pairs analyzed for the given age. (**b**) RNA was isolated from the epidermis of *Casp-8*$^{F/-}$*K5-Cre mice* (*empty circles*, values for individual mice born on different dates; *broken lines*, mean values for all individuals analyzed at a particular age) and of *Casp-8*$^{F+}$*K5-Cre* mice (*filled circles* and *solid lines*) 1 day before birth (D – 1) and at different times postnatally.

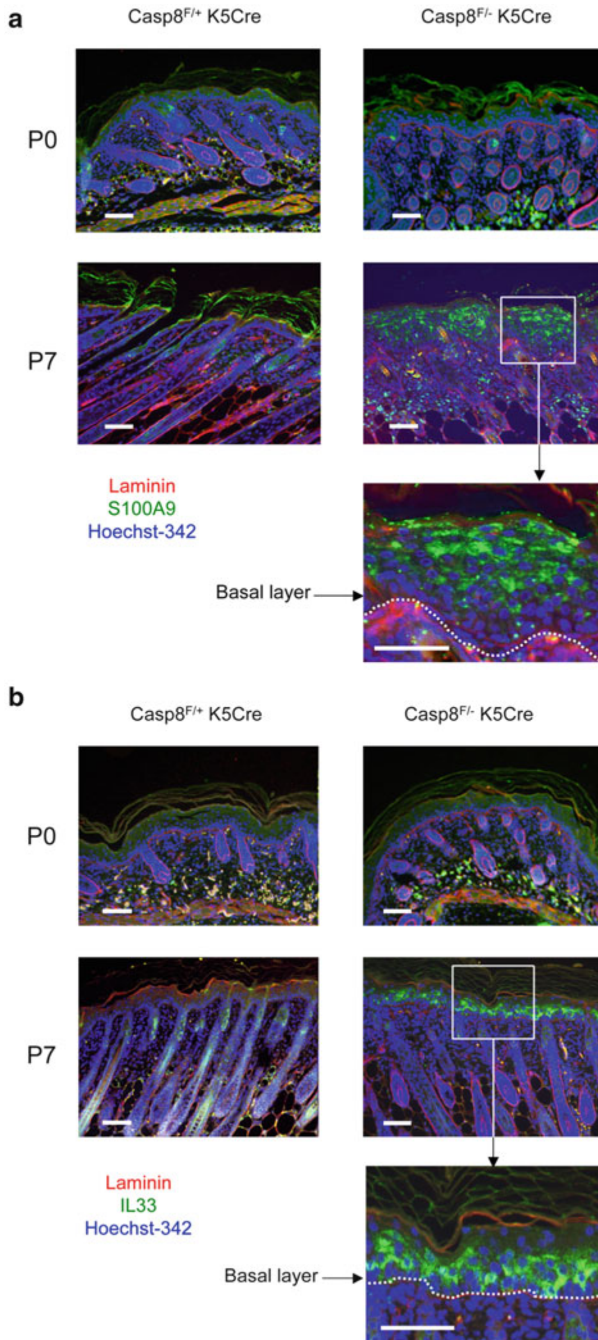

Fig. 7.8 Expression of inflammatory genes in caspase-8-deficient epidermis occurs mainly in the granular layer. In situ hybridization for spatial analysis of upregulated inflammatory genes in the caspase-8-deficient epidermis. Skin samples of *Caspase-8*$^{F/-}$*K5-Cre* and *Caspase-8*$^{F/+}$*K5-Cre* littermates at P0 and P7 were hybridized with DIG-labeled antisense RNA probe for mRNA of S100a9 (**a**) or IL33 (**b**) and then subjected to fluorescence detection (Cy2-anti-DIG, *green*). Nuclei were counterstained with Hoechst 342 (*blue*). Rabbit anti-laminin was used to visualize the basal membrane (*red*), *highlighted* in the magnified presentation of the *insets* by *dotted lines* and detected with Cy3-anti-rabbit Ig. Bars, 50 μm. From Kovalenko et al. (2009)

Taken at face value, these findings seem to suggest that caspase-8 deficiency triggers prenatal expression of some inflammatory genes and that the death of some cells in the skin observed days later is not the cause of the inflammation but rather a result of pathological changes inflicted on the skin. However, given the limitations of sensitivity of the techniques for detecting necrotic cells in vivo and the lack of means to distinguish necroptosis from other forms of necrotic cell death, we cannot exclude the possibility that necroptotic cell death occurring at a frequency lower than our detection limit does precede activation of inflammatory genes in the skin and is the initial cause of it.

7.6 Can Genetic Modulation of a Signaling Protein That Controls Necroptosis Induction Be Applied to Explore the Causal Role of Necroptosis in Inflammation?

The information underlying our current notions of the in vivo significance of necroptosis is fragmentary. There are two major gaps in our knowledge: (a) Necroptosis was defined in the framework of cell culture studies. We assume that some of the cells found to die necrotically in vivo have died by necroptosis. However, because necrotic death can also be inflicted merely by injury of the cells independently of activation of the necroptotic pathway and since there is currently no known molecular or histological parameter that distinguishes between cells that die through activation of the necroptotic pathway and cells in which necrotic death is inflicted in another way, we cannot clearly identify necroptotic cells in vivo. (b) Inflammation and necrotic cell death can each be the cause of the other. Inflammation can inflict necrotic cell death, for example, by arrest of the supply of nutrients and oxygen as a result of clogging of the vasculature or through effects of superoxide radicals and toxic proteins generated by activated granulocytes. (As stated above, the extent of similarity between death induced in this context and necroptosis is not yet known.) Conversely, cellular components released by cells when they die necrotically can trigger inflammation. At this stage we have no way of knowing which of the two sequences of events—induction of necrotic death by inflammation or triggering of inflammation by necrotic cells—has occurred in a given in vivo situation (Wallach et al. 2011).

As mentioned at the beginning of this article, on looking back at the early studies of the cellular basis for inflammation, we can now appreciate the limitation of using only histological analysis to affirm the occurrence and define the functional significance of necrotic cell death during inflammation. Deciphering of the molecular basis for cellular phenomena greatly advances our ability to study their in vivo significance. Molecules found to contribute to these phenomena can be used as probes for their detection in vivo, and mutation of these molecules in mice can allow exploration of their functional significance. As we now begin to acquire molecular knowledge of necroptosis, such tools and techniques, though not yet available to us,

can be expected to emerge. A major limitation of our current knowledge is that none of the necroptosis-regulating molecules identified so far serves exclusively for induction of this process. This article addresses the fact that occurrence of inflammation as a result of caspase-8 deficiency cannot be safely ascribed to necroptosis because there are other mechanisms besides facilitation of necroptosis by which caspase-8 can promote inflammation. The same limitation applies to all other signaling proteins so far found to participate in the initiation or the regulation of necroptosis—the receptors inducing it (the TNF receptor, TLRs, and others) and the signaling proteins activated by them (e.g., FADD, cIAP1 and cIAP2, CYLD, the kinase function of RIPK1). Our recent finding that three molecules known to participate further downstream in the induction of necroptosis—RIPK3, MLKL, and PGAM5—also mediate activation of the NLRP3 inflammasome independently of induction of cell death (Fig. 7.5 and Kang et al. 2013) implies that these molecules likewise cannot serve as exclusive markers for necroptosis. Probes that detect their activation will not necessarily provide a means to detect necroptosis, and functional changes resulting from genetic ablation of these proteins cannot be assumed to result from deficient or excessive necroptosis.

The above constraint does not mean, however, that specific molecular markers for necroptosis cannot be found. Even in the most pleiotropic signaling pathways, it may turn out to be possible to identify distal signaling molecules and molecular changes that are specific to a particular functional consequence of their activation. In the case of the pathways that trigger apoptotic cell death, for example, we currently believe that certain events are distinctive markers of this death, as exemplified by distal changes such as the cleavage of DNA down to nucleosomal units (Arends et al. 1990) or the caspase-mediated cleavage of I-CAD, the protein withholding the enzyme that mediates this DNA cleavage (Enari et al. 1998; Sakahira et al. 1998). Activation of the executioner caspases that mediate these distal events is less specific to this process because these enzymes can also have non-apoptotic functions, and—as demonstrated in this article with regard to the function of caspase-8—proteins acting upstream of the executioner caspases are even more pleiotropic. The terminal signaling enzymes and effector molecules in the necroptotic pathway are still unknown. We also lack reliable knowledge of the DAMPs generated by necroptotic cells in vivo, and indeed even the mere existence of such DAMPs has so far only been inferred from reports of their generation by cells in which necrotic death was imposed in other ways.

There is reason to hope that, as in the case of the assessment of apoptosis, once the distal events in necroptosis are identified, they will provide more reliable molecular tools to discern necroptosis in vivo and to define more clearly the causal relationship between this form of cell death and inflammation.

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References

Arends MJ, Morris RG, Wyllie AH (1990) Apoptosis. The role of the endonuclease. Am J Pathol 136:593–608

Ben Moshe T, Barash H, Kang TB, Kim JC, Kovalenko A, Gross E, Schuchmann M, Abramovitch R, Galun E, Wallach D (2007) Role of caspase-8 in hepatocyte response to infection and injury in mice. Hepatology 45:1014–1024

Biton S, Ashkenazi A (2011) NEMO and RIP1 control cell fate in response to extensive DNA damage via TNF-alpha feedforward signaling. Cell 145:92–103

Boldin MP, Goncharov TM, Goltsev YV, Wallach D (1996) Involvement of MACH, a novel MORT1/FADD-interacting protease, in Fas/APO-1- and TNF receptor-induced cell death. Cell 85:803–815

Bonnet MC, Preukschat D, Welz PS, van Loo G, Ermolaeva MA, Bloch W, Haase I, Pasparakis M (2011) The adaptor protein FADD protects epidermal keratinocytes from necroptosis in vivo and prevents skin inflammation. Immunity 35:572–582

Bossaller L, Chiang PI, Schmidt-Lauber C, Ganesan S, Kaiser WJ, Rathinam VA, Mocarski ES, Subramanian D, Green DR, Silverman N, Fitzgerald KA, Marshak-Rothstein A, Latz E (2012) Cutting edge: FAS (CD95) mediates noncanonical IL-1beta and IL-18 maturation via caspase-8 in an RIP3-independent manner. J Immunol 189:5508–5512

Cho YS, Challa S, Moquin D, Genga R, Ray TD, Guildford M, Chan FK (2009) Phosphorylation-driven assembly of the RIP1-RIP3 complex regulates programmed necrosis and virus-induced inflammation. Cell 137:1112–1123

Clarke PG, Clarke S (1996) Nineteenth century research on naturally occurring cell death and related phenomena. Anat Embryol 193:81–99

Degterev A, Hitomi J, Germscheid M, Ch'en IL, Korkina O, Teng X, Abbott D, Cuny GD, Yuan C, Wagner G, Hedrick SM, Gerber SA, Lugovskoy A, Yuan J (2008) Identification of RIP1 kinase as a specific cellular target of necrostatins. Nat Chem Biol 4:313–321

Devin A, Lin Y, Liu ZG (2003) The role of the death-domain kinase RIP in tumour-necrosis-factor-induced activation of mitogen-activated protein kinases. EMBO Rep 4:623–627

Enari M, Sakahira H, Yokoyama H, Okawa K, Iwamatsu A, Nagata S (1998) A caspase-activated DNase that degrades DNA during apoptosis, and its inhibitor ICAD. Nature 391:43–50

Fadok VA, Bratton DL, Konowal A, Freed PW, Westcott JY, Henson PM (1998) Macrophages that have ingested apoptotic cells in vitro inhibit proinflammatory cytokine production through autocrine/paracrine mechanisms involving TGF-beta, PGE2, and PAF. J Clin Invest 101: 890–898

Frelin C, Imbert V, Bottero V, Gonthier N, Samraj AK, Schulze-Osthoff K, Auberger P, Courtois G, Peyron JF (2008) Inhibition of the NF-kappaB survival pathway via caspase-dependent cleavage of the IKK complex scaffold protein and NF-kappaB essential modulator NEMO. Cell Death Differ 15:152–160

Green DR, Oberst A, Dillon CP, Weinlich R, Salvesen GS (2011) RIPK-dependent necrosis and its regulation by caspases: a mystery in five acts. Mol Cell 44:9–16

Gringhuis SI, Kaptein TM, Wevers BA, Theelen B, van der Vlist M, Boekhout T, Geijtenbeek TB (2012) Dectin-1 is an extracellular pathogen sensor for the induction and processing of IL-1beta via a noncanonical caspase-8 inflammasome. Nat Immunol 13:246

Gunther C, Martini E, Wittkopf N, Amann K, Weigmann B, Neumann H, Waldner MJ, Hedrick SM, Tenzer S, Neurath MF, Becker C (2011) Caspase-8 regulates TNF-alpha-induced epithelial necroptosis and terminal ileitis. Nature 477:335–339

Haslett C, Savill JS, Whyte MK, Stern M, Dransfield I, Meagher LC (1994) Granulocyte apoptosis and the control of inflammation. Philos Trans R Soc Lond B Biol Sci 345:327–333

He S, Wang L, Miao L, Wang T, Du F, Zhao L, Wang X (2009) Receptor interacting protein kinase-3 determines cellular necrotic response to TNF-alpha. Cell 137:1100–1111

Henkler F, Baumann B, Fotin-Mleczek M, Weingartner M, Schwenzer R, Peters N, Graness A, Wirth T, Scheurich P, Schmid JA, Wajant H (2003) Caspase-mediated cleavage converts the

tumor necrosis factor (TNF) receptor-associated factor (TRAF)-1 from a selective modulator of TNF receptor signaling to a general inhibitor of NF-kappaB activation. J Biol Chem 278:29216–29230

Henson PM, Johnston RB Jr (1987) Tissue injury in inflammation. Oxidants, proteinases, and cationic proteins. J Clin Invest 79:669–674

Holler N, Zaru R, Micheau O, Thome M, Attinger A, Valitutti S, Bodmer JL, Schneider P, Seed B, Tschopp J (2000) Fas triggers an alternative, caspase-8-independent cell death pathway using the kinase RIP as effector molecule. Nat Immunol 1:489–495

Kaiser WJ, Upton JW, Long AB, Livingston-Rosanoff D, Daley-Bauer LP, Hakem R, Caspary T, Mocarski ES (2011) RIP3 mediates the embryonic lethality of caspase-8-deficient mice. Nature 471:368–372

Kang TB, Oh G-S, Scandella E, Bolinger B, Ludewig B, Kovalenko A, Wallach D (2008) Mutation of a self-processing site in caspase-8 compromises its apoptotic but not its nonapoptotic functions in bacterial artificial chromosome-transgenic mice. J Immunol 181:2522–2532

Kang TB, Yang SH, Toth B, Kovalenko A, Wallach D (2013) Caspase-8 blocks kinase RIPK3-mediated activation of the NLRP3 inflammasome. Immunity 38:27–40

Kerr JF, Wyllie AH, Currie AR (1972) Apoptosis: a basic biological phenomenon with wide-ranging implications in tissue kinetics. Br J Cancer 26:239–257

Kovalenko A, Kim JC, Kang TB, Rajput A, Bogdanov K, Dittrich-Breiholz O, Kracht M, Brenner O, Wallach D (2009) Caspase-8 deficiency in epidermal keratinocytes triggers an inflammatory skin disease. J Exp Med 206:2161–2177

Levkau B, Scatena M, Giachelli CM, Ross R, Raines EW (1999) Apoptosis overrides survival signals through a caspase-mediated dominant-negative NF-kappa B loop. Nat Cell Biol 1: 227–233

Li C, Lasse S, Lee P, Nakasaki M, Chen SW, Yamasaki K, Gallo RL, Jamora C (2010) Development of atopic dermatitis-like skin disease from the chronic loss of epidermal caspase-8. Proc Natl Acad Sci U S A 107:22249–22254

Lin Y, Devin A, Rodriguez Y, Liu ZG (1999) Cleavage of the death domain kinase RIP by caspase-8 prompts TNF-induced apoptosis. Genes Dev 13:2514–2526

Lukens JR, Vogel P, Johnson GR, Kelliher MA, Iwakura Y, Lamkanfi M, Kanneganti TD (2013) RIP1-driven autoinflammation targets IL-1alpha independently of inflammasomes and RIP3. Nature 498:224–227

Majno G, La Gattuta M, Thompson TE (1960) Cellular death and necrosis: chemical, physical and morphologic changes in rat liver. Virchows Arch Pathol Anat Physiol Klin Med 333:421–465

Matzinger P (1994) Tolerance, danger, and the extended family. Annu Rev Immunol 12: 991–1045

Menkin V (1948) Newer concepts of inflammation. Charles C Thomas, Springfield, IL

Muzio M, Chinnaiyan AM, Kischkel FC, O'Rourke K, Shevchenko A, Ni J, Scaffidi C, Bretz JD, Zhang M, Gentz R, Mann M, Krammer PH, Peter ME, Dixit VM (1996) FLICE, a novel FADD-homologous ICE/CED-3-like protease, is recruited to the CD95 (Fas/APO-1) death-inducing signaling complex. Cell 85:817–827

O'Donnell MA, Perez-Jimenez E, Oberst A, Ng A, Massoumi R, Xavier R, Green DR, Ting AT (2011) Caspase 8 inhibits programmed necrosis by processing CYLD. Nat Cell Biol 13: 1437–1442

Rajput A, Kovalenko A, Bogdanov K, Yang SH, Kang TB, Kim JC, Du J, Wallach D (2011) RIG-I RNA helicase activation of IRF3 transcription factor is negatively regulated by caspase-8-mediated cleavage of the RIP1 protein. Immunity 34:340–351

Rock KL, Kono H (2008) The inflammatory response to cell death. Annu Rev Pathol 3:99–126

Sakahira H, Enari M, Nagata S (1998) Cleavage of CAD inhibitor in CAD activation and DNA degradation during apoptosis. Nature 391:96–99

Sun L, Wang H, Wang Z, He S, Chen S, Liao D, Wang L, Yan J, Liu W, Lei X, Wang X (2012) Mixed lineage kinase domain-like protein mediates necrosis signaling downstream of RIP3 kinase. Cell 148:213–227

Vandenabeele P, Galluzzi L, Vanden Berghe T, Kroemer G (2010) Molecular mechanisms of necroptosis: an ordered cellular explosion. Nat Rev Mol Cell Biol 11:700–714

Varfolomeev EE, Schuchmann M, Luria V, Chiannilkulchai N, Beckmann JS, Mett IL, Rebrikov D, Brodianski VM, Kemper OC, Kollet O, Lapidot T, Soffer D, Sobe T, Avraham KB, Goncharov T, Holtmann H, Lonai P, Wallach D (1998) Targeted disruption of the mouse Caspase 8 gene ablates cell death induction by the TNF receptors, Fas/Apo1, and DR3 and is lethal prenatally. Immunity 9:267–276

Vince JE, Wong WW, Gentle I, Lawlor KE, Allam R, O'Reilly L, Mason K, Gross O, Ma S, Guarda G, Anderton H, Castillo R, Hacker G, Silke J, Tschopp J (2012) Inhibitor of apoptosis proteins limit RIP3 kinase-dependent interleukin-1 activation. Immunity 36:215–227

Voll RE, Herrmann M, Roth EA, Stach C, Kalden JR, Girkontaite I (1997) Immunosuppressive effects of apoptotic cells. Nature 390:350–351

Wallach D, Kovalenko A, Kang TB (2011) 'Necrosome'-induced inflammation: must cells die for it? Trends Immunol 32:505

Wallach D, Varfolomeev EE, Malinin NL, Goltsev YV, Kovalenko AV, Boldin MP (1999) Tumor necrosis factor receptor and Fas signaling mechanisms. Annu Rev Immunol 17:331–367

Wang Z, Jiang H, Chen S, Du F, Wang X (2012) The mitochondrial phosphatase PGAM5 functions at the convergence point of multiple necrotic death pathways. Cell 148:228–243

Zhang DW, Shao J, Lin J, Zhang N, Lu BJ, Lin SC, Dong MQ, Han J (2009) RIP3, an energy metabolism regulator that switches TNF-induced cell death from apoptosis to necrosis. Science 325:332–336

Zhao J, Jitkaew S, Cai Z, Choksi S, Li Q, Luo J, Liu ZG (2012) Mixed lineage kinase domain-like is a key receptor interacting protein 3 downstream component of TNF-induced necrosis. Proc Natl Acad Sci U S A 109:5322–5327

Chapter 8
NOX1, Reactive Oxygen Species, JNK, and Necrotic Cell Death

Michael J. Morgan and You-Sun Kim

8.1 Cellular Reactive Oxygen Species (ROS)

Reactive oxygen species (ROS) are generally small-molecule, oxygen-containing compounds that are produced by the cell either as signaling intermediates, as a mechanism of cellular defense from pathogens, or as by-products of metabolic processes. While ROS are necessary for some cellular events in signal transduction or metabolism, cells are protected from inadvertent ROS damage by systems of antioxidant and repair proteins, such as thioredoxin and glutathione and their associated enzymes (Holmgren 2000; Rhee et al. 2005). Other enzymes directly eliminate specific kinds of ROS, such as superoxide dismutases, catalase, and peroxiredoxins. The cell also has many other nonenzymatic antioxidants, such as vitamins E and C, carotenoids, and oxy-carotenoids (Sies 1997). Thus, while ROS are essential for cellular function, under normal conditions, the cell is equipped with protective molecules such that the amounts of ROS are carefully controlled and managed to prevent cell damage and death.

A variety of ROS are produced in significant quantities in the cell. These include superoxide ($\cdot O_2^-$); hydrogen peroxide (H_2O_2) and lipid peroxides (ROOH); hydroxyl (·OH), alkyloxyl (RO·), and peroxyl (ROO·) radicals; hypochlorous acid (HOCl); singlet oxygen ($^1O_2$); as well as peroxynitrite ($ONOO^-$). ROS is mainly produced in

M.J. Morgan, Ph.D. (✉)
Department of Pharmacology, University of Colorado School of Medicine,
Anschutz Medical Campus, Mail Stop 8303, Research 1 South, 12801 E. 17th Avenue,
Rm. L18-6110, Aurora, CO 80045, USA
e-mail: michael.morgan@ucdenver.edu

Y.-S. Kim
Department of Biochemistry, Ajou University School of Medicine,
San 5, Wonchon-dong, Yeongtong-gu, Suwon 443-749, South Korea
e-mail: yousunkim@ajou.ac.kr

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the cell as superoxide or hydrogen peroxide, and these two species can react with other molecules to form many of the other ROS. While lipid membranes are relatively permeable to hydrogen peroxide, superoxide is not believed to diffuse across membranes due to its negative charge. When superoxide is present at high concentrations, its half-life is rather short due to spontaneous dismutation into O_2 and H_2O_2; however, since the dismutase reaction is a second-order reaction with respect to the initial superoxide concentration, its half-life can be on the order of hours when the superoxide concentration is low. Therefore, superoxide dismutases, which are highly efficient enzymes, are used by the cell to enzymatically increase the rate of this reaction. Within peroxisomes, the H_2O_2 is then broken down into oxygen and water by the enzyme catalase.

8.1.1 Intracellular Sources of ROS

Quantitative comparison of ROS sources was originally largely based on the pioneering work of Britton Chance in the 1950s–1970s, where his laboratory showed that various isolated subcellular fractions of rat liver, including mitochondrial, microsomal, peroxisomal, and soluble fractions, were all capable of producing H_2O_2 when supplied with various substrates (Boveris et al. 1972). H_2O_2 production was estimated to represent approximately 5 % of O_2 consumption in total, with approximately 1–2 % of electrons lost from mitochondrial electron transport chain complexes to form ROS (Boveris et al. 1972; Boveris and Chance 1973). The mitochondria have been assumed to be the largest intracellular source of ROS, but some have recently called this assumption into question (Brown and Borutaite 2012) and have suggested that peroxisomes are a greater source of cellular ROS during respiration of fatty acids. Since most quantitative comparisons are based on somewhat impure fractionations where an artificially high level of substrate is provided and without physiological conditions or stressors, it is difficult to say definitively which potential source may contribute the most to intracellular ROS. However, whether or not it is the greatest source of ROS, there are many places within mitochondrial respiratory chain that are capable of producing ROS (Turrens 2003; Orrenius et al. 2007), and since the mitochondria are closely associated with cell death, induced mitochondrial ROS during mitochondrial dysfunction is likely to play a significant role during programmed necrosis. Microsomal fractions from the previously cited work suggest that the ER may be another large source of intracellular ROS; the lysosome may also be another potential source when it comes to mediating necrotic cell death.

Another potentially large source of ROS is the NADPH oxidase family (NOX family), which produce superoxide from oxygen using NADPH as a substrate (Lambeth 2004; Brown and Griendling 2009). The superoxide from these oxidases (especially NOX2) is often used as a defense against pathogen infections by the cells of the innate immune system (Quinn et al. 2006). Other family members of the NOX family are expressed in more ubiquitous fashion, and it has recently been

more appreciated that these NOX enzymes contribute to signal transduction, many through being targeted to specific subcellular localization patterns (Ushio-Fukai 2009). Lastly, there are numerous other enzymes, including cytosolic enzymes such as xanthine oxidase that produce ROS within the cell, but these will not be discussed further here.

8.1.2 NADPH Oxidases (NOXs)

NADPH oxidases are some of few enzymes that mediate the deliberate production of ROS (Lambeth 2004; Brown and Griendling 2009). The most well characterized of the NOX enzymes is NOX2 (gp91phox), which is expressed mostly by cells of the innate immune system, such as neutrophils and macrophages, for pathogen defense, and is also expressed in smaller amounts in endothelial cells, vascular smooth muscle cells, cardiac myocytes, hepatocytes, and some fibroblasts (Lambeth 2004; Brown and Griendling 2009).

Other NADPH oxidases (NOX1, NOX3, NOX4, NOX5, DUOX1, and DUOX2) are found in many cell types, and some of these, such as NOX1 and NOX4, are more ubiquitously expressed (Lambeth 2004; Brown and Griendling 2009). Typically, these other members of the family generate smaller amounts of ROS that are thought to be used in signal transduction. NOX enzymes usually function as a heterodimer with the 22-kDa p22phox subunit, and many require activation by other subunits. Phosphorylation of p47phox leads to its binding to membrane phospholipids, where it associates with p22phox and then recruits p67phox. The p67phox protein activates the complex by recruiting and stabilizing the association of the complex with Rac1 or Rac2 small GTPase, which is necessary for superoxide production. The p41NOXO1 and p51NOXA1 homologues of these proteins can function in a similar manner to p47phox and p67phox, respectively, particularly in other oxidase complexes, such as NOX1 and NOX3 (Banfi et al. 2003; Geiszt et al. 2003; Takeya et al. 2003). Unlike p47phox, NOXO1 does not require a phosphorylation event for its membrane translocation and activity, suggesting it is regulated in a different manner. In studies utilizing overexpression of the phox proteins, p47phox and/or p67phox can be interchanged for NOXO1 and/or NOXA1, though this is not as efficient of a complex at generating superoxide production in most cases (Banfi et al. 2003; Geiszt et al. 2003; Takeya et al. 2003). Likewise, NOX1 is more efficient when utilizing NOXO1 and NOXA1. Thus, it is not completely clear as to how specific these subunits are in vivo, but mouse studies indicate that there may be some flexibility in subunit use by the different NOX proteins, though not in every case. Unlike other NOX enzymes, NOX4 appears to be constitutively active when bound to p22phox and does not require any other activating subunits. NOX4 also differs in that superoxide produced by this enzyme is immediately converted to hydrogen peroxide through an unknown mechanism (Brown and Griendling 2009). DUOX1 and DUOX2 do not require p22phox or other activation subunits for their activity and produce hydrogen peroxide directly via a two-electron reduction of oxygen without a superoxide intermediate (Brown and Griendling 2009).

8.2 Programmed Cell Death, Apoptosis, Autophagy, and Necrosis

We often define cell death processes using black or white terminology, which is in large measure due to the fact that death is an "all or none" state. Either a cell is dead or it is not dead. Either an organism is alive or it is dead. However, it is important to realize that cell death processes are made up of events that often occur together and cell death may occur abruptly due to a single set of events, or more slowly, and often going through a continuum of various processes that leads to a final cell demise. ROS is important in many different death processes, and so it is therefore important to understand the definitions, even if individual cells are undergoing multiple processes. The definitions of cell death processes have evolved largely over the past 30 years. In the late 1980s and 1990s, the cell death field evolved quickly owing to what could only be described as an "explosion" of literature and due largely to the realization that some cell death occurred in response to largely genetically defined processes that occurred as much due to the internal cell machinery of a dying cell as much as it did to the external circumstances characterizing the cellular surroundings. And thus the concept of "programmed cell death" (or PCD) was born, or rather reborn, since the phrase appears to be first coined in the 1960s by Lockshin and Williams upon the study of silkmoths (Lockshin and Williams 1964, 1965) and first investigated in tadpoles by Carl Vogt as early as 1842 (Cotter 2009). Likewise, the term "apoptosis" was resurrected from its forgotten use by Kerr, Wylie, and Currie in 1972 (Kerr et al. 1972). Apoptosis now took center stage, since many of the genetic components of the apoptotic machinery began to be identified, starting with discoveries in *C. elegans* (Cotter 2009).

Apoptosis was originally defined by the morphological criteria, including cellular shrinkage, chromatin condensation and nuclear fragmentation, membrane blebbing, and the formation of membrane-bounded bodies containing cellular structures and organelles, which were taken up by surrounding cells (Kerr et al. 1972). Kerr et al. particularly wanted to distiguish this form of death as an active, programmed process, as opposed to classical necrosis, which was typically thought of as a purely passive process and eventually characterized by eventual plasma membrane rupture after cellular swelling, organelle dysfunction, and extensive mitochondrial damage (Fiers et al. 1999; Kroemer et al. 2005). Apoptosis eventually was shown to involve caspase cysteine proteases and to occur without inflammation, which further set it apart from necrosis (Fiers et al. 1999; Kroemer et al. 2005). Since apoptosis was clearly a programmed death phenomenon and seemed to occur as the dominant form of cell death in most instances, the terms apoptosis and programmed cell death were often used for a time to mean exactly the same thing.

However, two discoveries showed that interchangeable usage of these terms was not warranted, although these discoveries were slow to be appreciated, due to the prominence of the apoptotic pathway. The first discovery was that although the newly characterized death receptor pathways by the Fas and TNFα ligands indeed induced caspase activation and programmed cell death in many cell types, in other

cell types they were capable of killing cells in a caspase-independent manner with a morphology that more closely resembled classical necrosis than apoptosis (Laster et al. 1988; Grooten et al. 1993; Vercammen et al. 1998; Holler et al. 2000; Denecker et al. 2001). The morphological distinction of the two types of death in L929 cells by the same ligand was an observation that had been made as early as 1972 in lymphotoxin-treated cells but, as with much in the cell death field, had been largely ignored (Russell et al. 1972). The second discovery, made by a number of groups, was that necrotic cell death induced by TNFα was often accompanied by and also required the production of reactive oxygen species (Schulze-Osthoff et al. 1992, 1993; Goossens et al. 1995; Vercammen et al. 1998; Fiers et al. 1999; Sakon et al. 2003; Lin et al. 2004; Ventura et al. 2004). These two discoveries seemed to confirm a different mechanism of death, for although ROS was known to also contribute to apoptosis, it was not essential in most cases, though caspases were essential.

By the end of the century, there was a great deal of evidence to suggest that necrotic cell death was a programmed phenomenon, however examples of programmed necrosis were mostly confined to a very limited number of cell lines: the murine fibrosarcoma cell lines L929 and WEHI164cl13. A third important discovery came with two findings. One was that MEF cell lines with genetic deletions of genes that affected NF-κB activity, such as p65/RelA, TRAF2, and TRAF5, became more susceptible to necrosis, while in order to detect necrosis in normal MEFs treated with TNFα, the addition of transcriptional or protein synthesis inhibitors was necessary (Sakon et al. 2003; Lin et al. 2004). NF-κB transcription, therefore, was shown to inhibit necrotic cell death to a large extent. In contrast to this, the deletion of RIP (now termed RIP1 or RIPK1), which also had a similar pronounced effect on NF-κB (Ting et al. 1996; Kelliher et al. 1998; Devin et al. 2000, 2001), had a completely opposite effect: complete inhibition of necrotic cell death, but not inhibition (and sometimes potentiation) of apoptosis (Holler et al. 2000; Lin et al. 2004). This was the first evidence that necrosis was influenced not only by external cell factors often produced by other cells (though certainly physiologically relevant), such as death receptor ligands, but also by an affected cell's own inherent genetic components. The "program" part of "programmed cell necrosis" was found as intrinsic as were the components of the apoptotic "program." Other essential/important genes involved in the process were identified in the intervening years, including RIP3 as an essential partner for RIP1 in inducing necrotic cell death (Cho et al. 2009; He et al. 2009; Zhang et al. 2009).

The final evidence that was lacking was that the necrotic cell death pathway was actually relevant in vivo in physiological and pathological conditions. This evidence arrived eventually from a small-molecule screen for inhibitors of cell death induced in U937 cells by TNFα and the pan-caspase inhibitor zVAD, which was now termed "necroptosis" by the authors (Degterev et al. 2005). This compound, termed necrostatin-1, inhibited ischemic brain injury in vivo (Degterev et al. 2005) and was later proven to be an inhibitor of RIP1 kinase activity (Degterev et al. 2008). Thus, programmed necrotic cell death occurs in vivo, under a condition, which, if not occurring under completely normal physiological circumstances, at least occurs under relevant pathological circumstances. Since that discovery, genetic deletion of

components of the necrotic cell death machinery, including RIP1 and RIP3, has been shown to rescue or ameliorate a variety of developmental defects, inflammatory conditions, and/or synthetic lethality of many different gene deletions, including FADD, caspase-8, cFLIP–FADD double knockout (but not cFLIP knockout alone), XIAP–cIAP1 double knockout, and cIAP1–cIAP2 double knockout, as well as TNF-induced systemic inflammatory response syndrome (SIRS), suggesting that necrotic machinery is quite fully functional in vivo but carefully controlled by other gene products (Bonnet et al. 2011; Ch'en et al. 2011; Duprez et al. 2011; Kaiser et al. 2011; Lu et al. 2011; Oberst et al. 2011; Zhang et al. 2011; Dillon et al. 2012; Moulin et al. 2012). Programmed necrotic cell death is therefore clearly a bona fide form of cell death and is clearly distinguished from apoptosis.

Programmed cell death is often classified into three types, with ROS participating in each type. Apoptosis is classified as type I programmed cell death, autophagic cell death classified as type II programmed cell death, and necrotic death classified as type III programmed cell death. ROS can potentially be involved in all three types. Type I programmed cell death (apoptosis) that has been described in the previous paragraphs requires caspase activation according to its purest modern definition. Type II death, or autophagic cell death, involves autophagy. Autophagy (Greek for self-eating) is an evolutionarily conserved process in which cells recycle macromolecules and organelles by targeting them for lysosomal degradation, thereby allowing components to be used in metabolic and catabolic processes. Autophagy promotes cell survival during nutrient starvation by providing amino acids, fatty acids, and ATP for cell functions. It also promotes resistance to oxidative stress by eliminating oxidized, misfolded, or aggregated proteins, and damaged organelles. Hence, it is relevant to the current matter at hand—that of ROS-mediated cell death—since ROS often induces such damage. The classification of type II death, or autophagic cell death, is quite confusing, since under the original morphological criteria it was defined as cell death with autophagic features. Because autophagy often occurs when a cell is undergoing stress and because cells that are dying are almost always under some form of stress, cells are often undergoing autophagy when dying of apoptosis or necrosis. Therefore, this original definition is of little use. Autophagy-dependent cell death, in which autophagy is required for cell death, is actually a much more of a rarity, since autophagy is more often a survival mechanism. But this definition also does not distinguish between cell death that requires autophagy in order for the cell to die through an apoptotic or necrotic mechanism and cell death that is actually mediated solely by the autophagic machinery (i.e., not apoptotic or necrotic), the latter being the most uncommon form of cell death of all. Making this distinction when talking about autophagic cell death is usually the most informative. Since most ATG proteins have both autophagic and non-autophagic functions that affect apoptosis (and probably necrosis as well), there can often be some confusion in interpreting the results of a single given experiment. Much of the literature classifying a cell death event as autophagic actually fits the original definition and is actually more suitably classified as either apoptotic or necrotic. Unless ROS is very limited in scope, it is also probably inducing autophagy whether the ROS is involved cell death of any type, and this may or may not influence the cell death mechanism.

And now we come to type III programmed cell death, or programmed necrosis, which is the main subject at hand and for which we have already given a historical perspective and justification for its classification as an alternate class of cell death. As mentioned above, the general term "necrosis" was originally used to refer to cell death that is characterized by specific morphological criteria. As used in the preceding paragraphs, the terms "programmed necrosis" and "programmed necrotic cell death" are used to distinguish an active death process, carried out by specific genetically encoded cellular apparatus, from the necrosis represented by the original classical concept of a passive or an injury-mediated process largely initiated by direct cellular damage, though both may have a similar general morphology. This distinction is an important one, especially when interpreting whether a result is physiologically relevant. However, when talking about the roles of ROS in cell death, the distinction is often blurry, especially when testing a hypothesis in the lab. For instance, some publications have indicated that modest amounts of ROS can induce apoptosis, whereas necrotic cell death can occur when cells of a similar type are exposed to high ROS levels (Takeda et al. 1999; Teramoto et al. 1999; Saito et al. 2006). While ROS is believed to play a substantial role in programmed necrotic cell death, once the level of ROS reaches a certain amount, classical non-programmed necrosis may occur in the same given cell type due to direct cellular damage.

This illustrates several of the complexities and difficulties in determining the role of ROS in cell death pathways. Since ROS can contribute both to signaling events and also to direct cellular damage, it is often hard to distinguish the difference in terms of a molecular mechanism for a given cell death event. Since some cell types have different abilities to respond to ROS and to repair damage, the actual concentration of actual ROS in one cell type may be different from the concentration in another cell type, even when presented with the same extracellular amount of ROS. Another difficulty lies in the localization of ROS within the cell, which can have a profound influence on both signaling events and the kind and consequences of ROS damage. Measurement of ROS in a given cellular localization is often problematic. Moreover, it is difficult, if not virtually impossible, to deliver a specified concentration of ROS to a given cellular location and equally difficult to quench ROS within a specifically designated cellular location. Therefore, it is also practically impossible to adequately mimic physiological ROS signaling events within a cell using extracellular delivery of ROS or ROS scavengers. Thus, while these agents are widely used in the field to examine biological questions, due to their availability and because they are easy to employ, in many cases effects are initiated or prevented by such agents outside of the given ROS signaling pathways that are being investigated.

8.3 The Necrotic Cell Death Machinery

The RIP1 protein was originally identified as being required for both TNFα-induced ROS production and caspase-independent cell death (Holler et al. 2000; Lin et al. 2004), identifying RIP1 as a central player in programmed necrotic cell death.

Although many of the functions of RIP1, such as its activation of NF-κB and the kinases, JNK and p38, do not require its kinase activity, the pronecrotic role of RIP1 requires its kinase function. Necrostatin-1, which is an inhibitor of the kinase function of RIP1, was identified in a small-molecule screen as a drug that prevents programmed necrotic cell death (Degterev et al. 2005, 2008). As mentioned, the term necroptosis was originally used to refer to programmed necrotic processes initiated in a death receptor setting and were inhibitable by necrostatin-1. Since this time, others have used the term loosely to refer to general programmed necrosis. However, although most forms of programmed necrotic cell death are RIP1 dependent, some similar necrotic cell death processes that can be classified as programmed are believed to occur in the absence of RIP1. Therefore, we shall use the term necroptosis to refer to only those processes mediated by death receptors that are known to require the activity of RIP1. While RIP1 was for some time the only protein known to be required for programmed necrotic cell death, in the past few years, there have been several other proteins identified that play a role in necrotic cell death. RIP3 is among the most important of these.

RIP3 was classified as a member of the RIP kinase family based on similarity between kinase domains. Three groups used RNA interference screens and differential microarray analysis to independently determine that RIP3 is a downstream component of the TNFα-induced necrotic pathway (Cho et al. 2009; He et al. 2009; Zhang et al. 2009). Unlike RIP1, RIP3 lacks a death domain but interacts with RIP1 through their homotypic interaction motifs (RHIM), forming a filamentous structure (Li et al. 2012). Assembly of the RIP1–RIP3 complex, which is required for necroptosis, appears to be linked to the kinase activity of both proteins (Cho et al. 2009). Thus, necrostatin-1 inhibits the formation of this complex. The downstream effects of RIP3 association are not completely clear at this point, but several proteins have been identified as RIP3 interactors. Zhang et al. found that seven different metabolic enzymes were found in complex with RIP3 as identified by mass spectrometry, including glycogen phosphorylase (PYGL), glutamate–ammonia ligase (GLUL), glutamate dehydrogenase 1 (GLUD1), fructose-1,6-bisphosphatase 2 (FBP2), fumarate hydratase (FH), glycosyltransferase 25 domain containing 1 (GLT25D1), and isocitrate dehydrogenase 1 (IDH1) (Zhang et al. 2009). They went on to verify that three of these seemed to interact with RIP3 when overexpressed (PYGL, GLUL, and GLUD1). These associations may suggest that RIP3 may regulate bioenergetic metabolic pathways, including some associated with glycolysis and some associated with the mitochondria. Upregulation in these pathways may lead to different kinds of ROS generation and cellular damage (Vandenabeele et al. 2010). More recently, two independent labs found that the mixed lineage kinase domain-like protein (MLKL) is an essential downstream interactor of RIP3 (Li et al. 2012; Zhao et al. 2012). MLKL is a RIP3 substrate and is required for formation of an expanded larger complex that is likely needed for downstream substrate recruitment. The drug necrosulfonamide prevents MLKL binding to the RIP1–RIP3 complex and thereby inhibits necrotic cell death (Li et al. 2012). Downstream of MLKL, it has been proposed that the PGAM5 mitochondrial phosphoglycerate mutase, which uses alternative catalytic activity to function as a Ser/Thr phosphatase

(Takeda et al. 2009), is recruited by MLKL, and PGAM5 recruits and activates the protein Drp1, which is involved in mitochondrial fission and which is also apparently involved in necrotic cell death (Wang et al. 2012). Since there are other studies that also suggest that mitochondrial fusion protects from necrotic cell death (Whelan et al. 2012), thus there is an implication that the mitochondrial dynamics are an important component required for necrotic cell death.

RIP1 is highly regulated by ubiquitination. The RING finger ligases TRAF2 and cIAP1/2 and E2 enzymes cooperate to catalyze RIP1 ubiquitination. Noncanonical K63 ubiquitination of RIP1 not only appears to be important for the ability of RIP1 to mediate NF-κB activation but also prevents RIP1 from interacting with the apoptotic complexes as well as the cellular machinery that mediated necrotic cell death and ROS production (Wang et al. 2008; Vanlangenakker et al. 2011; O'Donnell et al. 2012). Two deubiquitinases, therefore, are thought to be important for death signaling. CYLD, which is a tumor suppressor gene that represses NF-κB, removes K63-linked ubiquitin chains (Kovalenko et al. 2003), contributing to cell death. A20 also removes K63-linked chains but has a separate domain that adds K48-linked chains, resulting in protein degradation and inhibition of signaling (Wertz et al. 2004). While the stability of the RIP1–RIP3 is crucial to programmed necrotic cell death, RIP1 and its associated proteins are also downregulated by the apoptotic machinery when complexed with FADD, cFLIP, and caspase-8. Thus, while caspase inhibitors prevent apoptosis, inhibition of caspases potentiates necrotic cell death, due to inhibition of protein cleavage, including RIP1 (Lin et al. 1999), RIP3 (Feng et al. 2007), and CYLD deubiquitinase (O'Donnell et al. 2011) by caspase-8 during apoptosis (Lin et al. 1999). As mentioned before, in mice, the knockout lethality of FADD, caspase-8, cFLIP–FADD double knockout (but not cFLIP knockout alone), XIAP–cIAP1 double knockout, and cIAP1–cIAP2 double knockout is rescued to some degree by RIP1–RIP3 deficiency (Bonnet et al. 2011; Ch'en et al. 2011; Kaiser et al. 2011; Lu et al. 2011; Oberst et al. 2011; Zhang et al. 2011; Dillon et al. 2012; Moulin et al. 2012), suggesting that the repressive functions of these proteins are important to prevent RIP1–RIP3-mediated necrosis during development.

In addition to the RIP1–RIP3 complex proteins, cyclophilin D (Li et al. 2004; Baines et al. 2005; Nakagawa et al. 2005; Schinzel et al. 2005), PARPs (Los et al. 2002; Xu et al. 2006), AIF (Delavallee et al. 2011), and lysosomal proteases, such as cathepsins (Luke et al. 2007; Sato et al. 2008), and non-lysosomal proteases, such as calpains (Moubarak et al. 2007), may have functions in programmed necrosis. The stress-activated map kinase JNK may also have an important role in necrotic signaling (Shen and Liu 2006).

Jun-N-terminal kinases (JNKs) are one of three main family members of mitogen-activated protein kinases (MAPKs) (Seki et al. 2012). Of the three isoforms of JNK, two (JNK1 and JNK2) are ubiquitously expressed, while the third (JNK3) is expressed mainly in the nervous system, with some expression in the heart and testis. JNKs are mainly activated by the MKK4 and MKK7 MAP kinase kinases (MAP2Ks), which are activated by upstream MAP3Ks, among which there are at least 14 known to activate JNK (Seki et al. 2012), including MEKKs 1–4 (MAPK/ERK kinase kinases), MLK 2–3 (mixed lineage kinase-2/3), TAK1

(TGF-β-activated kinase-1), and ASK1 (apoptosis signal-regulating kinase-1). The MAP3Ks are often activated in response to cellular stress, as well as a number of cytokine, growth factor, pattern recognition, and death receptors.

The mitochondrial permeability transition pore (MPTP) channel is a nonspecific channel that spans both the inner and outer mitochondrial membranes, the protein composition of which is still highly debated but which may include the voltage-dependent anion channel (VDAC), the adenine nucleotide translocase (ANT), and cyclophilin D (CypD). The opening of this pore (which remains largely closed in unstressed cells) upon oxidative stress (which may or may not involve calcium overload as a stimulus for MPTP opening) results in an influx of ions and a loss in mitochondrial membrane potential, shutting down oxidative phosphorylation and energy (ATP) production. Opening can also be followed by water influx and lead to membrane rupture. Pore opening is regulated by the prolyl isomerase CypD. Though apoptotic signaling through Bcl-2 family members does not require CypD, CypD knockout cells are resistant to oxidative stress-mediated necrosis, while overexpression of CypD leads to ROS sensitization (Li et al. 2004; Baines et al. 2005; Nakagawa et al. 2005; Schinzel et al. 2005). This appears also to be true of ischemia-induced necrosis in vivo (Baines et al. 2005; Nakagawa et al. 2005; Schinzel et al. 2005). Thus, these data suggest that the opening of the mitochondrial permeability transition pore plays an essential role in necrotic cell death induced in response to ROS in several settings. In addition, the opening of the pores may lead to increased release of ROS into the cytosol, which could further amplify the response to the initial ROS. Triggering of this opening may be due to accumulation of p53 within the mitochondrial matrix and its physical interactions with CypD (Vaseva et al. 2012). Since p53 is a master sensor of various kinds of cell stress, this would connect oxidative damage with its cellular response.

PARP-1 is a pronecrotic molecule that is cleaved by caspases during apoptosis, thus preventing it from inducing a necrotic pathway. PARP-1 is activated by DNA damage initiated by ROS and catalyzes the covalent attachment of poly(ADP-ribose) (PAR) onto proteins from NAD^+. PARP-1 can have multiple effects on necrotic cell death: (1) it can cause NAD^+ depletion, which must then be replaced, and therefore, it causes the depletion of ATP from the cell, thus removing cellular energy; (2) NAD^+ is an important cofactor for glycolysis, so its depletion slows the glycolytic rate; (3) NAD^+ depletion by PARP-1 potentiates mitochondrial membrane depolarization through MPTP opening; and (4) it causes the release of AIF from the mitochondria. *N*-Methyl-*N*-nitro-*N*-nitrosoguanidine (MNNG), an alkylating agent that causes DNA damage, requires JNK activation downstream of RIP1 and TRAF2 to induce necrotic cell death (Xu et al. 2006). In this case, PARP-1 is required for cell death upstream RIP1, TRAF2, and JNK, and JNK may act cooperatively with the PAR polymer (formed by PARP-1), which then acts as a factor that causes the release of apoptosis-inducing factor (AIF) from the mitochondrial intermembrane space through a process that depends on calpain and Bax (Yu et al. 2006; Moubarak et al. 2007). Release from the mitochondria requires AIF binding directly to PAR (Wang et al. 2011). AIF translocates from the mitochondrial to nucleus, where it induces cell death characterized by chromatin condensation and

high molecular weight DNA fragmentation (Susin et al. 1999). PARP-1 has been reported to be required for TNFα-mediated (Los et al. 2002) as well as during TRAIL-mediated (Jouan-Lanhouet et al. 2012) necroptosis. Although most of the PARP activity in the cell is from PARP-1, PARP-2 was identified by siRNA screening as a protein that when knocked down causes some reduction of TNF-induced necrotic death, further suggesting that PARPs contribute to the necrotic process (Hitomi et al. 2008).

During some forms of necrotic death, lysosomal cathepsin proteases appear to mediate part of the downstream machinery involved in cell death execution. In *C. elegans*, where lysosomal function is required for necrotic cell death (Artal-Sanz et al. 2006), cathepsin release from the lysosome appears to play an important role in the process (Luke et al. 2007). The involvement of lysosomal proteases in necrosis may be somewhat conserved in mammalian cells and is thought to play a role under certain circumstances (Sato et al. 2008; Tu et al. 2009). Lysosomal rupture may at times occur in response to ROS damage to lysosomal lipids, thus releasing its proteolytic enzymes (Boya and Kroemer 2008).

As part of its downstream signaling machinery, TNFα appears to be an activator of NADPH oxidases in various cell types (Morgan et al. 2008). NOX1, NOXO1, and Rac1 interact with TNF receptor signaling components upon TNFα treatment leading to superoxide formation and contributing to necrotic cell death (Kim et al. 2007). RIP1 is essential for recruitment of this complex, apparently through a high-affinity interaction with NOXO1, while the polyproline-rich region of TRADD also interacts with the N-terminal SH3 domain of NOXO1 in an interaction that is proposed to support oxidase activation (Kim et al. 2007). RIP3 involvement in complex formation has not yet been investigated. NOX1-derived TNFα-induced superoxide contributes to necrotic cell death in L929 cells, since NOX1 knockdown significantly reduces both superoxide generation and cell death in response to TNFα (Kim et al. 2007). Death in this case was also dependent on JNK. NOX2 and NOX3 have also been shown to be activated by TNFα (Li et al. 2009, 2010; Anilkumar et al. 2008), as has NOX4 (St Hilaire et al. 2008), but the latter oxidase is likely regulated through induction of transcription (Moe et al. 2006), and it is not clear how much role these oxidases play in programmed necrosis.

NOX1 is also important for ROS induction by a DR4/DR5-specific KD548-Fc through the DR4 and DR5 TRAIL receptors (Park et al. 2012). Riboflavin kinase (RFK) is thought to contribute to NOX1 activation by TNFα and by DR5/DR5 (Yazdanpanah et al. 2009; Park et al. 2012). RFK binds directly to these receptors, as well as to p22phox (and possibly TRADD), and is important for NOX1 recruitment. Its enzymatic activity converts riboflavin (vitamin B2) to flavin mononucleotide (FMN), which FAD synthetase then converts to flavin adenine dinucleotide (FAD), which is a critical NADPH oxidase prosthetic group and is required for superoxide generation (Yazdanpanah et al. 2009). Thus, RFK activation enhances FAD loading, allowing for its activation of NOX1.

8.3.1 Mitochondrial Involvement: The Beginning, the Middle, and the End?

It is clear that in many cases, ROS is required for programmed necrotic cell death; however, the exact sources, the kinds of ROS that are involved, and the exact signaling pathways and cellular damage that are responsible are not completely clear in all cases. NADPH oxidase-derived ROS are only one source among many. The mitochondrion is a main cellular source of ROS during necrotic processes. Indeed the mitochondria may function as the initial source of ROS, the ROS amplification machinery, and one potential end target of ROS, leading to loss of mitochondrial function.

Hence, necrotic death stimuli are thought to involve mitochondria in multiple ways. ROS or other necrotic signaling events can result in the opening of the mitochondrial permeability transition pore (MPTP) and loss of mitochondrial membrane potential. Loss of membrane potential means that ATP is no longer being mitochondrially produced, since the transmembrane proton gradient drives the production of ATP by ATP synthase. However, in many cases during necroptosis, membrane potential actually is hyperpolarized for a time following a receptor stimulus (Goossens et al. 1999b; Vanden Berghe et al. 2010), which could be due to increased oxygen consumption (Goossens et al. 1999a) or to a RIP1-dependent inhibition of adenine nucleotide translocase (ANT) (Temkin et al. 2006), which exchanges cytosolic ADP for mitochondrially produced ATP. The latter has been proposed to lead to the reversal of the ATP synthase activity and the resultant pumping of protons into the mitochondrial matrix (Vanden Berghe et al. 2010). Inhibition of ANT could also potentially lead to extensive upregulation of glycolytic pathways, since they are sensitive to the falling levels of ATP available. High glycolysis may then lead to an increase methylglycol, which reacts with protein to form specific methylglycol-derived advanced glycation end-product damage (Van Herreweghe et al. 2002). The eventual loss of membrane potential that occurs during programmed necrosis and subsequent loss of available ATP energy contribute further to necrosis, as the cell is no longer able to carry out essential cellular functions. It also prevents apoptosis, which requires more energy. Loss of membrane potential can also lead to an influx of solutes, precipitating mitochondrial rupture.

In many instances, mitochondrial-derived ROS has been shown to be required for necroptosis (Schulze-Osthoff et al. 1992, 1993; Goossens et al. 1995, 1999a; Festjens et al. 2006; Vanden Berghe et al. 2010), and these largely come from the leakage of electrons from the mitochondrial electron transport chain complexes. As mentioned before, RIP1-dependent inhibition of adenine nucleotide translocase (ANT) (Temkin et al. 2006) leads to an inability of the mitochondria to take up ADP. In the absence of ADP, mitochondria are known to generate superoxide radicals, while in the presence of ADP the membrane potential is translated into ATP synthesis (Boveris et al. 1972). Without ADP, the membrane potential apparently rises. High mitochondrial membrane potential is an important factor in mitochondrial superoxide-derived H_2O_2 release (Korshunov et al. 1997; Votyakova and

Reynolds 2001; Lee et al. 2002; Lambert and Brand 2004), which is partially thought to be due to the reverse flow of electrons from succinate to complex II to complex I (Votyakova and Reynolds 2001; Liu et al. 2002). Complex I is the major source of ROS from the mitochondria, and the N2 iron–sulfur center was identified as one of the likely sources of ROS from this complex (Genova et al. 2001; Brand et al. 2004). Complex III may also contribute to ROS as well, in which case the main source seems to be the ubisemiquinone radical, which interacts with the iron–sulfur Rieske subunit and cytochrome b (Turrens et al. 1985; Brand et al. 2004). ROS produced by complex I is released into the matrix of the mitochondria, while ROS from complex III is released on both inside and outside of the inner mitochondrial membrane (Brand et al. 2004).

ROS generated by complex I is especially important during programmed necrosis, since rotenone has been shown to inhibit TNF necroptosis (Goossens et al. 1999b; Festjens et al. 2006; Vanden Berghe et al. 2010). Oxidative damage can cause the mitochondria to generate further ROS through damage to mitochondrial proteins, which can amplify ROS through a variety of mechanisms (Ott et al. 2007).

Reaction of ROS with mitochondrial aconitase, an iron–sulfur protein, can result in the release of free iron plus an equivalent mole of hydrogen peroxide, leading to the Fenton reaction production of the reactive hydroxyl radical, which can further oxidize mitochondrial components (Orrenius et al. 2007). Since mitochondrial aconitase plays a key role in the citric acid cycle, its deactivation may result in a decrease in the mitochondrial generation of energy.

The hydroxyl radical can extract hydrogen atoms from polyunsaturated fatty acyl chains of membrane phospholipids (Brand et al. 2004). This generates carbon-centered fatty acyl radicals that react with molecular oxygen to form peroxyl radicals, which then promulgate lipid peroxidation cascades (Brand et al. 2004). Damage of mitochondrial lipids can result in suppression of mitochondrial metabolism and alteration of important mitochondrial functions, as well as affect the mitochondrial permeability transition (Orrenius et al. 2007).

ROS can react with reactive nitrogen species to cause different kinds of damage. Mitochondrial complex I subunit NDUFB8 is a target of nitration predicted to come from nitrous oxide reaction in the presence of superoxide, thus probably involving a more reactive peroxynitrite intermediate (Davis et al. 2010). Nitration of NDUFB8, which occurs in a RIP1- and RIP3-dependent manner during necroptosis, apparently leads to inactivation of NDUFB8, which is essential for mitochondrial function and correlated with mitochondrial membrane potential loss (Davis et al. 2010). MnSOD is inactivated by peroxynitrite as well (Quijano et al. 2001), thus allowing superoxide to build up to toxic levels.

8.3.2 Linking of ROS Sources Together

While TNFα-induced ROS is believed to cause mitochondrial damage (Mariappan et al. 2009), whether this damage comes as a self-inflicted damage from

mitochondrial sources or from other sources may be context dependent. There is a large possibility that NOX1-produced superoxide and mitochondrial-produced ROS could be linked together in programmed necrosis (Morgan et al. 2008). For instance, superoxide produced by NADPH oxidases may lead to mitochondrial-produced ROS, or vice versa. There is actually a large body of evidence that shows that NOX-produced superoxide is capable of regulating ATP-regulated mitochondrial potassium channels (Dikalov 2011), an effect that in more physiological settings is a likely mechanism for ischemic preconditioning. For instance, NADPH oxidase-derived superoxide activated by angiotensin II can react with sulfhydryl groups of the ATP-regulated mitochondrial potassium channels, resulting in channel opening (Zhang et al. 2001). Opening of these channels has been proposed to increase potassium influx and cause matrix alkalinization, leading to ROS production, as well as mild mitochondrial uncoupling and swelling, bordering on membrane rupture (Costa et al. 2006). Thus, there are normal physiological connections between NADPH oxidases and the mitochondrial generation of ROS that may also be used by pathological pathways such as programmed necrosis to commit a cell to its own death, with the necrotic trigger being additional RIP1–RIP3-dependent signals. While NOX1-derived ROS likely would result in mitochondrial damage and the possibility of ROS amplification, NOX1 activation has also proposed to be downstream of mitochondrial ROS during necrotic cell death induced by serum withdrawal (Lee et al. 2006). This is consistent with other reports showing that hydrogen peroxide can induce superoxide production through a non-phagocytic NADPH oxidase (Li et al. 2001). NOX1 is proposed by some to be present at both the plasma membrane and the mitochondria (Byun et al. 2008), allowing for the possibility of translocation events or multiple places of NOX1-dependent superoxide formation.

In terms of how other cellular ROS sources are incorporated into the picture, we see a similar phenomenon: ROS can beget more ROS. For instance, H_2O_2 reacts with the xanthine oxidoreductase protein, transforming it from xanthine dehydrogenase into xanthine oxidase and thus converting it to a source of superoxide (McNally et al. 2005).

In certain cases, the lysosome may be a potential source of ROS, due to its large iron content and reducing equivalents without sufficient protective enzymes against H_2O_2, thus making H_2O_2 a potential target for conversion by the Fenton reaction into the more reactive hydroxyl radical (Kurz et al. 2006; Vandenabeele et al. 2010). The lysosome seems to be particularly important for necrosis induced by exogenous ROS, since lysosomal permeability precedes cell death and lysosomal permeabilization and necrosis are prevented by the iron chelator desferrioxamine (Vanden Berghe et al. 2010).

Overexpression of NOX1, NOXO1, and NOXA1 apparently results in TNFR1-dependent activation of JNK and induces TNFR1- and ASK1-dependent cell death (Pantano et al. 2007), indicating that in some cases ROS may also induce the feed-forward signaling at the receptor level.

In any case, ROS generation in large amounts by any cellular source may interact with other cellular sources of ROS and coupled with other signaling potentially coming from RIP1 can generate a vicious cycle of feed-forward ROS signaling (Fig. 8.1).

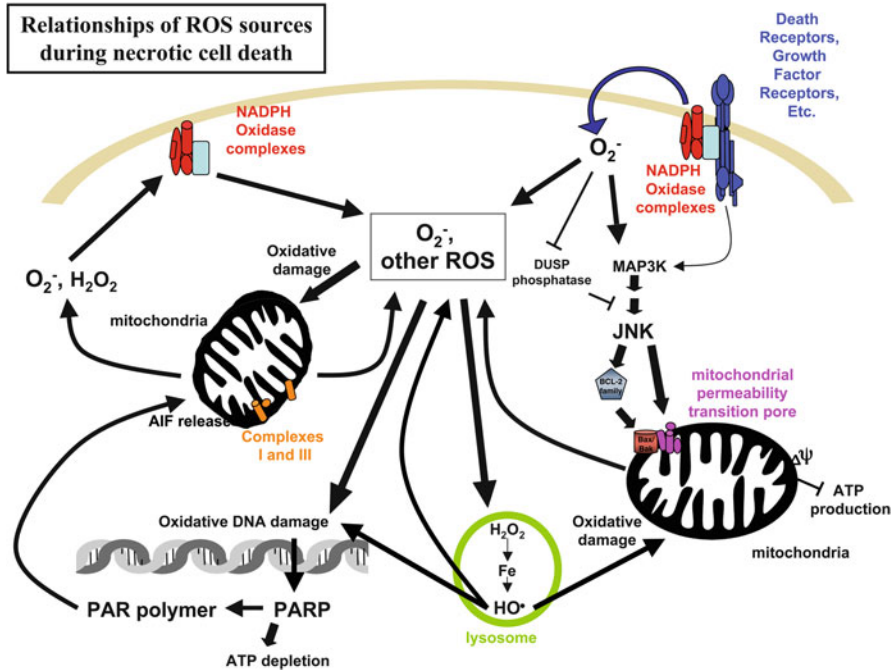

Fig. 8.1 Relationships of ROS sources during necrotic cell death. The various intercellular sources of ROS production are connected by a series of complex interrelationships. ROS, due to its ability to both influence cell signaling and cause direct oxidative damage, may cause the production of ROS by multiple other intercellular ROS sources. The end effect is the amplification of the ROS signal and extensive downstream damage, resulting in cell death

8.3.3 ROS Regulation of JNK

Unlike the receptor-mediated transient activation of JNK, which protects from cell death and is independent of ROS (Lamb et al. 2003; Ventura et al. 2006), prolonged JNK activation is known to be important for cell death in both apoptotic and necrotic settings (Tobiume et al. 2001; Sakon et al. 2003; Ventura et al. 2004, 2006; Kamata et al. 2005; Chang et al. 2006). Significant evidence supports the role of ROS-mediated JNK activation in necrotic cell death (Shen and Liu 2006). Exogenous ROS (H_2O_2) was proposed to require JNK for induction of necrotic cell death (Shen et al. 2004), and therefore JNK is proposed to be a mediator of necrotic cell death pathways (Shen and Pervaiz 2006). Prolonged JNK activity has also been intricately associated with ROS in a number of ways, in terms of both initial activation and potentiation of the active JNK signal.

The thiol groups of cysteine residues within the active sites of enzymes are often reactive with ROS due to their low pKA (Paulsen and Carroll 2010). This seems to be true of phosphatases. Not only can classical protein tyrosine phosphatases be

inactivated by catalytic cysteine oxidation by ROS, as has long been known (Nakashima et al. 2002, 2005; Groen et al. 2005), but ROS can also inactivate dual-specificity phosphatases (Kamata et al. 2005). Since many such phosphatases (such as DUSPs 1, 4, 8, 14, 15, 16, 19) are involved in dephosphorylating JNK (Boutros et al. 2008), the intensity and duration of the JNK signal are controlled in large part by whether these phosphatases are active. The oxidation of catalytic cysteines can be reversible or irreversible on the oxidation state of the cysteine (den Hertog et al. 2005; Groen et al. 2005). Initial oxidation of cysteines to sulfenic acid is usually reversible by the cellular antioxidant machinery through the formation of an S-glutathionated intermediate reduction by glutaredoxin. The further oxidation of the thiol to sulfinic and then to sulfonic acids almost always results in irreversible inactivation of the phosphatases (Paulsen and Carroll 2010), which can in some cases then function as a dominant negative molecule by displacing active phosphatases from molecular complexes.

ASK1 is a MAP3K that is essential for ROS-induced sustained JNK phosphorylation (Tobiume et al. 2001). Several mechanisms have been proposed for the influence of ROS on the activation of ASK1. In the first, reduced thioredoxin binds to ASK1 and inhibits its kinase activity in a large complex which is designated the ASK1 signalosome (Saitoh et al. 1998; Liu et al. 2000; Noguchi et al. 2005). When thioredoxin is oxidized, ASK1 is released from the complex and is then capable of being activated, in large part due to recruitment of TRAF2 and TRAF6, which facilitate the N-terminal homophilic interaction of ASK1 through its N-terminal coiled-coil domain, which is required for its activation (Noguchi et al. 2005; Fujino et al. 2007). Autophosphorylation of Thr845 (Thr 838 in human) is thought to be critical to activation of ASK, and this is negatively downregulated by the PPP5C phosphatase (Morita et al. 2001). Alternatively, thioredoxin was proposed to inhibit ASK1 activation through promoting its ubiquitination and subsequent degradation (Liu and Min 2002). The deubiquitinating enzyme USP9X is known to interact with activated ASK1 and prevent this degradation by removing the ubiquitin conjugates (Nagai et al. 2009). In the third proposed mechanism, SENP1, a SUMO-specific protease, forms an inactive complex with thioredoxin in resting endothelial cells. When oxidation of the complex occurs, SENP1 translocates to the nucleus where it desumoylates HIPK1. HIPK1 then translocates to the cytoplasm and activates ASK1 (Li et al. 2008). The last and simplest mechanism is proposed by Nadeau et al., which proposes that ASK1 is activated directly by ROS oxidation of cysteines which leads to multimerization of ASK1 through disulfide bonds (Nadeau et al. 2007). In this final model, thioredoxin reduces the ASK1, thus reducing its activity. Trx1 is bound to ASK1 through an interaction that occurs between Cys250 of ASK1 and Cys32 of Trx1 (Nadeau et al. 2009). Several of these models are consistent with NOX1-produced ROS at the TNF complex being a potential source of localized ROS that mediate ASK1 activity. Direct oxidation of ASK1 may also explain why this kinase is associated with prolonged JNK signaling (Tobiume et al. 2001), since the ASK1 would be covalently bonded to itself, unlike other kinases, which diffuse eventually from their activated signaling complexes. Since multiple mechanisms for ASK1 regulation by ROS have been proposed, its regulation may be cell type

specific, or it may in fact be regulated by a number of interrelated or overlapping mechanisms. Nevertheless, since ASK1$^{-/-}$ is required for sustained JNK activity and cell death initiated by H_2O_2 (Tobiume et al. 2001), it is believed to be a key component of the molecular machinery that translates ROS signals into JNK activation and oxidative stress-induced death.

The glutathione S-transferase Pi (GSTP) protein protects cells from ROS-mediated death at least in part through repression of JNK activity (Yin et al. 2000). It has been reported that monomeric GSTP binds to the C-terminus of JNK and thereby inhibits its activity (Wang et al. 2001), while ROS induces GSTP oligomerization, which prevents JNK binding (Adler et al. 1999). More recent data suggests that monomeric GSTP is unstable and that dimeric GSTP is capable of binding JNK (Gildenhuys et al. 2010). In either case, mice lacking GSTP have a high basal level of constitutive JNK activity, underscoring the ability of the protein to repress JNK activation (Elsby et al. 2003).

8.3.4 JNK Regulation of ROS: The Tables Are Turned

In some cases, ROS generation lies downstream of JNK. Though this may be partially due to the feed-forward mechanisms previously discussed, there are other cases where JNK more directly regulates ROS formation. One of the best evidences that JNK can potentiate TNF-stimulated necrosis and that it is also involved in reactive oxygen species generation comes from cells isolated from Mkk4$^{-/-}$ Mkk7$^{-/-}$ and Jnk1$^{-/-}$ Jnk2$^{-/-}$ mice (Ventura et al. 2004), though this study did not describe a mechanism. In some cases, JNK has been shown to activate the mitochondrial production of superoxide in a manner dependent on complex I (Chambers and LoGrasso 2011). Perhaps one key to this mechanism was observed when diallyl trisulfide treatment was shown to induce ROS generation in a JNK-dependent fashion (Antosiewicz et al. 2006). JNK was also required for the diallyl trisulfide-mediated degradation of ferritin L, an iron storage protein, thus increasing the labile iron pool and providing a mechanism of ROS generation through the iron-dependent Fenton reaction (Antosiewicz et al. 2006).

Subsequently, TNF was shown to induce the proteasomal degradation of ferritin L and increase the labile iron pool, which was dependent on JNK1, but not JNK2 (Antosiewicz et al. 2007). Thus, the degradation of iron-containing ferritin is thought to contribute to ROS generation through the release of free iron. Importantly, ferritin H-deficient L929 cells are resistant to TNF-induced necroptosis, while TNF cannot induce an increase of free iron levels in RIP1 knockout mouse embryonic fibroblast cells (Xie et al. 2005), suggesting that RIP1, possibly through its direct activation of JNK, is responsible for the changes in iron accessibility and ROS generation. However, others have not seen changes in TNF-induced L929 cell death in the presence of the iron chelator desferrioxamine (Vanden Berghe et al. 2010). The mechanism of JNK-dependent proteasomal degradation of ferritin L has been proposed to be mediated by phosphorylation of the mitochondrial-associated p66Shc at

serine 36 since mutation at this serine dominant negative mutant preventing labile iron pool accumulation and ROS formation was significantly attenuated in p66Shc$^{-/-}$ MEFs (Borkowska et al. 2011).

8.3.5 *Further Potential Downstream Consequences of JNK Activation*

As mentioned, JNK is largely proposed to contribute to ROS through mitochondrial amplification of ROS production (Chambers and LoGrasso 2011). Many members of the Bcl-2 family, including Bcl-2 itself, are targets of JNK kinase activity and may therefore mediate some of the effects of JNK and ROS (Bogoyevitch and Kobe 2006). For instance, JNK phosphorylation on serine 74 contributes to an increase in Bmf activity (Hubner et al. 2010), while knockdown of Bmf prevents TNFα-induced necrosis (Hitomi et al. 2008). JNK- and p38-mediated phosphorylation of Bax leads to Bax translocation to mitochondria (Kim et al. 2006). While Bax and Bak are typically thought of as being required only for apoptosis, some necrotic situations require these proteins, such as the release of AIF from the mitochondria during MNNG-mediated necrosis (Moubarak et al. 2007). This process also requires Bid (Cabon et al. 2012). Recent data suggest that deletion of Bax and Bak also reduces necrotic injury (myocardial infarction) in mice, while triple-knockout mice that further lack cyclophilin D are not further rescued (Whelan et al. 2012). The connection in this case between ROS, JNK, and Bax in necrotic death has not yet been tested. However, JNK2 has been proposed to be involved in ischemia/reperfusion injury after liver transplant via activation of the MPTP. Moreover, inhibition of JNK protects from ischemic brain injury in rats and mice (Benakis et al. 2010; Nijboer et al. 2010), while JNK-1$^{-/-}$ or JNK-2$^{-/-}$ mice are protected from cardiac ischemia/reperfusion injury (Kaiser et al. 2005), suggesting that there could be a connection between the JNK and Bcl-2 protein family-mediated opening of the mitochondrial permeability transition pore. Since necrostatin-mediated cardioprotection from ischemia/reperfusion (IR) injury in mice requires CypD (Lim et al. 2007), RIP1 may therefore be upstream of both JNK and the MPTP.

Another Bcl-2 family member, BNIP3, is upregulated by JNK through modulation of the transcription factor FOXO3a in pathological hypertrophy and in heart failure (Chaanine et al. 2012). Overexpression of BNIP3 causes the mitochondrial permeability transition (Kim et al. 2002) leading to necrotic-like cell death independent of caspases (Vande Velde et al. 2000). Dominant negative BNIP3 prevents loss of mitochondrial membrane potential and protects against TNFα-induced necrosis in L929 cells (Ghavami et al. 2009). Thus, BNIP3 may be an important connection between JNK and the MPTP. A related BNIP family member, Nix (BNIP3L), is also regulated by FOXO3a (Real et al. 2005), suggesting that JNK also regulates Nix expression. When found at the ER, Nix causes the release of calcium that then triggers the MPTP complex opening in a cyclophilin-D-dependent manner, resulting in

necrotic cell death, while when at the mitochondria it promotes cytochrome C release and apoptosis (Chen et al. 2010). Curiously, Nix activates JNK through its interactions with POSH, a JNK-scaffolding protein (Wilhelm et al. 2007), likely resulting in a feed-forward amplification loop. Both Nix and BNIP3 are also thought to function in mitophagy (the recycling of damaged mitochondria by the autophagy machinery) (Behrends and Fulda 2012), which may suggest that they are regulated in the roles that they will play in terms of what is happening at the mitochondria.

8.3.6 JNK and ROS in Necrotic Cell Death

We previously found that NOX1 mediates sustained activation of JNK by TNFα (Kim et al. 2007). Although NOX1 contributed to cell death in some circumstances, it is not absolutely required in many cases (Vanlangenakker et al. 2011). Both ROS and JNK are absolutely required in our hands in this context for the death of L929 cells (and to a lesser extent $p65^{-/-}$ MEFs), and we found that inhibition of either is sufficient to abrogate cell death. However, although the preceding paragraphs have illustrated the many ways that JNK and ROS are involved in a majority of cases, they do not always appear to be required for necrotic cell death in every circumstance either. For instance, ROS scavengers do not seem to prevent cell death in FADD-deficient Jurkat T cells (Degterev et al. 2005). Similarly, others have found that JNK is not required for necrotic cell death (Cho et al. 2011). Sustained JNK activation does not seem sufficient on its own to result in cell death, suggesting JNK is not the sole initiating signal for cell death and that other signals are also required for necrotic cell death. JNK and ROS generation are clearly important for the necrotic process, but the requirement for each appears to be context dependent.

8.4 Conclusions

In conclusion, ROS and JNK potentiate many of the programmed necrotic processes in diverse ways. ROS and JNK are highly integrated with ROS often inducing JNK activation and JNK-potentiating ROS generation. Both contribute to cellular damage, in a large part through their actions on the mitochondria. ROS have a mixture of roles in cell death since ROS may directly oxidize cellular components or can initiate cell death through activating various signaling pathways. The machinery that generates ROS, including NADPH oxidases, the mitochondria, and other cytoplasmic enzymes, can interact with each other in cooperative and mutually stimulatory ways, leading to a destructive amplification of ROS that can react with cellular components, including DNA, lipids, and proteins, and result in the necrotic death of the cell.

References

Adler V, Yin Z, Fuchs SY, Benezra M, Rosario L, Tew KD, Pincus MR, Sardana M, Henderson CJ, Wolf CR, Davis RJ, Ronai Z (1999) Regulation of JNK signaling by GSTp. EMBO J 18:1321–1334

Anilkumar N, Weber R, Zhang M, Brewer A, Shah AM (2008) Nox4 and nox2 NADPH oxidases mediate distinct cellular redox signaling responses to agonist stimulation. Arterioscler Thromb Vasc Biol 28:1347–1354

Antosiewicz J, Herman-Antosiewicz A, Marynowski SW, Singh SV (2006) c-Jun NH(2)-terminal kinase signaling axis regulates diallyl trisulfide-induced generation of reactive oxygen species and cell cycle arrest in human prostate cancer cells. Cancer Res 66:5379–5386

Antosiewicz J, Ziolkowski W, Kaczor JJ, Herman-Antosiewicz A (2007) Tumor necrosis factor-alpha-induced reactive oxygen species formation is mediated by JNK1-dependent ferritin degradation and elevation of labile iron pool. Free Radic Biol Med 43:265–270

Artal-Sanz M, Samara C, Syntichaki P, Tavernarakis N (2006) Lysosomal biogenesis and function is critical for necrotic cell death in Caenorhabditis elegans. J Cell Biol 173:231–239

Baines CP, Kaiser RA, Purcell NH, Blair NS, Osinska H, Hambleton MA, Brunskill EW, Sayen MR, Gottlieb RA, Dorn GW, Robbins J, Molkentin JD (2005) Loss of cyclophilin D reveals a critical role for mitochondrial permeability transition in cell death. Nature 434:658–662

Banfi B, Clark RA, Steger K, Krause KH (2003) Two novel proteins activate superoxide generation by the NADPH oxidase NOX1. J Biol Chem 278:3510–3513

Behrends C, Fulda S (2012) Receptor proteins in selective autophagy. Int J Cell Biol 2012:673290

Benakis C, Bonny C, Hirt L (2010) JNK inhibition and inflammation after cerebral ischemia. Brain Behav Immun 24:800–811

Bogoyevitch MA, Kobe B (2006) Uses for JNK: the many and varied substrates of the c-Jun N-terminal kinases. Microbiol Mol Biol Rev 70:1061–1095

Bonnet MC, Preukschat D, Welz PS, van Loo G, Ermolaeva MA, Bloch W, Haase I, Pasparakis M (2011) The adaptor protein FADD protects epidermal keratinocytes from necroptosis in vivo and prevents skin inflammation. Immunity 35:572–582

Borkowska A, Sielicka-Dudzin A, Herman-Antosiewicz A, Halon M, Wozniak M, Antosiewicz J (2011) P66Shc mediated ferritin degradation – a novel mechanism of ROS formation. Free Radic Biol Med 51:658–663

Boutros T, Chevet E, Metrakos P (2008) Mitogen-activated protein (MAP) kinase/MAP kinase phosphatase regulation: roles in cell growth, death, and cancer. Pharmacol Rev 60:261–310

Boveris A, Chance B (1973) The mitochondrial generation of hydrogen peroxide. General properties and effect of hyperbaric oxygen. Biochem J 134:707–716

Boveris A, Oshino N, Chance B (1972) The cellular production of hydrogen peroxide. Biochem J 128:617–630

Boya P, Kroemer G (2008) Lysosomal membrane permeabilization in cell death. Oncogene 27:6434–6451

Brand MD, Affourtit C, Esteves TC, Green K, Lambert AJ, Miwa S, Pakay JL, Parker N (2004) Mitochondrial superoxide: production, biological effects, and activation of uncoupling proteins. Free Radic Biol Med 37:755–767

Brown DI, Griendling KK (2009) Nox proteins in signal transduction. Free Radic Biol Med 47:1239–1253

Brown GC, Borutaite V (2012) There is no evidence that mitochondria are the main source of reactive oxygen species in mammalian cells. Mitochondrion 12:1–4

Byun HS, Won M, Park KA, Kim YR, Choi BL, Lee H, Hong JH, Piao L, Park J, Kim JM, Kweon GR, Kang SH, Han J, Hur GM (2008) Prevention of TNF-induced necrotic cell death by rottlerin through a Nox1 NADPH oxidase. Exp Mol Med 40:186–195

Cabon L, Galan-Malo P, Bouharrour A, Delavallee L, Brunelle-Navas MN, Lorenzo HK, Gross A, Susin SA (2012) BID regulates AIF-mediated caspase-independent necroptosis by promoting BAX activation. Cell Death Differ 19:245–256

Ch'en IL, Tsau JS, Molkentin JD, Komatsu M, Hedrick SM (2011) Mechanisms of necroptosis in T cells. J Exp Med 208:633–641

Chaanine AH, Jeong D, Liang L, Chemaly ER, Fish K, Gordon RE, Hajjar RJ (2012) JNK modulates FOXO3a for the expression of the mitochondrial death and mitophagy marker BNIP3 in pathological hypertrophy and in heart failure. Cell Death Dis 3:265

Chambers JW, LoGrasso PV (2011) Mitochondrial c-Jun N-terminal kinase (JNK) signaling initiates physiological changes resulting in amplification of reactive oxygen species generation. J Biol Chem 286:16052

Chang L, Kamata H, Solinas G, Luo JL, Maeda S, Venuprasad K, Liu YC, Karin M (2006) The E3 ubiquitin ligase itch couples JNK activation to TNFalpha-induced cell death by inducing c-FLIP(L) turnover. Cell 124:601–613

Chen Y, Lewis W, Diwan A, Cheng EH, Matkovich SJ, Dorn GW 2nd (2010) Dual autonomous mitochondrial cell death pathways are activated by Nix/BNip3L and induce cardiomyopathy. Proc Natl Acad Sci U S A 107:9035–9042

Cho Y, McQuade T, Zhang H, Zhang J, Chan FK (2011) RIP1-dependent and independent effects of necrostatin-1 in necrosis and T cell activation. PLoS One 6:e23209

Cho YS, Challa S, Moquin D, Genga R, Ray TD, Guildford M, Chan FK (2009) Phosphorylation-driven assembly of the RIP1-RIP3 complex regulates programmed necrosis and virus-induced inflammation. Cell 137:1112–1123

Costa AD, Quinlan CL, Andrukhiv A, West IC, Jaburek M, Garlid KD (2006) The direct physiological effects of mitoK(ATP) opening on heart mitochondria. Am J Physiol Heart Circ Physiol 290:H406–H415

Cotter TG (2009) Apoptosis and cancer: the genesis of a research field. Nat Rev Cancer 9:501–507

Davis CW, Hawkins BJ, Ramasamy S, Irrinki KM, Cameron BA, Islam K, Daswani VP, Doonan PJ, Manevich Y, Madesh M (2010) Nitration of the mitochondrial complex I subunit NDUFB8 elicits RIP1- and RIP3-mediated necrosis. Free Radic Biol Med 48:306–317

Degterev A, Hitomi J, Germscheid M, Ch'en IL, Korkina O, Teng X, Abbott D, Cuny GD, Yuan C, Wagner G, Hedrick SM, Gerber SA, Lugovskoy A, Yuan J (2008) Identification of RIP1 kinase as a specific cellular target of necrostatins. Nat Chem Biol 4:313–321

Degterev A, Huang Z, Boyce M, Li Y, Jagtap P, Mizushima N, Cuny GD, Mitchison TJ, Moskowitz MA, Yuan J (2005) Chemical inhibitor of nonapoptotic cell death with therapeutic potential for ischemic brain injury. Nat Chem Biol 1:112–119

Delavallee L, Cabon L, Galan-Malo P, Lorenzo HK, Susin SA (2011) AIF-mediated caspase-independent necroptosis: a new chance for targeted therapeutics. IUBMB Life 63:221–232

den Hertog J, Groen A, van der Wijk T (2005) Redox regulation of protein-tyrosine phosphatases. Arch Biochem Biophys 434:11–15

Denecker G, Vercammen D, Steemans M, Vanden Berghe T, Brouckaert G, Van Loo G, Zhivotovsky B, Fiers W, Grooten J, Declercq W, Vandenabeele P (2001) Death receptor-induced apoptotic and necrotic cell death: differential role of caspases and mitochondria. Cell Death Differ 8: 829–840

Devin A, Cook A, Lin Y, Rodriguez Y, Kelliher M, Liu Z (2000) The distinct roles of TRAF2 and RIP in IKK activation by TNF-R1: TRAF2 recruits IKK to TNF-R1 while RIP mediates IKK activation. Immunity 12:419–429

Devin A, Lin Y, Yamaoka S, Li Z, Karin M, Liu Z (2001) The alpha and beta subunits of IkappaB kinase (IKK) mediate TRAF2-dependent IKK recruitment to tumor necrosis factor (TNF) receptor 1 in response to TNF. Mol Cell Biol 21:3986–3994

Dikalov S (2011) Cross talk between mitochondria and NADPH oxidases. Free Radic Biol Med 51:1289–1301

Dillon CP, Oberst A, Weinlich R, Janke LJ, Kang TB, Ben-Moshe T, Mak TW, Wallach D, Green DR (2012) Survival function of the FADD-CASPASE-8-cFLIP(L) complex. Cell Rep 1:401–407

Duprez L, Takahashi N, Van Hauwermeiren F, Vandendriessche B, Goossens V, Vanden Berghe T, Declercq W, Libert C, Cauwels A, Vandenabeele P (2011) RIP kinase-dependent necrosis drives lethal systemic inflammatory response syndrome. Immunity 35:908–918

Elsby R, Kitteringham NR, Goldring CE, Lovatt CA, Chamberlain M, Henderson CJ, Wolf CR, Park BK (2003) Increased constitutive c-Jun N-terminal kinase signaling in mice lacking glutathione S-transferase Pi. J Biol Chem 278:22243–22249

Feng S, Yang Y, Mei Y, Ma L, Zhu DE, Hoti N, Castanares M, Wu M (2007) Cleavage of RIP3 inactivates its caspase-independent apoptosis pathway by removal of kinase domain. Cell Signal 19:2056–2067

Festjens N, Kalai M, Smet J, Meeus A, Van Coster R, Saelens X, Vandenabeele P (2006) Butylated hydroxyanisole is more than a reactive oxygen species scavenger. Cell Death Differ 13: 166–169

Fiers W, Beyaert R, Declercq W, Vandenabeele P (1999) More than one way to die: apoptosis, necrosis and reactive oxygen damage. Oncogene 18:7719–7730

Fujino G, Noguchi T, Matsuzawa A, Yamauchi S, Saitoh M, Takeda K, Ichijo H (2007) Thioredoxin and TRAF family proteins regulate reactive oxygen species-dependent activation of ASK1 through reciprocal modulation of the N-terminal homophilic interaction of ASK1. Mol Cell Biol 27:8152–8163

Geiszt M, Lekstrom K, Witta J, Leto TL (2003) Proteins homologous to p47phox and p67phox support superoxide production by NAD(P)H oxidase 1 in colon epithelial cells. J Biol Chem 278:20006–20012

Genova ML, Ventura B, Giuliano G, Bovina C, Formiggini G, Parenti Castelli G, Lenaz G (2001) The site of production of superoxide radical in mitochondrial Complex I is not a bound ubisemiquinone but presumably iron-sulfur cluster N2. FEBS Lett 505:364–368

Ghavami S, Eshraghi M, Kadkhoda K, Mutawe MM, Maddika S, Bay GH, Wesselborg S, Halayko AJ, Klonisch T, Los M (2009) Role of BNIP3 in TNF-induced cell death – TNF upregulates BNIP3 expression. Biochim Biophys Acta 1793:546–560

Gildenhuys S, Wallace LA, Burke JP, Balchin D, Sayed Y, Dirr HW (2010) Class Pi glutathione transferase unfolds via a dimeric and not monomeric intermediate: functional implications for an unstable monomer. Biochemistry 49:5074–5081

Goossens V, De Vos K, Vercammen D, Steemans M, Vancompernolle K, Fiers W, Vandenabeele P, Grooten J (1999a) Redox regulation of TNF signaling. Biofactors 10:145–156

Goossens V, Grooten J, De Vos K, Fiers W (1995) Direct evidence for tumor necrosis factor-induced mitochondrial reactive oxygen intermediates and their involvement in cytotoxicity. Proc Natl Acad Sci U S A 92:8115–8119

Goossens V, Stange G, Moens K, Pipeleers D, Grooten J (1999b) Regulation of tumor necrosis factor-induced, mitochondria- and reactive oxygen species-dependent cell death by the electron flux through the electron transport chain complex I. Antioxid Redox Signal 1:285–295

Groen A, Lemeer S, van der Wijk T, Overvoorde J, Heck AJ, Ostman A, Barford D, Slijper M, den Hertog J (2005) Differential oxidation of protein-tyrosine phosphatases. J Biol Chem 280:10298–10304

Grooten J, Goossens V, Vanhaesebroeck B, Fiers W (1993) Cell membrane permeabilization and cellular collapse, followed by loss of dehydrogenase activity: early events in tumour necrosis factor-induced cytotoxicity. Cytokine 5:546–555

He S, Wang L, Miao L, Wang T, Du F, Zhao L, Wang X (2009) Receptor interacting protein kinase-3 determines cellular necrotic response to TNF-alpha. Cell 137:1100–1111

Hitomi J, Christofferson DE, Ng A, Yao J, Degterev A, Xavier RJ, Yuan J (2008) Identification of a molecular signaling network that regulates a cellular necrotic cell death pathway. Cell 135:1311–1323

Holler N, Zaru R, Micheau O, Thome M, Attinger A, Valitutti S, Bodmer JL, Schneider P, Seed B, Tschopp J (2000) Fas triggers an alternative, caspase-8-independent cell death pathway using the kinase RIP as effector molecule. Nat Immunol 1:489–495

Holmgren A (2000) Antioxidant function of thioredoxin and glutaredoxin systems. Antioxid Redox Signal 2:811–820

Hubner A, Cavanagh-Kyros J, Rincon M, Flavell RA, Davis RJ (2010) Functional cooperation of the proapoptotic Bcl2 family proteins Bmf and Bim in vivo. Mol Cell Biol 30:98–105

Jouan-Lanhouet S, Arshad MI, Piquet-Pellorce C, Martin-Chouly C, Le Moigne-Muller G, Van Herreweghe F, Takahashi N, Sergent O, Lagadic-Gossmann D, Vandenabeele P, Samson M, Dimanche-Boitrel MT (2012) TRAIL induces necroptosis involving RIPK1/RIPK3-dependent PARP-1 activation. Cell Death Differ 19:2003–2014

Kaiser RA, Liang Q, Bueno O, Huang Y, Lackey T, Klevitsky R, Hewett TE, Molkentin JD (2005) Genetic inhibition or activation of JNK1/2 protects the myocardium from ischemia-reperfusion-induced cell death in vivo. J Biol Chem 280:32602–32608

Kaiser WJ, Upton JW, Long AB, Livingston-Rosanoff D, Daley-Bauer LP, Hakem R, Caspary T, Mocarski ES (2011) RIP3 mediates the embryonic lethality of caspase-8-deficient mice. Nature 471:368–372

Kamata H, Honda S, Maeda S, Chang L, Hirata H, Karin M (2005) Reactive oxygen species promote TNFalpha-induced death and sustained JNK activation by inhibiting MAP kinase phosphatases. Cell 120:649–661

Kelliher MA, Grimm S, Ishida Y, Kuo F, Stanger BZ, Leder P (1998) The death domain kinase RIP mediates the TNF-induced NF-kappaB signal. Immunity 8:297–303

Kerr JF, Wyllie AH, Currie AR (1972) Apoptosis: a basic biological phenomenon with wide-ranging implications in tissue kinetics. Br J Cancer 26:239–257

Kim BJ, Ryu SW, Song BJ (2006) JNK- and p38 kinase-mediated phosphorylation of Bax leads to its activation and mitochondrial translocation and to apoptosis of human hepatoma HepG2 cells. J Biol Chem 281:21256–21265

Kim JY, Cho JJ, Ha J, Park JH (2002) The carboxy terminal C-tail of BNip3 is crucial in induction of mitochondrial permeability transition in isolated mitochondria. Arch Biochem Biophys 398:147–152

Kim YS, Morgan MJ, Choksi S, Liu ZG (2007) TNF-induced activation of the Nox1 NADPH oxidase and its role in the induction of necrotic cell death. Mol Cell 26:675–687

Korshunov SS, Skulachev VP, Starkov AA (1997) High protonic potential actuates a mechanism of production of reactive oxygen species in mitochondria. FEBS Lett 416:15–18

Kovalenko A, Chable-Bessia C, Cantarella G, Israel A, Wallach D, Courtois G (2003) The tumour suppressor CYLD negatively regulates NF-kappaB signalling by deubiquitination. Nature 424:801–805

Kroemer G, El-Deiry WS, Golstein P, Peter ME, Vaux D, Vandenabeele P, Zhivotovsky B, Blagosklonny MV, Malorni W, Knight RA, Piacentini M, Nagata S, Melino G (2005) Classification of cell death: recommendations of the Nomenclature Committee on Cell Death. Cell Death Differ 12(Suppl 2):1463–1467

Kurz T, Gustafsson B, Brunk UT (2006) Intralysosomal iron chelation protects against oxidative stress-induced cellular damage. FEBS J 273:3106–3117

Lamb JA, Ventura JJ, Hess P, Flavell RA, Davis RJ (2003) JunD mediates survival signaling by the JNK signal transduction pathway. Mol Cell 11:1479–1489

Lambert AJ, Brand MD (2004) Superoxide production by NADH:ubiquinone oxidoreductase (complex I) depends on the pH gradient across the mitochondrial inner membrane. Biochem J 382:511–517

Lambeth JD (2004) NOX enzymes and the biology of reactive oxygen. Nat Rev Immunol 4:181–189

Laster SM, Wood JG, Gooding LR (1988) Tumor necrosis factor can induce both apoptotic and necrotic forms of cell lysis. J Immunol 141:2629–2634

Lee I, Bender E, Kadenbach B (2002) Control of mitochondrial membrane potential and ROS formation by reversible phosphorylation of cytochrome c oxidase. Mol Cell Biochem 234–235:63–70

Lee SB, Bae IH, Bae YS, Um HD (2006) Link between mitochondria and NADPH oxidase 1 isozyme for the sustained production of reactive oxygen species and cell death. J Biol Chem 281:36228–36235

Li J, McQuade T, Siemer AB, Napetschnig J, Moriwaki K, Hsiao YS, Damko E, Moquin D, Walz T, McDermott A, Chan FK, Wu H (2012) The RIP1/RIP3 necrosome forms a functional amyloid signaling complex required for programmed necrosis. Cell 150:339–350

Li L, He Q, Huang X, Man Y, Zhou Y, Wang S, Wang J, Li J (2010) NOX3-derived reactive oxygen species promote TNF-alpha-induced reductions in hepatocyte glycogen levels via a JNK pathway. FEBS Lett 584:995–1000

Li Q, Spencer NY, Oakley FD, Buettner GR, Engelhardt JF (2009) Endosomal Nox2 facilitates redox-dependent induction of NF-kappaB by TNF-alpha. Antioxid Redox Signal 11:1249–1263

Li WG, Miller FJ Jr, Zhang HJ, Spitz DR, Oberley LW, Weintraub NL (2001) H(2)O(2)-induced O(2) production by a non-phagocytic NAD(P)H oxidase causes oxidant injury. J Biol Chem 276:29251–29256

Li X, Luo Y, Yu L, Lin Y, Luo D, Zhang H, He Y, Kim YO, Kim Y, Tang S, Min W (2008) SENP1 mediates TNF-induced desumoylation and cytoplasmic translocation of HIPK1 to enhance ASK1-dependent apoptosis. Cell Death Differ 15:739–750

Li Y, Johnson N, Capano M, Edwards M, Crompton M (2004) Cyclophilin-D promotes the mitochondrial permeability transition but has opposite effects on apoptosis and necrosis. Biochem J 383:101–109

Lim SY, Davidson SM, Mocanu MM, Yellon DM, Smith CC (2007) The cardioprotective effect of necrostatin requires the cyclophilin-D component of the mitochondrial permeability transition pore. Cardiovasc Drugs Ther 21:467–469

Lin Y, Choksi S, Shen HM, Yang QF, Hur GM, Kim YS, Tran JH, Nedospasov SA, Liu ZG (2004) Tumor necrosis factor-induced nonapoptotic cell death requires receptor-interacting protein-mediated cellular reactive oxygen species accumulation. J Biol Chem 279:10822–10828

Lin Y, Devin A, Rodriguez Y, Liu ZG (1999) Cleavage of the death domain kinase RIP by caspase-8 prompts TNF-induced apoptosis. Genes Dev 13:2514–2526

Liu H, Nishitoh H, Ichijo H, Kyriakis JM (2000) Activation of apoptosis signal-regulating kinase 1 (ASK1) by tumor necrosis factor receptor-associated factor 2 requires prior dissociation of the ASK1 inhibitor thioredoxin. Mol Cell Biol 20:2198–2208

Liu Y, Fiskum G, Schubert D (2002) Generation of reactive oxygen species by the mitochondrial electron transport chain. J Neurochem 80:780–787

Liu Y, Min W (2002) Thioredoxin promotes ASK1 ubiquitination and degradation to inhibit ASK1-mediated apoptosis in a redox activity-independent manner. Circ Res 90:1259–1266

Lockshin R, Williams C (1964) Programmed cell death – II. Endocrine potentiation of the breakdown of the intersegmental muscles of silkmoths. J Insect Physiol 10:643

Lockshin R, Williams C (1965) Programmed cell death – I. Cytology of degeneration in the intersegmental muscles of the Pernyi silkmoth. J Insect Physiol 11:123

Los M, Mozoluk M, Ferrari D, Stepczynska A, Stroh C, Renz A, Herceg Z, Wang ZQ, Schulze-Osthoff K (2002) Activation and caspase-mediated inhibition of PARP: a molecular switch between fibroblast necrosis and apoptosis in death receptor signaling. Mol Biol Cell 13: 978–988

Lu JV, Weist BM, van Raam BJ, Marro BS, Nguyen LV, Srinivas P, Bell BD, Luhrs KA, Lane TE, Salvesen GS, Walsh CM (2011) Complementary roles of Fas-associated death domain (FADD) and receptor interacting protein kinase-3 (RIPK3) in T-cell homeostasis and antiviral immunity. Proc Natl Acad Sci U S A 108:15312–15317

Luke CJ, Pak SC, Askew YS, Naviglia TL, Askew DJ, Nobar SM, Vetica AC, Long OS, Watkins SC, Stolz DB, Barstead RJ, Moulder GL, Bromme D, Silverman GA (2007) An intracellular serpin regulates necrosis by inhibiting the induction and sequelae of lysosomal injury. Cell 130:1108–1119

Mariappan N, Elks CM, Fink B, Francis J (2009) TNF-induced mitochondrial damage: a link between mitochondrial complex I activity and left ventricular dysfunction. Free Radic Biol Med 46:462–470

McNally JS, Saxena A, Cai H, Dikalov S, Harrison DG (2005) Regulation of xanthine oxidoreductase protein expression by hydrogen peroxide and calcium. Arterioscler Thromb Vasc Biol 25:1623–1628

Moe KT, Aulia S, Jiang F, Chua YL, Koh TH, Wong MC, Dusting GJ (2006) Differential upregulation of Nox homologues of NADPH oxidase by tumor necrosis factor-alpha in human aortic smooth muscle and embryonic kidney cells. J Cell Mol Med 10:231–239

Morgan MJ, Kim YS, Liu ZG (2008) TNFalpha and reactive oxygen species in necrotic cell death. Cell Res 18:343–349

Morita K, Saitoh M, Tobiume K, Matsuura H, Enomoto S, Nishitoh H, Ichijo H (2001) Negative feedback regulation of ASK1 by protein phosphatase 5 (PP5) in response to oxidative stress. EMBO J 20:6028–6036

Moubarak RS, Yuste VJ, Artus C, Bouharrour A, Greer PA, Menissier-de Murcia J, Susin SA (2007) Sequential activation of poly(ADP-ribose) polymerase 1, calpains, and Bax is essential in apoptosis-inducing factor-mediated programmed necrosis. Mol Cell Biol 27:4844–4862

Moulin M, Anderton H, Voss AK, Thomas T, Wong WW, Bankovacki A, Feltham R, Chau D, Cook WD, Silke J, Vaux DL (2012) IAPs limit activation of RIP kinases by TNF receptor 1 during development. EMBO J 31:1679–1691

Nadeau PJ, Charette SJ, Landry J (2009) REDOX reaction at ASK1-Cys250 is essential for activation of JNK and induction of apoptosis. Mol Biol Cell 20:3628–3637

Nadeau PJ, Charette SJ, Toledano MB, Landry J (2007) Disulfide Bond-mediated multimerization of Ask1 and its reduction by thioredoxin-1 regulate H(2)O(2)-induced c-Jun NH(2)-terminal kinase activation and apoptosis. Mol Biol Cell 18:3903–3913

Nagai H, Noguchi T, Homma K, Katagiri K, Takeda K, Matsuzawa A, Ichijo H (2009) Ubiquitin-like sequence in ASK1 plays critical roles in the recognition and stabilization by USP9X and oxidative stress-induced cell death. Mol Cell 36:805–818

Nakagawa T, Shimizu S, Watanabe T, Yamaguchi O, Otsu K, Yamagata H, Inohara H, Kubo T, Tsujimoto Y (2005) Cyclophilin D-dependent mitochondrial permeability transition regulates some necrotic but not apoptotic cell death. Nature 434:652–658

Nakashima I, Kato M, Akhand AA, Suzuki H, Takeda K, Hossain K, Kawamoto Y (2002) Redox-linked signal transduction pathways for protein tyrosine kinase activation. Antioxid Redox Signal 4:517–531

Nakashima I, Takeda K, Kawamoto Y, Okuno Y, Kato M, Suzuki H (2005) Redox control of catalytic activities of membrane-associated protein tyrosine kinases. Arch Biochem Biophys 434:3–10

Nijboer CH, van der Kooij MA, van Bel F, Ohl F, Heijnen CJ, Kavelaars A (2010) Inhibition of the JNK/AP-1 pathway reduces neuronal death and improves behavioral outcome after neonatal hypoxic-ischemic brain injury. Brain Behav Immun 24:812–821

Noguchi T, Takeda K, Matsuzawa A, Saegusa K, Nakano H, Gohda J, Inoue J, Ichijo H (2005) Recruitment of tumor necrosis factor receptor-associated factor family proteins to apoptosis signal-regulating kinase 1 signalosome is essential for oxidative stress-induced cell death. J Biol Chem 280:37033–37040

O'Donnell MA, Hase H, Legarda D, Ting AT (2012) NEMO Inhibits Programmed Necrosis in an NFkappaB-Independent Manner by Restraining RIP1. PLoS One 7:e41238

O'Donnell MA, Perez-Jimenez E, Oberst A, Ng A, Massoumi R, Xavier R, Green DR, Ting AT (2011) Caspase 8 inhibits programmed necrosis by processing CYLD. Nat Cell Biol 13: 1437–1442

Oberst A, Dillon CP, Weinlich R, McCormick LL, Fitzgerald P, Pop C, Hakem R, Salvesen GS, Green DR (2011) Catalytic activity of the caspase-8-FLIP(L) complex inhibits RIPK3-dependent necrosis. Nature 471:363–367

Orrenius S, Gogvadze V, Zhivotovsky B (2007) Mitochondrial oxidative stress: implications for cell death. Annu Rev Pharmacol Toxicol 47:143–183

Ott M, Gogvadze V, Orrenius S, Zhivotovsky B (2007) Mitochondria, oxidative stress and cell death. Apoptosis 12:913–922

Pantano C, Anathy V, Ranjan P, Heintz NH, Janssen-Heininger YM (2007) Nonphagocytic oxidase 1 causes death in lung epithelial cells via a TNF-RI-JNK signaling axis. Am J Respir Cell Mol Biol 36:473–479

Park KJ, Lee CH, Kim A, Jeong KJ, Kim CH, Kim YS (2012) Death receptors 4 and 5 activate Nox1 NADPH oxidase through riboflavin kinase to induce reactive oxygen species-mediated apoptotic cell death. J Biol Chem 287:3313–3325

Paulsen CE, Carroll KS (2010) Orchestrating redox signaling networks through regulatory cysteine switches. ACS Chem Biol 5:47–62

Quijano C, Hernandez-Saavedra D, Castro L, McCord JM, Freeman BA, Radi R (2001) Reaction of peroxynitrite with Mn-superoxide dismutase. Role of the metal center in decomposition kinetics and nitration. J Biol Chem 276:11631–11638

Quinn MT, Ammons MC, Deleo FR (2006) The expanding role of NADPH oxidases in health and disease: no longer just agents of death and destruction. Clin Sci (Lond) 111:1–20

Real PJ, Benito A, Cuevas J, Berciano MT, de Juan A, Coffer P, Gomez-Roman J, Lafarga M, Lopez-Vega JM, Fernandez-Luna JL (2005) Blockade of epidermal growth factor receptors chemosensitizes breast cancer cells through up-regulation of Bnip3L. Cancer Res 65: 8151–8157

Rhee SG, Yang KS, Kang SW, Woo HA, Chang TS (2005) Controlled elimination of intracellular H(2)O(2): regulation of peroxiredoxin, catalase, and glutathione peroxidase via post-translational modification. Antioxid Redox Signal 7:619–626

Russell SW, Rosenau W, Lee JC (1972) Cytolysis induced by human lymphotoxin. Am J Pathol 69:103–118

Saito Y, Nishio K, Ogawa Y, Kimata J, Kinumi T, Yoshida Y, Noguchi N, Niki E (2006) Turning point in apoptosis/necrosis induced by hydrogen peroxide. Free Radic Res 40:619–630

Saitoh M, Nishitoh H, Fujii M, Takeda K, Tobiume K, Sawada Y, Kawabata M, Miyazono K, Ichijo H (1998) Mammalian thioredoxin is a direct inhibitor of apoptosis signal-regulating kinase (ASK) 1. EMBO J 17:2596–2606

Sakon S, Xue X, Takekawa M, Sasazuki T, Okazaki T, Kojima Y, Piao JH, Yagita H, Okumura K, Doi T, Nakano H (2003) NF-kappaB inhibits TNF-induced accumulation of ROS that mediate prolonged MAPK activation and necrotic cell death. EMBO J 22:3898–3909

Sato T, Machida T, Takahashi S, Murase K, Kawano Y, Hayashi T, Iyama S, Takada K, Kuribayashi K, Sato Y, Kobune M, Takimoto R, Matsunaga T, Kato J, Niitsu Y (2008) Apoptosis supercedes necrosis in mitochondrial DNA-depleted Jurkat cells by cleavage of receptor-interacting protein and inhibition of lysosomal cathepsin. J Immunol 181:197–207

Schinzel AC, Takeuchi O, Huang Z, Fisher JK, Zhou Z, Rubens J, Hetz C, Danial NN, Moskowitz MA, Korsmeyer SJ (2005) Cyclophilin D is a component of mitochondrial permeability transition and mediates neuronal cell death after focal cerebral ischemia. Proc Natl Acad Sci U S A 102:12005–12010

Schulze-Osthoff K, Bakker AC, Vanhaesebroeck B, Beyaert R, Jacob WA, Fiers W (1992) Cytotoxic activity of tumor necrosis factor is mediated by early damage of mitochondrial functions. Evidence for the involvement of mitochondrial radical generation. J Biol Chem 267:5317–5323

Schulze-Osthoff K, Beyaert R, Vandevoorde V, Haegeman G, Fiers W (1993) Depletion of the mitochondrial electron transport abrogates the cytotoxic and gene-inductive effects of TNF. EMBO J 12:3095–3104

Seki E, Brenner DA, Karin M (2012) A liver full of JNK: signaling in regulation of cell function and disease pathogenesis, and clinical approaches. Gastroenterology 143:307–320

Shen HM, Lin Y, Choksi S, Tran J, Jin T, Chang L, Karin M, Zhang J, Liu ZG (2004) Essential roles of receptor-interacting protein and TRAF2 in oxidative stress-induced cell death. Mol Cell Biol 24:5914–5922

Shen HM, Liu ZG (2006) JNK signaling pathway is a key modulator in cell death mediated by reactive oxygen and nitrogen species. Free Radic Biol Med 40:928–939

Shen HM, Pervaiz S (2006) TNF receptor superfamily-induced cell death: redox-dependent execution. FASEB J 20:1589–1598

Sies H (1997) Oxidative stress: oxidants and antioxidants. Exp Physiol 82:291–295

St Hilaire C, Koupenova M, Carroll SH, Smith BD, Ravid K (2008) TNF-alpha upregulates the A2B adenosine receptor gene: the role of NAD(P)H oxidase 4. Biochem Biophys Res Commun 375:292–296

Susin SA, Lorenzo HK, Zamzami N, Marzo I, Snow BE, Brothers GM, Mangion J, Jacotot E, Costantini P, Loeffler M, Larochette N, Goodlett DR, Aebersold R, Siderovski DP, Penninger

JM, Kroemer G (1999) Molecular characterization of mitochondrial apoptosis-inducing factor. Nature 397:441–446
Takeda K, Komuro Y, Hayakawa T, Oguchi H, Ishida Y, Murakami S, Noguchi T, Kinoshita H, Sekine Y, Iemura S, Natsume T, Ichijo H (2009) Mitochondrial phosphoglycerate mutase 5 uses alternate catalytic activity as a protein serine/threonine phosphatase to activate ASK1. Proc Natl Acad Sci U S A 106:12301–12305
Takeda M, Shirato I, Kobayashi M, Endou H (1999) Hydrogen peroxide induces necrosis, apoptosis, oncosis and apoptotic oncosis of mouse terminal proximal straight tubule cells. Nephron 81:234–238
Takeya R, Ueno N, Kami K, Taura M, Kohjima M, Izaki T, Nunoi H, Sumimoto H (2003) Novel human homologues of p47phox and p67phox participate in activation of superoxide-producing NADPH oxidases. J Biol Chem 278:25234–25246
Temkin V, Huang Q, Liu H, Osada H, Pope RM (2006) Inhibition of ADP/ATP exchange in receptor-interacting protein-mediated necrosis. Mol Cell Biol 26:2215–2225
Teramoto S, Tomita T, Matsui H, Ohga E, Matsuse T, Ouchi Y (1999) Hydrogen peroxide-induced apoptosis and necrosis in human lung fibroblasts: protective roles of glutathione. Jpn J Pharmacol 79:33–40
Ting AT, Pimentel-Muinos FX, Seed B (1996) RIP mediates tumor necrosis factor receptor 1 activation of NF-kappaB but not Fas/APO-1-initiated apoptosis. EMBO J 15:6189–6196
Tobiume K, Matsuzawa A, Takahashi T, Nishitoh H, Morita K, Takeda K, Minowa O, Miyazono K, Noda T, Ichijo H (2001) ASK1 is required for sustained activations of JNK/p38 MAP kinases and apoptosis. EMBO Rep 2:222–228
Tu HC, Ren D, Wang GX, Chen DY, Westergard TD, Kim H, Sasagawa S, Hsieh JJ, Cheng EH (2009) The p53-cathepsin axis cooperates with ROS to activate programmed necrotic death upon DNA damage. Proc Natl Acad Sci U S A 106:1093–1098
Turrens JF (2003) Mitochondrial formation of reactive oxygen species. J Physiol 552:335–344
Turrens JF, Alexandre A, Lehninger AL (1985) Ubisemiquinone is the electron-donor for superoxide formation by complex III of heart-mitochondria. Arch Biochem Biophys 237:408–414
Ushio-Fukai M (2009) Compartmentalization of redox signaling through NADPH oxidase-derived ROS. Antioxid Redox Signal 11:1289–1299
Van Herreweghe F, Mao J, Chaplen FW, Grooten J, Gevaert K, Vandekerckhove J, Vancompernolle K (2002) Tumor necrosis factor-induced modulation of glyoxalase I activities through phosphorylation by PKA results in cell death and is accompanied by the formation of a specific methylglyoxal-derived AGE. Proc Natl Acad Sci U S A 99:949–954
Vande Velde C, Cizeau J, Dubik D, Alimonti J, Brown T, Israels S, Hakem R, Greenberg AH (2000) BNIP3 and genetic control of necrosis-like cell death through the mitochondrial permeability transition pore. Mol Cell Biol 20:5454–5468
Vanden Berghe T, Vanlangenakker N, Parthoens E, Deckers W, Devos M, Festjens N, Guerin CJ, Brunk UT, Declercq W, Vandenabeele P (2010) Necroptosis, necrosis and secondary necrosis converge on similar cellular disintegration features. Cell Death Differ 17:922–930
Vandenabeele P, Galluzzi L, Vanden Berghe T, Kroemer G (2010) Molecular mechanisms of necroptosis: an ordered cellular explosion. Nat Rev Mol Cell Biol 11:700–714
Vanlangenakker N, Vanden Berghe T, Bogaert P, Laukens B, Zobel K, Deshayes K, Vucic D, Fulda S, Vandenabeele P, Bertrand MJ (2011) cIAP1 and TAK1 protect cells from TNF-induced necrosis by preventing RIP1/RIP3-dependent reactive oxygen species production. Cell Death Differ 18:656–665
Vaseva AV, Marchenko ND, Ji K, Tsirka SE, Holzmann S, Moll UM (2012) p53 opens the mitochondrial permeability transition pore to trigger necrosis. Cell 149:1536–1548
Ventura JJ, Cogswell P, Flavell RA, Baldwin AS Jr, Davis RJ (2004) JNK potentiates TNF-stimulated necrosis by increasing the production of cytotoxic reactive oxygen species. Genes Dev 18:2905–2915
Ventura JJ, Hubner A, Zhang C, Flavell RA, Shokat KM, Davis RJ (2006) Chemical genetic analysis of the time course of signal transduction by JNK. Mol Cell 21:701–710

Vercammen D, Beyaert R, Denecker G, Goossens V, Van Loo G, Declercq W, Grooten J, Fiers W, Vandenabeele P (1998) Inhibition of caspases increases the sensitivity of L929 cells to necrosis mediated by tumor necrosis factor. J Exp Med 187:1477–1485

Votyakova TV, Reynolds IJ (2001) DeltaPsi(m)-Dependent and -independent production of reactive oxygen species by rat brain mitochondria. J Neurochem 79:266–277

Wang L, Du F, Wang X (2008) TNF-alpha induces two distinct caspase-8 activation pathways. Cell 133:693–703

Wang T, Arifoglu P, Ronai Z, Tew KD (2001) Glutathione S-transferase P1-1 (GSTP1-1) inhibits c-Jun N-terminal kinase (JNK1) signaling through interaction with the C terminus. J Biol Chem 276:20999–21003

Wang Y, Kim NS, Haince JF, Kang HC, David KK, Andrabi SA, Poirier GG, Dawson VL, Dawson TM (2011) Poly(ADP-ribose) (PAR) binding to apoptosis-inducing factor is critical for PAR polymerase-1-dependent cell death (parthanatos). Sci Signal 4:ra20

Wang Z, Jiang H, Chen S, Du F, Wang X (2012) The mitochondrial phosphatase PGAM5 functions at the convergence point of multiple necrotic death pathways. Cell 148:228–243

Wertz IE, O'Rourke KM, Zhou H, Eby M, Aravind L, Seshagiri S, Wu P, Wiesmann C, Baker R, Boone DL, Ma A, Koonin EV, Dixit VM (2004) De-ubiquitination and ubiquitin ligase domains of A20 downregulate NF-kappaB signalling. Nature 430:694–699

Whelan RS, Konstantinidis K, Wei AC, Chen Y, Reyna DE, Jha S, Yang Y, Calvert JW, Lindsten T, Thompson CB, Crow MT, Gavathiotis E, Dorn GW, O'Rourke B, Kitsis RN (2012) Bax regulates primary necrosis through mitochondrial dynamics. Proc Natl Acad Sci U S A 109:6566–6571

Wilhelm M, Xu Z, Kukekov NV, Gire S, Greene LA (2007) Proapoptotic Nix activates the JNK pathway by interacting with POSH and mediates death in a Parkinson disease model. J Biol Chem 282:1288–1295

Xie C, Zhang N, Zhou H, Li J, Li Q, Zarubin T, Lin SC, Han J (2005) Distinct roles of basal steady-state and induced H-ferritin in tumor necrosis factor-induced death in L929 cells. Mol Cell Biol 25:6673–6681

Xu Y, Huang S, Liu ZG, Han J (2006) Poly(ADP-ribose) polymerase-1 signaling to mitochondria in necrotic cell death requires RIP1/TRAF2-mediated JNK1 activation. J Biol Chem 281: 8788–8795

Yazdanpanah B, Wiegmann K, Tchikov V, Krut O, Pongratz C, Schramm M, Kleinridders A, Wunderlich T, Kashkar H, Utermohlen O, Bruning JC, Schutze S, Kronke M (2009) Riboflavin kinase couples TNF receptor 1 to NADPH oxidase. Nature 460:1159–1163

Yin Z, Ivanov VN, Habelhah H, Tew K, Ronai Z (2000) Glutathione S-transferase p elicits protection against H2O2-induced cell death via coordinated regulation of stress kinases. Cancer Res 60:4053–4057

Yu SW, Andrabi SA, Wang H, Kim NS, Poirier GG, Dawson TM, Dawson VL (2006) Apoptosis-inducing factor mediates poly(ADP-ribose) (PAR) polymer-induced cell death. Proc Natl Acad Sci U S A 103:18314–18319

Zhang DW, Shao J, Lin J, Zhang N, Lu BJ, Lin SC, Dong MQ, Han J (2009) RIP3, an energy metabolism regulator that switches TNF-induced cell death from apoptosis to necrosis. Science 325:332–336

Zhang DX, Chen YF, Campbell WB, Zou AP, Gross GJ, Li PL (2001) Characteristics and superoxide-induced activation of reconstituted myocardial mitochondrial ATP-sensitive potassium channels. Circ Res 89:1177–1183

Zhang HB, Zhou XH, McQuade T, Li JH, Chan FKM, Zhang JK (2011) Functional complementation between FADD and RIP1 in embryos and lymphocytes. Nature 471:373

Zhao J, Jitkaew S, Cai Z, Choksi S, Li Q, Luo J, Liu ZG (2012) Mixed lineage kinase domain-like is a key receptor interacting protein 3 downstream component of TNF-induced necrosis. Proc Natl Acad Sci U S A 109:5322–5327

Chapter 9
PARP Activation and Necrotic Cell Death

Yongjun Fan and Wei-Xing Zong

9.1 Introduction

Among the 17 members, poly(ADP-ribose) polymerase (PARP) 1 is the first identified PARP family member (D'Amours et al. 1999). PARP1 (molecular weight of 116 kDa), like many other chromatin-binding and transcription-related proteins, has a structure comprising multiple independently folded domains. The major functional domains of PARP1 include an amino-terminal DNA-binding domain (DBD), a central automodification domain (AMD), and a carboxy-terminal catalytic domain (CD) (Hakme et al. 2008; Schreiber et al. 2006). The DBD contains two Cys-Cys-His-Cys zinc fingers (FI/Zn1 and FII/Zn2) that mediate PARP1 binding to DNA. A third zinc-binding domain (FIII/Zn3) mediates interdomain contacts important for DNA-dependent enzyme activation (Langelier et al. 2008, 2010), a nuclear localization signal (NLS), and a caspase-3/caspase-7 cleavage site at Asp214 (Hakme et al. 2008; Schreiber et al. 2006). The AMD contains a BRCA1 C terminal (BRCT) fold that mediates protein–protein interaction (e.g., with DNA repair enzymes). The CD, which is the most conserved domain across PARP family members, contains a PARP signature motif that binds NAD^+ and a "WGR" motif that is named after the most conserved amino acid sequence (Trp, Gly, Arg) with an unknown function.

PARP can be activated by several forms of DNA damage and catalyze polyADP-ribosylation (PARylation) of numerous proteins including histones, topoisomerase 1, DNA-dependent protein kinase, and PARP1 itself (Kim et al. 2005). Among the PARP family members, PARP1 accounts for 75–90 % of cellular PARylation in

Y. Fan • W.-X. Zong (✉)
Department of Molecular Genetics & Microbiology, Stony Brook University, Stony Brook, NY 11794, USA
e-mail: weixing.zong@stonybrook.edu

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response to DNA damage (Mangerich and Burkle 2011). The PAR chains on proteins can be hydrolyzed by poly(ADP-ribose) glycohydrolase (PARG).

PARylation is a common form of posttranslational modification of proteins. The dendroid PAR chains add negative charge to the substrate proteins that are often chromatin-binding proteins: hence, it is believed to help unwind and open chromatin via the static electric expelling force and facilitate the recruitment of other chromatin-binding proteins. In response to DNA damage, PARP binds to both single-strand breaks (SSBs) and double-strand breaks (DSBs) and becomes activated via a conformational change and dimerization (Ciccia and Elledge 2010; Beneke 2012). Protein PARylation is involved in numerous biological processes such as recognition and repair of damaged DNA, chromatin remodeling, transcription, programmed cell death, and mitosis. Owing to its critical function in various forms of DNA repair, including single-strand break repair (SSBR), base excision repair (BER), and double-strand break repair (DSBR), pharmacological inhibition of PARP is being developed as a promising anticancer agent, especially for cancers with loss-of-function mutations of other DNA repair enzymes such as BRCA1 and BRCA2 (Gibson and Kraus 2012). While PARP plays a critical role in DNA damage repair and other chromatin-related functions, its other prominent function is mediation of cell death upon excessive DNA damage. Two modes of PARP-mediated cell death have been described: necrotic cell death as a result of bioenergetic failure and apoptosis mediated by nuclear translocation of apoptosis-inducing factor (AIF) (Berger et al. 1983; Cregan et al. 2004; Dawson and Dawson 2004). While PARP1 is the major PARP member playing a crucial role in cell death, others are also involved including PARP2 and both tankyrase-PARPs TANK1 and TANK2.

9.2 PARP-Mediated Bioenergetic Failure

The involvement of PARP in necrosis was initially noticed in cells treated with DNA-damaging agents such as *N*-methyl-*N′*-nitro-*N*-nitrosoguanidine (MNNG), hydrogen peroxide (H_2O_2), or peroxynitrite (Berger et al. 1983; Sims et al. 1983). The morphological features of cells treated with these agents appeared to be necrotic. Pharmacological inhibition of PARP enzymatic activity or *Parp1* gene silencing blocks necrosis and provides protection against septic shock, diabetes, stroke, myocardial infarction, and ischemia in mouse models (Schreiber et al. 2006; Hassa et al. 2006; Cuzzocrea and Wang 2005; Jagtap and Szabo 2005). This necrotic cell death induced by hyperactivation of PARP upon DNA damage is attributed to the ability of PARP to consume cellular nicotinamide adenine dinucleotide (NAD). Since NAD is an essential cofactor for a number of metabolic enzymes, depletion of NAD leads to decreased activity of the enzymes involved in glycolysis, pentose phosphate shunt, and Krebs cycle, thereby preventing glucose-dependent ATP production (Berger et al. 1983). In an attempt to restore the NAD pools, 2–4 ATP molecules are used for the resynthesis of each NAD molecule. The combined effect of the inhibition of metabolic enzymes and the ATP consumption leads to a rapid drop

in the level of cellular ATP, leading to cell bioenergetic failure and necrotic cell death (Berger et al. 1983). The DNA damage-induced PARP-mediated NAD depletion is a rapid process which leads to near-complete depletion of cellular ATP within minutes, preceding by far the execution of the apoptotic process (D'Amours et al. 1999; Hassa et al. 2006). Since ATP is required for the execution of apoptosis, the bioenergetic failure induced by PARP hyperactivation can function to switch the death mode from apoptosis to necrosis (Decker and Muller 2002; Ying et al. 2005; Zong and Thompson 2006).

Cell sensitivity to PARP hyperactivation is largely determined by the cellular metabolic status (Zong et al. 2004; Ying et al. 2005). The massive generation of PAR in the nucleus by hyperactivation of PARP depletes the nuclear and cytosolic, but not the mitochondrial, pools of NAD, thereby directly affecting cytosolic metabolic events such as glycolysis but not mitochondrial events such as oxidative phosphorylation (Zong et al. 2004; Ying et al. 2005; Kofler et al. 2006). In cultured neurons, the cytosolic and mitochondrial NAD pools each account for about 50 % of total cellular NAD. However, only the cytosolic pool is directly consumed by hyperactivated PARP as cytosolic NAD can freely enter the nucleus via the nuclear pores (Alano et al. 2007). Restoration of cytosolic NAD alleviates the glycolytic inhibition, mitochondrial failure, and neuronal cell death. Bypassing the glycolytic inhibition by supplying cells with metabolic substrates pyruvate, acetoacetate, or hydroxybutyrate also prevents mitochondrial failure and neuron death. Conversely, depletion of cytosolic NAD with NAD glycohydrolase leads to glycolysis inhibition, mitochondrial depolarization, and neuron death, independently of PARP activation. These results establish NAD depletion as a causal event in PARP-mediated cell death.

Due to the preferential depletion of the cytosolic but not mitochondrial pool of NAD, a cell that relies on glycolysis for bioenergetic supply is more susceptible to PARP hyperactivation than a cell that can utilize other nutrient substrates such as fatty acids to fuel mitochondria (Zong et al. 2004; Cipriani et al. 2005; Ying et al. 2005). Thus, the cell sensitivity to PARP-mediated necrosis largely depends on the metabolic state of the cell, which has been shown in various types of endothelial and epithelial cells, as well as in several types of neuronal cells that are highly glycolytic (Virag and Szabo 2002; Virag 2005a; Hassa et al. 2006; Zong and Thompson 2006). This feature may also have a significant implication in cancer treatment, as many types of cancer cells use almost exclusively glucose through aerobic glycolysis for ATP production (Zong et al. 2004).

9.3 Regulation of PARP Activation by Phosphorylation

Although the precise mechanism for PARP hyperactivation upon DNA damage remains elusive, there exists a cross talk between PARP activation and several kinase signaling pathways (Kauppinen et al. 2006). In a rat model of hypoglycemic brain injury, PARP-mediated neuronal cell death induced by NMDA, peroxynitrite,

or DNA alkylation agents is blocked by pharmacological inhibition of the extracellular signal-regulated kinase-1 and -2 (ERK1/2) and by siRNA silencing of ERK2 (Kauppinen et al. 2006). Direct phosphorylation of PARP1 by ERK1/2 on S372 and T373 is required for maximal PARP1 activation in response to DNA damage. Inhibition of PARP1 phosphorylation by ERK1/2 was proposed to be the major mechanism by which inhibitors of the ERK2 signaling cascade reduce cell death rates following ischemia–reperfusion (Kauppinen et al. 2006). Furthermore, based on genetic approaches and pharmacological inhibition, it has been shown that c-Jun N-terminal kinase-1 (JNK-1), but not the other groups of mitogen-activated protein kinases (MAPK), is required for H_2O_2- and MNNG-induced PARP-mediated mitochondrial dysfunction and cell death (Xu et al. 2006; Zhang et al. 2007). Conversely, PARP-catalyzed PARylation can negatively affect cytoprotective kinase signaling pathways, which play a significant role in cell survival and cell death (Veres et al. 2003; Palfi et al. 2005; Tapodi et al. 2005; Kovacs et al. 2006). Several PARP inhibitors have been shown to enhance endotoxin- or ischemia–reperfusion-induced activation of phosphatidylinositol 3-kinase–AKT/protein kinase B (PKB) and p38-MAPK (Veres et al. 2003; Palfi et al. 2005; Tapodi et al. 2005; Kovacs et al. 2006). Moreover, PARP inhibition causes activation of ATM, a kinase that is involved in DNA DSB response (Aguilar-Quesada et al. 2007; Haince et al. 2007). Therefore, there exists a relationship between PARP activation and cell activation/survival pathways regulated by several kinases, although the exact molecular mechanism remains to be investigated.

9.4 PARP-Mediated Mitochondrion-to-Nucleus Translocation of AIF

In addition to necrosis induced by bioenergetic catastrophe, PARP has been found to mediate cell death via the mitochondria-to-nucleus translocation of AIF (Goto et al. 2002; Fossati et al. 2007). AIF is a mitochondrial flavoprotein that participates in cellular metabolism and cell death. It is synthesized as a 67 kDa precursor with three functional domains: an N-terminal region that bears a mitochondrial localization sequence (MLS), a central spacer sequence, and a C-terminal region that shares similarity with bacterial oxidoreductases and harbors two putative NLS. After the AIF precursor is imported into mitochondria, its N-terminal domain carrying the MLS is proteolytically cleaved off, giving rise to the 57 kDa mature form of AIF. Upon mitochondrial permeability transition (MPT), AIF translocates to the cytosol and then to the nucleus, where it induces peripheral chromatin condensation and high-molecular-weight (>50 kbp) DNA fragmentation. Pharmacological inhibition of PARP activity has been shown to decrease AIF nuclear translocation (Culmsee et al. 2005). PARP1-deficient neurons exhibit reduced AIF nuclear translocation and cell death in response to *N*-methyl-D-aspartic acid (NMDA) (Wang et al. 2004; Yuan et al. 2009).

The determinant for the choice of cell death via bioenergetic catastrophe or AIF translocation remains unclear, although these two processes are not mutually exclusive. One possible mechanism for the two types of cell death may lie in the differential cellular response to the extent of DNA damage. Profound NAD depletion (60–95 % of the normal level) and necrosis occur only under a very high level of DNA damage, while under a moderate level of DNA damage the intracellular NAD level decreases by only 5–10 %, which leads to AIF translocation and cell death (Hassa et al. 2006; Cregan et al. 2004; Dawson and Dawson 2004).

Although PARP enzymes were not shown to modify AIF directly, PAR has been found to play a critical role in mediating AIF translocation (Yu et al. 2002; Wang et al. 2004). Several mechanisms have been discussed in literature:

1. MPT upon cellular energetic catastrophe as a result of cytoplasmic and nuclear NAD/ATP depletion. Several studies support a model in which NAD depletion as well as ROS-induced mitochondrial dysfunction may lead to MPT and trigger AIF-induced cell death (Suh et al. 2002; Ying et al. 2003; Alano et al. 2007). PARP-dependent depletion of NAD/ATP appears to precede MPT and AIF translocation. Treatment of cells with PARP inhibitors, the MPT inhibitor cyclosporine A, or liposome-encapsulated NAD can preserve mitochondrial potential, rescue cellular respiration and ATP levels, and block nuclear translocation of AIF and subsequent cell death in cells undergoing hyper PARylation (Suh et al. 2002; Ying et al. 2003; Alano et al. 2007). These observations indicate that MPT resulting from NAD depletion contributes to PARylation-mediated AIF translocation and cell death.
2. A mechanism that prevents AIF nuclear translocation is through its retention in the cytoplasm by heat-shock protein 70 (Hsp70). Hsp70 has been found to antagonize AIF-mediated cell death by both inhibiting mitochondrial AIF release and retaining leaked AIF in the cytoplasm (Ravagnan et al. 2001; Gurbuxani et al. 2003). The expression level of Hsp70 is increased in several types of primary cells derived from *PARP1* knockout mice (Sevigny et al. 2003; Fossati et al. 2006). PARylation appears to repress heat-shock factor-1 (HSF-1) activity, which is required for Hsp70 gene expression, and the heat-shock response in these cells (Sevigny et al. 2003; Fossati et al. 2006).
3. PAR may act directly as a non-proteinaceous AIF-releasing factor and cell death signal (Andrabi et al. 2006; Yu et al. 2006). When exogenously added to neurons at a high concentration, PAR polymers cause AIF translocation and cell death. Exogenous delivery of PAR polymers can induce necrotic cell death in primary cortical neurons isolated from *PARP1* knockout mice, indicating a direct cell death-inducing function of PAR polymers. Furthermore, purified PAR polymers can induce AIF release from isolated mitochondria. The toxicity of PAR polymers appears to be dependent on its length, structure, and amount. Increasing length and complexity of PAR polymers are associated with higher toxicity (Andrabi et al. 2006; Yu et al. 2006). Consistent with the role of PAR in mediating AIF translocation and cell death, loss of PARG in *Drosophila melanogaster*

results in cytoplasmic accumulation of free or protein-associated PAR and leads to severe neurodegeneration (Hanai et al. 2004).

Despite the connection between PARP activation and AIF-mediated cell death, it is important to note that mitochondrial AIF release and shuttling to the nucleus are not exclusively dependent on PARylation. It was found that necrotic cell death caused by oxidative damage in other cell types, such as hepatocytes, does not depend on the PARylation reaction (van Wijk and Hageman 2005; Virag 2005b; Hassa and Hottiger 2008). High concentrations of MNNG (>100 μM) can directly induce MPT independently of PARP. Indeed, MNNG-induced NAD depletion and cell death can be prevented by inhibition of the permeability transition pore opening (Dodoni et al. 2004). Thus, mitochondrial dysfunction appears to be the cause rather than the consequence of MNNG-induced NAD depletion. The activities of other factors such as p53, caspase-2, and caspase-12 may also be involved in MPT and AIF translocation (Seth et al. 2005; Sanges et al. 2006). ROS production appears to be a key factor for these processes (van Wijk and Hageman 2005; Christophe and Nicolas 2006; Skulachev 2006). Therefore, PARP-mediated PARylation plays an important but not exclusive role in AIF-dependent cell death. The PARP/PARylation system and AIF may function together as a mitochondria–nucleus interconnected Yin/Yang-like sensor of metabolic or oxidative state of cells (Zhang 2003; Hassa and Hottiger 2008).

9.5 Other Cell Survival/Death-Related Molecular Events That Are Affected by PARP

In addition to the intensively studied NAD/ATP depletion and AIF translocation, there are several other molecular events that are regulated by PARP and affect cell survival/death. One such event is the shuttling of nuclear pro-necrotic factors (potentially PARylated) such as PARP1 itself and high-mobility group box 1 protein (HMGB1) into the cytoplasm or mitochondria. While most studies on PARP focus on its function as a nuclear protein, its existence and function in mitochondria and cytoplasm have also come into attention. Cytosolic and mitochondria-associated PARP and PARG activity has been reported in human primary hepatocytes, rat cortical neurons, and mouse fibroblasts (Thomassin et al. 1985; Jesser et al. 1993; Masmoudi et al. 1993). The possible existence of cytosolic and mitochondria-associated PARP activity may argue in favor of a direct interaction between PARP and AIF or other mitochondrial components involved in MPT. The validation and possible mechanism for the cytoplasmic presence of PARP need to be further investigated. In a study of the cytotoxicity of HIV-1 Vpr protein, it was found that PARP1 might translocate to the cytoplasm and mediate cell death in a glucocorticoid receptor complex-dependent manner (Muthumani et al. 2006).

HMGB1 is a nuclear nonhistone chromatin-associated protein which functions as a transcription factor (Muller et al. 2001). It is actively secreted by monocytes as a damage signal (alarmin) and functions as a late mediator of inflammation (Czura et al. 2001; Bianchi and Manfredi 2004; Raucci et al. 2007). The active secretion of HMGB1 by stimulated macrophages or monocytes requires acetylation of HMGB1, which in turn induces its relocalization to the cytosol into secretory lysosomes and its release into the extracellular environment (Scaffidi et al. 2002; Bonaldi et al. 2003).

In addition to the active secretion of HMGB1 during monocyte activation, upon DNA damage, HMGB1 undergoes nuclear-cytosolic relocalization and is passively released into the extracellular milieu (Czura et al. 2001; Bianchi and Manfredi 2004; Raucci et al. 2007). Like the monocyte secretion of HMGB1, the cytosolic translocation and extracellular release of HMGB1during necrosis also involve its acetylation. However, the acetylation-mediated HMGB1 release occurs only in necrotic cells, whereas during apoptosis HMGB1 is retained in the nucleus through its binding to the chromatin (Czura et al. 2001; Bianchi and Manfredi 2004; Raucci et al. 2007).

In addition to acetylation, the nuclear-cytosolic relocalization and extracellular release of HMGB1 are also regulated by several other posttranslational modifications including methylation, phosphorylation, glycosylation, and PARylation (Ulloa and Messmer 2006). PARylation of HMGB1 was found in DNA damage-induced necrosis (Ditsworth et al. 2007). Following MNNG treatment, relocalization of HMGB1 was observed in wild-type cells but not in PARP1-deficient cells or in the presence of PARP inhibitors. The release of HMGB1 from chromatin is likely promoted by the attachment of PAR to HMGB1, thereby destabilizing the association of HMGB1 with chromatin via the electric static force imposed by PAR.

Extracellular HMGB1 can act as a potent pro-inflammatory cytokine. HMGB1 signaling occurs via the receptor for advanced glycated end products and via members of the Toll-like receptor family (Czura et al. 2001; Muller et al. 2001; Bianchi and Manfredi 2004; Raucci et al. 2007)). HMGB1 has been shown to serve as a late mediator of endotoxin lethality in mice and as a mediator of post-ischemic brain damage by increasing excitotoxicity as well as ischemic neuronal death in vitro and in vivo (Tsung et al. 2005; Liu et al. 2007). Owing to these properties, HMGB1 has become a therapeutic target in experimental models of ischemia/reperfusion, rheumatoid arthritis, and endotoxin-induced septic shock. In a xenograft tumor model, HMGB1 was found to play an essential role in tumor regression upon chemotherapeutic treatment (Guerriero et al. 2011). Chemotherapy is found to induce sporadic necrosis and subsequent activation of the innate immune system that displays an antitumoral function. This chemotherapy-induced activation of innate immunity and tumor regression is largely compromised in tumors derived from HMGB1-deficient cells.

9.6 PARP Regulates Autophagy

In addition to cell death, there is also a molecular connection between PARP activation and induction of autophagy. Autophagy is a dynamic process of protein degradation, which is typically activated during nutrient limitation. Since autophagy is inducible by disturbance of cellular energy homeostasis, it is not surprising that hyperactivation of PARP1 can lead to autophagy through the depletion of cellular NAD and ATP. There are multiple mechanisms coupling PARP activation with autophagy induction. Among them, activation of AMP-activated protein kinase (AMPK) appears to be particularly relevant. Serving as a cellular fuel gauge, AMPK is readily activated with decreased ATP level and increased AMP/ATP ratio (Hardie 2007). AMPK is a critical positive regulator of autophagy via the following pathways: (1) AMPK activation leads to suppression of mTORC1 by phosphorylation and activation of TSC2 (Inoki et al. 2003), the negative regulator of mTORC1, or by direct phosphorylation of raptor, one of the key components of mTORC1 (Gwinn et al. 2008). (2) AMPK is able to directly phosphorylate and activate the Ulk1 complex (Egan et al. 2011; Kim et al. 2011). Several studies have demonstrated that DNA damage leads to sequential events including PARP activation, ATP depletion, AMPK activation, mTOR suppression, and induction of autophagy (Albert et al. 2007; Huang and Shen 2009; Munoz-Gamez et al. 2009; Alexander et al. 2010). Such inducible autophagy is a pro-survival force against cell death resulting from DNA damage-induced PARP activation. The exact outcome thus depends on the balance of these two opposite forces, which may differ depending on the cellular context, including the nature of DNA damage and status of cell metabolism.

9.7 Cross Talk Between PARP-Mediated Cell Death and Other Forms of Cell Death

Over the past decade, the focus on apoptosis as the primary mechanism of programmed cell death has dramatically shifted. Cell death mechanisms other than the conventional mitochondrial apoptosis have been uncovered or regained attention, one of which is PARP-mediated cell death upon excessive DNA damage. There exists a cross talk between PARP-mediated cell death and other forms of cell death such as conventional apoptosis and RIP kinase-mediated necroptosis.

A mutual inhibitory relationship exists between PARP-mediated necrosis and conventional apoptosis. As apoptosis is an energy-dependent process, PARP-mediated NAD and ATP depletion inhibits apoptosis. On the other hand, PARP1 is cleaved by caspases 3/7 into two distinct fragments during apoptosis: the 24 kDa N-terminal fragment that contains the DBD and the 89 kDa C-terminal fragment that contains the catalytic domain. While the N-terminal fragment retains strong DNA-binding activity, the C-terminal fragment loses its catalytic activity. Hence, the N-terminal fragment can function as a dominant-negative factor to inhibit NAD

hydrolysis and to maintain the energy level of the cells. This apoptotic cleavage of PARP1 facilitates apoptosis by preventing DNA repair and by blocking energy depletion-induced necrosis.

Although the molecular machinery for the initiation of RIP kinase-mediated necroptosis is quite well understood, the precise mechanism by which it is executed remains largely unclear. Currently, it seems that a larger number of processes downstream of the necrotic signaling platform including excessive metabolic activity, elevated ATP consumption, ROS formation, and lysosome membrane permeability transition tip the cell towards death. This bioenergetic catastrophe theory certainly overlaps with the downstream events of PARP hyperactivation. Therefore, while the value of PARP-mediated necrosis can be certainly appreciated for many pathological and physiological conditions, an understanding of its molecular mechanism also provides profound insights into the comprehension of other forms of cell demise.

References

Aguilar-Quesada R, Munoz-Gamez JA, Martin-Oliva D, Peralta A, Valenzuela MT, Matinez-Romero R, Quiles-Perez R, Menissier-de Murcia J, de Murcia G, Ruiz de Almodovar M, Oliver FJ (2007) Interaction between ATM and PARP-1 in response to DNA damage and sensitization of ATM deficient cells through PARP inhibition. BMC Mol Biol 8:29

Alano CC, Tran A, Tao R, Ying W, Karliner JS, Swanson RA (2007) Differences among cell types in NAD(+) compartmentalization: a comparison of neurons, astrocytes, and cardiac myocytes. J Neurosci Res 85:3378–3385

Albert JM, Cao C, Kim KW, Willey CD, Geng L, Xiao D, Wang H, Sandler A, Johnson DH, Colevas AD, Low J, Rothenberg ML, Lu B (2007) Inhibition of poly(ADP-ribose) polymerase enhances cell death and improves tumor growth delay in irradiated lung cancer models. Clin Cancer Res 13:3033–3042

Alexander A, Cai SL, Kim J, Nanez A, Sahin M, MacLean KH, Inoki K, Guan KL, Shen J, Person MD, Kusewitt D, Mills GB, Kastan MB, Walker CL (2010) ATM signals to TSC2 in the cytoplasm to regulate mTORC1 in response to ROS. Proc Natl Acad Sci U S A 107:4153–4158

Andrabi SA, Kim NS, Yu SW, Wang H, Koh DW, Sasaki M, Klaus JA, Otsuka T, Zhang Z, Koehler RC, Hurn PD, Poirier GG, Dawson VL, Dawson TM (2006) Poly(ADP-ribose) (PAR) polymer is a death signal. Proc Natl Acad Sci U S A 103:18308–18313

Beneke S (2012) Regulation of chromatin structure by poly(ADP-ribosyl)ation. Front Genet 3:169

Berger NA, Sims JL, Catino DM, Berger SJ (1983) Poly(ADP-ribose) polymerase mediates the suicide response to massive DNA damage: studies in normal and DNA-repair defective cells. Princess Takamatsu Symp 13:219–226

Bianchi ME, Manfredi A (2004) Chromatin and cell death. Biochim Biophys Acta 1677:181–186

Bonaldi T, Talamo F, Scaffidi P, Ferrera D, Porto A, Bachi A, Rubartelli A, Agresti A, Bianchi ME (2003) Monocytic cells hyperacetylate chromatin protein HMGB1 to redirect it towards secretion. EMBO J 22:5551–5560

Christophe M, Nicolas S (2006) Mitochondria: a target for neuroprotective interventions in cerebral ischemia-reperfusion. Curr Pharm Des 12:739–757

Ciccia A, Elledge SJ (2010) The DNA damage response: making it safe to play with knives. Mol Cell 40:179–204

Cipriani G, Rapizzi E, Vannacci A, Rizzuto R, Moroni F, Chiarugi A (2005) Nuclear poly (ADP-ribose) polymerase-1 rapidly triggers mitochondrial dysfunction. J Biol Chem 280: 17227–17234

Cozzi A, Cipriani G, Fossati S, Faraco G, Formentini L, Min W, Cortes U, Wang ZQ, Moroni F, Chiarugi A (2006) Poly(ADP-ribose) accumulation and enhancement of postischemic brain damage in 110-kDa poly(ADP-ribose) glycohydrolase null mice. J Cereb Blood Flow Metab 26:684–695

Cregan SP, Dawson VL, Slack RS (2004) Role of AIF in caspase-dependent and caspase-independent cell death. Oncogene 23:2785–2796

Culmsee C, Zhu C, Landshamer S, Becattini B, Wagner E, Pellecchia M, Blomgren K, Plesnila N (2005) Apoptosis-inducing factor triggered by poly(ADP-ribose) polymerase and Bid mediates neuronal cell death after oxygen-glucose deprivation and focal cerebral ischemia. J Neurosci 25:10262–10272

Cuzzocrea S, Wang ZQ (2005) Role of poly(ADP-ribose) glycohydrolase (PARG) in shock, ischemia and reperfusion. Pharmacol Res 52:100–108

Czura CJ, Wang H, Tracey KJ (2001) Dual roles for HMGB1: DNA binding and cytokine. J Endotoxin Res 7:315–321

D'Amours D, Desnoyers S, D'Silva I, Poirier GG (1999) Poly(ADP-ribosyl)ation reactions in the regulation of nuclear functions. Biochem J 342(Pt 2):249–268

Dawson VL, Dawson TM (2004) Deadly conversations: nuclear-mitochondrial cross-talk. J Bioenerg Biomembr 36:287–294

Decker P, Muller S (2002) Modulating poly (ADP-ribose) polymerase activity: potential for the prevention and therapy of pathogenic situations involving DNA damage and oxidative stress. Curr Pharm Biotechnol 3:275–283

Ditsworth D, Zong WX, Thompson CB (2007) Activation of poly(ADP)-ribose polymerase (PARP-1) induces release of the pro-inflammatory mediator HMGB1 from the nucleus. J Biol Chem 282:17845–17854

Dodoni G, Canton M, Petronilli V, Bernardi P, Di Lisa F (2004) Induction of the mitochondrial permeability transition by the DNA alkylating agent N-methyl-N′-nitro-N-nitrosoguanidine. Sorting cause and consequence of mitochondrial dysfunction. Biochim Biophys Acta 1658:58–63

Egan DF, Shackelford DB, Mihaylova MM, Gelino S, Kohnz RA, Mair W, Vasquez DS, Joshi A, Gwinn DM, Taylor R, Asara JM, Fitzpatrick J, Dillin A, Viollet B, Kundu M, Hansen M, Shaw RJ (2011) Phosphorylation of ULK1 (hATG1) by AMP-activated protein kinase connects energy sensing to mitophagy. Science 331:456–461

Fossati S, Cipriani G, Moroni F, Chiarugi A (2007) Neither energy collapse nor transcription underlie in vitro neurotoxicity of poly(ADP-ribose) polymerase hyper-activation. Neurochem Int 50:203–210

Fossati S, Formentini L, Wang ZQ, Moroni F, Chiarugi A (2006) Poly(ADP-ribosyl)ation regulates heat shock factor-1 activity and the heat shock response in murine fibroblasts. Biochem Cell Biol 84:703–712

Gibson BA, Kraus WL (2012) New insights into the molecular and cellular functions of poly(ADP-ribose) and PARPs. Nat Rev Mol Cell Biol 13:411–424

Goto W, Ota T, Morikawa N, Otori Y, Hara H, Kawazu K, Miyawaki N, Tano Y (2002) Protective effects of timolol against the neuronal damage induced by glutamate and ischemia in the rat retina. Brain Res 958:10–19

Guerriero JL, Ditsworth D, Catanzaro JM, Sabino G, Furie MB, Kew RR, Crawford HC, Zong WX (2011) DNA alkylating therapy induces tumor regression through an HMGB1-mediated activation of innate immunity. J Immunol 186(6):3517–3526

Gurbuxani S, Schmitt E, Cande C, Parcellier A, Hammann A, Daugas E, Kouranti I, Spahr C, Pance A, Kroemer G, Garrido C (2003) Heat shock protein 70 binding inhibits the nuclear import of apoptosis-inducing factor. Oncogene 22:6669–6678

Gwinn DM, Shackelford DB, Egan DF, Mihaylova MM, Mery A, Vasquez DS, Turk BE, Shaw RJ (2008) AMPK phosphorylation of raptor mediates a metabolic checkpoint. Mol Cell 30:214–226

Haince JF, Kozlov S, Dawson VL, Dawson TM, Hendzel MJ, Lavin MF, Poirier GG (2007) Ataxia telangiectasia mutated (ATM) signaling network is modulated by a novel poly(ADP-ribose)-dependent pathway in the early response to DNA-damaging agents. J Biol Chem 282:16441–16453

Hakme A, Wong HK, Dantzer F, Schreiber V (2008) The expanding field of poly(ADP-ribosyl) ation reactions. 'Protein modifications: beyond the usual suspects' review series. EMBO Rep 9:1094–1100

Hanai S, Kanai M, Ohashi S, Okamoto K, Yamada M, Takahashi H, Miwa M (2004) Loss of poly(ADP-ribose) glycohydrolase causes progressive neurodegeneration in Drosophila melanogaster. Proc Natl Acad Sci U S A 101:82–86

Hardie DG (2007) AMP-activated/SNF1 protein kinases: conserved guardians of cellular energy. Nat Rev Mol Cell Biol 8:774–785

Hassa PO, Haenni SS, Elser M, Hottiger MO (2006) Nuclear ADP-ribosylation reactions in mammalian cells: where are we today and where are we going? Microbiol Mol Biol Rev 70: 789–829

Hassa PO, Hottiger MO (2008) The diverse biological roles of mammalian PARPS, a small but powerful family of poly-ADP-ribose polymerases. Front Biosci 13:3046–3082

Huang Q, Shen HM (2009) To die or to live: the dual role of poly(ADP-ribose) polymerase-1 in autophagy and necrosis under oxidative stress and DNA damage. Autophagy 5:273–276

Inoki K, Li Y, Xu T, Guan KL (2003) Rheb GTPase is a direct target of TSC2 GAP activity and regulates mTOR signaling. Genes Dev 17:1829–1834

Jagtap P, Szabo C (2005) Poly(ADP-ribose) polymerase and the therapeutic effects of its inhibitors. Nat Rev Drug Discov 4:421–440

Jesser M, Chypre C, Hog F, Mandel P (1993) Cytoplasmic poly(ADP-ribose)polymerase from mouse plasmacytoma free messenger ribonucleoprotein particles: purification and characterization. Biochem Biophys Res Commun 195:558–564

Kauppinen TM, Chan WY, Suh SW, Wiggins AK, Huang EJ, Swanson RA (2006) Direct phosphorylation and regulation of poly(ADP-ribose) polymerase-1 by extracellular signal-regulated kinases 1/2. Proc Natl Acad Sci U S A 103:7136–7141

Kim J, Kundu M, Viollet B, Guan KL (2011) AMPK and mTOR regulate autophagy through direct phosphorylation of Ulk1. Nat Cell Biol 13:132–141

Kim MY, Zhang T, Kraus WL (2005) Poly(ADP-ribosyl)ation by PARP-1: 'PAR-laying' NAD+ into a nuclear signal. Genes Dev 19:1951–1967

Kofler J, Otsuka T, Zhang Z, Noppens R, Grafe MR, Koh DW, Dawson VL, de Murcia JM, Hurn PD, Traystman RJ (2006) Differential effect of PARP-2 deletion on brain injury after focal and global cerebral ischemia. J Cereb Blood Flow Metab 26:135–141

Kovacs K, Toth A, Deres P, Kalai T, Hideg K, Gallyas F Jr, Sumegi B (2006) Critical role of PI3-kinase/Akt activation in the PARP inhibitor induced heart function recovery during ischemia-reperfusion. Biochem Pharmacol 71:441–452

Langelier MF, Ruhl DD, Planck JL, Kraus WL, Pascal JM (2010) The Zn3 domain of human poly(ADP-ribose) polymerase-1 (PARP-1) functions in both DNA-dependent poly(ADP-ribose) synthesis activity and chromatin compaction. J Biol Chem 285:18877–18887

Langelier MF, Servent KM, Rogers EE, Pascal JM (2008) A third zinc-binding domain of human poly(ADP-ribose) polymerase-1 coordinates DNA-dependent enzyme activation. J Biol Chem 283:4105–4114

Liu K, Mori S, Takahashi HK, Tomono Y, Wake H, Kanke T, Sato Y, Hiraga N, Adachi N, Yoshino T, Nishibori M (2007) Anti-high mobility group box 1 monoclonal antibody ameliorates brain infarction induced by transient ischemia in rats. FASEB J 21:3904–3916

Mangerich A, Burkle A (2011) How to kill tumor cells with inhibitors of poly(ADP-ribosyl)ation. Int J Cancer 128:251–265

Masmoudi A, el-Fetouaki J, Weltin D, Belhadj O, Mandel P (1993) Association of mitochondrial ADP-ribosyl transferase activity with the DNA-protein complex. Biochem Mol Biol Int 29:77–83

Muller S, Scaffidi P, Degryse B, Bonaldi T, Ronfani L, Agresti A, Beltrame M, Bianchi ME (2001) New EMBO members' review: the double life of HMGB1 chromatin protein: architectural factor and extracellular signal. EMBO J 20:4337–4340

Munoz-Gamez JA, Rodriguez-Vargas JM, Quiles-Perez R, Aguilar-Quesada R, Martin-Oliva D, de Murcia G, Menissier de Murcia J, Almendros A, Ruiz de Almodovar M, Oliver FJ (2009) PARP-1 is involved in autophagy induced by DNA damage. Autophagy 5:61–74

Muthumani K, Choo AY, Zong WX, Madesh M, Hwang DS, Premkumar A, Thieu KP, Emmanuel J, Kumar S, Thompson CB, Weiner DB (2006) The HIV-1 Vpr and glucocorticoid receptor complex is a gain-of-function interaction that prevents the nuclear localization of PARP-1. Nat Cell Biol 8:170–179

Palfi A, Toth A, Kulcsar G, Hanto K, Deres P, Bartha E, Halmosi R, Szabados E, Czopf L, Kalai T, Hideg K, Sumegi B, Toth K (2005) The role of Akt and mitogen-activated protein kinase systems in the protective effect of poly(ADP-ribose) polymerase inhibition in Langendorff perfused and in isoproterenol-damaged rat hearts. J Pharmacol Exp Ther 315:273–282

Raucci A, Palumbo R, Bianchi ME (2007) HMGB1: a signal of necrosis. Autoimmunity 40: 285–289

Ravagnan L, Gurbuxani S, Susin SA, Maisse C, Daugas E, Zamzami N, Mak T, Jaattela M, Penninger JM, Garrido C, Kroemer G (2001) Heat-shock protein 70 antagonizes apoptosis-inducing factor. Nat Cell Biol 3:839–843

Sanges D, Comitato A, Tammaro R, Marigo V (2006) Apoptosis in retinal degeneration involves cross-talk between apoptosis-inducing factor (AIF) and caspase-12 and is blocked by calpain inhibitors. Proc Natl Acad Sci U S A 103:17366–17371

Scaffidi P, Misteli T, Bianchi ME (2002) Release of chromatin protein HMGB1 by necrotic cells triggers inflammation. Nature 418:191–195

Schreiber V, Dantzer F, Ame JC, de Murcia G (2006) Poly(ADP-ribose): novel functions for an old molecule. Nat Rev Mol Cell Biol 7:517–528

Seth R, Yang C, Kaushal V, Shah SV, Kaushal GP (2005) p53-dependent caspase-2 activation in mitochondrial release of apoptosis-inducing factor and its role in renal tubular epithelial cell injury. J Biol Chem 280:31230–31239

Sevigny M, Garnier P, Kauppinen T, Swanson R (2003) Heat shock-induced Hsp70 expression in murine astrocytes does not require poly(ADP-ribose) polymerase activity. Cell Physiol Biochem 13:297–300

Sims JL, Berger SJ, Berger NA (1983) Poly(ADP-ribose) Polymerase inhibitors preserve nicotinamide adenine dinucleotide and adenosine 5′-triphosphate pools in DNA-damaged cells: mechanism of stimulation of unscheduled DNA synthesis. Biochemistry 22:5188–5194

Skulachev VP (2006) Bioenergetic aspects of apoptosis, necrosis and mitoptosis. Apoptosis 11:473–485

Suh J, Payvandi F, Edelstein LC, Amenta PS, Zong WX, Gelinas C, Rabson AB (2002) Mechanisms of constitutive NF-kappaB activation in human prostate cancer cells. Prostate 52:183–200

Tapodi A, Debreceni B, Hanto K, Bognar Z, Wittmann I, Gallyas F Jr, Varbiro G, Sumegi B (2005) Pivotal role of Akt activation in mitochondrial protection and cell survival by poly(ADP-ribose)polymerase-1 inhibition in oxidative stress. J Biol Chem 280:35767–35775

Thomassin H, Niedergang C, Mandel P (1985) Characterization of the poly(ADP-ribose) polymerase associated with free cytoplasmic mRNA-protein particles. Biochem Biophys Res Commun 133:654–661

Tsung A, Sahai R, Tanaka H, Nakao A, Fink MP, Lotze MT, Yang H, Li J, Tracey KJ, Geller DA, Billiar TR (2005) The nuclear factor HMGB1 mediates hepatic injury after murine liver ischemia-reperfusion. J Exp Med 201:1135–1143

Ulloa L, Messmer D (2006) High-mobility group box 1 (HMGB1) protein: friend and foe. Cytokine Growth Factor Rev 17:189–201

van Wijk SJ, Hageman GJ (2005) Poly(ADP-ribose) polymerase-1 mediated caspase-independent cell death after ischemia/reperfusion. Free Radic Biol Med 39:81–90

Veres B, Gallyas F Jr, Varbiro G, Berente Z, Osz E, Szekeres G, Szabo C, Sumegi B (2003) Decrease of the inflammatory response and induction of the Akt/protein kinase B pathway by poly-(ADP-ribose) polymerase 1 inhibitor in endotoxin-induced septic shock. Biochem Pharmacol 65:1373–1382

Virag L (2005a) The expanding universe of poly(ADP-ribosyl)ation. Cell Mol Life Sci 62:719–720

Virag L (2005b) Structure and function of poly(ADP-ribose) polymerase-1: role in oxidative stress-related pathologies. Curr Vasc Pharmacol 3:209–214

Virag L, Szabo C (2002) The therapeutic potential of poly(ADP-ribose) polymerase inhibitors. Pharmacol Rev 54:375–429

Wang H, Yu SW, Koh DW, Lew J, Coombs C, Bowers W, Federoff HJ, Poirier GG, Dawson TM, Dawson VL (2004) Apoptosis-inducing factor substitutes for caspase executioners in NMDA-triggered excitotoxic neuronal death. J Neurosci 24:10963–10973

Xu Y, Huang S, Liu ZG, Han J (2006) Poly(ADP-ribose) polymerase-1 signaling to mitochondria in necrotic cell death requires RIP1/TRAF2-mediated JNK1 activation. J Biol Chem 281:8788–8795

Ying W, Alano CC, Garnier P, Swanson RA (2005) NAD+ as a metabolic link between DNA damage and cell death. J Neurosci Res 79:216–223

Ying W, Garnier P, Swanson RA (2003) NAD + repletion prevents PARP-1-induced glycolytic blockade and cell death in cultured mouse astrocytes. Biochem Biophys Res Commun 308: 809–813

Yu SW, Andrabi SA, Wang H, Kim NS, Poirier GG, Dawson TM, Dawson VL (2006) Apoptosis-inducing factor mediates poly(ADP-ribose) (PAR) polymer-induced cell death. Proc Natl Acad Sci U S A 103:18314–18319

Yu SW, Wang H, Poitras MF, Coombs C, Bowers WJ, Federoff HJ, Poirier GG, Dawson TM, Dawson VL (2002) Mediation of poly(ADP-ribose) polymerase-1-dependent cell death by apoptosis-inducing factor. Science 297:259–263

Yuan M, Siegel C, Zeng Z, Li J, Liu F, McCullough LD (2009) Sex differences in the response to activation of the poly (ADP-ribose) polymerase pathway after experimental stroke. Exp Neurol 217:210–218

Zhang J (2003) Are poly(ADP-ribosyl)ation by PARP-1 and deacetylation by Sir2 linked? Bioessays 25:808–814

Zhang S, Lin Y, Kim YS, Hande MP, Liu ZG, Shen HM (2007) c-Jun N-terminal kinase mediates hydrogen peroxide-induced cell death via sustained poly(ADP-ribose) polymerase-1 activation. Cell Death Differ 14:1001–1010

Zong WX, Ditsworth D, Bauer DE, Wang ZQ, Thompson CB (2004) Alkylating DNA damage stimulates a regulated form of necrotic cell death. Genes Dev 18:1272–1282

Zong WX, Thompson CB (2006) Necrotic death as a cell fate. Genes Dev 20:1–15

Chapter 10
Programmed Necrosis in Immunity and Inflammatory Diseases

Kenta Moriwaki and Francis Ka-Ming Chan

10.1 Introduction

Necrotic cell death is characterized by extensive organelle and cell swelling and rupture of the plasma membrane. These morphological changes are entirely distinct from those of apoptotic cell death, which show organelle and cell shrinking, nuclear chromatin condensation, and nuclear and cytoplasmic blebbing to form membrane-bound fragments known as apoptotic bodies (Kerr et al. 1972; Schweichel and Merker 1973). Necrosis was once considered to be an accidental and unregulated type of cell injury. However, emerging evidence shows that necrosis can be induced in a regulated manner like apoptosis. Regulated necrosis has been called "programmed necrosis" or "necroptosis" to distinguish it from necrosis induced by physical trauma (Vandenabeele et al. 2010). Programmed necrosis can be induced by plasma membrane-associated death receptors in the TNF receptor (TNFR) superfamily (Laster et al. 1988; Vercammen et al. 1998a, b; Holler et al. 2000), T cell receptor (TCR) (Ch'en et al. 2008, 2011; Cho et al. 2011), and toll-like receptors (TLRs) (He et al. 2011; Fortes et al. 2012; McComb et al. 2012). Necrotic cell death is pro-inflammatory because it releases intracellular contents or the so-called danger-associated molecular patterns (DAMPs) (Kono and Rock 2008). The released DAMPs from necrotic cells such as HMGB1 can activate TLRs on the surface of innate immune effector cells to promote inflammatory cytokine expression (Lamkanfi et al. 2010; Yang et al. 2010). These observations imply that programmed necrosis is an important cell death module in the immune system. In fact, recent studies show that the programmed necrosis is closely associated with

K. Moriwaki • F.K.-M. Chan (✉)
Department of Pathology, Immunology and Microbiology Program,
University of Massachusetts Medical School, Worcester, MA, USA
e-mail: francis.chan@umassmed.edu

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infectious and noninfectious inflammatory diseases. In this chapter, we discuss the emerging roles of programmed necrosis in biology with a specific emphasis on its role in immunity and inflammation. For simplicity sake, we will use the term necrosis to refer to regulated programmed necrosis hereafter.

10.2 Molecular Regulation of Necrosis

The most extensively characterized pathway leading to necrosis is initiated by ligation of TNF receptor 1 (TNFR-1/TNFRSF1a/CD120a). We will therefore use the pathway regulated by TNFR-1 ligation to illustrate the salient principles that govern necrosis. When TNF binds to TNFR-1, the membrane-associated TNFR-1 signaling complex termed "Complex I" is formed. Complex I comprises multiple protein adaptors including TNFR-associated death domain (TRADD), receptor-interacting protein kinase 1 (RIPK1), cellular inhibitor of apoptosis 1 (cIAP1), cIAP2, TNFR-associated factor 2 (TRAF2), and linear ubiquitin chain assembly complex (LUBAC) (Micheau and Tschopp 2003) (Fig. 10.1). This complex primarily triggers the NF-κB signaling pathway. RIPK1 ubiquitination is an essential event that mediates NF-κB activation (Walczak 2011). Although early reports show that K63 ubiquitination of RIPK1 at K377 is essential for recruitment of NEMO and activation of the IKK complex (Ea et al. 2006; Li et al. 2006), recent studies indicate that ubiquitination at sites other than K377 as well as other types of ubiquitin linkages can also occur (Dynek et al. 2010; Gerlach et al. 2011). RIPK1 ubiquitination prevents assembly of the cytoplasmic death-inducing signaling complex, also known as "Complex II," through NF-κB-dependent and -independent mechanisms (O'Donnell et al. 2007). Consistent with an inhibitory role for RIPK1 ubiquitination in cell death signaling, ubiquitin hydrolases such as cylindromatosis (CYLD) have been shown to facilitate apoptotic and necrotic responses (Hitomi et al. 2008; Vanlangenakker et al. 2011) (Fig. 10.1).

Caspase activity is a critical parameter that controls necrosis. Early studies show that the broad caspase inhibitor zVAD-fmk can facilitate RIPK1-dependent necrosis in certain cell types (Vercammen et al. 1998a; Holler et al. 2000). However, RIPK1 does not act alone to drive necrosis. Another serine/threonine kinase, RIPK3, was identified in several RNA interference screens to be a critical partner of RIPK1 in necrosis (Cho et al. 2009; He et al. 2009). Caspase 8 inhibits necrosis by cleaving and inactivating RIPK1, RIPK3, and CYLD (Lin et al. 1999; Chan et al. 2003; Feng et al. 2007; O'Donnell et al. 2011). When the activity of caspase 8 is inhibited or in caspase $8^{-/-}$ or $Fadd^{-/-}$ cells, the integrity of RIPK1 and RIPK3 is preserved. This allows the two kinases to form a tight and stable complex termed the "necrosome" (Cho et al. 2009; He et al. 2009). Necrosome formation requires the RIP homotypic interaction motif (RHIM) that is present in both RIPK1 and RIPK3 (Sun et al. 2002). *Trans*-phosphorylation of RIPK1 and RIPK3 appears to be crucial for necrosome assembly, as kinase-inactive RIPK1 or RIPK3, and the RIPK1-specific inhibitor necrostatin-1 potently inhibits TNF-induced necrosis (Degterev et al. 2008).

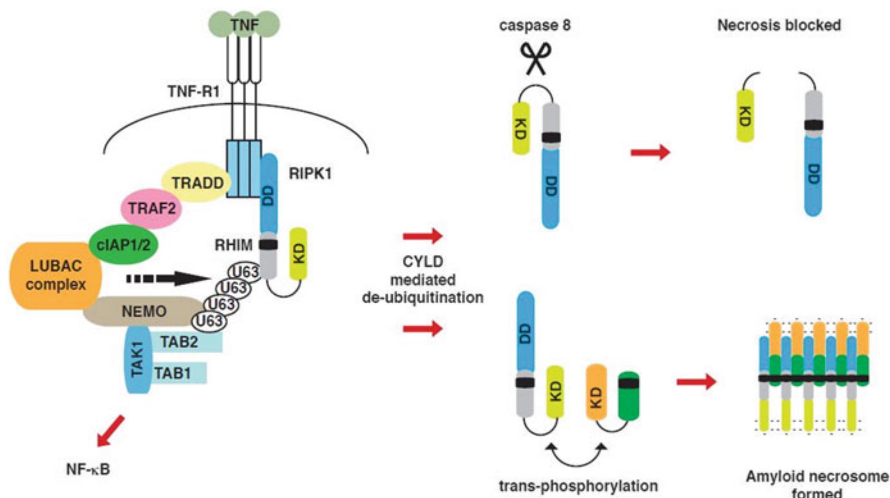

Fig. 10.1 The necrosis signaling pathway is regulated by protein ubiquitination, phosphorylation, and proteolytic cleavage. The TNFR-1-associated membrane complex (Complex I) is composed of many adaptors. Many of the molecular interactions within this complex require protein ubiquitination. RIPK1 ubiquitination through K63 linkage (U63), which is crucial for downstream NF-κB activation, is *highlighted*. Removal of ubiquitin chains from RIPK1 by the de-ubiquitinase cylindromatosis (CYLD) is important for transition of Complex I to the cytosol and assembly of Complex II. In the presence of active caspase 8, RIPK1, RIPK3, and CYLD are cleaved and inactivated (only RIPK1 cleavage is shown for simplicity sake). Cleavage of RIPK1 removes the kinase domain, thereby preventing phosphorylation of downstream substrates (e.g., RIPK3) that are important for necrosis induction. When the integrity of RIPK1 and RIPK3 is preserved, they *trans-phosphorylate* each other. The resulting negative charge may be critical in "opening up" the RHIM domain to facilitate amyloid fibril assembly and recruitment of downstream RIPK3 substrates

Hence, necrosis is regulated by at least three distinct mechanisms: protein ubiquitination, caspase cleavage, and phosphorylation.

The RHIM is an emerging protein–protein interaction domain found in several other adaptors including TIR domain-containing adaptor molecule 1 (TICAM1/TRIF) and DNA-dependent activator of interferon regulatory transcription factors (DAI/ZBP1) (Moquin and Chan 2010). Thus, the RHIM-containing adaptors all have important functions in innate immune and cell death signaling. The RHIM is defined by a highly conserved tetra-peptide core sequence of mostly hydrophobic residues that are predicted to be β-sheet (IQIG for RIPK1 and VQVG for RIPK3). Recent biophysical studies show that the RHIMs of RIPK1 and RIPK3 assemble in an amyloid-like filamentous fibrillar complex (Li et al. 2012). Mutagenesis of the RHIM core sequences shows that this amyloidal assembly is crucial for activation of RIPK1 and RIPK3 kinase activity, necrosome cluster formation, and necrosis induction (Fig. 10.1).

Although amyloid fibrils are toxic to neurons, the RHIM amyloid fibril appears to be an intermediary that does not directly elicit cell damage. Rather, it has a crucial function in recruitment of downstream RIPK3 substrates. One such substrate is the

mixed lineage kinase domain-like (MLKL), which was identified by biochemical purification and by shRNA screen (Sun et al. 2012; Zhao et al. 2012). Phosphorylation of MLKL by RIPK3 is critical for necrosis induction. The significance of MLKL in necrosis is further bolstered by identification of a small-molecule inhibitor called “necrosulfonamide” (NSA). NSA inhibits TNF-induced necrosis by covalently modifying human MLKL. Surprisingly, NSA or siRNA knockdown of MLKL did not interfere with RIPK1–RIPK3 necrosome formation. Hence, MLKL is a key regulator of necrosis downstream of RIPK3 (Sun et al. 2012).

Another RIPK3 substrate is the mitochondrial protein phosphoglycerate mutase family member 5 (PGAM5). Both isoforms of PGAM5, $PGAM5_S$ and $PGAM5_L$, were reported to be downstream effectors involved in necrosis induction (Wang et al. 2012). NSA prevented the recruitment of $PGAM5_S$, but not MLKL, to the necrosome. MLKL therefore appears to function as a key adaptor that links the RIPK1–RIPK3 necrosome to downstream effectors. Interestingly, PGAM5 is a phosphatase (Takeda et al. 2009) that can dephosphorylate and activate the mitochondria fission factor Drp-1 (Wang et al. 2012). This raises the interesting possibility that the necrosome can engage the mitochondria fission machinery to execute necrosis. In addition to TNF-induced necrosis, PGAM5 also appears to have broader roles in mediating death receptor-independent necrosis, such as that induced by reactive oxygen species (ROS) or calcium ionophore (Wang et al. 2012). Whether MLKL and PGAM5 are physiologically relevant RIPK3 substrates in vivo will require examination in the relevant mutant animals.

10.3 Role of Necrosis in Innate Inflammatory Responses

10.3.1 Viral Infections

Necrotic cells are characterized by organelle and cell swelling that eventually cumulate in plasma membrane leakage. The release of endogenous adjuvants from necrotic cells is known to be immuno-stimulatory. As we have alluded to in the previous section, many protein adaptors of innate immune signaling pathways contain RHIM domains. This molecular signature suggests that the RIP kinases may have broad roles in innate immunity and inflammation. Further evidence that supports this notion comes from the fact that interferons, which are critical cytokines against viral pathogens, can greatly sensitize cellular necrosis (Kalai et al. 2002).

The first example that highlights this emerging paradigm comes from a study of host defense against vaccinia virus infection. Vaccinia virus, like other poxviruses, encodes many immune evasion genes (Moss and Shisler 2001), including those that inhibit inflammatory cytokines and TLRs (Reading et al. 2002; Harte et al. 2003; Stack et al. 2005). Despite the inhibition of inflammatory signaling, vaccinia virus elicits a strong inflammatory response in infected mice.

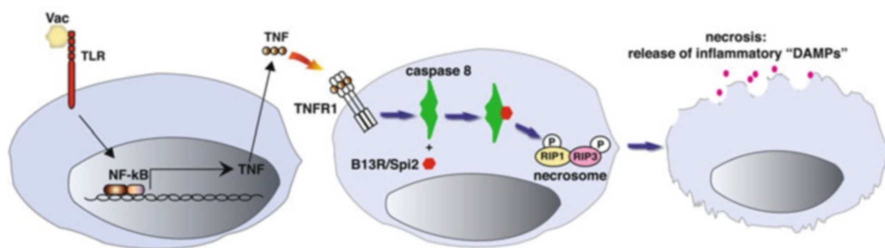

Fig. 10.2 RIP kinase-dependent necrosis is an important innate immune defense mechanism against vaccinia virus. Vaccinia virus is a large DNA virus that has been shown to activate multiple TLRs. Engagement of TLRs results in expression of inflammatory cytokines including TNF. TNF can elicit the cell death program upon binding to the receptor on the cell surface of an infected cell. Because of the virus-encoded caspase inhibitor B13/Spi2, caspase 8 is inhibited and apoptosis is suppressed. This allows assembly of the RIPK1–RIPK3 necrosome. The induction of necrosis may be advantageous in two ways. First, it serves to limit the viral factory before adaptive immunity is launched. Secondly, the release of DAMPs from the necrotic cells may further promote the antiviral inflammatory reaction

One of the immune evasion genes encoded by vaccinia virus is B13R or Spi2, which is a serpin that inhibits caspase 1 and caspase 8 and is functionally similar to the cytokine response modifier A (CrmA) from cowpox virus (Zhou et al. 1997). Despite inhibition of caspase 8 by B13R/Spi2, vaccinia virus-infected cells are still sensitive to the cytotoxic effect of TNF (Li and Beg 2000). TNF-induced cell death of vaccinia virus-infected cells exhibits morphology that resembles necrosis and is dependent on intact RIPK1 and RIPK3 functions (Chan et al. 2003; Cho et al. 2009) (Fig. 10.2). Consistent with results from in vitro infections, RIPK3$^{-/-}$ mice exhibit reduced necrosis and inflammation and greatly increased viral replication in multiple tissues. Eventually, RIPK3$^{-/-}$ mice succumb to the infection 4–5 days post-infection (Cho et al. 2009). In wild-type mice, elevated TNF expression was detected by 24 h post-infection, which coincided with the appearance of RIPK1–RIPK3 complex in the liver (Cho et al. 2009). Because these events occur prior to induction of adaptive T cell responses, which usually peaks at days 7–8 post-infection, we conclude that RIPK3 is critically important for innate immune protection against vaccinia virus. This early control of viral replication is likely to be crucial for host control of the viral factory before virus-specific T and B cells are mobilized in high enough number to fully eradicate the virus.

For vaccinia virus, host cell necrosis is an effective innate immune antiviral defense. Could viruses have developed strategies to inhibit necrosis as a means to escape elimination from the host? Murine cytomegalovirus (MCMV) encodes three different types of viral cell death inhibitors, vICA (inhibitor of caspase 8-induced apoptosis), vMIA (mitochondria inhibitor of apoptosis), and vIRA (inhibitor of RIP activation) (reviewed in Mocarski et al. 2012). Productive infection and replication of viral progenies require the action of all three inhibitors. Although no enzymatic activity can be detected (Lembo et al. 2004), vIRA or M45 exhibits homology to ribonucleotide reductase (Brune et al. 2001). Interestingly, M45 contains a RHIM at the amino terminus that is crucial for binding to RHIM-containing cellular adaptors

Fig. 10.3 Different RHIM domain-containing adaptors can partner with RIPK3 to induce necrosis. The three known RHIM-mediated interactions that lead to necrosis are shown on the *left*. In the case of TRIF–RIPK3, genetic and pharmacological evidence suggests that RIPK1 is also involved. However, biochemical evidence for RIPK1–RIPK3–TRIF complex is lacking at present. Hence, RIPK1 is not included in the necrotic TRIF–RIPK3 complex. On the *right*, the different types of RHIM-mediated interactions that regulate NF-κB activation are shown. Note that RIPK3 has been shown to have positive and negative effects on NF-κB activation by different complexes (Kaiser et al. 2008; Rebsamen et al. 2009)

including RIPK1, RIPK3, and DAI (Kaiser et al. 2008; Upton et al. 2008, 2012; Rebsamen et al. 2009). Recombinant virus that encodes a defective vIRA with tetra-alanine substitutions within the core RHIM sequence fails to establish productive infection in cells and in mice due to premature cell death by necrosis. Significantly, productive infection is restored when the RHIM mutant MCMV infects RIPK3$^{-/-}$ mice (Upton et al. 2010). Surprisingly, the necrotic cell death induced upon mutant MCMV infection is not driven by TNF or RIPK1. Instead, RIPK3 pairs with another RHIM-containing adaptor DAI to induce necrosis (Upton et al. 2012). Hence, similar to poxviruses, MCMV infection sensitizes cells to necrosis. However, unlike vaccinia virus, MCMV has developed an effective strategy to inhibit host cell necrosis to ensure productive viral replication within the infected host. It will be of great interest to determine if similar viral inhibition of necrosis also occurs in human CMV infection. The MCMV studies also reveal that other than RIPK1, other RHIM-containing adaptors can partner with RIPK3 to induce necrosis (Fig. 10.3). It is noteworthy that similar RHIM-mediated interactions between RIPK1–TRIF and RIPK1–DAI/ZBP1 have been shown to mediate NF-κB activation (Meylan et al. 2004; Kaiser and Offermann 2005; Rebsamen et al. 2009) (Fig. 10.3). It will be important to determine how the different types of RHIM complexes can mediate cell survival and cell death signaling under different conditions.

10.3.2 Viral Necrosis Inhibitors

The vIRA/M45 story reveals that active suppression of host cell necrosis can be an important immune evasion mechanism for viruses. In fact, vIRA/M45 is not the first viral necrosis inhibitor identified. Viral FLICE-like inhibitor proteins (FLIPs) are orthologs of cellular caspase 8 and caspase 10. They contain tandem death effector domains but lack the enzymatic domains. Hence, they were first recognized as caspase and apoptosis inhibitors (Bertin et al. 1997; Hu et al. 1997; Thome et al. 1997). In 2003, a subset of vFLIPs, namely, MC159 from molluscum contagiosum virus and E8 from equine herpesvirus, were found to also inhibit TNF-induced necrosis (Chan et al. 2003). In contrast to M45, which inhibits necrosis through RHIM-mediated interaction with RIPK3 (Upton et al. 2010), the molecular basis by which vFLIPs inhibit necrosis is not fully understood. Because vFLIPs and vIRA/M45 are structurally unrelated, these results indicate that viral inhibitors of necrosis can come in different flavors. It will be interesting to see if additional classes of viral necrosis inhibitors will be identified in the future.

10.3.3 Bacterial Infections

Macrophages are sentinels against bacterial infections. Recent studies indicate that in the presence of caspase inhibition, the TLR4 agonist bacterial lipopolysaccharides (LPS) can induce RIPK3-dependent necrosis in macrophages (He et al. 2011). In addition, Smac mimetics, which target cIAP1, cIAP2, and XIAP for proteasomal degradation (Varfolomeev et al. 2007; Vince et al. 2007; Bertrand et al. 2008), can induce RIPK1- and RIPK3-dependent macrophage necrosis (McComb et al. 2012). Besides RIPK1 and RIPK3, another RHIM-containing adaptor TRIF also plays a crucial role in macrophage necrosis (He et al. 2011). TRIF is a TIR domain-containing adaptor that mediates type I interferon expression in response to TLR3 and TLR4 signaling (Yamamoto et al. 2002; Oshiumi et al. 2003). Like RIPK1 and RIPK3, TRIF can induce apoptosis under certain conditions (Kaiser and Offermann 2005; Weber et al. 2010). Treatment with LPS and zVAD-fmk, which mimics bacterial septic shock, causes an inflammatory cytokine storm and extensive macrophage necrosis. These effects were greatly ameliorated in $RIP3^{-/-}$ and $TRIF^{lps2/lps2}$ mutant mice (He et al. 2011). Moderately reduced inflammatory cytokine production in response to LPS was also observed in $RIP3^{-/-}$ mice treated with LPS alone (Newton et al. 2004), suggesting that necrosis-induced inflammation can occur in vivo without pharmacologic inhibition of caspases.

TNF is a major inflammatory cytokine that mediates the systemic effects of LPS-induced septic shock. Consistent with a role for TNF in bacterial sepsis, $RIPK3^{-/-}$ mice are protected from TNF-induced systemic inflammatory response syndrome (SIRS) (Duprez et al. 2011; Linkermann et al. 2012a) and cecal ligation puncture-induced sepsis (Duprez et al. 2011). However, results obtained using the RIPK1

inhibitor necrostatin-1 (Nec-1) were less definitive than those obtained with RIPK3$^{-/-}$ mice. While one report shows protection by Nec-1, another report indicates that Nec-1 exacerbates TNF-induced SIRS (Duprez et al. 2011; Linkermann et al. 2012a). These opposing observations may be due to off-target effects of Nec-1 (Cho et al. 2011). Unfortunately, genetic model to assess RIPK1 function in these inflammatory diseases is currently not available because RIPK1$^{-/-}$ mice exhibit neonatal lethality (Kelliher et al. 1998). Conditional RIPK1$^{-/-}$ mice will be invaluable tools to dissect the in vivo role of RIPK1 in inflammatory diseases.

10.3.4 Necrosis in Sterile Inflammation

Besides its role in pathogen-induced inflammation, necrosis can also promote sterile inflammation. For example, retinal detachment-induced photoreceptor necrosis is blocked in RIPK3$^{-/-}$ cells (Trichonas et al. 2010). Because caspase inhibition greatly sensitizes cells to necrosis, it is no surprise that a large number of studies on necrosis-induced sterile inflammation have been performed using caspase 8$^{-/-}$ or mice deficient in FADD, an upstream adaptor that is essential for caspase 8 recruitment and activation. Similar to caspase inhibition in tissue culture, caspase 8$^{-/-}$ or FADD$^{-/-}$ mice are highly sensitive to necrosis induction. Most remarkably, germline inactivation of these genes results in extensive necrosis during embryogenesis, which results in lethality on E9.5. Embryonic lethality of caspase 8$^{-/-}$ or FADD$^{-/-}$ mice is rescued by deletion of RIPK1 or RIPK3 (Kaiser et al. 2011; Oberst et al. 2011; Zhang et al. 2011; Dillon et al. 2012). Keratinocyte- or intestinal epithelium-specific deletion of FADD or caspase 8 causes severe spontaneous inflammation in the respective tissues that can be corrected by deletion of RIPK3 (Kovalenko et al. 2009; Bonnet et al. 2011; Gunther et al. 2011; Welz et al. 2011). While the more popular view is that the inflammatory disease is caused by increased necrosis, the possibility that FADD, caspase 8, RIPK1, and RIPK3 can directly regulate innate inflammatory signaling cannot be overlooked (see below) (Rajput et al. 2011a; Wallach et al. 2011).

In addition to caspase 8$^{-/-}$ or FADD$^{-/-}$ mice, necrosis-induced sterile injury and inflammation have also been detected in wild-type animals with normal FADD and caspase 8 functions. For instance, repeated doses of cerulein can cause RIPK3-dependent acinar cell necrosis and acute pancreatitis in wild-type mice (He et al. 2009; Zhang et al. 2009). Administration of the RIPK1 inhibitor Nec-1 significantly ameliorates tissue damage in animal models of myocardial infarction, ischemia-induced brain injury, and renal ischemia/reperfusion injury (Degterev et al. 2005; Lim et al. 2007; Smith et al. 2007; Northington et al. 2011; Linkermann et al. 2012b), indicating that RIPK1-dependent necrosis is activated under these conditions in wild-type animals. Although TNF and other inflammatory cytokines are often elevated in ischemia/reperfusion-induced injury (Watters and O'Connor 2011; Lambertsen et al. 2012), it is not clear if they are the direct triggers for necrosis in these diseases. If necrosis is induced without death receptor engagement in these situations, it will be analogous to "intrinsic" apoptosis induced in response to genotoxic stress.

10.4 Direct Roles for RIPK1 and RIPK3 in Inflammation Signaling

As we have discussed in previous sections, promoting inflammation via NF-κB was the first function ascribed to RIPK1. In addition to TNFR, RIPK1 also mediates NF-κB activation by certain innate immune receptors such as TLR3 (Meylan et al. 2004), TLR4 (Cusson-Hermance et al. 2005; Ermolaeva et al. 2008) and RIG-I (Michallet et al. 2008; Rajput et al. 2011b). In contrast to RIPK1, RIPK3$^{-/-}$ cells exhibit normal NF-κB induction in response to TNFR-1 and several TLR agonists (Newton et al. 2004). However, early reports show that over-expression of RIPK3 can often inhibit or promote NF-κB activation (Sun et al. 1999; Kasof et al. 2000; Meylan et al. 2004; Kaiser and Offermann 2005). Hence, it remains possible that RIPK3 can modulate NF-κB responses in specific scenarios.

Recent evidence suggests that RIPK3 has a surprising function in driving maturation of the pro-inflammatory cytokine IL-1β (Vince et al. 2012). Production of IL-1β requires two signals. The first signal, which can be provided by activation of innate immune receptors such as TNFR-1 or TLR4, activates de novo synthesis of pro-IL-1β in an NF-κB-dependent manner. Release of mature IL-1β requires a second signal that involves activation of the inflammasome and caspase-mediated processing of pro-IL-1β (reviewed in Rathinam et al. 2012a). In most cases, caspase 1 is the enzyme responsible for processing of pro-IL-1β and related cytokines such as pro-IL-18. However, noncanonical activation of the inflammasome can result in activation of caspase 8 or caspase 11 (Kayagaki et al. 2011; Gringhuis et al. 2012; Pierini et al. 2012; Rathinam et al. 2012b). Vince and colleagues show that in LPS-primed macrophages, Smac mimetics induces IL-1β processing and maturation through canonical NLRP3–caspase 1 and noncanonical NLRP3–caspase 8 inflammasome activation. Surprisingly, RIPK3 and ROS production are also required for Smac mimetic-induced IL-1β maturation. Consistent with the effects of Smac mimetics, LPS-primed cIAP1$^{-/-}$cIAP2$^{-/-}$XIAP$^{-/-}$ macrophages exhibit spontaneous IL-1β processing (Vince et al. 2012). These results suggest the tantalizing possibility that RIPK3 can promote inflammation through multiple means. On one hand, release of DAMPs from necrotic cells can activate TLRs to promote inflammatory gene expression. On the other hand, RIPK3 can directly engage the inflammasome to promote the expression of IL-1-like inflammatory cytokines.

10.5 Necrosis in Adaptive Immunity

10.5.1 T Cell Tolerance

The maintenance of immune homeostasis is critically dependent on proper cell death regulation. T cells recognize antigenic peptides bound to self major histocompatibility complex (MHC) through their TCRs. Because antigen receptors on T and

B cells are generated by random gene rearrangement, T cells that express TCRs of different affinities to MHC are generated. Lymphocytes that express TCR with little affinity for MHC are eliminated through "death by neglect" in a process termed "positive selection." T cells that survive positive selection are further subjected to "negative selection," a process that eliminates potentially autoreactive T cells with TCR that bind too strongly to self peptide–MHC complexes. The cumulative effect of positive and negative selection is a TCR repertoire that is largely devoid of autoreactive cells (reviewed in Stritesky et al. 2012). Death receptors in the TNFR superfamily do not appear to play significant roles in the thymic selection processes, since animals deficient in these receptors undergo normal thymic selection.

Once T cells leave the thymus to populate the peripheral organs, additional mechanisms, collectively termed "peripheral tolerance," are required to prevent activation of any autoreactive T cells that managed to escape thymic negative selection. In contrast to thymic selection, the death receptors Fas/CD95/APO-1 and, to a lesser extent, TNFR-1 and TNFR-2 play key roles in peripheral tolerance (Zheng et al. 1995; Lenardo et al. 1999). Naïve T cells undergo clonal expansion upon TCR engagement. However, repeated TCR stimulation can result in death of the activated T cells (Zheng et al. 1998). This phenomenon is often referred to as "activation-induced cell death" (AICD) or more appropriately as "restimulation-induced cell death" (RICD) (Snow et al. 2009). Both TCR restimulation and T cell trophic factor IL-2 can greatly enhance Fas and Fas ligand (FasL) expression in activated T cells (Zheng et al. 1998). As a result, activated T cells are eliminated through FasL–Fas interaction in a paracrine fashion. As such, deficiency in the receptor or the ligand leads to defective RICD and lymphoproliferative diseases. The well-known mouse models for autoimmunity *lpr* and *gld* are caused by mutations in Fas and FasL, respectively (Watanabe-Fukunaga et al. 1992; Lynch et al. 1994). In human, similar mutations lead to similar systemic autoimmune disease termed the autoimmune lymphoproliferation syndromes (ALPS) (Puck and Sneller 1997).

Because Fas–FasL-induced lymphocyte cell death exhibits classical features of apoptosis (e.g., chromatin condensation, caspase activation), it is widely believed that apoptosis is the cell death module that controls peripheral tolerance. However, this notion was challenged when mice with T cell-specific deletion of FADD or caspase 8 were found to be immunodeficient rather than developing *lpr*-like autoimmune disease (Zhang et al. 1998, 2005; Ch'en et al. 2008). Similarly, human patients with caspase 8 mutations also exhibit immunodeficiency rather than ALPS-like systemic autoimmunity (Chun et al. 2002). Although these defects were originally attributed to defective TCR-induced NF-κB activation (Su et al. 2005), subsequent experiments show that TCR-induced NF-κB activation was normal in caspase $8^{-/-}$ T cells (Ch'en et al. 2008). Further examination revealed that $FADD^{-/-}$ or caspase $8^{-/-}$ T cells undergo extensive necrosis-like cell death upon stimulation through the TCR (Walsh et al. 1998; Kennedy et al. 1999; Hueber et al. 2000). Consistent with the notion that necrosis underlies the proliferative defect, treatment with Nec-1 restored normal T cell proliferation (Osborn et al. 2010). Moreover, $FADD^{-/-}RIPK1^{-/-}$ and caspase $8^{-/-}RIPK3^{-/-}$ T cells show normal TCR-induced proliferation in vitro, virus-induced clonal expansion in vivo, and cytokine expression (Kaiser et al. 2011;

Zhang et al. 2011). Results obtained from RIPK3$^{-/-}$ mice expressing a dominant negative FADD also show similar phenotypes (Lu et al. 2011). Most remarkably, mice deficient in FADD/caspase 8 and RIPK3 developed lpr-like autoimmune disease that is more aggressive than lpr itself (Ch'en et al. 2011; Kaiser et al. 2011; Oberst et al. 2011), possibly because both Fas- and TNFR-1-induced cell deaths are inhibited. These results revealed an unexpected pro-survival function for FADD and caspase 8 during T cell clonal expansion. They also highlight the fact that caspase-dependent apoptosis and RIP kinase-dependent necrosis are both required to enforce T cell tolerance and homeostasis.

10.5.2 B Cell Responses

In contrast to T cells, B cell proliferation through the antigen receptor or CD40 is unaffected in FADD$^{-/-}$ and caspase 8$^{-/-}$ B cells (Beisner et al. 2005; Imtiyaz et al. 2006). By contrast, TLR3- and TLR4-induced B cell proliferation, but not B cell proliferation induced by the TLR9 agonist CpG DNA, is impaired in FADD$^{-/-}$ and caspase 8$^{-/-}$ B cells (Beisner et al. 2005; Imtiyaz et al. 2006). Unlike TCR-induced proliferation, defective FADD$^{-/-}$ B cell proliferation was not restored in FADD$^{-/-}$RIPK1$^{-/-}$ B cells (Zhang et al. 2011). Because TLR3 and TLR4 share the unique signaling adaptor TRIF and that TRIF has been shown to interact with RIPK1 to mediate NF-κB activation (Meylan et al. 2004; Vivarelli et al. 2004), the defective TLR3/4-induced proliferation in FADD$^{-/-}$RIPK1$^{-/-}$ B cells can be attributed to defective NF-κB signaling. Taken together, these results illustrate that RIPK1 and RIPK3 have differential roles in regulating antigen receptor-induced proliferation in T and B cells.

10.6 Concluding Remarks

Genetic experiments have clearly demonstrated that the RIP kinase-driven necrosis is a biologically relevant cell death module. However, key questions remained to be answered. For example, why are RIPK1 and RIPK3 expression highly induced during T cell activation (Cho et al. 2009, 2011)? It seems counterintuitive that death-promoting molecules are upregulated at a time when lymphocyte expansion is a priority. Similarly, expression of RIPK3 was highly induced during embryogenesis (Zhang et al. 2011). The potential inflammation and damage that necrosis can lead to, such as that seen in FADD$^{-/-}$ and caspase 8$^{-/-}$ animals, is unlikely to be a desired outcome during embryogenesis. In light of these observations, one can envision that RIPK1 and RIPK3 have important biological functions other than necrosis. Discovering and deciphering the non-necrotic or normal physiological functions of the RIP kinases will be of critical relevance as the scientific community ponders the therapeutic potential of manipulating necrosis in the clinics.

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References

Beisner DR, Ch'en IL, Kolla RV, Hoffmann A, Hedrick SM (2005) Cutting edge: innate immunity conferred by B cells is regulated by caspase-8. J Immunol 175:3469–3473

Bertin J, Armstrong RC, Ottilie S, Martin DA, Wang Y, Banks S, Wang GH, Senkevich TG, Alnemri ES, Moss B et al (1997) Death effector domain-containing herpesvirus and poxvirus proteins inhibit both Fas- and TNFR1-induced apoptosis. Proc Natl Acad Sci U S A 94:1172–1176

Bertrand MJ, Milutinovic S, Dickson KM, Ho WC, Boudreault A, Durkin J, Gillard JW, Jaquith JB, Morris SJ, Barker PA (2008) cIAP1 and cIAP2 facilitate cancer cell survival by functioning as E3 ligases that promote RIP1 ubiquitination. Mol Cell 30:689–700

Bonnet MC, Preukschat D, Welz PS, van Loo G, Ermolaeva MA, Bloch W, Haase I, Pasparakis M (2011) The adaptor protein FADD protects epidermal keratinocytes from necroptosis in vivo and prevents skin inflammation. Immunity 35:572–582

Brune W, Menard C, Heesemann J, Koszinowski UH (2001) A ribonucleotide reductase homolog of cytomegalovirus and endothelial cell tropism. Science 291:303–305

Ch'en IL, Beisner DR, Degterev A, Lynch C, Yuan J, Hoffmann A, Hedrick SM (2008) Antigen-mediated T cell expansion regulated by parallel pathways of death. Proc Natl Acad Sci U S A 105:17463–17468

Ch'en IL, Tsau JS, Molkentin JD, Komatsu M, Hedrick SM (2011) Mechanisms of necroptosis in T cells. J Exp Med 208(4):633–641

Chan FK, Shisler J, Bixby JG, Felices M, Zheng L, Appel M, Orenstein J, Moss B, Lenardo MJ (2003) A role for tumor necrosis factor receptor-2 and receptor-interacting protein in programmed necrosis and antiviral responses. J Biol Chem 278:51613–51621

Cho Y, McQuade T, Zhang HB, Zhang JK, Chan FKM (2011) RIP1-dependent and independent effects of necrostatin-1 in necrosis and T cell activation. PLoS One 6

Cho YS, Challa S, Moquin D, Genga R, Ray TD, Guildford M, Chan FK (2009) Phosphorylation-driven assembly of the RIP1-RIP3 complex regulates programmed necrosis and virus-induced inflammation. Cell 137:1112–1123

Chun HJ, Zheng L, Ahmad M, Wang J, Speirs CK, Siegel RM, Dale JK, Puck J, Davis J, Hall CG et al (2002) Pleiotropic defects in lymphocyte activation caused by caspase-8 mutations lead to human immunodeficiency. Nature 419:395–399

Cusson-Hermance N, Khurana S, Lee TH, Fitzgerald KA, Kelliher MA (2005) Rip1 mediates the Trif-dependent toll-like receptor 3- and 4-induced NF-{kappa}B activation but does not contribute to interferon regulatory factor 3 activation. J Biol Chem 280:36560–36566

Degterev A, Hitomi J, Germscheid M, Ch'en IL, Korkina O, Teng X, Abbott D, Cuny GD, Yuan C, Wagner G et al (2008) Identification of RIP1 kinase as a specific cellular target of necrostatins. Nat Chem Biol 4:313–321

Degterev A, Huang Z, Boyce M, Li Y, Jagtap P, Mizushima N, Cuny GD, Mitchison TJ, Moskowitz MA, Yuan J (2005) Chemical inhibitor of nonapoptotic cell death with therapeutic potential for ischemic brain injury. Nat Chem Biol 1:112–119

Dillon CP, Oberst A, Weinlich R, Janke LJ, Kang TB, Ben-Moshe T, Mak TW, Wallach D, Green DR (2012) Survival function of the FADD-CASPASE-8-cFLIPL complex. Cell Rep 1(5): 401–407

Duprez L, Takahashi N, Van Hauwermeiren F, Vandendriessche B, Goossens V, Vanden Berghe T, Declercq W, Libert C, Cauwels A, Vandenabeele P (2011) RIP kinase-dependent necrosis drives lethal systemic inflammatory response syndrome. Immunity 35:908–918

Dynek JN, Goncharov T, Dueber EC, Fedorova AV, Izrael-Tomasevic A, Phu L, Helgason E, Fairbrother WJ, Deshayes K, Kirkpatrick DS et al (2010) c-IAP1 and UbcH5 promote K11-linked polyubiquitination of RIP1 in TNF signalling. EMBO J 29:4198–4209

Ea CK, Deng L, Xia ZP, Pineda G, Chen ZJ (2006) Activation of IKK by TNFalpha requires site-specific ubiquitination of RIP1 and polyubiquitin binding by NEMO. Mol Cell 22:245–257

Ermolaeva MA, Michallet MC, Papadopoulou N, Utermohlen O, Kranidioti K, Kollias G, Tschopp J, Pasparakis M (2008) Function of TRADD in tumor necrosis factor receptor 1 signaling and in TRIF-dependent inflammatory responses. Nat Immunol 9:1037–1046

Feng S, Yang Y, Mei Y, Ma L, Zhu DE, Hoti N, Castanares M, Wu M (2007) Cleavage of RIP3 inactivates its caspase-independent apoptosis pathway by removal of kinase domain. Cell Signal 19:2056–2067

Fortes GB, Alves LS, de Oliveira R, Dutra FF, Rodrigues D, Fernandez PL, Souto-Padron T, De Rosa MJ, Kelliher M, Golenbock D et al (2012) Heme induces programmed necrosis on macrophages through autocrine TNF and ROS production. Blood 119:2368–2375

Gerlach B, Cordier SM, Schmukle AC, Emmerich CH, Rieser E, Haas TL, Webb AI, Rickard JA, Anderton H, Wong WW et al (2011) Linear ubiquitination prevents inflammation and regulates immune signalling. Nature 471:591–596

Gringhuis SI, Kaptein TM, Wevers BA, Theelen B, van der Vlist M, Boekhout T, Geijtenbeek TB (2012) Dectin-1 is an extracellular pathogen sensor for the induction and processing of IL-1beta via a noncanonical caspase-8 inflammasome. Nat Immunol 13:246–254

Gunther C, Martini E, Wittkopf N, Amann K, Weigmann B, Neumann H, Waldner MJ, Hedrick SM, Tenzer S, Neurath MF et al (2011) Caspase-8 regulates TNF-alpha-induced epithelial necroptosis and terminal ileitis. Nature 477:335–339

Harte MT, Haga IR, Maloney G, Gray P, Reading PC, Bartlett NW, Smith GL, Bowie A, O'Neill LA (2003) The poxvirus protein A52R targets Toll-like receptor signaling complexes to suppress host defense. J Exp Med 197:343–351

He S, Liang Y, Shao F, Wang X (2011) Toll-like receptors activate programmed necrosis in macrophages through a receptor-interacting kinase-3-mediated pathway. Proc Natl Acad Sci U S A 108:20054–20059

He S, Wang L, Miao L, Du F, Zhao L, Wang X (2009) Receptor interacting protein kinase-3 determines cellular necrotic response to TNF-α. Cell 137:1100–1111

Hitomi J, Christofferson DE, Ng A, Yao J, Degterev A, Xavier RJ, Yuan J (2008) Identification of a molecular signaling network that regulates a cellular necrotic cell death pathway. Cell 135:1311–1323

Holler N, Zaru R, Micheau O, Thome M, Attinger A, Valitutti S, Bodmer JL, Schneider P, Seed B, Tschopp J (2000) Fas triggers an alternative, caspase-8-independent cell death pathway using the kinase RIP as effector molecule. Nat Immunol 1:489–495

Hu S, Vincenz C, Buller M, Dixit VM (1997) A novel family of viral death effector domain-containing molecules that inhibit both CD-95- and tumor necrosis factor receptor-1-induced apoptosis. J Biol Chem 272:9621–9624

Hueber AO, Zornig M, Bernard AM, Chautan M, Evan G (2000) A dominant negative Fas-associated death domain protein mutant inhibits proliferation and leads to impaired calcium mobilization in both T-cells and fibroblasts. J Biol Chem 275:10453–10462

Imtiyaz HZ, Rosenberg S, Zhang Y, Rahman ZS, Hou YJ, Manser T, Zhang J (2006) The Fas-associated death domain protein is required in apoptosis and TLR-induced proliferative responses in B cells. J Immunol 176:6852–6861

Kaiser WJ, Offermann MK (2005) Apoptosis induced by the toll-like receptor adaptor TRIF is dependent on its receptor interacting protein homotypic interaction motif. J Immunol 174:4942–4952

Kaiser WJ, Upton JW, Long AB, Livingston-Rosanoff D, Daley-Bauer LP, Hakem R, Caspary T, Mocarski ES (2011) RIP3 mediates the embryonic lethality of caspase-8-deficient mice. Nature 471:368–372

Kaiser WJ, Upton JW, Mocarski ES (2008) Receptor-interacting protein homotypic interaction motif-dependent control of NF-kappa B activation via the DNA-dependent activator of IFN regulatory factors. J Immunol 181:6427–6434

Kalai M, Van Loo G, Vanden Berghe T, Meeus A, Burm W, Saelens X, Vandenabeele P (2002) Tipping the balance between necrosis and apoptosis in human and murine cells treated with interferon and dsRNA. Cell Death Differ 9:981–994

Kasof GM, Prosser JC, Liu D, Lorenzi MV, Gomes BC (2000) The RIP-like kinase, RIP3, induces apoptosis and NF-kappaB nuclear translocation and localizes to mitochondria. FEBS Lett 473:285–291

Kayagaki N, Warming S, Lamkanfi M, Vande Walle L, Louie S, Dong J, Newton K, Qu Y, Liu J, Heldens S et al (2011) Non-canonical inflammasome activation targets caspase-11. Nature 479:117–121

Kelliher MA, Grimm S, Ishida Y, Kuo F, Stanger BZ, Leder P (1998) The death domain kinase RIP mediates the TNF-induced NF-kappaB signal. Immunity 8:297–303

Kennedy NJ, Kataoka T, Tschopp J, Budd RC (1999) Caspase activation is required for T cell proliferation. J Exp Med 190:1891–1896

Kerr JF, Wyllie AH, Currie AR (1972) Apoptosis: a basic biological phenomenon with wide-ranging implications in tissue kinetics. Br J Cancer 26:239–257

Kono H, Rock KL (2008) How dying cells alert the immune system to danger. Nat Rev Immunol 8:279–289

Kovalenko A, Kim JC, Kang TB, Rajput A, Bogdanov K, Dittrich-Breiholz O, Kracht M, Brenner O, Wallach D (2009) Caspase-8 deficiency in epidermal keratinocytes triggers an inflammatory skin disease. J Exp Med 206:2161–2177

Lambertsen KL, Biber K, Finsen B (2012) Inflammatory cytokines in experimental and human stroke. J Cereb Blood Flow Metab 32(9):1677–1698

Lamkanfi M, Sarkar A, Vande Walle L, Vitari AC, Amer AO, Wewers MD, Tracey KJ, Kanneganti TD, Dixit VM (2010) Inflammasome-dependent release of the alarmin HMGB1 in endotoxemia. J Immunol 185:4385–4392

Laster SM, Wood JG, Gooding LR (1988) Tumor necrosis factor can induce both apoptotic and necrotic forms of cell lysis. J Immunol 141:2629–2634

Lembo D, Donalisio M, Hofer A, Cornaglia M, Brune W, Koszinowski U, Thelander L, Landolfo S (2004) The ribonucleotide reductase R1 homolog of murine cytomegalovirus is not a functional enzyme subunit but is required for pathogenesis. J Virol 78:4278–4288

Lenardo M, Chan KM, Hornung F, McFarland H, Siegel R, Wang J, Zheng L (1999) Mature T lymphocyte apoptosis–immune regulation in a dynamic and unpredictable antigenic environment. Annu Rev Immunol 17:221–253

Li H, Kobayashi M, Blonska M, You Y, Lin X (2006) Ubiquitination of RIP is required for tumor necrosis factor alpha-induced NF-kappaB activation. J Biol Chem 281:13636–13643

Li J, McQuade T, Siemer AB, Napetschnig J, Moriwaki K, Hsiao YS, Damko E, Moquin D, Walz T, McDermott A et al (2012) The RIP1/RIP3 necrosome forms a functional amyloid signaling complex required for programmed necrosis. Cell 150:339–350

Li M, Beg AA (2000) Induction of necrotic-like cell death by tumor necrosis factor alpha and caspase inhibitors: novel mechanism for killing virus-infected cells. J Virol 74:7470–7477

Lim SY, Davidson SM, Mocanu MM, Yellon DM, Smith CC (2007) The cardioprotective effect of necrostatin requires the cyclophilin-D component of the mitochondrial permeability transition pore. Cardiovasc Drugs Ther 21:467–469

Lin Y, Devin A, Rodriguez Y, Liu ZG (1999) Cleavage of the death domain kinase RIP by caspase-8 prompts TNF-induced apoptosis. Genes Dev 13:2514–2526

Linkermann A, Brasen JH, De Zen F, Weinlich R, Schwendener RA, Green DR, Kunzendorf U, Krautwald S (2012a) Dichotomy between RIP1- and RIP3-mediated necroptosis in tumor necrosis factor alpha-induced shock. Mol Med 18:577–586

Linkermann A, Brasen JH, Himmerkus N, Liu S, Huber TB, Kunzendorf U, Krautwald S (2012b) Rip1 (receptor-interacting protein kinase 1) mediates necroptosis and contributes to renal ischemia/reperfusion injury. Kidney Int 81:751–761

Lu JV, Weist BM, van Raam BJ, Marro BS, Nguyen LV, Srinivas P, Bell BD, Luhrs KA, Lane TE, Salvesen GS et al (2011) Complementary roles of Fas-associated death domain (FADD) and

receptor interacting protein kinase-3 (RIPK3) in T-cell homeostasis and antiviral immunity. Proc Natl Acad Sci U S A 108:15312–15317
Lynch DH, Watson ML, Alderson MR, Baum PR, Miller RE, Tough T, Gibson M, Davis-Smith T, Smith CA, Hunter K et al (1994) The mouse Fas-ligand gene is mutated in gld mice and is part of a TNF family gene cluster. Immunity 1:131–136
McComb S, Cheung HH, Korneluk RG, Wang S, Krishnan L, Sad S (2012) cIAP1 and cIAP2 limit macrophage necroptosis by inhibiting Rip1 and Rip3 activation. Cell Death Differ 19(11): 1791–1801
Meylan E, Burns K, Hofmann K, Blancheteau V, Martinon F, Kelliher M, Tschopp J (2004) RIP1 is an essential mediator of Toll-like receptor 3-induced NF-kappa B activation. Nat Immunol 5:503–507
Michallet MC, Meylan E, Ermolaeva MA, Vazquez J, Rebsamen M, Curran J, Poeck H, Bscheider M, Hartmann G, Konig M et al (2008) TRADD protein is an essential component of the RIG-like helicase antiviral pathway. Immunity 28:651–661
Micheau O, Tschopp J (2003) Induction of TNF receptor I-mediated apoptosis via two sequential signaling complexes. Cell 114:181–190
Mocarski ES, Upton JW, Kaiser WJ (2012) Viral infection and the evolution of caspase 8-regulated apoptotic and necrotic death pathways. Nat Rev Immunol 12:79–88
Moquin D, Chan FK (2010) The molecular regulation of programmed necrotic cell injury. Trends Biochem Sci 35:434–441
Moss B, Shisler JL (2001) Immunology 101 at poxvirus U: immune evasion genes. Semin Immunol 13:59–66
Newton K, Sun X, Dixit VM (2004) Kinase RIP3 is dispensable for normal NF-kappa Bs, signaling by the B-cell and T-cell receptors, tumor necrosis factor receptor 1, and Toll-like receptors 2 and 4. Mol Cell Biol 24:1464–1469
Northington FJ, Chavez-Valdez R, Graham EM, Razdan S, Gauda EB, Martin LJ (2011) Necrostatin decreases oxidative damage, inflammation, and injury after neonatal HI. J Cereb Blood Flow Metab 31:178–189
O'Donnell MA, Legarda-Addison D, Skountzos P, Yeh WC, Ting AT (2007) Ubiquitination of RIP1 regulates an NF-kappaB-independent cell-death switch in TNF signaling. Curr Biol 17:418–424
O'Donnell MA, Perez-Jimenez E, Oberst A, Ng A, Massoumi R, Xavier R, Green DR, Ting AT (2011) Caspase 8 inhibits programmed necrosis by processing CYLD. Nat Cell Biol 13: 1437–1442
Oberst A, Dillon CP, Weinlich R, McCormick LL, Fitzgerald P, Pop C, Hakem R, Salvesen GS, Green DR (2011) Catalytic activity of the caspase-8-FLIP(L) complex inhibits RIPK3-dependent necrosis. Nature 471:363–367
Osborn SL, Diehl G, Han SJ, Xue L, Kurd N, Hsieh K, Cado D, Robey EA, Winoto A (2010) Fas-associated death domain (FADD) is a negative regulator of T-cell receptor-mediated necroptosis. Proc Natl Acad Sci U S A 107:13034–13039
Oshiumi H, Matsumoto M, Funami K, Akazawa T, Seya T (2003) TICAM-1, an adaptor molecule that participates in Toll-like receptor 3-mediated interferon-beta induction. Nat Immunol 4:161–167
Pierini R, Juruj C, Perret M, Jones CL, Mangeot P, Weiss DS, Henry T (2012) AIM2/ASC triggers caspase-8-dependent apoptosis in Francisella-infected caspase-1-deficient macrophages. Cell Death Differ 19(10):1709–1721
Puck JM, Sneller MC (1997) ALPS: an autoimmune human lymphoproliferative syndrome associated with abnormal lymphocyte apoptosis. Semin Immunol 9:77–84
Rajput A, Kang TB, Bogdanov K, Kim JC, Ben-Moshe T, Kovalenko A, Wallach D (2011a) Anti-inflammatory functions of caspase-8. Adv Exp Med Biol 691:253–260
Rajput A, Kovalenko A, Bogdanov K, Yang SH, Kang TB, Kim JC, Du J, Wallach D (2011b) RIG-I RNA helicase activation of IRF3 transcription factor is negatively regulated by caspase-8-mediated cleavage of the RIP1 protein. Immunity 34:340–351

Rathinam VA, Vanaja SK, Fitzgerald KA (2012a) Regulation of inflammasome signaling. Nat Immunol 13:333–342

Rathinam VA, Vanaja SK, Waggoner L, Sokolovska A, Becker C, Stuart LM, Leong JM, Fitzgerald KA (2012b) TRIF licenses caspase-11-dependent NLRP3 inflammasome activation by gram-negative bacteria. Cell 150(3):606–619

Reading PC, Khanna A, Smith GL (2002) Vaccinia virus CrmE encodes a soluble and cell surface tumor necrosis factor receptor that contributes to virus virulence. Virology 292:285–298

Rebsamen M, Heinz LX, Meylan E, Michallet MC, Schroder K, Hofmann K, Vazquez J, Benedict CA, Tschopp J (2009) DAI/ZBP1 recruits RIP1 and RIP3 through RIP homotypic interaction motifs to activate NF-kappaB. EMBO Rep 10:916–922

Schweichel JU, Merker HJ (1973) The morphology of various types of cell death in prenatal tissues. Teratology 7:253–266

Smith CC, Davidson SM, Lim SY, Simpkin JC, Hothersall JS, Yellon DM (2007) Necrostatin: a potentially novel cardioprotective agent? Cardiovasc Drugs Ther 21:227–233

Snow AL, Marsh RA, Krummey SM, Roehrs P, Young LR, Zhang K, van Hoff J, Dhar D, Nichols KE, Filipovich AH et al (2009) Restimulation-induced apoptosis of T cells is impaired in patients with X-linked lymphoproliferative disease caused by SAP deficiency. J Clin Invest 119:2976–2989

Stack J, Haga IR, Schroder M, Bartlett NW, Maloney G, Reading PC, Fitzgerald KA, Smith GL, Bowie AG (2005) Vaccinia virus protein A46R targets multiple Toll-like-interleukin-1 receptor adaptors and contributes to virulence. J Exp Med 201:1007–1018

Stritesky GL, Jameson SC, Hogquist KA (2012) Selection of self-reactive T cells in the thymus. Annu Rev Immunol 30:95–114

Su H, Bidere N, Zheng L, Cubre A, Sakai K, Dale J, Salmena L, Hakem R, Straus S, Lenardo M (2005) Requirement for caspase-8 in NF-kappaB activation by antigen receptor. Science 307:1465–1468

Sun L, Wang H, Wang Z, He S, Chen S, Liao D, Wang L, Yan J, Liu W, Lei X et al (2012) Mixed lineage kinase domain-like protein mediates necrosis signaling downstream of RIP3 kinase. Cell 148:213–227

Sun X, Lee J, Navas T, Baldwin DT, Stewart TA, Dixit VM (1999) RIP3, a novel apoptosis-inducing kinase. J Biol Chem 274:16871–16875

Sun X, Yin J, Starovasnik MA, Fairbrother WJ, Dixit VM (2002) Identification of a novel homotypic interaction motif required for the phosphorylation of receptor-interacting protein (RIP) by RIP3. J Biol Chem 277:9505–9511

Takeda K, Komuro Y, Hayakawa T, Oguchi H, Ishida Y, Murakami S, Noguchi T, Kinoshita H, Sekine Y, Iemura S et al (2009) Mitochondrial phosphoglycerate mutase 5 uses alternate catalytic activity as a protein serine/threonine phosphatase to activate ASK1. Proc Natl Acad Sci U S A 106:12301–12305

Thome M, Schneider P, Hofmann K, Fickenscher H, Meinl E, Neipel F, Mattmann C, Burns K, Bodmer JL, Schroter M et al (1997) Viral FLICE-inhibitory proteins (FLIPs) prevent apoptosis induced by death receptors. Nature 386:517–521

Trichonas G, Murakami Y, Thanos A, Morizane Y, Kayama M, Debouck CM, Hisatomi T, Miller JW, Vavvas DG (2010) Receptor interacting protein kinases mediate retinal detachment-induced photoreceptor necrosis and compensate for inhibition of apoptosis. Proc Natl Acad Sci U S A 107(50):21695–21700

Upton JW, Kaiser WJ, Mocarski ES (2008) Cytomegalovirus M45 cell death suppression requires receptor-interacting protein (RIP) homotypic interaction motif (RHIM)-dependent interaction with RIP1. J Biol Chem 283:16966–16970

Upton JW, Kaiser WJ, Mocarski ES (2010) Virus inhibition of RIP3-dependent necrosis. Cell Host Microbe 7:302–313

Upton JW, Kaiser WJ, Mocarski ES (2012) DAI/ZBP1/DLM-1 complexes with RIP3 to mediate virus-induced programmed necrosis that is targeted by murine cytomegalovirus vIRA. Cell Host Microbe 11:290–297

Vandenabeele P, Galluzzi L, Vanden Berghe T, Kroemer G (2010) Molecular mechanisms of necroptosis: an ordered cellular explosion. Nat Rev Mol Cell Biol 11:700–714
Vanlangenakker N, Vanden Berghe T, Bogaert P, Laukens B, Zobel K, Deshayes K, Vucic D, Fulda S, Vandenabeele P, Bertrand MJ (2011) cIAP1 and TAK1 protect cells from TNF-induced necrosis by preventing RIP1/RIP3-dependent reactive oxygen species production. Cell Death Differ 18:656–665
Varfolomeev E, Blankenship JW, Wayson SM, Fedorova AV, Kayagaki N, Garg P, Zobel K, Dynek JN, Elliott LO, Wallweber HJ et al (2007) IAP antagonists induce autoubiquitination of c-IAPs, NF-kappaB activation, and TNFalpha-dependent apoptosis. Cell 131:669–681
Vercammen D, Beyaert R, Denecker G, Goossens V, Van Loo G, Declercq W, Grooten J, Fiers W, Vandenabeele P (1998a) Inhibition of caspases increases the sensitivity of L929 cells to necrosis mediated by tumor necrosis factor. J Exp Med 187:1477–1485
Vercammen D, Brouckaert G, Denecker G, Van de Craen M, Declercq W, Fiers W, Vandenabeele P (1998b) Dual signaling of the Fas receptor: initiation of both apoptotic and necrotic cell death pathways. J Exp Med 188:919–930
Vince JE, Wong WW, Gentle I, Lawlor KE, Allam R, O'Reilly L, Mason K, Gross O, Ma S, Guarda G et al (2012) Inhibitor of apoptosis proteins limit RIP3 kinase-dependent interleukin-1 activation. Immunity 36:215–227
Vince JE, Wong WW, Khan N, Feltham R, Chau D, Ahmed AU, Benetatos CA, Chunduru SK, Condon SM, McKinlay M et al (2007) IAP antagonists target cIAP1 to induce TNFalpha-dependent apoptosis. Cell 131:682–693
Vivarelli MS, McDonald D, Miller M, Cusson N, Kelliher M, Geha RS (2004) RIP links TLR4 to Akt and is essential for cell survival in response to LPS stimulation. J Exp Med 200:399–404
Walczak H (2011) TNF and ubiquitin at the crossroads of gene activation, cell death, inflammation, and cancer. Immunol Rev 244:9–28
Wallach D, Kovalenko A, Kang TB (2011) 'Necrosome'-induced inflammation: must cells die for it? Trends Immunol 32:505–509
Walsh CM, Wen BG, Chinnaiyan AM, O'Rourke K, Dixit VM, Hedrick SM (1998) A role for FADD in T cell activation and development. Immunity 8:439–449
Wang Z, Jiang H, Chen S, Du F, Wang X (2012) The mitochondrial phosphatase PGAM5 functions at the convergence point of multiple necrotic death pathways. Cell 148:228–243
Watanabe-Fukunaga R, Brannan CI, Copeland NG, Jenkins NA, Nagata S (1992) Lymphoproliferation disorder in mice explained by defects in Fas antigen that mediates apoptosis. Nature 356:314–317
Watters O, O'Connor JJ (2011) A role for tumor necrosis factor-alpha in ischemia and ischemic preconditioning. J Neuroinflammation 8:87
Weber A, Kirejczyk Z, Besch R, Potthoff S, Leverkus M, Hacker G (2010) Proapoptotic signalling through Toll-like receptor-3 involves TRIF-dependent activation of caspase-8 and is under the control of inhibitor of apoptosis proteins in melanoma cells. Cell Death Differ 17:942–951
Welz PS, Wullaert A, Vlantis K, Kondylis V, Fernandez-Majada V, Ermolaeva M, Kirsch P, Sterner-Kock A, van Loo G, Pasparakis M (2011) FADD prevents RIP3-mediated epithelial cell necrosis and chronic intestinal inflammation. Nature 477:330–334
Yamamoto M, Sato S, Mori K, Hoshino K, Takeuchi O, Takeda K, Akira S (2002) Cutting edge: a novel Toll/IL-1 receptor domain-containing adapter that preferentially activates the IFN-beta promoter in the Toll-like receptor signaling. J Immunol 169:6668–6672
Yang H, Hreggvidsdottir HS, Palmblad K, Wang H, Ochani M, Li J, Lu B, Chavan S, Rosas-Ballina M, Al-Abed Y et al (2010) A critical cysteine is required for HMGB1 binding to Toll-like receptor 4 and activation of macrophage cytokine release. Proc Natl Acad Sci U S A 107:11942–11947
Zhang DW, Shao J, Lin J, Zhang N, Lu BJ, Lin SC, Dong MQ, Han J (2009) RIP3, an energy metabolism regulator that switches TNF-induced cell death from apoptosis to necrosis. Science 325(5938):332–336
Zhang H, Zhou X, McQuade T, Li J, Chan FK, Zhang J (2011) Functional complementation between FADD and RIP1 in embryos and lymphocytes. Nature 471:373–376

Zhang J, Cado D, Chen A, Kabra NH, Winoto A (1998) Fas-mediated apoptosis and activation-induced T-cell proliferation are defective in mice lacking FADD/Mort1. Nature 392:296–300

Zhang Y, Rosenberg S, Wang H, Imtiyaz HZ, Hou YJ, Zhang J (2005) Conditional Fas-associated death domain protein (FADD): GFP knockout mice reveal FADD is dispensable in thymic development but essential in peripheral T cell homeostasis. J Immunol 175:3033–3044

Zhao J, Jitkaew S, Cai Z, Choksi S, Li Q, Luo J, Liu ZG (2012) Mixed lineage kinase domain-like is a key receptor interacting protein 3 downstream component of TNF-induced necrosis. Proc Natl Acad Sci U S A 109:5322–5327

Zheng L, Fisher G, Miller RE, Peschon J, Lynch DH, Lenardo MJ (1995) Induction of apoptosis in mature T cells by tumour necrosis factor. Nature 377:348–351

Zheng L, Trageser CL, Willerford DM, Lenardo MJ (1998) T cell growth cytokines cause the superinduction of molecules mediating antigen-induced T lymphocyte death. J Immunol 160: 763–769

Zhou Q, Snipas S, Orth K, Muzio M, Dixit VM, Salvesen GS (1997) Target protease specificity of the viral serpin CrmA. Analysis of five caspases. J Biol Chem 272:7797–7800

Chapter 11
p53 Opens the Mitochondrial Permeability Transition Pore to Trigger Necrosis in Response to Oxidative Damage

Katharina Zirngibl and Ute M. Moll

11.1 Established Roles of p53

11.1.1 p53: A General Stress Sensor Mediating Cell Cycle Arrest, Apoptosis, and Autophagy

The p53 protein has a central role in preventing damaged, potentially tumorigenic cells from propagating into daughter cells, as established by a massive amount of functional and genetic cell-, mouse model-, and human tumor-based work over the last 30 years. Hence, p53 acts as the most powerful tumor suppressor known in human cancer. In response to a broad spectrum of potentially mutagenic cell stress, p53 orchestrates various cellular processes including transient or permanent cell cycle regulation (cell cycle arrest or senescence, depending on the permanence and severity of the inciting stress signal) as well as apoptotic and autophagic forms of cell death. This prevents cells with genome instability, DNA mutations, and aneuploidy from uncontrolled growth (Lane 1992; Levine 1997; Wahl et al. 1997). In unstressed healthy cells, basal levels of p53 are kept very low due to constitutive rapid turnover (degradation) by E3 ubiquitin ligase(s). Physiologically, p53 degradation is mainly controlled by its negative regulator *h*uman *d*ouble *m*inute 2 (HDM2 in human, MDM2 in mice), itself a p53 target gene and hence linked to p53 in a negative feedback loop to keep unstressed levels low. Thus, the half-life of p53 in

K. Zirngibl
Department of Pathology, Stony Brook University, Stony Brook, NY 11794, USA

U.M. Moll (✉)
Department of Pathology, Stony Brook University, Stony Brook, NY 11794, USA

Department of Molecular Oncology, University of Göttingen, 37077 Göttingen, Germany
e-mail: ute.moll@sbumed.org

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most healthy tissues is only 20–30 min. Upon stress, the protein-protein complex between p53 and HDM2 becomes rapidly broken due to bilateral stress-induced modifications (mainly phosphorylations) and possibly stress-induced HDM2 degradation, ensuring a rapid increase in cellular p53 levels in case of acute need (Shieh et al. 1997; Prives 1998; Fuster et al. 2007; Dai and Gu 2010). p53 is capable of sensitizing a surprisingly broad variety of stress signals derived from all types of DNA damage (single- and double-strand breaks, alkylating and oxidative DNA adducts, DNA intercalation, translocation, chromosomal aneuploidy, telomere attrition, and collapsed DNA replication forks) as well as by oncogenic signaling, signaling from oxidative and nitric oxide damage (generating reactive oxygen species, ROS), and hypoxia (Pluquet and Hainaut 2001). These stress signals all converge on p53, thereby activating it via rapid stabilization and posttranslational modification including phosphorylation, acetylation, and ubiquitination and by engaging it in new protein–protein interactions (Dai and Gu 2010). Dependent on cell type, nature of the damaging agent, and severity of the insult, once activated p53 induces various cellular downstream effector pathways regulating reversible growth arrest and DNA repair, permanent cellular senescence, or cell death programs such as apoptosis and autophagy (Rotter et al. 1994; Vogelstein et al. 2000). Biochemically, p53 executes these roles by functioning as a major transcriptional activator in the nucleus as well as by directly exerting transcription-independent functions via interactions with other proteins at mitochondria and in the cytoplasm (Laptenko and Prives 2006; Fuster et al. 2007).

11.1.2 p53 as Stress-Induced Transcriptional Regulator of Target Genes

p53's function as transcriptional activator of target genes was discovered first in the context of DNA damage-induced cell cycle arrest. A central pathway that blocks cell cycle progression in response to genotoxic drugs is the p53-dependent transcription of p21 and Gadd45 genes, direct inhibitors of the cyclin-dependent kinases CDK1, CDK2, and CDK4/6 that delay cell cycle progression at the G1/S, intra-S, and G2/M checkpoints (Kastan et al. 1991). In addition, p53 is a transcriptional repressor of cyclin B, a regulatory timing subunit of CDK1 at the G2/M checkpoint (Abraham 2001; Taylor and Stark 2001). Furthermore, p53 also possesses DNA repair functions. For example, during DNA damage-induced cytostatic arrest, a direct protein–protein interaction between p53 and components of the DNA replication and repair machinery during S-phase progression occurs with Rad51, involved in homologous recombination and DNA repair (Ford and Hanawalt 1995; Sengupta et al. 2003).

Moreover, the transcription-dependent role of p53 in stress-induced apoptosis is well established. Thus, under many stress conditions p53 induces the transcription of a spectrum of pro-apoptotic genes that cooperatively exert apoptotic cell death via the extrinsic death receptor pathway (e.g., induction of CD95/FasR and the

related death receptor Trail/DR5) and/or the intrinsic mitochondrial death pathway (e.g., induction of the pro-apoptotic BH3-only regulators Puma, Noxa, Bid and Bad and to a lesser extent the BH123 effector Bax (Sax et al. 2002; Jiang et al. 2006; Riley et al. 2008).

Besides the prominent apoptosis-inducing role, it was recently found that autophagy can also be transcriptionally promoted by p53 in certain circumstances. Several transcriptional targets of p53 can lead to autophagy either directly (e.g., via AMPK, which inhibits mTOR, a critical promoter of protein synthesis and suppressor of autophagy) or indirectly (e.g., via Sestrin2, a positive regulator of AMPK). Of note, there is considerable overlap between p53-induced autophagic and apoptotic target genes. An example is *d*amage-*r*egulated *a*utophagy *m*odulator (DRAM), a lysosomal protein that induces macroautophagy but is also an inducer of apoptosis (Crighton et al. 2006). Another overlap between autophagic and apoptotic pathways occurs via the p53-mediated transcriptional induction of PUMA and Noxa that primarily contribute to the induction of apoptosis but are also involved in the autophagic pathway (Vousden and Ryan 2009).

11.1.3 Transcription-Independent Function of p53 in Apoptosis

In addition to its apoptotic activity exerted via transcriptional mechanisms, p53 also has a transcription-independent activity in inducing apoptosis, which was established in the last 10 years. p53 can directly induce apoptotic cell death via multiple protein–protein interactions with Bcl2 family members at the outer mitochondrial membrane (OMM) that all lead to mitochondrial outer membrane permeabilization (MOMP), the hallmark of the mitochondrial apoptotic pathway. Mechanistically, it is the cytosolic p53 pool that exerts this function via stress-induced translocation to the mitochondrial outer membrane (MOM) (Marchenko et al. 2000). At the mitochondria p53 interacts with anti-apoptotic BclxL and Bcl2 and neutralizes their inhibitory effects on pro-apoptotic Bax and Bak (i.e., liberates them). Bax and Bak are the only members of the Bcl2 family able to oligomerize and form lipid pores in the MOM. p53 interaction with BclxL also liberates pro-apoptotic tBid from its inhibitory complex with BclxL which is now free to activate Bax and Bak. Moreover, p53 interacts directly with Bak, liberating Bak from a preexisting inhibitory complex with anti-apoptotic Mcl-1. Taken together, p53 acts like a "super" BH3-only protein, combining both "enabling" and "activating" BH3-only functions. Cytosolic p53 was also proposed to interact with Bax in a "hit-and-run" manner, which stimulates mitochondrial translocation of Bax, its oligomerization, and pore formation (Mihara et al. 2003; Chipuk et al. 2004; Leu et al. 2004; Jiang et al. 2006). All these Bcl-directed activities in turn lead to Bax/Bak oligomerization and lipid pore formation, causing MOMP. MOMP then suddenly releases all pro-apoptotic factors from the intermembranous space, including cytochrome *c*, Smac/Diablo, HtrA2, AIF, and EndoG. This subsequently triggers apoptosis by activating the preassembled cytosolic

enzymatic caspase 9/3 cascade and by causing chromatin degradation (Vaseva and Moll 2009). This direct p53-mediated mitochondrial apoptosis program occurs for example in radiosensitive tissues in vivo such as in thymus, spleen, testis, and brain but not in radioresistant tissues such as liver and kidney (Erster and Moll 2004). The transcription-independent apoptotic function of p53 is faster than the transcription-dependent apoptotic function, providing a "jump start" upon stress in vivo. Both pathways are largely independent of each other, although it appears that in some circumstances Puma might be a link between them. It was proposed that after genotoxic stress, BclxL sequesters cytosolic p53, while stabilized nuclear p53 causes Puma expression (transcriptionally), which in turn displaces cytosolic p53 from BclxL, thereby liberating p53 to induce mitochondrial permeabilization (Chipuk et al. 2005).

In addition, a transcription-independent role of p53 was also found in autophagy. Basal levels of cytosolic p53 can inhibit autophagy by directly inhibiting autophagosomes. How this cytosolic inhibitory role is integrated into p53's transcriptional pro-autophagic activity (exerted by nuclear p53) is the subject of ongoing debate (Vousden and Ryan 2009).

11.2 New Role of p53 in Necrotic Cell Death

11.2.1 Oxidative Stress Is One of the Major Triggers of Mitochondrial Permeability Transition

There are several environmental noxious agents including UV light, chemical agents, hypoxia/ischemia, oxidative stress, and genotoxic DNA damage that induce intrinsic cell death if repair of the damaged cells is not possible. This can save cellular resources and prevents propagation of damaged cells to limit harm to the organism. The three known types of cell death are apoptosis, necrosis, and autophagy (Kroemer et al. 2009). Which cell death mode is chosen depends on the type and severity of the noxious agent and the type of cell/tissue. All three death modes have in common that they exhibit an extrinsic pathway mediated by cell surface death receptors that can be activated by extracellular signaling and an intrinsic pathway involving mitochondria and/or the endoplasmic reticulum which responds to intracellular perturbations (Konstantinidis et al. 2012). Ischemic tissue necrosis is of paramount pathophysiologic significance for catastrophic tissue loss in human health, dwarfing the other death modes. The main and interconnected triggers of ischemic necrosis are Ca^{2+} overload and ROS, leading to complete bioenergetic failure. These signals may also trigger apoptosis in some circumstances, and the extent to which ROS and Ca^{2+} induce mitochondria-dependent necrosis versus apoptosis and autophagy is still under debate (Zong and Thompson 2006; Baines 2010a; Webster 2012).

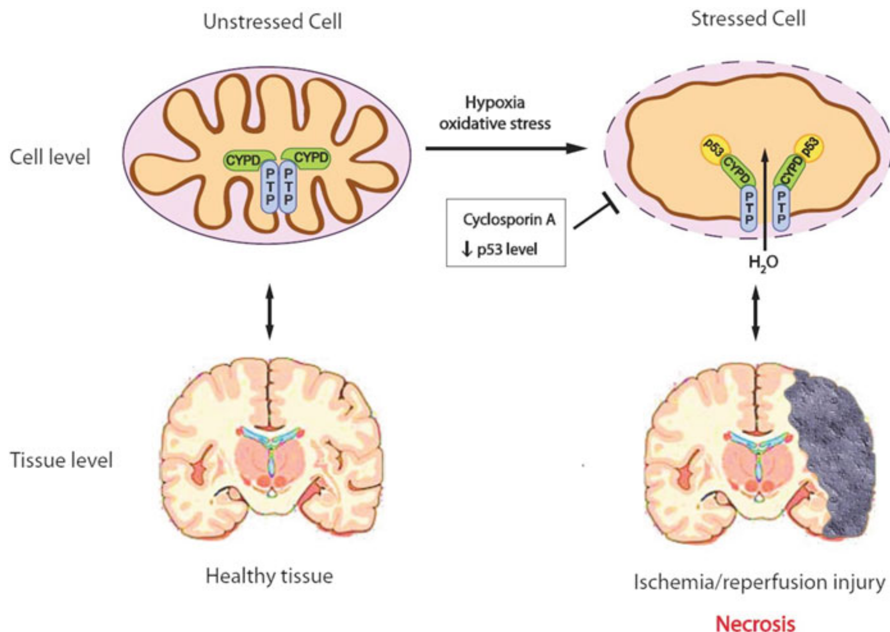

Fig. 11.1 p53 promotes necrosis. During ischemia, p53 accumulates in the mitochondrial matrix of neurons and other brain cells and triggers opening of the permeability transition pore by binding CypD, causing mitochondria to swell and rupture. Ultimately this causes stroke (see Vaseva et al. 2012)

Necrosis is the irreversible tissue destruction due to energy depletion. Necrosis is central to ischemia/reperfusion injury and oxidative damage and underlies cerebral stroke and myocardial infarction. The fundamental difference to apoptosis is the rapid loss of cellular membrane potentials due to energy depletion and ion pump/channel failures, leading to swelling, rupture, and cytolysis. Mediators of necrosis are excess Ca^{2+} and ROS levels in the mitochondrial matrix, leading to mitochondrial rupture and bioenergetic failure. Rather than being a passive event, necrosis has emerged as a controlled cell death that induces an inflammatory response to stimulate tissue repair by selectively releasing factors like HMGB1 and HDGF from dying cells (Zong and Thompson 2006). Necrosis in ischemic tissues, which is experimentally modeled by H_2O_2 treatment of cultured cells, depends on cyclophilin D (CypD, see below), the key regulator of the mitochondrial permeability transition pore (mPTP) at the inner membrane whose opening leads to cell death (Halestrap 2005). The mPTP is a regulated nonselective water- and solute-passing protein channel spanning the inner (IMM) and outer mitochondrial membranes at points of contact (Fig. 11.1).

H^+ pumping out of the matrix creates the proton gradient $\Delta\Psi m$ across the IMM necessary to maintain mitochondrial respiration and ATP production. In unstressed cells, mPTP is closed and the IMM impermeable to ions. Upon oxidative stress,

sudden mPTP opening causes massive ion influx that dissipates ΔΨm and shuts down oxidative phosphorylation and ATP production. This is called mitochondrial permeability transition (mPT). Concomitantly, water influx causes matrix swelling, rupture of the rigid OMM, and release of all sequestered cell death factors. mPT is triggered by mitochondrial matrix sequestration of high levels of cytosolic Ca^{2+} and ROS generated during oxidative damage. Clinically, CypD-triggered mPTP opening and mPT is the driving pathophysiological force behind cerebral stroke, myocardial infarction, and other vascular catastrophes that are leading causes of death. How CypD becomes activated to induce mPT remains unclear but depends on its prolyl isomerase activity. Cyclosporine A (CsA) potently and specifically prevents mPT by binding to CypD, inhibiting its isomerase activity and displacing it from the mPTP (Kroemer et al. 2007).

For completeness it should be added that some older evidence exists suggesting that mPT can also occur in the context of apoptosis, and consequently the relative contribution of mPT to necrosis or apoptosis is under debate by some (Halestrap 2005; Schinzel et al. 2005; Kroemer et al. 2007; Baines 2010a). However, recent results support a necrotic consequence of mPTP opening, especially if the opening is persistent (Zong and Thompson 2006; Baines 2010b).

11.2.2 CypD is the Major Regulator of Oxidative Stress-Induced Necrosis by Regulating Permeability Transition Pore Opening

The structural composition of the mPTP remains unclear. The originally proposed components, i.e., the voltage-dependent anion channel (VDAC) in the outer membrane and the adenine nucleotide translocase (ANT) in the inner membrane (Szabo et al. 1993; Crompton et al. 1998; Vyssokikh et al. 2001), turned out not to be indispensible, since mitochondria that genetically lack either or both of these components can still undergo mPT and necrotic cell death, albeit a stronger stimulus is needed. However, evidence exists for ANT as an obligatory co-regulator of mPTP (Kokoszka et al. 2004; Krauskopf et al. 2006; Baines et al. 2007). Another recently suggested structural candidate for the mPTP pore is the inorganic phosphate carrier (PiC) at the IMM, possibly in complex with ANT. At least PiC has not been conclusively excluded since clean genetic knockout mice do not yet exist, although significant knockdown of PiC by RNAi again did not prevent mPTP (Baines 2010a; Kung et al. 2011). As discussed above, only CypD (encoded by the Ppif gene), a peptidylprolyl *cis–trans* isomerase located in the mitochondrial matrix and a member of the highly conserved family of cyclophilins, has been unequivocally proven to be *the* crucial and only known trigger for ischemic mPTP opening. CypD absence in cells and mitochondria from Ppif−/− mice causes a strong resistance to ischemia, oxidative stress, and Ca^{2+}-induced mPT and necrotic cell death. As shown by four independently generated strains, $CypD^{-/-}$ mice are resistant to ischemia-induced

necrosis in myocardial infarction and stroke, and CypD-deficient mitochondria and cells are resistant to Ca^{2+}- and H_2O_2-induced cell death. But notably, they remain sensitive to Bcl2 family-driven apoptosis, emphasizing the two functionally distinct mitochondrial death systems (Baines et al. 2005; Basso et al. 2005; Nakagawa et al. 2005; Schinzel et al. 2005). Conversely, re-expression of wild-type CypD resensitizes *Ppif* knockout cells to mPT and necrotic cell death, whereas an isomerase dead point mutant of CypD failed to rescue the phenotype. Thus, while not a constitutive structural component of mPTP, CypD is a stress-induced key regulator of the mPTP. Moreover, its peptidylprolyl isomerase function is essential for CypD's ability to regulate mPTP opening (Baines et al. 2005; Baines 2010a). Thus, CypD functions as an enzyme (PPIase activity). CypD causes sudden mPTP opening by binding to the actual pore-forming protein(s), likely inducing their conformational change via prolyl isomerization to form a channel. It is important to note that CypD is the *only* cyclophilin in mitochondria. CsA (a cyclic undecapeptide (11-mer) which binds to and inhibits the catalytic site of all cyclophilins A–D) potently and specifically prevents mitochondrial permeability transition by binding to CypD's catalytic site, inhibiting its isomerase activity and blocking CypD binding to mPTP pore-forming components, thereby preventing pore opening (Kroemer et al. 2007).

A current mechanistic working model of the mPTP suggested by Halestrap proposes that CypD is the regulatory component of the mPTP and exerts its role as pore regulator by binding to PiC, the actual pore-forming component. The PPIase activity of CypD greatly facilitates a conformational change of pore components, triggered by high matrix levels of Ca^{2+} and needed for pore opening. CypD acts by increasing the Ca^{2+} sensitivity of the pore, specifically lowering the threshold for opening. Oxidative stress enhances CypD binding to PiC. In contrast, CsA prevents CypD binding to PiC via a higher affinity interaction to CypD. This is in line with the findings that neither CsA nor CypD deficiency can completely prevent pore opening if the stimulus is too great (Basso et al. 2008). Recently uncovered negative *physiologic* regulators of CypD probably work via the same principle, i.e., by displacing CypD from PiC. These include Hsp90–TRAP1–CypD interactions (Kang et al. 2007) and the desensitizing agent GSK-3beta (Juhaszova et al. 2004). Importantly though, until our recent work described below, a *positive* physiologic regulator of CypD in response to oxidative stress remained elusive.

11.2.3 p53 Triggers mPTP Opening Upon Oxidative Stress

We previously discovered the *direct* (transcription independent) p53 apoptosis program, which comprises stress-induced translocation of p53 to mitochondria and acts by p53 directly targeting Bcl2 family members at the outer membrane (Mihara et al. 2003; Wolff et al. 2008; Vaseva and Moll 2009). In addition to genotoxic and oncogenic stress, we also found that oxidative stress and hypoxia rapidly induce p53 stabilization and concomitant p53 translocation to mitochondria. Next, we took a closer look at the apoptogenic factors released from isolated healthy mitochondria

in response to exposure to purified p53 protein. Interestingly, in addition to soluble apoptogenic factors such as CytoC and Smac that are readily released from the intermembranous space upon MOMP, p53 is also able to release large amounts of nonsoluble proteins such as noncleaved apoptosis-inducing factor (AIF) and Endo G which are firmly tethered to the IMM (Vaseva et al. 2012). Conversely tBid, the prototypical direct activator of the Bax/Bak pore which *exclusively* acts via MOMP (Wolff et al. 2008), is able to release *only* soluble factors. This result indicates a broader, more disruptive mitochondrial permeabilization action of p53 compared to tBid, characterized by severe disruption of the outer *and* inner mitochondrial membrane by p53. This severe loss of mitochondrial membrane integrity induced by p53 is reminiscent of an engagement of the mPTP pore leading to necrosis. To investigate this hypothesis, we tested the ability of p53 to directly open the mPTP. This was done by adding purified p53 to mitochondria freshly isolated from healthy $Bax^{-/-}Bak^{-/-}$ (double knockout, DKO) mouse embryo fibroblasts (MEFs), thereby ensuring complete independence from the Bax/Bak apoptotic pathway. mPTP opening in these DKO MEFs was directly measured by the fluorescent dye calcein, a highly selective indicator of sustained mPTP opening in vivo (Kroemer et al. 2007). Indeed, p53—but not tBid—was able to induce calcein release from DKO mitochondria, indicating that p53 is able to trigger mPTP opening and induce mPT while tBid is not (Vaseva et al. 2012).

11.2.4 p53 Directly Interacts with the Critical mPTP Regulator CypD

Assuming a regulatory function of p53 in mPTP opening and mPT, the next task was to find if p53 has a putative interaction partner through which it mediates mPTP opening. Thus, VDAC and ANT and CypD, facultative or essential components of mPTP formation or regulation, respectively, were tested for stress-induced interaction with p53. Indeed, upon oxidative stress via H_2O_2 treatment, an endogenous complex between p53 and CypD was detected by co-immunoprecipitation in cells (Vaseva et al. 2012). Using recombinant purified proteins confirmed that this complex is a *direct* interaction between p53 and CypD. Moreover, this interaction is competitively blocked by CsA. CsA is a potent and within mitochondria highly specific inhibitor of the CypD isomerase activity by binding to the catalytic domain of CypD (Vaseva et al. 2012). The CsA sensitivity of the complex further suggests that the p53–CypD interaction is a specific event in the p53-mediated opening of the mPTP pore. To structurally investigate this interaction, we mapped the CypD interaction on p53 by a series of p53 deletion mutants. The p53 region between aa 80 and 220, which roughly comprises the first half of the DNA-binding site of p53, was identified as critical for the CypD interaction (Vaseva et al. 2012). In contrast, none of the previously identified contact domains required for the BclxL/Bcl2 interaction was required (Mihara et al. 2003), further underlining the separate mechanisms of

the p53 apoptotic and necrotic mitochondrial pathways. Since CypD is located in the matrix, presumably in close contact with the IMM, we next tested whether p53 not only accumulates at the outer mitochondrial membrane but also within the mitochondrial matrix in cells upon oxidative stress. Careful submitochondrial fractionation of untreated and H_2O_2-treated mitochondria confirmed that p53 in fact does accumulate in the mitochondrial matrix upon oxidative stress where CypD is located. The additional pools of p53 proteins found in the intermembranous space and in the inner membrane are presumably transport intermediates on their way to the matrix (Vaseva et al. 2012).

11.2.5 Oxidative Stress Induces Mitochondrial Permeability Transition in a p53-Dependent Manner

The next question was whether the observed interaction between p53 and CypD plays a direct causal role in triggering mPTP opening. To this end, purified p53 protein was added to either freshly isolated healthy mitochondria from CypD$^{-/-}$ MEFs or to mitochondria from WT mice and incubated for 30 min. Then mPTP opening was assessed by measuring induction of mitochondrial swelling. p53 was able to induce mPTP opening in WT mitochondria but not in CypD-deficient mitochondria. This indicates that the mPT-regulatory activity of p53 in response to oxidative stress concomitantly depends on CypD function. Moreover, it indicates that in response to oxidative stress, p53 is epistatic to CypD in mPTP regulation (Vaseva et al. 2012). This finding also holds true for mPTP opening in living cells, indicated by a complete insensitivity of p53-deficient cells to H_2O_2-induced collapse of the electrochemical potential across the inner membrane when treated with the $\Delta\Psi$m-sensitive potentiometric in vivo dye TMRM. In contrast, WT cells are fully sensitive. p53-deficient cells—as well as CsA-pretreated WT cells—were completely resistant to H_2O_2-induced loss of $\Delta\Psi$m. These results were further confirmed by direct in situ visualization of mPTP pore opening via calcein release (Vaseva et al. 2012). Taken together, oxidative stress-induced mPTP opening concomitantly depends on p53 and CypD. Moreover, both proteins act in the same biochemical pathway where p53 is an upstream regulator of CypD. In contrast, p53 protein does not accumulate in cells nor at mitochondria in response to calcium overload in the mitochondrial matrix, and conversely, calcium overload-induced PTP opening and subsequent necrosis do not require p53 (Vaseva et al. 2012). Overall, these results fit well into the current Halestrap model of mPTP opening whereby p53 takes on the role of the positive signal transducer bringing about oxidative stress-induced enhancement of CypD isomerase binding to structural pore components, which in turn induces conformational changes in the structural components that facilitate pore opening. Thus p53, by binding to CypD, drastically lowers the threshold for pore opening.

11.2.6 Oxidative Stress-Induced Necrosis Concomitantly Depends on CypD and p53

After we proved the p53-mediated regulation of CypD during oxidative stress-induced mPTP opening, we next examined the downstream cell death effects of this pathway and specifically whether it induces necrosis *versus* apoptosis. To this end, $p53^{-/-}$ and $CypD^{-/-}$ versus WT MEFs were treated with H_2O_2 and induction of apoptosis was measured by TUNEL assay. While cell viability readily declined in primary WT but not in p53−/− nor CypD−/− MEFs treated with H_2O_2, apoptotic death of WT cells was negligible in all three genotypes. Instead, WT MEFs showed all morphologic hallmarks of necrosis by electron microscopy, including loss of plasma membrane integrity, organelle swelling, massive intracellular vacuoles and lack of nuclear fragmentation (Vaseva et al. 2012). Thus, the H_2O_2-induced type of cell death is mainly necrosis. Moreover, H_2O_2 induced necrosis in a strongly p53-dependent manner in an isogenic pair of HCT116 $p53^{+/+}$ *versus* $p53^{-/-}$ human colon cancer cells. Here, necrosis of WT cells was measured by a specific biochemical marker. Again, no significant apoptosis was detectable, indicated by the complete absence of cleaved PARP. Instead, cell death induced in WT HCT116 cells was accompanied by release of large quantities of nuclear chromatin-associated *high-mobility g*roup *b*ox 1 (HMGB1) protein into the culture medium in a dose-dependent, p53-dependent, and CypD-dependent manner (Vaseva et al. 2012). Released HMGB1 is the classical biochemical hallmark specific for necrosis. In contrast, in apoptotic cells HMGB1 binds irreversibly to the condensed chromatin in the nucleus (Rossoni et al. 2004). On the other hand, lack of p53 or silencing of CypD by shRNA, as well as both defects combined, caused significant resistance to H_2O_2-induced cell death. In sum, these data indicate that the predominant mode of cell death in response to H_2O_2 in primary and transformed cells is p53/CypD-mediated necrotic cell death.

11.2.7 p53 Regulation of Necrotic Cell Death via mPTP Opening Is Largely Transcription Independent

The above data strongly suggest a *direct* necrosis-activating action of p53 at mitochondria. To look for direct evidence and to clearly distinguish this mechanism from p53's nuclear action as transcription factor that might also contribute to regulate the observed H_2O_2-induced CypD-dependent necrosis, we first used transcriptional inhibitors in combination with oxidative stress. Indeed, neither PFTα, a specific inhibitor of p53-mediated transcription, nor α-amanitin, a powerful general transcriptional inhibitor of RNA polymerase II, could significantly prevent oxidative stress-induced mPTP opening and necrotic cell death in WT MEFs (Vaseva et al. 2012). To further confirm the largely transcription-independent necrotic effect of p53 and to relate it again to regulation of CypD, we used the strategy of directly

targeting p53 to the mitochondrial matrix. This was achieved by ectopic expression of wild-type p53 protein fused to the classic amphipathic mitochondrial import leader sequence of ornithine transcarbamylase, a resident protein of the mitochondrial matrix (called Lp53). Lp53 bypasses the nucleus and is devoid of transcriptional activity (as assayed by highly sensitive reporter assays and endogenous gene transcription) but instead gets specifically and exclusively delivered to the mitochondrial matrix where the leader sequence is cleaved off by resident endopeptidases (Mihara et al. 2003). Indeed, adenoviral delivery of Lp53 into WT MEFs caused a major loss of ΔΨm, indicated by loss of TMRM fluorescence. In contrast, $CypD^{-/-}$ MEFs were completely resistant (Vaseva et al. 2012). Next, to further prove the ability of Lp53 to directly mediate oxidative stress-induced necrosis and to exclude any interference from endogenous p53, HCT116 $p53^{-/-}$ cells were employed. Indeed, at an early time point when H_2O_2 had not yet killed many cells (12 h), Lp53 was found to sensitize these cells towards necrotic cell death in a H_2O_2 dose- and CypD-dependent manner. Again, apoptosis did not play a role in this system (Vaseva et al. 2012). Moreover, Bax−/−Bak−/− DKO MEFs exhibit exactly the same H_2O_2 dose-dependent and CypD-dependent sensitization for necrotic death by Lp53, as do WT MEFs. These data again confirm the Bax/Bak independence of this pathway. A parallel analysis of the factors released into the culture medium confirms the necrotic nature of cell death, as indicated by the huge release of HMGB but concomitant absence of cleaved Casp3 and PARP (Vaseva et al. 2012).

11.2.8 Clinical Relevance: Pathologic p53–CypD Complex Formation Correlates with Ischemic Stroke-Induced Brain Tissue Necrosis

We hypothesized that this newly implicated role of p53 in oxidative stress-induced, CypD/mPTP-mediated necrosis may also play an essential role in ischemic tissue damage. Ischemic tissue damage constitutes the leading cause of human disease due to irreversible catastrophic tissue destruction. One prominent example of oxidative stress-induced necrosis is cerebral stroke, i.e., irreversible ischemia–reperfusion injury of the brain due to a local thrombotic or embolic event. The depletion of glucose and oxygen following brain ischemia causes a spike in intracellular Ca^{2+} in the cytosol, leading to mitochondrial dysfunction and a sharp decline in ATP levels. Subsequent brain reperfusion (by spontaneous partial thrombolysis) then generates the conditions required for mPTP and mPT, as mitochondria repolarize just enough to sequester the excess cytosolic Ca^{2+} that accumulated during the ischemic period into the mitochondrial matrix and generate huge amounts of ROS and thus oxidative stress. In this scenario brain cells (mainly neurons but also glia) in the center of the stroke die largely by necrosis, although in the peripheral penumbra apoptosis also takes place. Importantly, as discussed above, genetic CypD deficiency in knockout mice confers significant protection against ischemia–reperfusion injury of the heart

(Baines et al. 2005; Basso et al. 2005; Nakagawa et al. 2005) and the brain in permanent and transient ischemic stroke models in mice (Schinzel et al. 2005; Wang et al. 2009).

To test our hypothesis, we investigated the pathophysiological impact of p53 with respect to mPTP regulation *in vivo* in a classic murine stroke model of transient brain ischemia/reperfusion. To this end, WT mice, p53$^{+/-}$ mice, and WT control mice pretreated with CsA were subjected to carefully controlled unilateral acute middle cerebral artery occlusion (MCAO) for 1 h, followed by 24 h of reperfusion. Infarct size was measured by staining coronal brain slices with triphenyltetrazolium chloride (TTC), a dye that is oxidized by intact mitochondrial dehydrogenase to yield the red product formazan. In the infarcted white areas, mitochondria are uncoupled and dysfunctional and no longer stain red with TTC. As expected, WT brains were grossly infarcted. Intriguingly, however, p53$^{+/-}$ brains were strongly protected from stroke, similar to WT control mice protected by intraperitoneal injection of CsA 15 min prior to MCAO (Vaseva et al. 2012). Importantly, a stroke-associated pathologic p53–CypD complex was robustly induced in infarcted WT brains after MCAO, as shown by co-immunoprecipitation with a specific p53 antibody but not with nonspecific control IgG. Most intriguingly, the induced p53–CypD complex was undetectable in the stroke-protected CsA-pretreated WT control mice. Moreover, the pathologic p53–CypD complex was also essentially undetectable in p53+/− mice, whose stroke/oxidative stress-mediated p53 induction in the brain did *not* exceed the baseline p53 level of *un*treated WT control mice (Schinzel et al. 2005). Notably, these p53+/− mice were also strongly protected from infarction. Thus, a perfect association exists between the formation of the pathologic p53–CypD complex and ischemia–reperfusion brain infarction. In sum, a p53–CypD complex is formed in necrotic brain tissue in a stroke model in mice. Conversely, blocking formation of the destructive p53–CypD complex is associated with stroke protection. Taken together, these data strongly support the idea that this novel p53–CypD axis is a critical pathophysiologic contributor to ischemic stroke. Moreover, our data suggest that acute blockade of the p53–CypD complex by clinically well-tolerated CsA-type inhibitors may be a therapeutic strategy to limit the extent of ischemic stroke in patients.

11.3 Outlook

We identify an unexpected critical function of wild-type p53 in activating ischemic necrosis in normal tissues. In response to oxidative stress, p53 accumulates in the mitochondrial matrix and triggers opening of the permeability transition pore at the inner membrane, leading to collapse of the electrochemical gradient and cell necrosis. p53 acts via physical interaction with the critical mPTP regulator CypD. This p53 action occurs in multiple cell types and in ischemic stroke in mice. Conversely, ischemic brain tissue is strongly protected from necrosis when the destructive p53–CypD complex is prevented from forming. Our data suggest that acute

blockade of the p53–CypD complex by clinically well-tolerated CsA-type inhibitors may be a therapeutic strategy to limit the extent of ischemic stroke in patients. Our findings provide genetic, biochemical, and pharmacological evidence that fundamentally expands our understanding of p53-mediated cell death networks.

In sum, our discovery represents major progress in our understanding of the mechanism and signaling pathway that underlies oxidative stress-induced mPTP opening and ischemic necrosis. However, as always, many questions remain to be answered. Aside from brain, is this mechanism also critical for other tissues undergoing ischemic necrosis? Also, a deeper analysis of the newly discovered link between matrix p53 and induction of mPTP opening will be important. For example, structure–function analysis as well as biophysical and enzymatic characterization of the p53–CypD interaction will shed light onto the precise mechanism by which p53 activates CypD upon oxidative stress. Also the pathway by which p53 gets imported into the mitochondrial matrix upon oxidative stress needs to be defined in more detail. Obtaining (the long awaited) definitive elucidation of the structural components of the mPTP pore would be an enormous advance in this regard and may also provide novel perspectives towards the development of new, more specific tissue-protective substances in p53/CypD-dependent ischemic diseases. In this context our data suggest that acute temporary blockade of the destructive p53–CypD complex by CsA-type inhibitors may be a therapeutic strategy to limit infarct extent in the rising number of those ischemic stroke patients where reperfusion of the occluded artery can be quickly reestablished by interventional thrombolysis. The new p53–CypD complex as drug target for small-molecule inhibitors and direction for future studies might hold enormous clinical potential.

References

Abraham RT (2001) Cell cycle checkpoint signaling through the ATM and ATR kinases. Genes Dev 15:2177–2196

Baines CP (2010a) The cardiac mitochondrion: nexus of stress. Annu Rev Physiol 72:61–80

Baines CP (2010b) Role of the mitochondrion in programmed necrosis. Front Physiol 1:156

Baines CP, Kaiser RA, Purcell NH, Blair NS, Osinska H, Hambleton MA, Brunskill EW, Sayen MR, Gottlieb RA, Dorn GW, Robbins J, Molkentin JD (2005) Loss of cyclophilin D reveals a critical role for mitochondrial permeability transition in cell death. Nature 434:658–662

Baines CP, Kaiser RA, Sheiko T, Craigen WJ, Molkentin JD (2007) Voltage-dependent anion channels are dispensable for mitochondrial-dependent cell death. Nat Cell Biol 9:550–555

Basso E, Fante L, Fowlkes J, Petronilli V, Forte MA, Bernardi P (2005) Properties of the permeability transition pore in mitochondria devoid of Cyclophilin D. J Biol Chem 280: 18558–18561

Basso E, Petronilli V, Forte MA, Bernardi P (2008) Phosphate is essential for inhibition of the mitochondrial permeability transition pore by cyclosporin A and by cyclophilin D ablation. J Biol Chem 283:26307–26311

Chipuk JE, Bouchier-Hayes L, Kuwana T, Newmeyer DD, Green DR (2005) PUMA couples the nuclear and cytoplasmic proapoptotic function of p53. Science 309:1732–1735

Chipuk JE, Kuwana T, Bouchier-Hayes L, Droin NM, Newmeyer DD, Schuler M, Green DR (2004) Direct activation of Bax by p53 mediates mitochondrial membrane permeabilization and apoptosis. Science 303:1010–1014

Crighton D, Wilkinson S, O'Prey J, Syed N, Smith P, Harrison PR, Gasco M, Garrone O, Crook T, Ryan KM (2006) DRAM, a p53-induced modulator of autophagy, is critical for apoptosis. Cell 126:121–134

Crompton M, Virji S, Ward JM (1998) Cyclophilin-D binds strongly to complexes of the voltage-dependent anion channel and the adenine nucleotide translocase to form the permeability transition pore. Eur J Biochem/FEBS 258:729–735

Dai C, Gu W (2010) p53 post-translational modification: deregulated in tumorigenesis. Trends Mol Med 16:528–536

Erster S, Moll UM (2004) Stress-induced p53 runs a direct mitochondrial death program: its role in physiologic and pathophysiologic stress responses in vivo. Cell Cycle 3:1492–1495

Ford JM, Hanawalt PC (1995) Li-Fraumeni syndrome fibroblasts homozygous for p53 mutations are deficient in global DNA repair but exhibit normal transcription-coupled repair and enhanced UV resistance. Proc Natl Acad Sci U S A 92:8876–8880

Fuster JJ, Sanz-Gonzalez SM, Moll UM, Andres V (2007) Classic and novel roles of p53: prospects for anticancer therapy. Trends Mol Med 13:192–199

Halestrap A (2005) Biochemistry: a pore way to die. Nature 434:578–579

Jiang P, Du W, Heese K, Wu M (2006) The Bad guy cooperates with good cop p53: bad is transcriptionally up-regulated by p53 and forms a Bad/p53 complex at the mitochondria to induce apoptosis. Mol Cell Biol 26:9071–9082

Juhaszova M, Zorov DB, Kim SH, Pepe S, Fu Q, Fishbein KW, Ziman BD, Wang S, Ytrehus K, Antos CL, Olson EN, Sollott SJ (2004) Glycogen synthase kinase-3beta mediates convergence of protection signaling to inhibit the mitochondrial permeability transition pore. J Clin Invest 113:1535–1549

Kang BH, Plescia J, Dohi T, Rosa J, Doxsey SJ, Altieri DC (2007) Regulation of tumor cell mitochondrial homeostasis by an organelle-specific Hsp90 chaperone network. Cell 131:257–270

Kastan MB, Onyekwere O, Sidransky D, Vogelstein B, Craig RW (1991) Participation of p53 protein in the cellular response to DNA damage. Cancer Res 51:6304–6311

Kokoszka JE, Waymire KG, Levy SE, Sligh JE, Cai J, Jones DP, MacGregor GR, Wallace DC (2004) The ADP/ATP translocator is not essential for the mitochondrial permeability transition pore. Nature 427:461–465

Konstantinidis K, Whelan RS, Kitsis RN (2012) Mechanisms of cell death in heart disease. Arterioscler Thromb Vasc Biol 32:1552–1562

Krauskopf A, Eriksson O, Craigen WJ, Forte MA, Bernardi P (2006) Properties of the permeability transition in VDAC1(−/−) mitochondria. Biochim Biophys Acta 1757:590–595

Kroemer G, Galluzzi L, Brenner C (2007) Mitochondrial membrane permeabilization in cell death. Physiol Rev 87:99–163

Kroemer G, Galluzzi L, Vandenabeele P, Abrams J, Alnemri ES, Baehrecke EH, Blagosklonny MV, El-Deiry WS, Golstein P, Green DR, Hengartner M, Knight RA, Kumar S, Lipton SA, Malorni W, Nunez G, Peter ME, Tschopp J, Yuan J, Piacentini M, Zhivotovsky B, Melino G, Nomenclature Committee on Cell D (2009) Classification of cell death: recommendations of the Nomenclature Committee on Cell Death 2009. Cell Death Differ 16:3–11

Kung G, Konstantinidis K, Kitsis RN (2011) Programmed necrosis, not apoptosis, in the heart. Circ Res 108:1017–1036

Lane DP (1992) Cancer. p53, guardian of the genome. Nature 358:15–16

Laptenko O, Prives C (2006) Transcriptional regulation by p53: one protein, many possibilities. Cell Death Differ 13:951–961

Leu JI, Dumont P, Hafey M, Murphy ME, George DL (2004) Mitochondrial p53 activates Bak and causes disruption of a Bak-Mcl1 complex. Nat Cell Biol 6:443–450

Levine AJ (1997) p53, the cellular gatekeeper for growth and division. Cell 88:323–331

Marchenko ND, Zaika A, Moll UM (2000) Death signal-induced localization of p53 protein to mitochondria. A potential role in apoptotic signaling. J Biol Chem 275:16202–16212

Mihara M, Erster S, Zaika A, Petrenko O, Chittenden T, Pancoska P, Moll UM (2003) p53 has a direct apoptogenic role at the mitochondria. Mol Cell 11:577–590

Nakagawa T, Shimizu S, Watanabe T, Yamaguchi O, Otsu K, Yamagata H, Inohara H, Kubo T, Tsujimoto Y (2005) Cyclophilin D-dependent mitochondrial permeability transition regulates some necrotic but not apoptotic cell death. Nature 434:652–658

Pluquet O, Hainaut P (2001) Genotoxic and non-genotoxic pathways of p53 induction. Cancer Lett 174:1–15

Prives C (1998) Signaling to p53: breaking the MDM2-p53 circuit. Cell 95:5–8

Riley T, Sontag E, Chen P, Levine A (2008) Transcriptional control of human p53-regulated genes. Nat Rev Mol Cell Biol 9:402–412

Rossoni G, Manfredi B, Del Soldato P, Berti F (2004) The nitric oxide-releasing naproxen derivative displays cardioprotection in perfused rabbit heart submitted to ischemia-reperfusion. J Pharmacol Exp Ther 310:555–562

Rotter V, Aloni-Grinstein R, Schwartz D, Elkind NB, Simons A, Wolkowicz R, Lavigne M, Beserman P, Kapon A, Goldfinger N (1994) Does wild-type p53 play a role in normal cell differentiation? Semin Cancer Biol 5:229–236

Sax JK, Fei P, Murphy ME, Bernhard E, Korsmeyer SJ, El-Deiry WS (2002) BID regulation by p53 contributes to chemosensitivity. Nat Cell Biol 4:842–849

Schinzel AC, Takeuchi O, Huang Z, Fisher JK, Zhou Z, Rubens J, Hetz C, Danial NN, Moskowitz MA, Korsmeyer SJ (2005) Cyclophilin D is a component of mitochondrial permeability transition and mediates neuronal cell death after focal cerebral ischemia. Proc Natl Acad Sci U S A 102:12005–12010

Sengupta S, Linke SP, Pedeux R, Yang Q, Farnsworth J, Garfield SH, Valerie K, Shay JW, Ellis NA, Wasylyk B, Harris CC (2003) BLM helicase-dependent transport of p53 to sites of stalled DNA replication forks modulates homologous recombination. EMBO J 22:1210–1222

Shieh SY, Ikeda M, Taya Y, Prives C (1997) DNA damage-induced phosphorylation of p53 alleviates inhibition by MDM2. Cell 91:325–334

Szabo I, De Pinto V, Zoratti M (1993) The mitochondrial permeability transition pore may comprise VDAC molecules. II. The electrophysiological properties of VDAC are compatible with those of the mitochondrial megachannel. FEBS Lett 330:206–210

Taylor WR, Stark GR (2001) Regulation of the G2/M transition by p53. Oncogene 20:1803–1815

Vaseva AV, Marchenko ND, Ji K, Tsirka SE, Holzmann S, Moll UM (2012) p53 opens the mitochondrial permeability transition pore to trigger necrosis. Cell 149:1536–1548

Vaseva AV, Moll UM (2009) The mitochondrial p53 pathway. Biochim Biophys Acta 1787: 414–420

Vogelstein B, Lane D, Levine AJ (2000) Surfing the p53 network. Nature 408:307–310

Vousden KH, Ryan KM (2009) p53 and metabolism. Nat Rev Cancer 9:691–700

Vyssokikh MY, Katz A, Rueck A, Wuensch C, Dorner A, Zorov DB, Brdiczka D (2001) Adenine nucleotide translocator isoforms 1 and 2 are differently distributed in the mitochondrial inner membrane and have distinct affinities to cyclophilin D. Biochem J 358:349–358

Wahl GM, Linke SP, Paulson TG, Huang LC (1997) Maintaining genetic stability through TP53 mediated checkpoint control. Cancer Surv 29:183–219

Wang X, Carlsson Y, Basso E, Zhu C, Rousset CI, Rasola A, Johansson BR, Blomgren K, Mallard C, Bernardi P, Forte MA, Hagberg H (2009) Developmental shift of cyclophilin D contribution to hypoxic-ischemic brain injury. J Neurosci 29:2588–2596

Webster KA (2012) Mitochondrial membrane permeabilization and cell death during myocardial infarction: roles of calcium and reactive oxygen species. Future Cardiol 8:863–884

Wolff S, Erster S, Palacios G, Moll UM (2008) p53's mitochondrial translocation and MOMP action is independent of Puma and Bax and severely disrupts mitochondrial membrane integrity. Cell Res 18:733–744

Zong WX, Thompson CB (2006) Necrotic death as a cell fate. Genes Dev 20:1–15

Chapter 12
Autophagic Cell Death: A Real Killer, an Accomplice, or an Innocent Bystander?

Shi-Hao Tan and Han-Ming Shen

12.1 Introduction

Macroautophagy (referred to as autophagy hereafter) is an evolutionarily conserved and regulated catabolic process that sequesters cellular proteins, lipids and organelles from the cytoplasm in double membrane vesicles called autophagosomes before fusing with lysosomal compartments so that the sequestered cargo can be degraded by lysosomal enzymes (Mizushima 2007; Mizushima et al. 2008). Our knowledge of the molecular machinery of autophagy has been greatly facilitated by studies done on the budding yeast *Saccharomyces cerevisiae* in which more than 30 *ATG* (autophagy-related) genes have been identified and many orthologs have been discovered in mammals as well (He and Klionsky 2009). This has allowed the dissection of autophagy into several sequential steps that are regulated by the hierarchical function of the Atg proteins. These steps include: (1) induction or initiation that depends on a complex consisting of ULK1 (ATG1 homologue), ATG13 and

S.-H. Tan
Department of Physiology, Yong Loo Lin School of Medicine, National University of Singapore, MD9, 2 Medical Drive, Singapore 117597, Republic of Singapore

NUS Graduate School for Integrative Sciences and Engineering, National University of Singapore, Singapore, Republic of Singapore

H.-M. Shen (✉)
Department of Physiology, Yong Loo Lin School of Medicine, National University of Singapore, Singapore, Singapore
e-mail: han-ming_shen@nuhs.edu.sg

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FIP2000 proteins which leads to the formation of the phagophore and is negatively regulated by mammalian target of rapamycin (mTOR); (2) nucleation, which is mediated by the Beclin 1 and hVps34/class III phosphoinositide 3-kinase (PI3K) complex that produces phosphatidylinositol-3-phosphate (PtdIns3P) and also recruits various proteins to the autophagosome; (3) elongation, which is required for the completion of autophagosomes by making use of two ubiquitin-like conjugation systems (Atg12–Atg5 system and the LC3/Atg8 system) that results in the covalent binding of phosphatidylethanolamine (PE) to the carboxyl-terminal glycine of LC3/Atg8 protein which is then localized to the autophagosomal membrane; and finally (4) maturation and degradation, which involves fusion of the completed autophagosomes with endosomes–lysosomes to form autolysosomes whereby the cargoes sequestered can be degraded by the lysosomal hydrolases (Nakatogawa et al. 2009; Yang and Klionsky 2010).

Low basal level of autophagy is essential for maintaining the cellular homeostasis by preventing accumulation of unfolded proteins and damaged organelles. Autophagy can also be induced during times of nutrient deprivation and cellular stresses. The widely accepted regulatory pathway of autophagy induction is the PI3K-Akt-mTOR pathway (Mizushima 2007). The mTOR pathway is one of the convergent points of signaling pathways originating from growth factors like insulin and also amino acids. The mTOR protein has also been identified as a key negative regulator directly upstream of the ULK1/Atg1 complex (Jung et al. 2009; Mizushima 2010). During nutrient deprivation, autophagy is up-regulated to degrade components within the cells and the products are then fed into metabolic pathways to keep processes like protein synthesis and energy production ongoing and thus contribute to cell survival. Apart from mediating cell survival and metabolism, autophagy has also been shown to play a role other biological processes like cell death, development, aging, infection and immunity (Levine and Klionsky 2004). Recent studies have also brought into focus the role of autophagy in the development of various human diseases like cancer, neurodegenerative diseases and metabolic disorders (Mizushima et al. 2008; Meijer and Codogno 2009). For example, mouse models with Atg gene knockout has been developed and shown to promote the development of neurodegenerative conditions like Huntington's disease, Parkinson's disease and Alzheimer's disease (Hara et al. 2006; Komatsu et al. 2006; Sarkar and Rubinsztein 2008; Yue et al. 2009; Funderburk et al. 2010a). Furthermore, autophagy has also been implicated to have a positive effect on tumorigenesis and cancer development in many studies (Mathew et al. 2007; Levine and Kroemer 2008; Apel et al. 2009; Chen and Debnath 2010).

The role of autophagy as a cell death or cell survival mechanisms in cells has been the subject of much debate and also an area of intense research. Therefore, the understanding of how autophagy contributes to cell death or survival will be essential for the development of suitable therapeutics against the various diseases mentioned.

Programmed cell death (PCD) was first used to describe cell death observed in silk moths in the 1960s (Lockshin and Zakeri 2001) and it was not until 1970s before the term "apoptosis" was first used to differentiate between different forms

of cell death, namely apoptosis and necrosis based on their morphological differences (Kerr et al. 1972). Subsequently, cell death was further classified into three main types including: (1) Type I cell death (apoptosis), (2) Type II cell death (autophagic cell death, ACD) and (3) Type III cell death (necrosis) based on the morphological appearances of the dying cells (Galluzzi et al. 2012). Type I cell death or apoptosis is the most well studied form of cell death and apoptotic cells usually display characteristic morphological features like nuclear fragmentation and the condensation of the cell bodies. The molecular machinery involved in this process has been well established ever since the discovery of the apoptotic process in *Caenorhabditis elegans* (Horvitz et al. 1994) and also the caspase cascade in mammalian cells (Nicholson et al. 1995). Type II cell death or ACD was coined to describe cell death in dying cells that displayed the morphological characteristics of increase in numbers of autophagosomes which was taken as a feature of upregulation of the autophagic process in these cells. In contrast, Type III cell death or necrosis was initially thought of as a kind of unprogrammed or accidental cell death and characterized morphologically by the loss of plasma membrane integrity and swelling of intracellular organelles. Necrosis normally arises as a result of loss of bioenergetic homeostasis caused by various toxic insults or physical damages (Zong and Thompson 2006). Recent advances into the molecular mechanisms of necrosis have shown that there are indeed other forms of necrosis which are controlled and not "accidental" as previously thought. This type of programmed necrosis is therefore a type of PCD that displays non-apoptotic features with cell death occurring independent of the normal apoptotic molecular machinery. Terms like necroptosis have been used to describe this type of programmed necrosis and various studies have also reported the importance of this type of PCD in various physiological and pathological scenarios (Degterev et al. 2005; Hitomi et al. 2008; Vandenabeele et al. 2010). In order to reduce the confusion within the field due to the ever increasing number of non-apoptotic PCD being defined, Yuan et al. have proposed to classify non-apoptotic PCD into three different categories based on how they are initiated, the signaling pathways governing these type of cell deaths and also their physiological and pathological significances including: (1) necroptosis, (2) PARP-mediated cell death and (3) ACD (Degterev and Yuan 2008). Figure 12.1 summarizes the intricate relationship between various forms of cell death and in this chapter, we focus on ACD.

Of all the type of cell deaths mentioned, ACD has re-emerged recently as one of the key areas of research after being overshadowed by its other counterparts like apoptosis and necrosis for decades. This can be attributed to the increase in interest on the field of autophagy in the past decade (Edinger and Thompson 2004; Levine and Yuan 2005; Tsujimoto and Shimizu 2005; Galluzzi et al. 2008; Kroemer and Levine 2008). In this chapter, we discuss the original definition of ACD and propose a new definition of ACD based on a new set of criteria. We take a look at some of the evidence in different model systems suggesting the presence of ACD and the current controversies on the existence of ACD in mammalian cells. Lastly we discuss the physiological or pathological relevance of ACD to human health and disease, especially on cancer and neurodegenerative diseases.

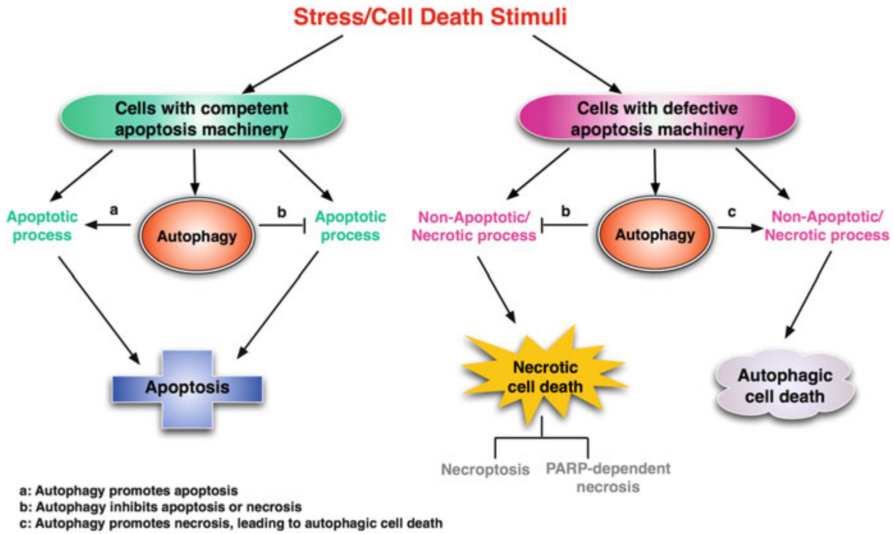

Fig. 12.1 The intricate relationship between different forms of cell death in which autophagy serves as either a pro-death (pathway a and c) or as a pro-survival mechanism (pathway b)

12.2 The Current Definition of ACD

As mentioned above, ACD was first used to describe a kind of cell death where the dying cells exhibited increased in numbers of autophagosomes when their morphological features were examined. In these cells, killing of the cells was thought to be carried out via the autophagic process.

12.2.1 Pitfalls of Current Definition

Relying on a definition formulated solely based on morphological features has contributed to a significant number of issues and discrepancies within the area of ACD research because there are many areas which the definition has not taken into consideration. This has certainly contributed to the confusion in which data regarding ACD has been interpreted. One of the most obvious problems with the definition is that the number of autophagosomes cannot be used definitively as a marker for increase in autophagic activity since it gives us only a measurement of the steady state of autophagic process. Recall that autophagy is a dynamic process (involving initiation, nucleation, elongation, maturation and degradation), and thus, the increase in the number of autophagosomes in cells can actually indicate two phenomena. Firstly, the increase in autophagosome formation could well and truly indicate an increase in the rate of autophagy activity. Secondly, the increase can also

suggest that the autophagic process is actually inhibited. It is now well established that disruption of the maturation step of the autophagy process will lead to accumulation of autophagosomes as well since these double membrane structures cannot be fused to the lysosomes and be degraded (Edinger and Thompson 2004; White 2008). Therefore, in this scenario an increase of autophagosome numbers actually indicates a blockage of autophagy. With the advancement of research in autophagy, it is now a norm to measure what is known as the autophagic flux of the cells. Autophagic flux is defined as the quantity of degradable material that is transported from the autophagosome to the lysosome and therefore is essential to tell us whether the whole autophagic process has occurred to full completion the lysosome (Meijer 2009). Without this piece of crucial evidence, we cannot definitively conclude that autophagy has been induced or inhibited in the cells. Another major issue with the current definition of ACD is that it completely ignores the role which autophagy plays throughout the cell death process. It is well established that cellular stresses that can ultimately lead to cell death are also capable of inducing autophagy as a form of cell survival mechanism that could help to delay the onset of cell death. Therefore, this type of observation can be interpreted as cell death with autophagy where the actual cause of cell death is not by autophagy but by other cell death mechanisms like apoptosis. To fit in with the original idea of ACD, we should be looking for cell death that occurs due to autophagy process as the main executioner and not as an accompanying process. The other obvious pitfall with the current definition is that no single biochemical test was suggested to be used to assess the occurrence of ACD. The advantages of using biochemical tests to assess cell death over morphological features have been adequately covered in the recent review article from the Nomenclature Committee on Cell Death (Galluzzi et al. 2012). As mentioned earlier, the measurement of autophagic flux is one of the essential biochemical assays that should be done to confirm the induction or inhibition of autophagy in the cells before any other conclusions regarding cell death can be made. The various types of biochemical assays that are used for monitoring autophagic flux and other aspect of autophagy have been covered in several other reviews and inclusion of these assays will no doubt help to confirm the accuracy of the observations made in the dying cells (Mizushima et al. 2010; Klionsky et al. 2012). As mentioned, ACD has recently been classified as a type of "programmed necrotic cell death" that is independent of the apoptotic machinery. Hence we can also make use of biochemical assays to confirm the absence of apoptotic markers in dying cells that shows upregulation of autophagy as another important marker for identifying instances of ACD. This will help greatly in defining the functional role of autophagy in cell death and help to differentiate between cells that are dying with autophagy and cells that are dying due to autophagy.

12.2.2 The New Definition of ACD

Based on the discussions above, recently we have proposed a new definition of ACD (Shen and Codogno 2011): ACD should refer to a form of programmed cell death in

which autophagy per se serves as a cell death mechanism. Furthermore, there are three criteria which will need to be fulfilled before cells can be classified as dying by ACD: (1) the dying cells should not exhibit any forms of apoptotic cell death machinery such as activation of the caspase cascade; (2) autophagic flux should be measured and shown to have increased instead of just looking at the steady state levels of autophagic markers in the dying cells; (3) cell death can be prevented via pharmacological inhibitors of autophagy and also by using genetic approaches like *ATG* gene knockdowns or over-expression of dominant negative forms of *ATG* genes.

It is well established that there is an intimate relationship between autophagy and apoptosis. In some cases where both autophagy and apoptosis are occurring concurrently in dying cells, any cell death effects exerted by autophagy would most probably be carried out via the apoptotic machinery instead cell death (Cecconi and Levine 2008). Therefore, this type of cell death should not be classified as ACD since autophagy is not executing the so called death signal. Furthermore, most of the established models of ACD have been in systems where the models are deficient in apoptosis, with the soil amoeba *Dictyostelium discoideum* (Calvo-Garrido et al. 2010) and the Bax−/−Bak−/− double knockout MEFs (Shimizu et al. 2004) prime examples of displaying ACD in the absence of apoptosis. Therefore, by taking into account the classification of ACD as a non-apoptotic PCD and the function of autophagy as a cell death mechanism, we believe that exclusion of apoptosis is critical for defining ACD.

Another issue that has been troubling the field of ACD is the lack of experiments showing the levels of autophagic flux as an evidence for induction or inhibition of autophagic flux. Proving the existence of increased autophagic activity is one the most fundamental issues that have to be answered before ACD can even be suggested, thus making this criteria essential in fulfilling the new definition. There are examples of studies where no biochemical assays for autophagic flux was carried out leading to flawed conclusions regarding the role of autophagy in their systems. For example, it was suggested that ACD played a part in the pathogenesis of Parkinson Disease due to increased numbers of autophagosomes observed in the nigral neuronal cells from patients (Ruberg et al. 1997). However, it is now known that the increase in autophagosomes merely represented a blockage in the maturation step of the autophagic process and this was something that could have been detected with a simple assay to detect autophagic flux. Another example can be found in studies of detailing zVAD induced cell death in L929 cells (Yu et al. 2004). The authors interpreted the increase in autophagic markers as an indication of increased autophagic activity during zVAD treatment. However, zVAD was shown to be able to inhibit activity of cathepsin enzymes which make up the lysosomal proteases and thus lead to inhibition of autophagy at the degradation step (Rozman-Pungercar et al. 2003; Scaringi et al. 2004; Wu et al. 2008). Understanding the meaning behind autophagic flux assays and how the inhibitors used in these assays work is also essential for researches in this field to avoid flawed conclusions in their studies. For example there has been reports suggesting that Chloroquine (CQ) or another derivative hydroxychloroquine (HCQ) which is commonly used in flux assays to block lysosomal degradation autophagic flux (Mizushima et al. 2010) can

induced ACD in the cells they investigated (Ramser et al. 2009; Geng et al. 2010). The authors provided evidence of increased autophagic markers like LC3-II and increased number of autophagosomes suggesting that cell death was due to autophagy. However, the fact is that CQ or HCQ are actually able to block autophagy by inhibition of lysosomal function and degradation. Therefore, the examples given strongly suggest the need to conduct assays looking at autophagic flux before any conclusion can be made on whether the autophagic process in any system plays a cell survival or cell death function.

The third criteria we proposed for fulfilling the new function of ACD was to block cell death from occurring with pharmacological inhibitors of autophagy or using genetic approaches to inhibit autophagy. Pharmacological inhibitors for autophagy can be divided into two major types. The first types used are typically the Class III PI3K inhibitors like 3-methyladenine (3-MA) and wortmannin which suppresses autophagy at the early nucleation stage while the second types are typically inhibitors that suppress autophagy at the late maturation stage by blocking autophagosome and lysosome fusion (Bafilomycin A1) or blocking lysosomal degradation by neutralizing the lysosomal pH (CQ, Ammonium Chloride). However, recent advances have brought to light issues regarding the specificities of these inhibitors. For example, 3-MA has been used as a classical autophagy inhibitor that suppresses class III PI3K (Petiot et al. 2000) since being discovered via screening of purine-related substances using isolated hepatocytes from starved rats (Seglen and Gordon 1982). Recent work in our lab however showed that 3-MA can promote autophagy when treated in full medium for a prolonged period while still retaining its ability to inhibit starvation-induced autophagy (Wu et al. 2010). The dual effects of 3-MA are due to its ability to persistently inhibit Class I PI3K throughout the whole treatment and thus induce autophagy when used for long time points while its ability to inhibit Class III PI3K is transient and only occurs at very early time points (Wu et al. 2010). This discovery has thus raised questions on the validity of previous studies since no evidences were shown to address the effect of 3-MA on Class III PI3K and autophagic flux when the inhibitor was added to cells concurrently with other cytotoxic agents (Kanzawa et al. 2004; Ito et al. 2005; Gao et al. 2008; Longo et al. 2008; Li et al. 2009; Xiong et al. 2010) to inhibit autophagy at time points that exceeded 24 h in total.

Questions have also been raised about the use of inhibitors that block autophagosome and lysosome fusion and also lysosomal protein degradation (Juhasz 2012). This is due to the fact that recent publications have shown that the mTOR protein localizes to lysosomes and activation of mTOR is dependent on amino acid that is generated from within the lysosomal lumen (Sancak et al. 2010; Zoncu et al. 2011). Therefore, use of Bafilomycin A1 or CQ to block autophagic process is very likely to have inhibitory effects on the activity of mTOR and thus interfere with the validity of the results we obtain. Without the availability of more specific inhibitors currently, researchers will have to interpret the results they obtain with more caution and instead of depending on one single pharmacological inhibitor, should use multiple inhibitors to prove that the same outcome when autophagy is inhibited can be obtained. Use of genetic approaches to suppress autophagy which will be discussed next is also a must to support and confirm the findings with the pharmacological inhibitors.

The use of genetic approaches like knockdown and over-expression of mutant forms of the gene of interest has become essential in almost all scientific studies since the effects of these approaches are believed to be much more specific than the use of pharmacological inhibitors. Thus, it is important to provide evidences in conjunction with results from pharmacological inhibitors in studies to show that the knockdown of essential *ATG* genes like Atg5, Atg7 and Beclin 1 can rescue the cell death caused by autophagy before any conclusions can be made regarding ACD in any systems. Like all other techniques however, genetic approaches can have their own drawbacks especially since many of the *ATG* genes have functions that are independent of autophagy. Proteins like Atg3 and Atg5 for example has been shown in a few studies to be directly involved in apoptosis apart from its role in autophagy (Codogno and Meijer 2006; Yousefi et al. 2006; Radoshevich et al. 2010). A very recent study has also uncovered an autophagy independent role of Atg7 in modulating the activity of p53 and cell cycle induction during metabolic stress (Lee et al. 2012). Another important example will be beclin 1 which has been known to function in other vesicle trafficking pathways and might also have a autophagy independent role in cell death due to its ability to bind to the apoptotic protein Bcl-2 (Funderburk et al. 2010b). Furthermore, non-canonical autophagy which occurs independently of beclin 1 protein has also been described to be involved in cell death (Scarlatti et al. 2008a, b; Gao et al. 2010; Smith et al. 2010). Therefore, results regarding cell death from knockdown of Beclin 1 will have to be interpreted with caution. Lastly, the discovery of Atg5 and Atg7-independent alternative macroautophagy (Nishida et al. 2009) has further highlighted the fact that we cannot rely solely on the results of a single gene knockdown during any studies involving autophagy and cell death.

As discussed in this section, we believe that by adhering to the new definition proposed for ACD and the three criteria listed a lot of the current controversies in this particular field can be cleared up. Although there are still disadvantages that one has to take note of while using the pharmacological inhibitors or genetic approaches to study ACD, the results obtained can still be convincing as long as both methods are utilized to support the findings of each other.

12.2.3 The Reality of ACD

After a lengthy discussion on the definition of ACD and how we should go about studying this phenomenon, the most important question we need to ask ourselves is that: Does ACD really exist? The most common answer that one would probably receive is that the topic is still very controversial as there have been no confirmative evidences to prove or disprove the existence of ACD.

12.2.3.1 ACD in *D. discoideum*

The widely accepted model for ACD has come from the lower eukaryotes like the *D. discoideum* which is defective in apoptosis. When starved, autophagy and cell

death occurs and the loss of ATG1 function will lead to reduction of ACD (Kosta et al. 2004). It is important to take note that in this organism, autophagy induction by starvation alone does not induce cell death Instead another independent signal, differentiation-inducing factor-1 (DIF-1) is also essential before ACD can be observed (Luciani et al. 2009) The two signals have to work in tandem before cell death can be observed as the presence of either autophagy or DIF-1 alone does not kill the cells. Furthermore, inhibition of autophagy when both signals are present does not totally block cell death as the cells can still die by necrosis (Kosta et al. 2004). The requirement of a second factor apart from autophagy to induce ACD and the fact that inhibition of autophagy does not rescue the cells from cell death poses a conundrum for us as to whether autophagy does indeed play a direct role in killing of the cells or is it required as part of the mechanism for cell death to occur.

12.2.3.2 ACD in *Drosophila melanogaster*

Another lower eukaryote that has been established as a model organism for studying ACD is *Drosophila melanogaster*. ACD is most prominent during the development of this organism (Berry and Baehrecke 2007; McPhee et al. 2010). Removal of the larval midgut section occurs during the transition from the larval to pupal stage of the fly has been shown to be dependent on ACD (Denton et al. 2009). Although the caspase machinery has been found to be activated during midgut cell death, inhibition of caspase activity and thus apoptosis does not rescue the cells from dying (Denton et al. 2010). Instead, over-expression of loss of function mutants of *Atg2* and *Atg18* or knockdown of *Atg1* and *Atg18* results in rescue of cell death and delayed clearance of the midgut section (Denton et al. 2009, 2010). Therefore, these studies have been taken as evidences that autophagy is indeed a cell death mechanism during the midgut development of the *Drosophila*. The problem with this conclusion is that although autophagy inhibition does rescue the cells from dying, it merely delays the clearance of the midgut cells, thus raising questions again on whether autophagy is the sole cell death mechanism in play or it is merely one of the PCD mechanisms that are taking place during the midgut development. The role of autophagy in cell death in the salivary gland of the *Drosophila* is not as clear cut. It was shown that dying salivary glands during metamorphosis exhibited both the features of apoptosis like activation of caspases and also increased autophagosome formation suggesting activation of autophagy (Martin and Baehrecke 2004; Berry and Baehrecke 2007). Blocking apoptosis by inhibition of caspases or blocking autophagy using loss of function *Atg* genes alone merely delayed the degradation of the salivary glands suggesting that both processes are required for efficient clearance of the salivary glands. Indeed, inhibition of both processes resulted in a greater effect in delaying the clearance of the glands (Berry and Baehrecke 2007) and thus again suggests that ACD might only be an accomplice in the removal of the glands and not the sole cell death mechanism. Similar phenomenon can also be observed in the late *D. melanogaster* oogenesis during development. It was shown that degradation of *Drosophila* inhibitor of apoptosis (IAP) dBruce by autophagy will ultimately

lead to caspase-dependent apoptotic cell death in the nurse cells (Nezis et al. 2010). Extensive studies have presented evidences that autophagy has an important cell death function during the development of *D. melanogaster*. However, it seems that the role of autophagy in cell death or survival depends greatly on the type of cells and when the process occurs in the organism. The pro survival role of autophagy is most prominent in the larval fat body of *D. melanogaster* as studies have shown that upregulation of autophagy in the fat body has cytoprotective effects during periods of starvation and mitochondria or endoplasmic reticulum stresses (Scott et al. 2004; Arsham and Neufeld 2009). Thus, it is clear autophagy plays a role in cell death in *D. melanogaster* only specific periods during development and is also limited to a few specific tissue and cell types. Furthermore, all the evidences show clearly that ACD is not the sole mechanism of cell death in these tissues but in fact work in tandem with other PCD like apoptosis.

12.2.3.3 ACD in Mammals

So far there have been no definitive reports of ACD in any in vivo models of mammalian systems whether under physiological or pathological situations. Indeed, there seems to be overwhelming evidences showing that disruption of autophagy process does not disrupt cell death in many mouse models, For example, apart from the *BECLIN 1* knockout mice which displayed increased embryonic lethality due to enhanced apoptosis (Yue et al. 2003), no disruption of cell death was observed in the various *ATG* knockout mouse models that have been generated including *ATG5* (Kuma et al. 2004; Qu et al. 2007) and *ATG7* (Komatsu et al. 2005). Indeed supporting evidences for the presence of ACD has come mainly from in vitro studies on cell culture systems only. However, a quick glance across these studies would reveal that ACD can only be found in some specific cell lines and stimuli specific as well. This is especially true in cells where there is the absence of the normal apoptotic pathway and the most classic example is the Bax−/−Bak−/− double knockout MEFs. Bax and Bak proteins belong to the pro-apoptotic Bcl-2 family and these double knockout MEFs are known to be resistant to apoptotic cell death. When stimulated with cytotoxic reagents like etoposide and staurosporine the Bax−/−Bak−/− double knockout MEFs died via ACD and the cell death could be rescued by knockdown of *Atg5* or *Beclin 1* expression (Shimizu et al. 2004, 2010). However, an interesting phenomenon is that other stress stimuli like X-ray irradiation or TNF+cycloheximide treatment failed to induce ACD in these cells (Tsujimoto and Shimizu 2005), suggesting that the ability of autophagy to induce cell death in the absence of apoptosis might be dependent on various factors like the levels of autophagy induced by the stimuli. Another example commonly discussed is the use of zVAD to block the activation of the caspase machinery in cells like L929 and U937 cells leading to ACD (Yu et al. 2004, 2006). However, as mentioned earlier zVAD used at high concentrations led to inhibition of cathepsin activity as well and thus inhibited autophagy at the degradation step (Rozman-Pungercar et al. 2003; Scaringi et al. 2004; Wu et al. 2008).

Further studies have suggested that cell death induced by zVAD in these cells is actually via necroptosis that is mediated by autocrine production of TNFa and its downstream effector molecules such as RIP1 and RIP3 (Wu et al. 2011). Furthermore, autophagy was shown to play a pro survival role during zVAD induced cell death instead of being the cell death mechanism as suggested by the previous studies survival function (Wu et al. 2008, 2009).

Another mammalian model that has been reported to exhibit ACD would be in cells with over-expression of oncogenic Ras. One study in human ovarian surface epithelial cells reported that over-expression of H-Ras^{V12} resulted in cell death without caspase activation. It was shown that cell death was caused by induction of autophagy and knockdown of Atg5, Atg7 or Beclin 1 could rescue the cells from dying (Elgendy et al. 2011). However, in another study involving over-expression of oncogenic Ras in immortalized baby mouse kidney (iBMK) cells, the authors showed that autophagy induction was necessary for cells over-expressing Ras to survive in times of nutrient deprivation. Furthermore, tumourigenesis of these Ras expressing cells were also reduced when Atg5 or Atg7 was knockdown (Guo et al. 2011). This cytoprotective function of autophagy was also shown in other studies where Ras was over-expressed in different cancer cell lines and MEFs (Kim et al. 2011; Lock et al. 2011).

Recently a study was published suggesting that autophagy rarely, if ever, serves as a executioner of cell death in cells and that most evidences in the field suggest that autophagy is cytoprotective instead (Shen et al. 2011). In this particular study, the authors screened more than 1,400 cytotoxic compounds that were known to have anti-tumour capabilities in a panel of cancer cells and found that out of the 59 compounds that induced an increase in autophagic flux, none was able to induce ACD as knockdown of Atg5 or Atg7 by shRNA failed to rescue cells from dying although autophagy was proven to be inhibited (Shen et al. 2011). Instead, the authors found that autophagy inhibition resulted in increased cell death in the cells treated with these autophagy inducing compounds, thus suggesting that autophagy is a cytoprotective mechanism. In a subsequent article published by the same group, the authors went on to suggest that the results above might signal the end of ACD (Shen et al. 2012) and again stressed that most cases of ACD are actually cells dying with autophagy, but not by autophagy. Furthermore, they also suggested that for the identification of true ACD process, they should also fulfil another criterion which is ACD must involve dismantling of the cell by the autophagic process. In a response to the above study (Shen et al. 2011, 2012), Clarke and Puyal argued that (Clarke and Puyal 2012) addition of another this particular criterion is simply too excessive and clearing of cell debris by autophagy would not help in enhancing cell death. The authors went on to suggest that cancer cells might not be a good model for investigating ACD due to three reasons: (1) ACD seem to be rare in mammals, (3) dividing and postmitotic cells are sensitive to apoptosis and thus ACD might not occur and lastly (2) cancer cell lines harbour multiple mutations and might not be representative of normal cells in animals.

12.3 ACD in Health and Disease

12.3.1 ACD in Cancer

The role which autophagy plays in cancer, either as a cell survival or cell death pathway remains a hot area of debate within the field. However, the general consensus within the field is that autophagy can both suppress cancer initiation but also promote the growth of established tumours (Liu and Ryan 2012; White 2012) and many studies have been formulated to try and evaluate autophagy modulation as a form of therapeutic for cancer (Cheong et al. 2012; Liu and Ryan 2012; Rubinsztein et al. 2012). Examples of ACD in cancer therapeutics and also the various evidences used to confirm the involvement of autophagy are summarized in Table 12.1.

Most of the data suggesting that ACD occurs in cancer cells have come from in vitro data on various cancer cell lines and some of them has been discussed above. In addition to those studies, there is a recent report that suggests that cytosolic FoxO1 is able to kill cancer cells via induction of ACD in cell lines like HCT116 and Hela independently of the transcriptional activity of FoxO1. The result was also replicated in a tumour xenograft model and provides evidence for ACD as a cancer therapeutic (Zhao et al. 2010). However, further in depth studies will need to be carried out since the authors did not look at whether the apoptotic machinery was involved in the cell death mechanism and also did not provide any mechanistic studies on how autophagy induction by cytosolic FoxO1 led to cell death.

Interestingly, arsenic trioxide which exhibit potent anti-tumour effects was shown in a few studies to cause cytotoxicity to cells via the induction of ACD in glioma and leukaemia cell lines (Kanzawa et al. 2003, 2005; Goussetis et al. 2010). In the studies using glioma cell lines, the authors were able to show that arsenic trioxide induced cell death was independent of caspase activation but dependent on autophagy as autophagy inhibition by Bafilomycin A1 was able to prevent the cells from dying. The authors also went on to show that autophagy induction and cell death was dependent upon expression of BNIP3 as over-expression of dominant negative BNIP3 inhibited autophagy induction and rescued cell death (Kanzawa et al. 2003, 2005). In the other study with leukaemia cell lines, the authors also demonstrated that the cells died by ACD after arsenic trioxide as knockdown of *Beclin 1* or *Atg7* rescued the cells from dying. However, the authors failed to demonstrated if apoptosis was involved in the cell death mechanism as apoptosis has been shown to be induced during arsenic trioxide treatment in cells (Goussetis et al. 2010).

Another interesting chemical under considerations as a cancer therapeutic agent is the plant phytoalexin, resveratrol. In a study using several human ovarian carcinoma cell lines, there was a large increase in the number of autophagosomes in the cancer cells following resveratrol treatment. Although activation of the caspase cascade was observed, inhibition of caspases using zVAD or over-expression of antiapoptotic proteins like Bcl-2 and Bcl-XL did not rescue the cells from dying, leading the authors to conclude that autophagy is the cause of cell death

Table 12.1 Autophagic cell death induced by various cancer therapeutic agents

Compounds/stimulus	Mechanism/target	Cancer type/cell lines	Evidence for involvement of autophagy	References
Cytosolic Foxo1	Acetylated Foxo1 interacts with Atg7	Lung carcinoma, colorectal carcinoma, cervical cancer	↑ Autophagic flux TEM Use of 3-MA Atg5 and Atg7 knockdown	Zhao et al. (2010)
Arsenic trioxide	Inhibits thioredoxin reductase, Activates P53	Glioma, acute myelogenous leukaemia	↑ Autophagic flux TEM Use of 3-MA, CQ DN-BNIP3 Beclin1 and Atg7 Knockdown	Kanzawa et al., (2003); Kanzawa et al. (2005); Goussetis et al. (2010)
Resveratrol	Antioxidant	Ovarian carcinoma, chronic myelogenous leukaemia	TEM Use of 3-MA Atg5, LC3 and p62 knockdown	Opipari et al., (2004); Puissant et al. (2010)
Sodium Butyrate, SAHA, OSU-HDAC42	Inhibition of histone deacetylase	Hepatocellular carcinoma, cervical cancer	↑ Autophagic flux TEM Use of 3-MA, Bafilomycin A1 Atg5 knockdown	Shao et al. (2004); Liu et al. (2010)
Rapamycin RAD001	Inhibition of mTORC1	Glioma, papillary thyroid cancer	TEM Use of 3-MA Atg5 and Beclin 1 knockdown	Iwamaru et al. (2007); Lin et al. (2010)
Dexamethasone	Glucocorticoid	Lymphoid Leukaemia	TEM ↑ Autophagic flux Use of 3-MA Beclin 1 knockdown	Laane et al. (2009)

(Opipari et al. 2004). However, no experiments were done to explore whether knockdown of *Atg* genes could rescue the cells from dying while no autophagic flux data was also shown upon resveratrol treatment. A recent study on chronic myelogenous leukaemia cell lines also concluded that resveratrol was able to induce ACD in the cells and cell death can be rescued by knocking down *atg5* in the cells (Puissant et al. 2010). However, the authors also showed that autophagy was upstream of caspase activation and apoptosis. Therefore, this study clearly presents data suggesting that autophagy does not execute cell death directly, but does it through activation of apoptosis.

Another group of anti-cancer agent currently being linked to initiation of ACD in cancer cells are the HDAC inhibitors (HDACis). HDACis have been shown to be able to inhibit tumour cell survival and some have been used in clinical treatment of haematological malignancies (Bolden et al. 2006). One initial study had shown that treatment with HDACis like butyrate and suberoylanilide hydroxamic acid (SAHA) were able to induce activation of the caspase cascade and cell death in Hela cells (Shao et al. 2004). However, the authors later showed that blocking caspase activation by caspase inhibitors or over-expression of Bcl-XL did not prevent cell death in the Hela cells treated with the HDACis. Instead, an increased number of autophagosomes were also observed during treatment, suggesting that cell death was mediated through autophagy. However, the study stopped short of looking at whether autophagy inhibition by pharmacological inhibition or genetic knockdown of *Atg* genes could rescue the cells from cell death, and thus, the functional role of autophagy remains to be defined. Similar observations were also obtained from another group who showed that treatment with HDACis in hepatocellular carcinoma (HCC) cell lines like HepG2 was able to induce autophagy and cell death as well (Liu et al. 2010). Inhibition of autophagy by 3-MA or in *Atg5* knockout cells were also shown to be able to reduce cell death caused by HDACis. However, the authors also showed that apoptosis was induced during treatment and zVAD treatment could reduce cell death by apoptosis as well, and thus, it would be crucial to investigate the how autophagy and apoptosis interact with each other during cell death before conclusions can be made about the functional role of autophagy in cell death. Furthermore, there are other studies suggesting that autophagy induction caused by HDACis treatment actually plays a cell survival role in cancer cells like glioblastomas and chronic myelogenous leukaemia as inhibition of autophagy can potentiate the cytotoxicity of these inhibitors (Carew et al. 2007; Gammoh et al. 2012). These studies again stress that ACD might occur only in certain specific cells and treatment with the same group of chemical inhibitors might lead to different conclusions about the role of autophagy in cell death and survival.

12.3.2 ACD in Neurodegenerative Diseases

The cytoprotective role of autophagy in neurodegenerative diseases has been well documented in many previous studies (Hara et al. 2006; Komatsu et al. 2006; Sarkar and Rubinsztein 2008; Yue et al. 2009; Funderburk et al. 2010a). Mice with

neuron-specific knockout of *Atg5* display accumulation of cytoplasmic inclusion bodies and progressive loss of motor functions (Hara et al. 2006). In another study, knockout of *Atg7* in the purkinje cells resulted in progressive dystrophy and degeneration of the axon terminals implicating autophagy dysfunction as the possible mechanism for axonal pathologies during neurodegeneration (Komatsu et al. 2007). Inhibition of autophagy was also recently implicated in a new Parkinson's disease model where conditional knockout of *Atg7* in the dopamine neurons of the substantia nigra pars compacta, other regions of the midbrain and the hindbrain in mice led to age related loss of dopaminergic neurons and accumulation of alpha-synuclein and ubiquitin protein aggregates, which are all typical features of Parkinson's disease (Ahmed et al. 2012). Intriguingly, the more convincing evidences showing the occurrence of ACD in mammalian systems have also come from studies in neuronal cells where despite the presence of intact apoptotic machinery have been suggested to die via ACD. Both neonatal and adult mice with conditional *Atg7* knockdown in the central nervous system (CNS) had reduced cell death in the pyramidal neuronal cells and hippocampal damage was almost completely blocked after hypoxic ischemic brain injuries (Koike et al. 2008). Further studies by other groups using similar mouse models and the Class III PI3K inhibitor, 3-MA have also shown similar results (Puyal et al. 2009; Zhang et al. 2012). However, it has to be mentioned that apoptotic machinery is also known to be activated during hypoxic ischemic injuries and in the original study, caspase 3 activation were observed in the neonatal and adult mice (Koike et al. 2008), while cell death was shown to be caspase independent only in the adult mice. Therefore, more studies would have to be conducted to find out whether autophagy is the executioner of cell death in this model or only a contributing factor. Dysfunctions in the endosomal sorting complex required for transport-III (ESCRT-III) have been implicated in frontotemporal dementia linked to chromosome 3 with accumulation of excessive autophagosomes. Furthermore, autophagy inhibition using 3-MA or knockdown of *Atg5* and *Atg7* was able to delay but not completely suppress neuronal cell loss (Lee et al. 2009). The fact that neuronal lost was only delayed again suggests that autophagy process might not be the direct cause of cell death and might only be required to work in tandem with other PCD like apoptosis. Hippocampal neural (HCN) stem cells in culture were also shown to undergo ACD upon withdrawal of insulin from the medium (Yu et al. 2008; Baek et al. 2009). Increase in autophagic flux was observed in the HCN stem cells after insulin withdrawal while cell death was also observed. Interestingly, cell death was shown to be independent of caspase activation despite the presence of intact apoptotic machinery in these cells. Furthermore, cell death upon insulin withdrawal could be rescued by inhibition of autophagy via knockdown of *Atg7*, providing strong evidence supporting ACD in this particular model. Despite the growing evidences for involvement of ACD as a cause of neurodegenerative diseases, caution still has to be taken when evaluating the results from the various experiments. One recent example regarding the role of ACD in the *lurcher* mutant mice demonstrates this point very well. It was shown previously that neuronal loss due to gain of function mutation of the delta2 glutamate receptors in cerebellar Purkinje cells led to cell death that was dependent on autophagy. This conclusion was made as there was increase in the number of autophagosomes observed and cell death could

be rescued by treatment with 3-MA (Yuzaki 2003; Vogel et al. 2007). However, this conclusion was recently challenge by another study which showed that inhibition of autophagy by a dominant negative form of AMPK was not able to rescue cell death caused by *lurcher* mutation (Nishiyama et al. 2010). Furthermore, loss of Purkinje cells in the *lurcher* mice could not be rescued in offspring generated from crossing with conditional *Atg5* knockout mice. The authors thus concluded that cell death in this case was not by autophagy, but rather with the presence of autophagy (Nishiyama et al. 2010; Nishiyama and Yuzaki 2010). Therefore, evidences from all these different studies suggest that the occurrence of ACD in neurodegenerative diseases depend on (1) the specific type of neuronal cells and (2) the particular type of stimuli present for the induction of autophagy in the neurons. However, more studies will have to be conducted to truly identify the functional role of autophagy in this cell death: i.e. autophagy the sole and direct cell death mechanism or is it contributing to cell death by enhancing the efficacy of other cell death modalities like apoptosis.

12.4 Conclusions

The interest in modulation of autophagy as a form of therapeutic in various diseases has been greatly elevated in the recent decade as our understanding of both the biological functions of autophagy and the intimate relationship autophagy plays in the aetiology of the major diseases like cancer, neurodegenerative diseases and metabolic disorders. To fulfill its potential as a therapeutic target, it is critical for researchers to be able to define the role of autophagy in cell death or survival in the particular disease model being investigated. In this review we have attempted to address some of the common issues and controversies concerning ACD with the new definition and additional criteria that should be used for studies concerning ACD. Although not comprehensive by any means, some of the case studies mentioned in this review have highlighted the importance of having certain guidelines and criteria to adhere to when studying ACD. Only by adhering to these criteria can we avoid the problems of inconsistencies and contradicting results that have been plaguing the field. However, it is clear that current evidence supports the notion that any pro-cell death function of autophagy works by either (1) helping to promote apoptosis and (2) direct execution of cell death independent of apoptotic machinery. A clear distinction of these two functions of autophagy would likely help to better elucidate the physiological and pathological role of ACD in health and disease.

References

Ahmed I, Liang Y, Schools S, Dawson VL, Dawson TM, Savitt JM (2012) Development and characterization of a new Parkinson's disease model resulting from impaired autophagy. J Neurosci 32:16503–16509

Apel A, Zentgraf H, Buchler MW, Herr I (2009) Autophagy—a double-edged sword in oncology. Int J Cancer 125:991–995

Arsham AM, Neufeld TP (2009) A genetic screen in Drosophila reveals novel cytoprotective functions of the autophagy-lysosome pathway. PLoS One 4:e6068

Baek SH, Kim EK, Goudreau JL, Lookingland KJ, Kim SW, Yu SW (2009) Insulin withdrawal-induced cell death in adult hippocampal neural stem cells as a model of autophagic cell death. Autophagy 5:277–279

Berry DL, Baehrecke EH (2007) Growth arrest and autophagy are required for salivary gland cell degradation in Drosophila. Cell 131:1137–1148

Bolden JE, Peart MJ, Johnstone RW (2006) Anticancer activities of histone deacetylase inhibitors. Nat Rev Drug Discov 5:769–784

Calvo-Garrido J, Carilla-Latorre S, Kubohara Y, Santos-Rodrigo N, Mesquita A, Soldati T, Golstein P, Escalante R (2010) Autophagy in Dictyostelium: genes and pathways, cell death and infection. Autophagy 6:686–701

Carew JS, Nawrocki ST, Kahue CN, Zhang H, Yang C, Chung L, Houghton JA, Huang P, Giles FJ, Cleveland JL (2007) Targeting autophagy augments the anticancer activity of the histone deacetylase inhibitor SAHA to overcome Bcr-Abl-mediated drug resistance. Blood 110: 313–322

Cecconi F, Levine B (2008) The role of autophagy in mammalian development: cell makeover rather than cell death. Dev Cell 15:344–357

Chen N, Debnath J (2010) Autophagy and tumorigenesis. FEBS Lett 584:1427–1435

Cheong H, Lu C, Lindsten T, Thompson CB (2012) Therapeutic targets in cancer cell metabolism and autophagy. Nat Biotechnol 30:671–678

Clarke PG, Puyal J (2012) Autophagic cell death exists. Autophagy 8:867–869

Codogno P, Meijer AJ (2006) Atg5: more than an autophagy factor. Nat Cell Biol 8:1045–1047

Degterev A, Yuan J (2008) Expansion and evolution of cell death programmes. Nat Rev Mol Cell Biol 9:378–390

Degterev A, Huang Z, Boyce M, Li Y, Jagtap P, Mizushima N, Cuny GD, Mitchison TJ, Moskowitz MA, Yuan J (2005) Chemical inhibitor of nonapoptotic cell death with therapeutic potential for ischemic brain injury. Nat Chem Biol 1:112–119

Denton D, Shravage B, Simin R, Mills K, Berry DL, Baehrecke EH, Kumar S (2009) Autophagy, not apoptosis, is essential for midgut cell death in Drosophila. Curr Biol 19:1741–1746

Denton D, Shravage B, Simin R, Baehrecke EH, Kumar S (2010) Larval midgut destruction in Drosophila: not dependent on caspases but suppressed by the loss of autophagy. Autophagy 6:163–165

Edinger AL, Thompson CB (2004) Death by design: apoptosis, necrosis and autophagy. Curr Opin Cell Biol 16:663–669

Elgendy M, Sheridan C, Brumatti G, Martin SJ (2011) Oncogenic Ras-induced expression of Noxa and Beclin-1 promotes autophagic cell death and limits clonogenic survival. Mol Cell 42:23–35

Funderburk SF, Marcellino BK, Yue Z (2010a) Cell "self-eating" (autophagy) mechanism in Alzheimer's disease. Mt Sinai J Med 77:59–68

Funderburk SF, Wang QJ, Yue Z (2010b) The Beclin 1–VPS34 complex–at the crossroads of autophagy and beyond. Trends Cell Biol 20:355–362

Galluzzi L, Vicencio JM, Kepp O, Tasdemir E, Maiuri MC, Kroemer G (2008) To die or not to die: that is the autophagic question. Curr Mol Med 8:78–91

Galluzzi L, Vitale I, Abrams JM, Alnemri ES, Baehrecke EH, Blagosklonny MV, Dawson TM, Dawson VL, El-Deiry WS, Fulda S, Gottlieb E, Green DR, Hengartner MO, Kepp O, Knight RA, Kumar S, Lipton SA, Lu X, Madeo F, Malorni W, Mehlen P, Nunez G, Peter ME, Piacentini M, Rubinsztein DC, Shi Y, Simon HU, Vandenabeele P, White E, Yuan J, Zhivotovsky B, Melino G, Kroemer G (2012) Molecular definitions of cell death subroutines: recommendations of the Nomenclature Committee on Cell Death 2012. Cell Death Differ 19:107–120

Gammoh N, Lam D, Puente C, Ganley I, Marks PA, Jiang X (2012) Role of autophagy in histone deacetylase inhibitor-induced apoptotic and nonapoptotic cell death. Proc Natl Acad Sci U S A 109:6561–6565

Gao M, Yeh PY, Lu YS, Hsu CH, Chen KF, Lee WC, Feng WC, Chen CS, Kuo ML, Cheng AL (2008) OSU-03012, a novel celecoxib derivative, induces reactive oxygen species-related autophagy in hepatocellular carcinoma. Cancer Res 68:9348–9357

Gao P, Bauvy C, Souquere S, Tonelli G, Liu L, Zhu Y, Qiao Z, Bakula D, Proikas-Cezanne T, Pierron G, Codogno P, Chen Q, Mehrpour M (2010) The BH3-mimetic gossypol induces both beclin 1-dependent and beclin 1-independent cytoprotective autophagy in cancer cells. J Biol Chem 285:25570–25581

Geng Y, Kohli L, Klocke BJ, Roth KA (2010) Chloroquine-induced autophagic vacuole accumulation and cell death in glioma cells is p53 independent. Neuro Oncol 12:473–481

Goussetis DJ, Altman JK, Glaser H, McNeer JL, Tallman MS, Platanias LC (2010) Autophagy is a critical mechanism for the induction of the antileukemic effects of arsenic trioxide. J Biol Chem 285:29989–29997

Guo JY, Chen HY, Mathew R, Fan J, Strohecker AM, Karsli-Uzunbas G, Kamphorst JJ, Chen G, Lemons JM, Karantza V, Coller HA, Dipaola RS, Gelinas C, Rabinowitz JD, White E (2011) Activated Ras requires autophagy to maintain oxidative metabolism and tumorigenesis. Genes Dev 25:460–470

Hara T, Nakamura K, Matsui M, Yamamoto A, Nakahara Y, Suzuki-Migishima R, Yokoyama M, Mishima K, Saito I, Okano H, Mizushima N (2006) Suppression of basal autophagy in neural cells causes neurodegenerative disease in mice. Nature 441:885–889

He C, Klionsky DJ (2009) Regulation mechanisms and signaling pathways of autophagy. Annu Rev Genet 43:67–93

Hitomi J, Christofferson DE, Ng A, Yao J, Degterev A, Xavier RJ, Yuan J (2008) Identification of a molecular signaling network that regulates a cellular necrotic cell death pathway. Cell 135:1311–1323

Horvitz HR, Shaham S, Hengartner MO (1994) The genetics of programmed cell death in the nematode *Caenorhabditis elegans*. Cold Spring Harb Symp Quant Biol 59:377–385

Ito H, Daido S, Kanzawa T, Kondo S, Kondo Y (2005) Radiation-induced autophagy is associated with LC3 and its inhibition sensitizes malignant glioma cells. Int J Oncol 26:1401–1410

Iwamaru A, Kondo Y, Iwado E, Aoki H, Fujiwara K, Yokoyama T, Mills GB, Kondo S (2007) Silencing mammalian target of rapamycin signaling by small interfering RNA enhances rapamycin-induced autophagy in malignant glioma cells. Oncogene 26:1840–1851

Juhasz G (2012) Interpretation of bafilomycin, pH neutralizing or protease inhibitor treatments in autophagic flux experiments: novel considerations. Autophagy 8

Jung CH, Jun CB, Ro SH, Kim YM, Otto NM, Cao J, Kundu M, Kim DH (2009) ULK-Atg13-FIP200 complexes mediate mTOR signaling to the autophagy machinery. Mol Biol Cell 20:1992–2003

Kanzawa T, Kondo Y, Ito H, Kondo S, Germano I (2003) Induction of autophagic cell death in malignant glioma cells by arsenic trioxide. Cancer Res 63:2103–2108

Kanzawa T, Germano IM, Komata T, Ito H, Kondo Y, Kondo S (2004) Role of autophagy in temozolomide-induced cytotoxicity for malignant glioma cells. Cell Death Differ 11:448–457

Kanzawa T, Zhang L, Xiao L, Germano IM, Kondo Y, Kondo S (2005) Arsenic trioxide induces autophagic cell death in malignant glioma cells by upregulation of mitochondrial cell death protein BNIP3. Oncogene 24:980–991

Kerr JF, Wyllie AH, Currie AR (1972) Apoptosis: a basic biological phenomenon with wide-ranging implications in tissue kinetics. Br J Cancer 26:239–257

Kim MJ, Woo SJ, Yoon CH, Lee JS, An S, Choi YH, Hwang SG, Yoon G, Lee SJ (2011) Involvement of autophagy in oncogenic K-Ras-induced malignant cell transformation. J Biol Chem 286:12924–12932

Klionsky DJ, Abdalla FC, Abeliovich H, Abraham RT, Acevedo-Arozena A, Adeli K, Agholme L, Agnello M, Agostinis P, Aguirre-Ghiso JA, Ahn HJ, Ait-Mohamed O, Ait-Si-Ali S, Akematsu T, Akira S, Al-Younes HM, Al-Zeer MA, Albert ML, Albin RL, Alegre-Abarrategui J, Aleo MF, Alirezaei M, Almasan A, Almonte-Becerril M, Amano A, Amaravadi R, Amarnath S, Amer AO, Andrieu-Abadie N, Anantharam V, Ann DK, Anoopkumar-Dukie S, Aoki H,

Apostolova N, Arancia G, Aris JP, Asanuma K, Asare NY, Ashida H, Askanas V, Askew DS, Auberger P, Baba M, Backues SK, Baehrecke EH, Bahr BA, Bai XY, Bailly Y, Baiocchi R, Baldini G, Balduini W, Ballabio A, Bamber BA, Bampton ET, Banhegyi G, Bartholomew CR, Bassham DC, Bast RC Jr, Batoko H, Bay BH, Beau I, Bechet DM, Begley TJ, Behl C, Behrends C, Bekri S, Bellaire B, Bendall LJ, Benetti L, Berliocchi L, Bernardi H, Bernassola F, Besteiro S, Bhatia-Kissova I, Bi X, Biard-Piechaczyk M, Blum JS, Boise LH, Bonaldo P, Boone DL, Bornhauser BC, Bortoluci KR, Bossis I, Bost F, Bourquin JP, Boya P, Boyer-Guittaut M, Bozhkov PV, Brady NR, Brancolini C, Brech A, Brenman JE, Brennand A, Bresnick EH, Brest P, Bridges D, Bristol ML, Brookes PS, Brown EJ, Brumell JH et al (2012) Guidelines for the use and interpretation of assays for monitoring autophagy. Autophagy 8:445–544

Koike M, Shibata M, Tadakoshi M, Gotoh K, Komatsu M, Waguri S, Kawahara N, Kuida K, Nagata S, Kominami E, Tanaka K, Uchiyama Y (2008) Inhibition of autophagy prevents hippocampal pyramidal neuron death after hypoxic-ischemic injury. Am J Pathol 172:454–469

Komatsu M, Waguri S, Ueno T, Iwata J, Murata S, Tanida I, Ezaki J, Mizushima N, Ohsumi Y, Uchiyama Y, Kominami E, Tanaka K, Chiba T (2005) Impairment of starvation-induced and constitutive autophagy in Atg7-deficient mice. J Cell Biol 169:425–434

Komatsu M, Waguri S, Chiba T, Murata S, Iwata J, Tanida I, Ueno T, Koike M, Uchiyama Y, Kominami E, Tanaka K (2006) Loss of autophagy in the central nervous system causes neurodegeneration in mice. Nature 441:880–884

Komatsu M, Wang QJ, Holstein GR, Friedrich VL Jr, Iwata J, Kominami E, Chait BT, Tanaka K, Yue Z (2007) Essential role for autophagy protein Atg7 in the maintenance of axonal homeostasis and the prevention of axonal degeneration. Proc Natl Acad Sci U S A 104:14489–14494

Kosta A, Roisin-Bouffay C, Luciani MF, Otto GP, Kessin RH, Golstein P (2004) Autophagy gene disruption reveals a non-vacuolar cell death pathway in Dictyostelium. J Biol Chem 279:48404–48409

Kroemer G, Levine B (2008) Autophagic cell death: the story of a misnomer. Nat Rev Mol Cell Biol 9:1004–1010

Kuma A, Hatano M, Matsui M, Yamamoto A, Nakaya H, Yoshimori T, Ohsumi Y, Tokuhisa T, Mizushima N (2004) The role of autophagy during the early neonatal starvation period. Nature 432:1032–1036

Laane E, Tamm KP, Buentke E, Ito K, Kharaziha P, Oscarsson J, Corcoran M, Bjorklund AC, Hultenby K, Lundin J, Heyman M, Soderhall S, Mazur J, Porwit A, Pandolfi PP, Zhivotovsky B, Panaretakis T, Grander D (2009) Cell death induced by dexamethasone in lymphoid leukemia is mediated through initiation of autophagy. Cell Death Differ 16:1018–1029

Lee JA, Liu L, Gao FB (2009) Autophagy defects contribute to neurodegeneration induced by dysfunctional ESCRT-III. Autophagy 5:1070–1072

Lee IH, Kawai Y, Fergusson MM, Rovira II, Bishop AJ, Motoyama N, Cao L, Finkel T (2012) Atg7 modulates p53 activity to regulate cell cycle and survival during metabolic stress. Science 336:225–228

Levine B, Klionsky DJ (2004) Development by self-digestion: molecular mechanisms and biological functions of autophagy. Dev Cell 6:463–477

Levine B, Kroemer G (2008) Autophagy in the pathogenesis of disease. Cell 132:27–42

Levine B, Yuan J (2005) Autophagy in cell death: an innocent convict? J Clin Invest 115:2679–2688

Li J, Hou N, Faried A, Tsutsumi S, Takeuchi T, Kuwano H (2009) Inhibition of autophagy by 3-MA enhances the effect of 5-FU-induced apoptosis in colon cancer cells. Ann Surg Oncol 16:761–771

Lin CI, Whang EE, Donner DB, Du J, Lorch J, He F, Jiang X, Price BD, Moore FD Jr, Ruan DT (2010) Autophagy induction with RAD001 enhances chemosensitivity and radiosensitivity through Met inhibition in papillary thyroid cancer. Mol Cancer Res 8:1217–1226

Liu EY, Ryan KM (2012) Autophagy and cancer—issues we need to digest. J Cell Sci 125: 2349–2358

Liu YL, Yang PM, Shun CT, Wu MS, Weng JR, Chen CC (2010) Autophagy potentiates the anti-cancer effects of the histone deacetylase inhibitors in hepatocellular carcinoma. Autophagy 6:1057–1065

Lock R, Roy S, Kenific CM, Su JS, Salas E, Ronen SM, Debnath J (2011) Autophagy facilitates glycolysis during Ras-mediated oncogenic transformation. Mol Biol Cell 22:165–178

Lockshin RA, Zakeri Z (2001) Programmed cell death and apoptosis: origins of the theory. Nat Rev Mol Cell Biol 2:545–550

Longo L, Platini F, Scardino A, Alabiso O, Vasapollo G, Tessitore L (2008) Autophagy inhibition enhances anthocyanin-induced apoptosis in hepatocellular carcinoma. Mol Cancer Ther 7:2476–2485

Luciani MF, Kubohara Y, Kikuchi H, Oshima Y, Golstein P (2009) Autophagic or necrotic cell death triggered by distinct motifs of the differentiation factor DIF-1. Cell Death Differ 16:564–570

Martin DN, Baehrecke EH (2004) Caspases function in autophagic programmed cell death in Drosophila. Development 131:275–284

Mathew R, Karantza-Wadsworth V, White E (2007) Role of autophagy in cancer. Nat Rev Cancer 7:961–967

McPhee CK, Logan MA, Freeman MR, Baehrecke EH (2010) Activation of autophagy during cell death requires the engulfment receptor Draper. Nature 465:1093–1096

Meijer AJ (2009) Autophagy research: lessons from metabolism. Autophagy 5:3–5

Meijer AJ, Codogno P (2009) Autophagy: regulation and role in disease. Crit Rev Clin Lab Sci 46:210–240

Mizushima N (2007) Autophagy: process and function. Genes Dev 21:2861–2873

Mizushima N (2010) The role of the Atg1/ULK1 complex in autophagy regulation. Curr Opin Cell Biol 22:132–139

Mizushima N, Levine B, Cuervo AM, Klionsky DJ (2008) Autophagy fights disease through cellular self-digestion. Nature 451:1069–1075

Mizushima N, Yoshimori T, Levine B (2010) Methods in mammalian autophagy research. Cell 140:313–326

Nakatogawa H, Suzuki K, Kamada Y, Ohsumi Y (2009) Dynamics and diversity in autophagy mechanisms: lessons from yeast. Nat Rev Mol Cell Biol 10:458–467

Nezis IP, Shravage BV, Sagona AP, Lamark T, Bjorkoy G, Johansen T, Rusten TE, Brech A, Baehrecke EH, Stenmark H (2010) Autophagic degradation of dBruce controls DNA fragmentation in nurse cells during late *Drosophila melanogaster* oogenesis. J Cell Biol 190:523–531

Nicholson DW, Ali A, Thornberry NA, Vaillancourt JP, Ding CK, Gallant M, Gareau Y, Griffin PR, Labelle M, Lazebnik YA et al (1995) Identification and inhibition of the ICE/CED-3 protease necessary for mammalian apoptosis. Nature 376:37–43

Nishida Y, Arakawa S, Fujitani K, Yamaguchi H, Mizuta T, Kanaseki T, Komatsu M, Otsu K, Tsujimoto Y, Shimizu S (2009) Discovery of Atg5/Atg7-independent alternative macroautophagy. Nature 461:654–658

Nishiyama J, Yuzaki M (2010) Excitotoxicity and autophagy: Lurcher may not be a model of "autophagic cell death". Autophagy 6

Nishiyama J, Matsuda K, Kakegawa W, Yamada N, Motohashi J, Mizushima N, Yuzaki M (2010) Reevaluation of neurodegeneration in lurcher mice: constitutive ion fluxes cause cell death with, not by, autophagy. J Neurosci 30:2177–2187

Opipari AW Jr, Tan L, Boitano AE, Sorenson DR, Aurora A, Liu JR (2004) Resveratrol-induced autophagocytosis in ovarian cancer cells. Cancer Res 64:696–703

Petiot A, Ogier-Denis E, Blommaart EF, Meijer AJ, Codogno P (2000) Distinct classes of phosphatidylinositol 3'-kinases are involved in signaling pathways that control macroautophagy in HT-29 cells. J Biol Chem 275:992–998

Puissant A, Robert G, Fenouille N, Luciano F, Cassuto JP, Raynaud S, Auberger P (2010) Resveratrol promotes autophagic cell death in chronic myelogenous leukemia cells via JNK-mediated p62/SQSTM1 expression and AMPK activation. Cancer Res 70:1042–1052

Puyal J, Vaslin A, Mottier V, Clarke PG (2009) Postischemic treatment of neonatal cerebral ischemia should target autophagy. Ann Neurol 66:378–389

Qu X, Zou Z, Sun Q, Luby-Phelps K, Cheng P, Hogan RN, Gilpin C, Levine B (2007) Autophagy gene-dependent clearance of apoptotic cells during embryonic development. Cell 128: 931–946

Radoshevich L, Murrow L, Chen N, Fernandez E, Roy S, Fung C, Debnath J (2010) ATG12 conjugation to ATG3 regulates mitochondrial homeostasis and cell death. Cell 142:590–600

Ramser B, Kokot A, Metze D, Weiss N, Luger TA, Bohm M (2009) Hydroxychloroquine modulates metabolic activity and proliferation and induces autophagic cell death of human dermal fibroblasts. J Invest Dermatol 129:2419–2426

Rozman-Pungercar J, Kopitar-Jerala N, Bogyo M, Turk D, Vasiljeva O, Stefe I, Vandenabeele P, Bromme D, Puizdar V, Fonovic M, Trstenjak-Prebanda M, Dolenc I, Turk V, Turk B (2003) Inhibition of papain-like cysteine proteases and legumain by caspase-specific inhibitors: when reaction mechanism is more important than specificity. Cell Death Differ 10:881–888

Ruberg M, France-Lanord V, Brugg B, Lambeng N, Michel PP, Anglade P, Hunot S, Damier P, Faucheux B, Hirsch E, Agid Y (1997) Neuronal death caused by apoptosis in Parkinson disease. Rev Neurol (Paris) 153:499–508

Rubinsztein DC, Codogno P, Levine B (2012) Autophagy modulation as a potential therapeutic target for diverse diseases. Nat Rev Drug Discov 11:709–730

Sancak Y, Bar-Peled L, Zoncu R, Markhard AL, Nada S, Sabatini DM (2010) Ragulator-Rag complex targets mTORC1 to the lysosomal surface and is necessary for its activation by amino acids. Cell 141:290–303

Sarkar S, Rubinsztein DC (2008) Huntington's disease: degradation of mutant huntingtin by autophagy. FEBS J 275:4263–4270

Scaringi L, Cornacchione P, Ayroldi E, Corazzi L, Capodicasa E, Rossi R, Marconi P (2004) Omeprazole induces apoptosis in jurkat cells. Int J Immunopathol Pharmacol 17:331–342

Scarlatti F, Maffei R, Beau I, Codogno P, Ghidoni R (2008a) Role of non-canonical Beclin 1-independent autophagy in cell death induced by resveratrol in human breast cancer cells. Cell Death Differ 15:1318–1329

Scarlatti F, Maffei R, Beau I, Ghidoni R, Codogno P (2008b) Non-canonical autophagy: an exception or an underestimated form of autophagy? Autophagy 4:1083–1085

Scott RC, Schuldiner O, Neufeld TP (2004) Role and regulation of starvation-induced autophagy in the Drosophila fat body. Dev Cell 7:167–178

Seglen PO, Gordon PB (1982) 3-Methyladenine: specific inhibitor of autophagic/lysosomal protein degradation in isolated rat hepatocytes. Proc Natl Acad Sci U S A 79:1889–1892

Shao Y, Gao Z, Marks PA, Jiang X (2004) Apoptotic and autophagic cell death induced by histone deacetylase inhibitors. Proc Natl Acad Sci U S A 101:18030–18035

Shen HM, Codogno P (2011) Autophagic cell death: Loch Ness monster or endangered species? Autophagy 7:457–465

Shen S, Kepp O, Michaud M, Martins I, Minoux H, Metivier D, Maiuri MC, Kroemer RT, Kroemer G (2011) Association and dissociation of autophagy, apoptosis and necrosis by systematic chemical study. Oncogene 30:4544–4556

Shen S, Kepp O, Kroemer G (2012) The end of autophagic cell death? Autophagy 8:1–3

Shimizu S, Kanaseki T, Mizushima N, Mizuta T, Arakawa-Kobayashi S, Thompson CB, Tsujimoto Y (2004) Role of Bcl-2 family proteins in a non-apoptotic programmed cell death dependent on autophagy genes. Nat Cell Biol 6:1221–1228

Shimizu S, Konishi A, Nishida Y, Mizuta T, Nishina H, Yamamoto A, Tsujimoto Y (2010) Involvement of JNK in the regulation of autophagic cell death. Oncogene 29:2070–2082

Smith DM, Patel S, Raffoul F, Haller E, Mills GB, Nanjundan M (2010) Arsenic trioxide induces a beclin-1-independent autophagic pathway via modulation of SnoN/SkiL expression in ovarian carcinoma cells. Cell Death Differ 17(12):1867–1881

Tsujimoto Y, Shimizu S (2005) Another way to die: autophagic programmed cell death. Cell Death Differ 12(Suppl 2):1528–1534

Vandenabeele P, Galluzzi L, Vanden Berghe T, Kroemer G (2010) Molecular mechanisms of necroptosis: an ordered cellular explosion. Nat Rev Mol Cell Biol 11:700–714
Vogel MW, Caston J, Yuzaki M, Mariani J (2007) The Lurcher mouse: fresh insights from an old mutant. Brain Res 1140:4–18
White E (2008) Autophagic cell death unraveled: pharmacological inhibition of apoptosis and autophagy enables necrosis. Autophagy 4:399–401
White E (2012) Deconvoluting the context-dependent role for autophagy in cancer. Nat Rev Cancer 12:401–410
Wu YT, Tan HL, Huang Q, Kim YS, Pan N, Ong WY, Liu ZG, Ong CN, Shen HM (2008) Autophagy plays a protective role during zVAD-induced necrotic cell death. Autophagy 4: 457–466
Wu YT, Tan HL, Huang Q, Ong CN, Shen HM (2009) Activation of the PI3K-Akt-mTOR signaling pathway promotes necrotic cell death via suppression of autophagy. Autophagy 5:824–834
Wu YT, Tan HL, Shui G, Bauvy C, Huang Q, Wenk MR, Ong CN, Codogno P, Shen HM (2010) Dual role of 3-methyladenine in modulation of autophagy via different temporal patterns of inhibition on class I and III phosphoinositide 3-kinase. J Biol Chem 285:10850–10861
Wu YT, Tan HL, Huang Q, Sun XJ, Zhu X, Shen HM (2011) zVAD-induced necroptosis in L929 cells depends on autocrine production of TNFalpha mediated by the PKC-MAPKs-AP-1 pathway. Cell Death Differ 18(1):26–37
Xiong HY, Guo XL, Bu XX, Zhang SS, Ma NN, Song JR, Hu F, Tao SF, Sun K, Li R, Wu MC, Wei LX (2010) Autophagic cell death induced by 5-FU in Bax or PUMA deficient human colon cancer cell. Cancer Lett 288(1):68–74
Yang Z, Klionsky DJ (2010) Mammalian autophagy: core molecular machinery and signaling regulation. Curr Opin Cell Biol 22:124–131
Yousefi S, Perozzo R, Schmid I, Ziemiecki A, Schaffner T, Scapozza L, Brunner T, Simon HU (2006) Calpain-mediated cleavage of Atg5 switches autophagy to apoptosis. Nat Cell Biol 8:1124–1132
Yu L, Alva A, Su H, Dutt P, Freundt E, Welsh S, Baehrecke EH, Lenardo MJ (2004) Regulation of an ATG7-beclin 1 program of autophagic cell death by caspase-8. Science 304:1500–1502
Yu L, Wan F, Dutta S, Welsh S, Liu Z, Freundt E, Baehrecke EH, Lenardo M (2006) Autophagic programmed cell death by selective catalase degradation. Proc Natl Acad Sci U S A 103:4952–4957
Yu SW, Baek SH, Brennan RT, Bradley CJ, Park SK, Lee YS, Jun EJ, Lookingland KJ, Kim EK, Lee H, Goudreau JL, Kim SW (2008) Autophagic death of adult hippocampal neural stem cells following insulin withdrawal. Stem Cells 26:2602–2610
Yue Z, Jin S, Yang C, Levine AJ, Heintz N (2003) Beclin 1, an autophagy gene essential for early embryonic development, is a haploinsufficient tumor suppressor. Proc Natl Acad Sci U S A 100:15077–15082
Yue Z, Friedman L, Komatsu M, Tanaka K (2009) The cellular pathways of neuronal autophagy and their implication in neurodegenerative diseases. Biochim Biophys Acta 1793:1496–1507
Yuzaki M (2003) The delta2 glutamate receptor: 10 years later. Neurosci Res 46:11–22
Zhang J, Zhang Y, Li J, Xing S, Li C, Li Y, Dang C, Fan Y, Yu J, Pei Z, Zeng J (2012) Autophagosomes accumulation is associated with beta-amyloid deposits and secondary damage in the thalamus after focal cortical infarction in hypertensive rats. J Neurochem 120:564–573
Zhao Y, Yang J, Liao W, Liu X, Zhang H, Wang S, Wang D, Feng J, Yu L, Zhu WG (2010) Cytosolic FoxO1 is essential for the induction of autophagy and tumour suppressor activity. Nat Cell Biol 12:665–675
Zoncu R, Bar-Peled L, Efeyan A, Wang S, Sancak Y, Sabatini DM (2011) mTORC1 senses lysosomal amino acids through an inside-out mechanism that requires the vacuolar H(+)-ATPase. Science 334:678–683
Zong WX, Thompson CB (2006) Necrotic death as a cell fate. Genes Dev 20:1–15

Chapter 13
Autophagy in Necrosis: A Force for Survival

Han-Ming Shen and Patrice Codogno

13.1 Introduction

Autophagy, which includes macroautophagy, microautophagy, and chaperone-mediated autophagy, is a highly conserved degradation pathway for bulk cellular components (Mizushima and Komatsu 2011). Macroautophagy (hereafter referred to autophagy in this review), the most prevalent form of autophagy, is morphologically featured by the formation of double-membrane autophagosomes which sequester impaired organelles or unwanted cellular components and deliver them to lysosomes for degradation and recycling (Klionsky and Emr 2000).

Although there is basal level of autophagy in all living organisms, autophagy is readily induced by various cellular factors, including deprivation of nutrients (amino acids and glucose), withdrawal of growth factors, and many other stress conditions, such as hypoxia, oxidative stress, endoplasmic reticulum (ER) stress, DNA damage, etc. (Yang and Klionsky 2010). It has been established that the autophagic process is tightly controlled by a group of autophagy-related genes (Atg). To date, in yeast, more than 30 ATG s have been discovered, and many of their mammalian orthologs

H.-M. Shen (✉)
Department of Physiology, Yong Loo Lin School of Medicine,
National University of Singapore, Singapore, Singapore
e-mail: han-ming_shen@nuhs.edu.sg

P. Codogno
French Institute of Health and Medical Research, University Paris-Descartes,
75015 Paris, France

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have also been identified as the machinery to execute the autophagic process (Rubinsztein et al. 2007; Yang and Klionsky 2009). One important concept is that autophagy is a dynamic process consisting of several consecutive stages: (1) induction or initiation, which involves formation of phagophore and is negatively regulated by mammalian target of rapamycin (mTOR) via the ULK1/Atg1 complex; (2) nucleation, which is mediated by the Beclin 1 and hVps34/class III PI3K complex; (3) elongation, which is a critical step in forming the complete autophagosomes controlled by two ubiquitin-like conjugation systems (Atg12–Atg5 system and the LC3/Atg8 system); and finally (4) maturation and degradation, which involves fusion with endosomes–lysosomes to form autolysosomes and degradation of the inner membrane together with its luminal contents (Mizushima 2007; Chan and Tooze 2009; Orsi et al. 2010).

Autophagy has been demonstrated to be implicated in a wide variety of physiological and pathological processes, including cell survival, cell death, cell metabolism, development, infection and immunity, and aging (Mizushima 2009; Mehrpour et al. 2010). Recently, the critical role of autophagy in energy metabolism, cellular homeostasis, and cell and tissue renovation has also been increasingly appreciated (Mizushima and Komatsu 2011). More importantly, autophagy is closely involved in the etiology of many important human diseases, including cancer, neurodegenerative diseases and metabolic disorders (Mizushima et al. 2008; Meijer and Codogno 2009; White et al. 2010).

Among various biological functions of autophagy, its involvement in cell death/cell survival is the one deserving particular attention. It appears that autophagy has a rather complicated relationship with the cell death program. On the one hand, autophagy has been well established as an important cell survival mechanism, especially in cells under stress conditions such as starvation (Cecconi and Levine 2008; Kroemer and Levine 2008). On the other hand, autophagy has been implicated in the cell death process, either in apoptosis, or in non-apoptotic or necrotic cell death, including autophagic cell death in which autophagy itself serves as the cell death machinery (Lenardo et al. 2009; Giusti et al. 2010; Shimizu et al. 2010; Shen and Codogno 2011). At present, there are numerous excellent reviews focusing on relationship between autophagy and apoptosis (Luo and Rubinsztein 2007; Maiuri et al. 2007; Levine et al. 2008; Corcelle et al. 2009; Eisenberg-Lerner et al. 2009; Djavaheri-Mergny et al. 2010; Fimia and Piacentini 2010; Giansanti et al. 2011). Nevertheless, the interplay between autophagy and non-apoptotic or necrotic cell death has not been well discussed. In this chapter we focus on the role of autophagy in necrotic cell death. We first present the evidence showing the anti-necrosis function of autophagy, and then discuss the biological significance of the anti-necrosis function of autophagy in cancer and ischemia–reperfusion injury. Based on the current literature, we believe that one important aspect of the pro-survival function of autophagy is achieved via its ability to block various forms of necrotic cell death.

13.2 The Pro-death Function of Autophagy

At present, it is still a rather controversial topic in the field of autophagy study regarding the role of autophagy in the cell death program. Evidence coexists for the pro-death and pro-survival function of autophagy. The involvement of autophagy in apoptotic cell death is relatively better studied and understood, while there are more doubts and disputes regarding the relationship between autophagy and non-apoptotic cell death, especially about the concept of "autophagic cell death."

13.2.1 Autophagy Promotes Apoptosis

The interplay between autophagy and apoptosis, the most well-studied form of programmed cell death (PCD), has been extensively discussed. Due to the presence of intricate interplays, it is not uncommon to see that a variety of stimuli such as ROS, ER stress, and DNA damage agents can activate apoptosis and autophagy simultaneously, and promotion of autophagy is accompanied by the induction of apoptosis.

Such outcomes are determined by the cross talks between autophagy and apoptosis at different levels. First, both autophagy and apoptosis are mediated by a common set of proteins. The tumor suppressor p53, the death-associated protein kinase-1 (DAPK), the proapoptotic "BH3-only" proteins, and several Atg proteins are known to play a "positive" role in promoting both autophagy and apoptosis, Conversely, some factors including the anti-apoptosis Bcl-2 family members, such as Bcl-2, Bcl-xL, and Mcl-1, are known to play a "negative" role in suppressing both autophagy and apoptosis. For instance, the cleaved form of Atg5 by calpains has been found to translocate from cytosol to mitochondria to promote cytochrome c release and intrinsic apoptosis pathway (Yousefi et al. 2006). A recent report from Kimchi's group demonstrated that the essential autophagy protein Atg12 acts as a positive mediator of mitochondrial apoptosis via direct binding and inactivating the prosurvival Bcl-2 family proteins (Bcl-2 and Mcl-1) (Rubinstein et al. 2011).

The second level of interaction is based on the fact that there are proteins having opposite function in autophagy and apoptosis. One good example is that caspases, essential for apoptosis, are able to suppress autophagy through cleavage of Beclin-1 and Atg4D (Betin and Lane 2009; Wirawan et al. 2010).

The third level of interaction between autophagy and apoptosis is more at the functional level. Qu et al. found that autophagy and apoptosis are partners in embryonic cavitation as autophagy is required to clear up cell corpses from the apoptotic cell death, an important process in embryonic development (Qu et al. 2007).

13.2.2 *Autophagic Cell Death*

The concept of autophagic cell death was first developed based on the morphologic features: the presence of autophagosomes in dying cells, suggesting that autophagy is closely linked to cell death (Schweichel and Merker 1973; Bursch 2001). At present, the most convincing evidence supporting autophagic cell death is from model organisms, other than mammals (Shen and Codogno 2011). For instance, autophagy has been convincingly demonstrated as a cell death mechanism in *Dictyostelium discoideum*, a soil amoeba which is known to be defective in the apoptosis machinery, as *ATG1*-mutation decreases autophagy and suppresses cell death (Kosta et al. 2004). Also it has been well reported that the midgut cell death in *Drosophila* is autophagy-dependent, based on the observations that suppression of autophagy by overexpression of *ATG2* and *ATG18* mutants or by *ATG1* and *ATG18* knockdown caused significant delay in midgut removal (Denton et al. 2009). In mammalian cells, most of the reported autophagic cell death was from in vitro cell culture models in which the apoptosis machinery is impaired or suppressed. For instance, Shimizu et al. found that in response to DNA damage caused by etoposide, the Bax−/− and Bak−/− double knockout mouse embryonic fibroblasts (MEFs) underwent non-apoptotic cell death that was autophagy-dependent (Shimizu et al. 2004). Another group reported that zVAD, a pan-caspase inhibitor, induced autophagic cell death in a number of cell lines such as fibrosarcoma L929 cells (Yu et al. 2004).

However, with the increasing understanding of the autophagy, especially the dynamic process of autophagy, it came to the realization that the mere presence of autophagosomes or the increase of autophagic markers in the dying cells could not confer a conclusion that autophagy is indeed a cell death mechanism. There are two important aspects that need to be determined and differentiated: (1) cell death **with** autophagy and (2) cell death **by** autophagy (Galluzzi et al. 2008; Kroemer and Levine 2008). To make the situation even more complicated, it is also important to note that the increase of autophagic markers also does not necessary mean the increase of autophagic level, as blockage of the autophagic flux at the late stage (such as the suppression of lysosomal function) will markedly enhance the autophagic makers in the cell (Klionsky et al. 2008; Mizushima et al. 2010). Therefore, in defining autophagic cell death, it is critically important to address the following two points: (1) whether there is a real increase of autophagic flux level, (2) whether the increased autophagy really contributes to cell death.

In order to clarify this point, recently we have proposed the following criteria for defining autophagic cell death (Shen and Codogno 2011): (1) Cell death occurs without the involvement of the apoptosis machinery, such as caspase activation; (2) There is an increase of autophagic flux, and not just an increase of the autophagic markers, in the dying cells; and (3) Suppression of autophagy via both pharmacological inhibitors and genetic approaches (either ATG siRNA knockdown, or overexpression of dominant negative ATG genes) is able to rescue or prevent cell death. We believe that setting up such criteria will help to clarify the controversies, and it would not be surprised to see many of the earlier reported autophagic cell

death fail to reach these criteria, and thus, it is subject to further confirmation to see whether cells really die via “autophagic cell death.” In fact, very recent discoveries from Kroeme’s lab even challenged the presence of autophagic cell death in mammalian cells (Shen et al. 2011, 2012). Based on their study, among approximately 1,400 compounds screened in a panel of human cancer cells, there is not even a single compound that would induce cell death by autophagy, indicating the possibility that cell death is rarely, if ever, executed by autophagy in human cells (Shen et al. 2011).

13.3 The Pro-survival Function of Autophagy

13.3.1 Autophagy Blocks Apoptosis

Although there is accumulating evidence suggesting the role of autophagy in the cell death process, as discussed above, autophagy has been generally recognized as pro-survival mechanism especially for cells and organisms under stress conditions such as starvation. The most convincing evidence supporting the pro-survival function of autophagy is from animal models in which the Atgs are deleted. For instance, Atg5/Atg7 defective mice are unable to survive through neonatal period, demonstrating the crucial pro-survival function of autophagy (Kuma et al. 2004; Komatsu et al. 2005). Generally, the pro-survival function of autophagy could be achieved via (1) blockage of apoptosis and (2) suppression of non-apoptotic/necrotic cell death. We have briefly discussed the pro-apoptotic function of autophagy in Sect. 2.1 above. It is believed that whether autophagy acts to promote or block apoptosis is context-dependent, including the nature of the stimuli, the cell type, and level of autophagic flux. At present, the mechanisms for the anti-apoptotic function of autophagy are less studied. One possibility is that autophagy promotes the degradation of apoptotic proteins such as caspases. One such example is TRAIL-mediated apoptosis. It has been reported that activated caspase 8 was targeted to autophagosomes and subsequently degraded in lysosomes, preventing activation of downstream apoptotic effectors in TRAIL-treated cancer cells (Hou et al. 2010). Importantly, the anti-apoptotic function of autophagy has also been demonstrated in other systems in which apoptosis was induced in various cancer cells by epidermal growth factor receptor tyrosine kinase inhibitors (gefitinib) or COX-2 inhibitor (celecoxib) (Huang and Sinicrope 2010; Han et al. 2011b). Therefore, understanding the anti-apoptotic function of autophagy is important for developing novel cancer therapeutic strategies by suppression of autophagy. For instance, inhibition of autophagy by chloroquine was able to sensitize apoptosis-resistant tumor cells to DNA damage agents in chronic myelogenous leukemia (CML) cell lines, primary CML, and a mouse model of lymphoma (Amaravadi et al. 2007; Carew et al. 2007). Several ongoing clinical trials using chloroquine alone or in combination with some cancer therapeutic agents (http://www.clinicaltrials.gov/ct2/results?term=chloroquine%2C+cancer&Search=Search) are also partly based on such a rationale.

13.3.2 Autophagy Suppresses Necrosis/Non-apoptotic Cell Death

Necrosis or non-apoptosis has been historically considered as an accidental and uncontrolled form of cell death. However, in the past decade, necrosis has been discovered to be also a programmed and regulated cell death process (Fiers et al. 1999; Edinger and Thompson 2004; Zong and Thompson 2006). Among various forms of necrotic cell death, necroptosis and PARP-mediated necrosis have emerged as two important forms with increased understanding of the molecular mechanisms and biological functions. Here we present a more detailed discussion on the functional interaction between autophagy and these two forms of necrotic cell death.

13.3.2.1 Autophagy Suppresses Necroptosis

Necroptosis refers to a specific form of caspase-independent, non-apoptotic or necrotic cell death, with the following features: (1) it is mediated by cell death ligands (TNF-α and FasL) via their respective cell death receptors; (2) it is controlled via a unique downstream signaling pathway involving RIP1-RIP3 and (3) it is susceptible to the inhibitory effect of a specific RIP1 inhibitor, necrostatin (Degterev and Yuan 2008; Hitomi et al. 2008; Christofferson and Yuan 2010). More recently, Wang's group has identified two important target proteins mixed lineage kinase domain-like protein (MLKL) and mitochondrial phosphatase PGAM5 as downstream targets of RIP1 and RIP3 in both TNFR-triggered necroptosis, as well as in intrinsic necrosis induced by reactive oxygen species (Sun et al. 2012; Wang et al. 2012). At present, the physiological and pathological function of necroptosis has also been increasingly understood. For instance, necroptosis has been implicated in inflammatory response against virus infections, embryogenesis, and lymphocyte function (Cho et al. 2009; Kaiser et al. 2011; Zhang et al. 2011).

There is a rather complicated relationship between autophagy and necroptosis, a topic covered by one of our recent reviews (Shen and Codogno 2012). Although majority reports support the notion that autophagy serves as an anti-necroptosis function, there is evidence suggesting otherwise. For instance, a recent study reported that palmitic acid (PA) mediates autophagy which contributes to necroptosis in human umbilical vein endothelial cells (Khan et al. 2012). However, such a conclusion is contradictory to our findings that PA induces autophagy to protect against the lipotoxicity of PA (Tan et al. 2012). Here we would like to summarize the intricate relationship between autophagy and necroptosis using zVAD-mediated cell death as an example. zVAD is a short peptide and widely used as general caspase inhibitor for apoptosis study. Intriguingly, zVAD has also been shown to either sensitize TNF-mediated necrosis or itself is able to induce cell death in a selected number of cells (Vercammen et al. 1998; Holler et al. 2000). There were two conflicting explanations for zVAD-mediated cell death: zVAD induces autophagic cell death (Yu et al. 2004) or necroptosis (Degterev et al. 2008). Recently we have

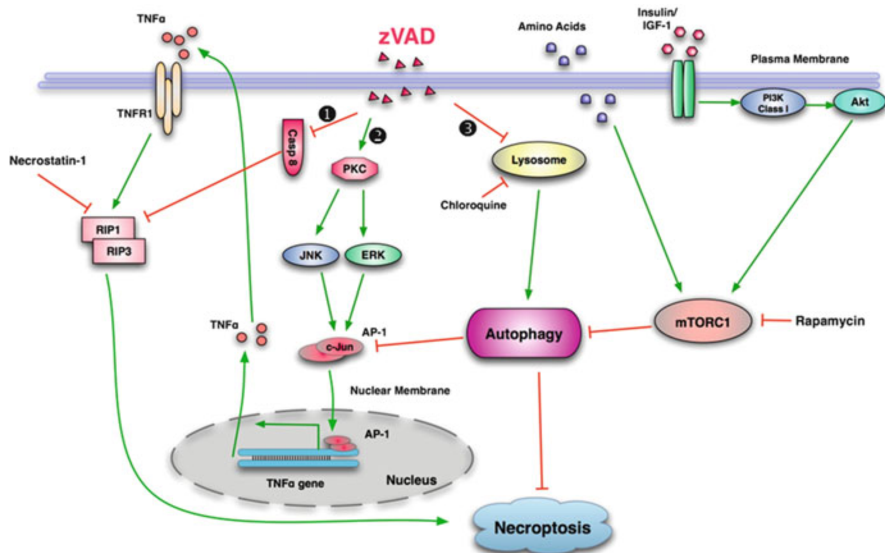

Fig. 13.1 Autophagy is a pro-survival mechanism in zVAD-induced necroptosis. zVAD has 3 major effects: (*1*) suppression of apoptosis via inhibition of the caspase cascade, (*2*) induction of necroptosis via promotion of TNFa autocrine mediated by PKC-MAPK-AP-1 pathway, and (*3*) suppression of autophagy via inhibition of the lysosomal cathepsins

attempted to elucidate the molecular mechanisms underlying zVAD-induced cell death and its relationship with autophagy via a series of work (Wu et al. 2008, 2009, 2011), with the following observations (1) zVAD induces necroptosis, but not autophagic cell death in L929 cells. (2) autophagy is a pro-survival mechanism that acts to protect against zVAD-induced necroptosis. (3) zVAD does not induce autophagy, rather it can block autophagy via its inhibitory effect on lysosomal cathepsins. (4) zVAD-induced necroptosis was that zVAD-induced necroptosis is depending on the TNFα autocrine which is mediated via a pathway involving nuclear transcription factor activating protein-1 (AP-1) but not NF-κB, downstream of two mitogen-activated kinases (MAPKs), JNK and extracellular signal-regulated kinase (ERK). Figure 13.1 summarizes the intricate relationship between autophagy and zVAD-induced necroptosis. It is believed that understanding the pro-survival function of autophagy against zVAD-induced necroptosis offers a new line of evidence for the pro-survival function of autophagy.

13.3.2.2 Autophagy Suppresses PARP-Mediated Necrosis

PARP is a family of nuclear enzymes with multiple functions in chromatin structure, transcription, and genomic integrity that are achieved through poly(ADP-ribosyl) ation (Jagtap and Szabo 2005; Krishnakumar and Kraus 2010). It is known that

Table 13.1 Protective role of autophagy in PARP-mediated necrotic cell death

Refs	DNA damage agents	Cell type	Autophagy level	Autophagy function	Main mechanisms
(Zhou et al. 2013)	MNNG	Bax-Bak DKO MEFs	Induced	Protective	PARP activation and AMPK-mediated mTOR suppression
(Ethier et al. 2012)	MNNG	Human embryonic kidney HEK293	Induced	Protective	PARP activation and AMPK-mediated mTOR suppression
(Alexander et al. 2010)	H_2O_2	MCF7, HEK293	Induced	Protective	ATM-mediated mTOR suppression
(Munoz-Gamez et al. 2009)	Doxorubicin	NIH3T3 MEFs	Induced	Protective	PARP activation and mTOR suppression
(Yang et al. 2011)	Gelomulide K	Human breast cancer cells	Induced	Protective	ROS and PARP activation
(Hwang et al. 2010)	Cigarette smoke	Lung epithelial cells	Induced	Protective	SIRT1 and PARP activation
(Albert et al. 2007)	Ionizing radiation	H460 lung cancer cells	Induced	Protective	PARP activation

poly(ADP-ribosyl)ation is an energetically expensive process, causing rapid depletion of intracellular ATP, and eventually necrotic cell death (Ha and Snyder 1999; Zong et al. 2004). PARP-mediated cell death is considered as another important form of programmed necrosis (Degterev and Yuan 2008), and it has recently been classified as "Parthanatos" based on the new recommendation by the Nomenclature Committee on Cell Death (NCCD) (Galluzzi et al. 2012).

So far, there is substantial evidence showing the functional link between PARP-mediated necrosis and autophagy. PARP is readily activated by DNA damage, mainly DNA strand breaks in response to oxidative stress/ROS or DNA damage agents such as DNA alkylating agents (Martin et al. 2000; Zong et al. 2004). Since autophagy is inducible by disturbance of the cellular energy homeostasis, the involvement of autophagy in PARP-mediated cell death has thus attracted substantial attention. Table 13.1 summarizes some of the relevant studies showing the pro-survival function of autophagy in PARP-mediated necrosis. For instance, two recent studies including one from our own laboratory have provided clear evidence that a DNA Alkylating Agent (MNNG) induces PARP1 activation, ATP depletion, and AMP-activated protein kinase (AMPK) activation, leading to autophagy which acts as a protective mechanism to reduce cell death (Ethier et al. 2012; Zhou et al. 2013).

Among the various mechanisms linking PARP activation with autophagy induction, activation of AMPK appears to be particularly relevant. AMPK is a key sensor

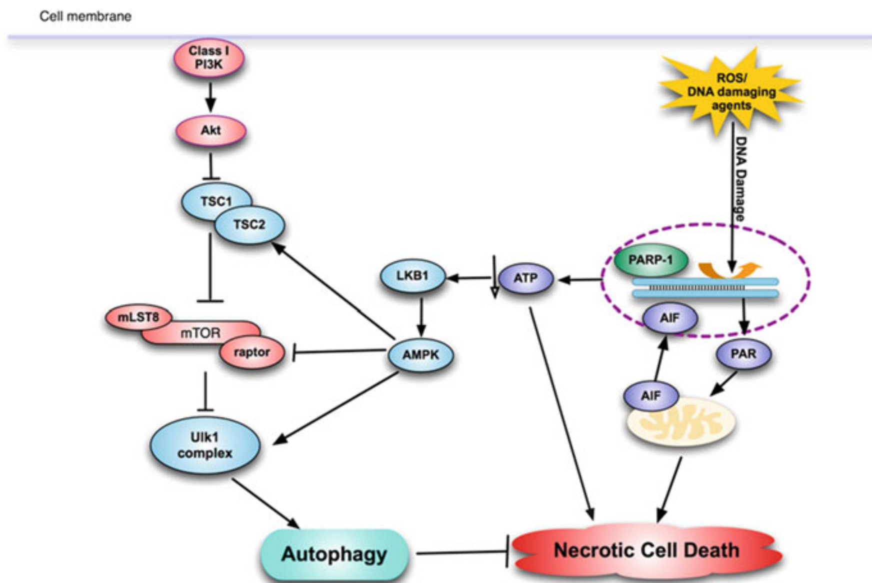

Fig. 13.2 The pro-survival function of autophagy in PAPR-mediated necrotic cell death. In cells under oxidative stress or treated with DNA damage agents, DNA damage (strand breaks) leads to massive PARP activation and rapid ATP depletion, which then activates autophagy mainly via AMPK activation and mTOR suppression. Such inducible autophagy protects against cell death

for cellular energy status and is readily activated with decreased ATP levels and increased AMP:ATP ratio (Hardie 2007). AMPK is known to play a key role in autophagy, via the following pathways: (1) AMPK activation leads to suppression of mTORC1 by either phosphorylation of TSC2 or raptor (Inoki et al. 2003) (Gwinn et al. 2008). (2) AMPK is able to directly phosphorylate and activate the Ulk1 complex (Egan et al. 2011; Kim et al. 2011). Therefore, it is believed that PARP activation has a dual role in deciding the cell fate: (1) induction of necrotic cell death due to acute ATP depletion and AIF nuclear translocation, and (2) protection against cell death via induction of autophagy. The exact outcome for cell death or survival is thus depending on the balance of these two opposite forces, which may differ depending on the cellular context, including the nature of DNA damage agents and status of PARP function. Figure 13.2 summarizes the molecular mechanisms mediating PARP-induced induction of autophagy and its functional impact on cell death under DNA damage induced by either ROS or DNA damage agents.

Understand the pro-survival function of autophagy in PARP-mediated cell death is very relevant to cancer chemotherapy. At present, PARP is a promising cancer therapeutic target and suppression of PRAP by small chemical inhibitors has found to be an effective therapeutic strategy, especially in breast cancer with mutant BRCA1/2 (Lord and Ashworth 2008; Anders et al. 2010). It remains to be tested

whether suppression of PARP will affect the autophagic response in those cancer cells, and whether modulation of autophagy will cast any positive or negative effect on the therapeutic efficacy of those PARP inhibitors.

13.3.3 Autophagy Blocks Other Forms of Non-apoptotic Cell Death

Besides necroptosis and PARP-mediated cell death as described above, cells employ other non-apoptotic machineries to execute cell death such as mitotic catastrophe and glyceraldehyde-3-phosphate dehydrogenase (GAPDH)-related caspase-independent cell death (CICD).

Mitotic catastrophe, originally described as a result of aberrant chromosome segregation in yeast, is defined as a type of cell death that occurs during mitosis (Vakifahmetoglu et al. 2008; Vitale et al. 2011). At present, the signaling cascade controlling mitotic catastrophe is poorly understood. As a form of non-apoptotic cell death, the main function of mitotic catastrophe is to eliminate cells with mitotic defects and thus to maintain the genome stability and suppress oncogenesis (Vitale et al. 2011). Induction of mitotic catastrophe is also becoming an effective approach in cancer therapy as many of the chemotherapeutic agents are found to induce of mitotic catastrophe in human cancer cells (Jin et al. 2007; Portugal et al. 2010). However, little is known about the relationship between autophagy and mitotic catastrophe. There are some preliminary findings indicating the presence of these two events in cancer cells under chemotherapy, while no clue is given about the possible functional interaction between them (O'Sullivan-Coyne et al. 2009; Grzanka et al. 2010). Moreover, there are other studies showing mitotic catastrophe induced either by a UV-inactivated parvovirus or by a polynuclear platinum compound is not associated with autophagy (Shingu et al. 2010; Fragkos and Beard 2011). Therefore, further studies are needed to address the functional relationship between these two important events in deciding the cell fate in response to DNA damage and cell cycle arrest.

GAPDH is a house-keeping gene that has been increasingly appreciated as an important regulator in many processes including glucose metabolism, cell death, and tumor progress (Colell et al. 2009; Tristan et al. 2011). Earlier studies from Green's group have demonstrated the pro-survival function of autophagy in a form of CICD. In their studies, ectopic expression GAPDH in HeLa cells offer significant protection against CICD when cells were treated with staurosporine (STS), etoposide (Etop.), or actinomycin D (Act D) in the presence of a pan-caspase inhibitor qVD-oph (Colell et al. 2007). Moreover, the protective effect of GAPDH is found to be associated with autophagy based on the evidence that GAPDH is able to up-regulate Atg12 transcription and suppression of autophagy by chemical inhibitors or stable knockdown of Atg5 abolished the protective effect of GAPDH (Colell et al. 2007). Therefore, understanding the anti-necrosis effect of autophagy mediated by GAPDH provides another example illustrating the pro-survival function of autophagy.

13.4 The Biological Significance of the Anti-necrosis Function of Autophagy

Accumulating evidence has clearly indicated that programmed necrosis, as a conserved cell death pathway, plays essential roles in many physiological and pathological processes including tissue injury, immune system regulation, infection, inflammation, septic shock, neurodegenerative diseases and tumorigenesis (Christofferson and Yuan 2010; Han et al. 2011a; Wu et al. 2012). Therefore, modulation of necrosis has emerged as a potential therapeutic strategy for a number of diseases (Sodeoka and Dodo 2010). On the one hand, suppression of necrosis can save cells from ischemia-reperfusion injury such as cardiac infarct and brain injury (Degterev et al. 2005; Tavernarakis 2007; Sims and Muyderman 2010). On the other hand, acceleration of necrosis can serve as a backup route to execute cell death for tumor cells where the apoptotic machinery is defective or impaired (de Bruin and Medema 2008; Speirs et al. 2011). Therefore, understanding the pro-survival function of autophagy against various forms of necrosis is important for development of such novel therapeutic approaches.

13.4.1 Anti-necrosis Function of Autophagy in Cancer

Establishing the anti-necrosis function of autophagy has two important implications in cancer. First, the pro-survival function autophagy against necrosis is closely related to its anticancer ability. A series studies from White's group have provided convincing evidence that during the tumorigenesis process, there is progressive adverse conditions including oxidative stress and metabolic stress that result in necrotic cell death (especially in those cells with defective apoptosis machinery), and occurrence of such necrotic cell death would lead to inflammation, DNA, genome instability, all contributing to the tumorigenic process (Degenhardt et al. 2006; Karantza-Wadsworth et al. 2007; Mathew et al. 2007b), as summarized in Fig. 13.3 below. Therefore, the anti-necrotic function of autophagy is able to stop this vicious cycle and block and delay the tumorigenesis and accordingly, promotion of autophagy is believed to be conducive for cancer prevention.

The second important implication of the anti-necrosis function of autophagy is related to cancer therapy. Nowadays it has been well recognized that autophagy is a double edged sword in cancer, i.e., the pro-survival function of autophagy may give the cancer cells an edge to resist the killing effects of therapeutic agents (Mathew et al. 2007a; White and DiPaola 2009). Moreover, many of the cancer therapeutic agents are capable of induce autophagy via various pathways and suppression of such inducible autophagy is able to enhance cell death and therapeutic efficacy (Rosenfeldt and Ryan 2009; White and DiPaola 2009; Chen and Debnath 2010; Dikic et al. 2010). Although most of existing evidence demonstrates that suppression of autophagy will enhance the susceptibility of cancer cells to apoptosis, there

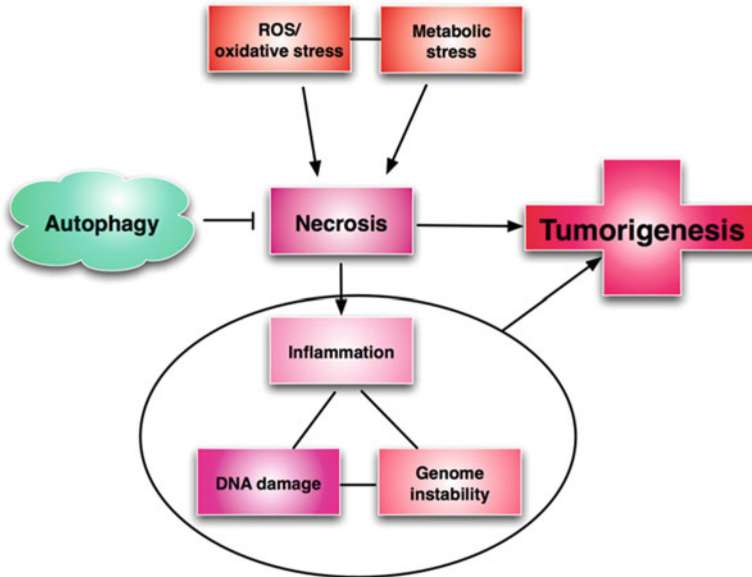

Fig. 13.3 The anti-necrosis effect of autophagy is implicated in its anticancer function. During tumorigenesis process, those cells with deficient or impaired apoptotic machinery undergo necrotic cell death in response to oxidative stress and metabolic stress, leading to sequent events including inflammation, DNA damage and genome instability and eventually tumorigenesis. Autophagy is able to stop this vicious cycle via suppression of necrosis

are cases showing that suppression of autophagy promotes necrotic cell death in response to cancer therapeutics. For example, in tumor cells, chemical blockade of autophagy or genetic knockdown of Beclin 1 aggravated necrotic cell death ovarian cancer cells under treatment of FTY720, a sphingosine analog with immune-suppressing activity (Zhang et al. 2010). Such findings indicate that the anti-necrotic function of autophagy could be exploited as a novel cancer therapeutic strategy.

13.4.2 Anti-necrosis Function of Autophagy in Ischemia–Reperfusion Injury

Ischemia–reperfusion injury is an important mechanism for tissue damage in several pathological conditions, including stroke, heart attack, and organ transplantation (Laskowski et al. 2000; Zhao 2007; Kosieradzki and Rowinski 2008). Necrosis is one of the cell death modalities induced by ischemia–reperfusion injury, together with apoptotic cell death (van Wijk and Hageman 2005; Iliodromitis et al. 2007). Importantly, autophagy is induced by ischemia–reperfusion via multiple pathways including ROS/oxidative stress and AMPK activation (Matsui et al. 2007; Hariharan et al. 2011). Consistent with the well-reported pro-survival function of autophagy as

discussed in the earlier sections of this review, the increased level of autophagy under ischemia–reperfusion condition is believed to serve as an important protective mechanism in mitigating the injury (Hamacher-Brady et al. 2006; Sadoshima 2008; Jiang et al. 2010; Jin et al. 2010). Therefore, tools for activating autophagy have the therapeutic value in various clinical settings, including stroke, heart attack and organ transplantation.

13.4.3 Anti-necrosis Function of Autophagy in Inflammation

One important consequence following necrosis is the induction of inflammation at the site of tissue injury and damage, as a result of release of the pro-inflammatory substances from ruptured necrotic cells (Patel et al. 2009). High-mobility group box 1 (HMGB1), a chromatin-binding nuclear protein and damage-associated molecular pattern molecule is one of such important factors that is released from the necrotic cells and contributes to the inflammatory responses (Bustin 2002; Ditsworth et al. 2007; Raucci et al. 2007). More importantly, recent studies have also linked autophagy, necrosis and inflammation via HMGB1. In the course of autophagy induced by starvation, rapamycin, or oxidative stress, HMGB1 is translocated from nucleoli to cytosol and the cytosolic HMGB1 is able to promote autophagy via binding to Beclin 1 (Kang et al. 2010; Tang et al. 2010a, b). Therefore, it is reasonable to speculate that in the course of necrosis, the enhanced level of cytosolic HMGB1 due to translocation of HMGB1 from nucleoli to cytosol would elicit the autophagic response, as a self-protective mechanism to limiting the necrotic and inflammatory response. Intriguingly, sustained autophagy is also found to stimulate HMGB1 release from the tumor cells treated with epidermal growth factor receptor-targeted diphtheria toxin (DT-EGF) (Thorburn et al. 2009). As discussed above (Fig. 13.3), the anti-necrosis function of autophagy will lead to suppression of inflammation and tumorigenesis. With the understanding of the relationship between HMGB1 and autophagy, it is believed that anti-necrosis and anti-inflammatory function of autophagy is implicated in wider spectrum of pro-inflammatory responses. Further studies are needed to examine the anti-inflammatory function of autophagy in association with necrosis and HMGB1, especially under an in vivo model.

13.5 Summary and Perspectives

With the increasing understanding of biological functions of autophagy, the involvement of autophagy in the cell death and cell survival process becomes a critical point of concern. Here we have attempted to address some of the emerging issues related to the relation between autophagy and necrosis. Autophagy first serves as a cell survival mechanism, via its suppressive role on necrotic cell death, such as necroptosis and PARP-mediated cell death. More importantly, the anti-necrosis

function of autophagy has been found to have important biological functions in various pathological processes and diseases, including cancer and ischemia–reperfusion injury. Therefore, it is reasonable to speculate that modulation of the anti-necrosis function of autophagy should be considered as novel preventive or therapeutic approaches for a number of necrosis-related human diseases. The future work should focus on the further illustration of the detailed molecular mechanisms underlying the cross talks between necrosis and autophagy, and establishment of appropriate animal models for studying the role of autophagy and necrosis in diseases.

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References

Albert JM, Cao C, Kim KW, Willey CD, Geng L, Xiao D, Wang H, Sandler A, Johnson DH, Colevas AD, Low J, Rothenberg ML, Lu B (2007) Inhibition of poly(ADP-ribose) polymerase enhances cell death and improves tumor growth delay in irradiated lung cancer models. Clin Cancer Res 13:3033–3042

Alexander A, Cai SL, Kim J, Nanez A, Sahin M, MacLean KH, Inoki K, Guan KL, Shen J, Person MD, Kusewitt D, Mills GB, Kastan MB, Walker CL (2010) ATM signals to TSC2 in the cytoplasm to regulate mTORC1 in response to ROS. Proc Natl Acad Sci U S A 107:4153–4158

Amaravadi RK, Yu D, Lum JJ, Bui T, Christophorou MA, Evan GI, Thomas-Tikhonenko A, Thompson CB (2007) Autophagy inhibition enhances therapy-induced apoptosis in a Myc-induced model of lymphoma. J Clin Invest 117:326–336

Anders CK, Winer EP, Ford JM, Dent R, Silver DP, Sledge GW, Carey LA (2010) Poly(ADP-Ribose) polymerase inhibition: "targeted" therapy for triple-negative breast cancer. Clin Cancer Res 16:4702–4710

Betin VM, Lane JD (2009) Caspase cleavage of Atg4D stimulates GABARAP-L1 processing and triggers mitochondrial targeting and apoptosis. J Cell Sci 122:2554–2566

Bursch W (2001) The autophagosomal-lysosomal compartment in programmed cell death. Cell Death Differ 8:569–581

Bustin M (2002) At the crossroads of necrosis and apoptosis: signaling to multiple cellular targets by HMGB1. Sci STKE 2002:pe39

Carew JS, Nawrocki ST, Kahue CN, Zhang H, Yang C, Chung L, Houghton JA, Huang P, Giles FJ, Cleveland JL (2007) Targeting autophagy augments the anticancer activity of the histone deacetylase inhibitor SAHA to overcome Bcr-Abl-mediated drug resistance. Blood 110(1): 313–322

Cecconi F, Levine B (2008) The role of autophagy in mammalian development: cell makeover rather than cell death. Dev Cell 15:344–357

Chan EY, Tooze SA (2009) Evolution of Atg1 function and regulation. Autophagy 5:758–765

Chen N, Debnath J (2010) Autophagy and tumorigenesis. FEBS Lett 584:1427–1435

Cho YS, Challa S, Moquin D, Genga R, Ray TD, Guildford M, Chan FK (2009) Phosphorylation-driven assembly of the RIP1-RIP3 complex regulates programmed necrosis and virus-induced inflammation. Cell 137:1112–1123

Christofferson DE, Yuan J (2010) Necroptosis as an alternative form of programmed cell death. Curr Opin Cell Biol 22:263–268

Colell A, Ricci JE, Tait S, Milasta S, Maurer U, Bouchier-Hayes L, Fitzgerald P, Guio-Carrion A, Waterhouse NJ, Li CW, Mari B, Barbry P, Newmeyer DD, Beere HM, Green DR (2007) GAPDH and autophagy preserve survival after apoptotic cytochrome c release in the absence of caspase activation. Cell 129:983–997
Colell A, Green DR, Ricci JE (2009) Novel roles for GAPDH in cell death and carcinogenesis. Cell Death Differ 16:1573–1581
Corcelle EA, Puustinen P, Jaattela M (2009) Apoptosis and autophagy: targeting autophagy signalling in cancer cells—'trick or treats'? FEBS J 276:6084–6096
de Bruin EC, Medema JP (2008) Apoptosis and non-apoptotic deaths in cancer development and treatment response. Cancer Treat Rev 34:737–749
Degenhardt K, Mathew R, Beaudoin B, Bray K, Anderson D, Chen G, Mukherjee C, Shi Y, Gelinas C, Fan Y, Nelson DA, Jin S, White E (2006) Autophagy promotes tumor cell survival and restricts necrosis, inflammation, and tumorigenesis. Cancer Cell 10:51–64
Degterev A, Yuan J (2008) Expansion and evolution of cell death programmes. Nat Rev Mol Cell Biol 9:378–390
Degterev A, Huang Z, Boyce M, Li Y, Jagtap P, Mizushima N, Cuny GD, Mitchison TJ, Moskowitz MA, Yuan J (2005) Chemical inhibitor of nonapoptotic cell death with therapeutic potential for ischemic brain injury. Nat Chem Biol 1:112–119
Degterev A, Hitomi J, Germscheid M, Ch'en IL, Korkina O, Teng X, Abbott D, Cuny GD, Yuan C, Wagner G, Hedrick SM, Gerber SA, Lugovskoy A, Yuan J (2008) Identification of RIP1 kinase as a specific cellular target of necrostatins. Nat Chem Biol 4:313–321
Denton D, Shravage B, Simin R, Mills K, Berry DL, Baehrecke EH, Kumar S (2009) Autophagy, not apoptosis, is essential for midgut cell death in Drosophila. Curr Biol 19:1741–1746
Dikic I, Johansen T, Kirkin V (2010) Selective autophagy in cancer development and therapy. Cancer Res 70:3431–3434
Ditsworth D, Zong WX, Thompson CB (2007) Activation of poly(ADP)-ribose polymerase (PARP-1) induces release of the pro-inflammatory mediator HMGB1 from the nucleus. J Biol Chem 282:17845–17854
Djavaheri-Mergny M, Maiuri MC, Kroemer G (2010) Cross talk between apoptosis and autophagy by caspase-mediated cleavage of Beclin 1. Oncogene 29:1717–1719
Edinger AL, Thompson CB (2004) Death by design: apoptosis, necrosis and autophagy. Curr Opin Cell Biol 16:663–669
Egan DF, Shackelford DB, Mihaylova MM, Gelino S, Kohnz RA, Mair W, Vasquez DS, Joshi A, Gwinn DM, Taylor R, Asara JM, Fitzpatrick J, Dillin A, Viollet B, Kundu M, Hansen M, Shaw RJ (2011) Phosphorylation of ULK1 (hATG1) by AMP-activated protein kinase connects energy sensing to mitophagy. Science 331:456–461
Eisenberg-Lerner A, Bialik S, Simon HU, Kimchi A (2009) Life and death partners: apoptosis, autophagy and the cross-talk between them. Cell Death Differ 16:966–975
Ethier C, Tardif M, Arul L, Poirier GG (2012) PARP-1 modulation of mTOR signaling in response to a DNA alkylating agent. PLoS One 7:e47978
Fiers W, Beyaert R, Declercq W, Vandenabeele P (1999) More than one way to die: apoptosis, necrosis and reactive oxygen damage. Oncogene 18:7719–7730
Fimia GM, Piacentini M (2010) Regulation of autophagy in mammals and its interplay with apoptosis. Cell Mol Life Sci 67:1581–1588
Fragkos M, Beard P (2011) Mitotic catastrophe occurs in the absence of apoptosis in p53-null cells with a defective G1 checkpoint. PLoS One 6:e22946
Galluzzi L, Vicencio JM, Kepp O, Tasdemir E, Maiuri MC, Kroemer G (2008) To die or not to die: that is the autophagic question. Curr Mol Med 8:78–91
Galluzzi L, Vitale I, Abrams JM, Alnemri ES, Baehrecke EH, Blagosklonny MV, Dawson TM, Dawson VL, El-Deiry WS, Fulda S, Gottlieb E, Green DR, Hengartner MO, Kepp O, Knight RA, Kumar S, Lipton SA, Lu X, Madeo F, Malorni W, Mehlen P, Nunez G, Peter ME, Piacentini M, Rubinsztein DC, Shi Y, Simon HU, Vandenabeele P, White E, Yuan J, Zhivotovsky B, Melino G, Kroemer G (2012) Molecular definitions of cell death subroutines: recommendations of the Nomenclature Committee on Cell Death 2012. Cell Death Differ 19:107–120

Giansanti V, Torriglia A, Scovassi AI (2011) Conversation between apoptosis and autophagy: “Is it your turn or mine?”. Apoptosis 16:321–333

Giusti C, Luciani MF, Ravens S, Gillet A, Golstein P (2010) Autophagic cell death in dictyostelium requires the receptor histidine kinase DhkM. Mol Biol Cell 21:1825–1835

Grzanka D, Grzanka A, Izdebska M, Gackowska L, Stepien A, Marszalek A (2010) Actin reorganization in CHO AA8 cells undergoing mitotic catastrophe and apoptosis induced by doxorubicin. Oncol Rep 23:655–663

Gwinn DM, Shackelford DB, Egan DF, Mihaylova MM, Mery A, Vasquez DS, Turk BE, Shaw RJ (2008) AMPK phosphorylation of raptor mediates a metabolic checkpoint. Mol Cell 30:214–226

Ha HC, Snyder SH (1999) Poly(ADP-ribose) polymerase is a mediator of necrotic cell death by ATP depletion. Proc Natl Acad Sci U S A 96:13978–13982

Hamacher-Brady A, Brady NR, Gottlieb RA (2006) Enhancing macroautophagy protects against ischemia/reperfusion injury in cardiac myocytes. J Biol Chem 281:29776–29787

Han J, Zhong CQ, Zhang DW (2011a) Programmed necrosis: backup to and competitor with apoptosis in the immune system. Nat Immunol 12:1143–1149

Han W, Pan H, Chen Y, Sun J, Wang Y, Li J, Ge W, Feng L, Lin X, Wang X, Jin H (2011b) EGFR tyrosine kinase inhibitors activate autophagy as a cytoprotective response in human lung cancer cells. PLoS One 6:e18691

Hardie DG (2007) AMP-activated/SNF1 protein kinases: conserved guardians of cellular energy. Nat Rev Mol Cell Biol 8:774–785

Hariharan N, Zhai P, Sadoshima J (2011) Oxidative stress stimulates autophagic flux during ischemia/reperfusion. Antioxid Redox Signal 14:2179–2190

Hitomi J, Christofferson DE, Ng A, Yao J, Degterev A, Xavier RJ, Yuan J (2008) Identification of a molecular signaling network that regulates a cellular necrotic cell death pathway. Cell 135: 1311–1323

Holler N, Zaru R, Micheau O, Thome M, Attinger A, Valitutti S, Bodmer JL, Schneider P, Seed B, Tschopp J (2000) Fas triggers an alternative, caspase-8-independent cell death pathway using the kinase RIP as effector molecule. Nat Immunol 1:489–495

Hou W, Han J, Lu C, Goldstein LA, Rabinowich H (2010) Autophagic degradation of active caspase-8: a crosstalk mechanism between autophagy and apoptosis. Autophagy 6:891–900

Huang S, Sinicrope FA (2010) Celecoxib-induced apoptosis is enhanced by ABT-737 and by inhibition of autophagy in human colorectal cancer cells. Autophagy 6:256–269

Hwang JW, Chung S, Sundar IK, Yao H, Arunachalam G, McBurney MW, Rahman I (2010) Cigarette smoke-induced autophagy is regulated by SIRT1-PARP-1-dependent mechanism: implication in pathogenesis of COPD. Arch Biochem Biophys 500:203–209

Iliodromitis EK, Lazou A, Kremastinos DT (2007) Ischemic preconditioning: protection against myocardial necrosis and apoptosis. Vasc Health Risk Manag 3:629–637

Inoki K, Zhu T, Guan KL (2003) TSC2 mediates cellular energy response to control cell growth and survival. Cell 115:577–590

Jagtap P, Szabo C (2005) Poly(ADP-ribose) polymerase and the therapeutic effects of its inhibitors. Nat Rev Drug Discov 4:421–440

Jiang M, Liu K, Luo J, Dong Z (2010) Autophagy is a renoprotective mechanism during in vitro hypoxia and in vivo ischemia-reperfusion injury. Am J Pathol 176:1181–1192

Jin S, DiPaola RS, Mathew R, White E (2007) Metabolic catastrophe as a means to cancer cell death. J Cell Sci 120:379–383

Jin Y, Wang H, Cui X, Xu Z (2010) Role of autophagy in myocardial reperfusion injury. Front Biosci (Elite Ed) 2:1147–1153

Kaiser WJ, Upton JW, Long AB, Livingston-Rosanoff D, Daley-Bauer LP, Hakem R, Caspary T, Mocarski ES (2011) RIP3 mediates the embryonic lethality of caspase-8-deficient mice. Nature 471:368–372

Kang R, Livesey KM, Zeh HJ, Loze MT, Tang D (2010) HMGB1: a novel Beclin 1-binding protein active in autophagy. Autophagy 6:1209–1211

Karantza-Wadsworth V, Patel S, Kravchuk O, Chen G, Mathew R, Jin S, White E (2007) Autophagy mitigates metabolic stress and genome damage in mammary tumorigenesis. Genes Dev 21: 1621–1635

Khan MJ, Rizwan Alam M, Waldeck-Weiermair M, Karsten F, Groschner L, Riederer M, Hallstrom S, Rockenfeller P, Konya V, Heinemann A, Madeo F, Graier WF, Malli R (2012) Inhibition of autophagy rescues palmitic acid-induced necroptosis of endothelial cells. J Biol Chem 287:21110–21120

Kim J, Kundu M, Viollet B, Guan KL (2011) AMPK and mTOR regulate autophagy through direct phosphorylation of Ulk1. Nat Cell Biol 13:132–141

Klionsky DJ, Emr SD (2000) Autophagy as a regulated pathway of cellular degradation. Science 290:1717–1721

Klionsky DJ, Abeliovich H, Agostinis P, Agrawal DK, Aliev G, Askew DS, Baba M, Baehrecke EH, Bahr BA, Ballabio A, Bamber BA, Bassham DC, Bergamini E, Bi X, Biard-Piechaczyk M, Blum JS, Bredesen DE, Brodsky JL, Brumell JH, Brunk UT, Bursch W, Camougrand N, Cebollero E, Cecconi F, Chen Y, Chin LS, Choi A, Chu CT, Chung J, Clarke PG, Clark RS, Clarke SG, Clave C, Cleveland JL, Codogno P, Colombo MI, Coto-Montes A, Cregg JM, Cuervo AM, Debnath J, Demarchi F, Dennis PB, Dennis PA, Deretic V, Devenish RJ, Di Sano F, Dice JF, Difiglia M, Dinesh-Kumar S, Distelhorst CW, Djavaheri-Mergny M, Dorsey FC, Droge W, Dron M, Dunn WA Jr, Duszenko M, Eissa NT, Elazar Z, Esclatine A, Eskelinen EL, Fesus L, Finley KD, Fuentes JM, Fueyo J, Fujisaki K, Galliot B, Gao FB, Gewirtz DA, Gibson SB, Gohla A, Goldberg AL, Gonzalez R, Gonzalez-Estevez C, Gorski S, Gottlieb RA, Haussinger D, He YW, Heidenreich K, Hill JA, Hoyer-Hansen M, Hu X, Huang WP, Iwasaki A, Jaattela M, Jackson WT, Jiang X, Jin S, Johansen T, Jung JU, Kadowaki M, Kang C, Kelekar A, Kessel DH, Kiel JA, Kim HP, Kimchi A, Kinsella TJ, Kiselyov K, Kitamoto K, Knecht E et al (2008) Guidelines for the use and interpretation of assays for monitoring autophagy in higher eukaryotes. Autophagy 4:151–175

Komatsu M, Waguri S, Ueno T, Iwata J, Murata S, Tanida I, Ezaki J, Mizushima N, Ohsumi Y, Uchiyama Y, Kominami E, Tanaka K, Chiba T (2005) Impairment of starvation-induced and constitutive autophagy in Atg7-deficient mice. J Cell Biol 169:425–434

Kosieradzki M, Rowinski W (2008) Ischemia/reperfusion injury in kidney transplantation: mechanisms and prevention. Transplant Proc 40:3279–3288

Kosta A, Roisin-Bouffay C, Luciani MF, Otto GP, Kessin RH, Golstein P (2004) Autophagy gene disruption reveals a non-vacuolar cell death pathway in Dictyostelium. J Biol Chem 279: 48404–48409

Krishnakumar R, Kraus WL (2010) The PARP side of the nucleus: molecular actions, physiological outcomes, and clinical targets. Mol Cell 39:8–24

Kroemer G, Levine B (2008) Autophagic cell death: the story of a misnomer. Nat Rev Mol Cell Biol 9:1004–1010

Kuma A, Hatano M, Matsui M, Yamamoto A, Nakaya H, Yoshimori T, Ohsumi Y, Tokuhisa T, Mizushima N (2004) The role of autophagy during the early neonatal starvation period. Nature 432:1032–1036

Laskowski I, Pratschke J, Wilhelm MJ, Gasser M, Tilney NL (2000) Molecular and cellular events associated with ischemia/reperfusion injury. Ann Transplant 5:29–35

Lenardo MJ, McPhee CK, Yu L (2009) Autophagic cell death. Methods Enzymol 453:17–31

Levine B, Sinha S, Kroemer G (2008) Bcl-2 family members: dual regulators of apoptosis and autophagy. Autophagy 4:600–606

Lord CJ, Ashworth A (2008) Targeted therapy for cancer using PARP inhibitors. Curr Opin Pharmacol 8:363–369

Luo S, Rubinsztein DC (2007) Atg5 and Bcl-2 provide novel insights into the interplay between apoptosis and autophagy. Cell Death Differ 14(7):1247–1250

Maiuri MC, Zalckvar E, Kimchi A, Kroemer G (2007) Self-eating and self-killing: crosstalk between autophagy and apoptosis. Nat Rev Mol Cell Biol 8:741–752

Martin DS, Bertino JR, Koutcher JA (2000) ATP depletion + pyrimidine depletion can markedly enhance cancer therapy: fresh insight for a new approach. Cancer Res 60:6776–6783

Mathew R, Karantza-Wadsworth V, White E (2007a) Role of autophagy in cancer. Nat Rev Cancer 7:961–967

Mathew R, Kongara S, Beaudoin B, Karp CM, Bray K, Degenhardt K, Chen G, Jin S, White E (2007b) Autophagy suppresses tumor progression by limiting chromosomal instability. Genes Dev 21:1367–1381

Matsui Y, Takagi H, Qu X, Abdellatif M, Sakoda H, Asano T, Levine B, Sadoshima J (2007) Distinct roles of autophagy in the heart during ischemia and reperfusion: roles of AMP-activated protein kinase and Beclin 1 in mediating autophagy. Circ Res 100:914–922

Mehrpour M, Esclatine A, Beau I, Codogno P (2010) Autophagy in health and disease. 1. Regulation and significance of autophagy: an overview. Am J Physiol Cell Physiol 298:C776–C785

Meijer AJ, Codogno P (2009) Autophagy: regulation and role in disease. Crit Rev Clin Lab Sci 46:210–240

Mizushima N (2007) Autophagy: process and function. Genes Dev 21:2861–2873

Mizushima N (2009) Physiological functions of autophagy. Curr Top Microbiol Immunol 335:71–84

Mizushima N, Komatsu M (2011) Autophagy: renovation of cells and tissues. Cell 147:728–741

Mizushima N, Levine B, Cuervo AM, Klionsky DJ (2008) Autophagy fights disease through cellular self-digestion. Nature 451:1069–1075

Mizushima N, Yoshimori T, Levine B (2010) Methods in mammalian autophagy research. Cell 140:313–326

Munoz-Gamez JA, Rodriguez-Vargas JM, Quiles-Perez R, Aguilar-Quesada R, Martin-Oliva D, de Murcia G, Menissier de Murcia J, Almendros A, Ruiz de Almodovar M, Oliver FJ (2009) PARP-1 is involved in autophagy induced by DNA damage. Autophagy 5:61–74

Orsi A, Polson HE, Tooze SA (2010) Membrane trafficking events that partake in autophagy. Curr Opin Cell Biol 22:150–156

O'Sullivan-Coyne G, O'Sullivan GC, O'Donovan TR, Piwocka K, McKenna SL (2009) Curcumin induces apoptosis-independent death in oesophageal cancer cells. Br J Cancer 101:1585–1595

Patel VA, Lee DJ, Longacre-Antoni A, Feng L, Lieberthal W, Rauch J, Ucker DS, Levine JS (2009) Apoptotic and necrotic cells as sentinels of local tissue stress and inflammation: response pathways initiated in nearby viable cells. Autoimmunity 42:317–321

Portugal J, Mansilla S, Bataller M (2010) Mechanisms of drug-induced mitotic catastrophe in cancer cells. Curr Pharm Des 16:69–78

Qu X, Zou Z, Sun Q, Luby-Phelps K, Cheng P, Hogan RN, Gilpin C, Levine B (2007) Autophagy gene-dependent clearance of apoptotic cells during embryonic development. Cell 128:931–946

Raucci A, Palumbo R, Bianchi ME (2007) HMGB1: a signal of necrosis. Autoimmunity 40:285–289

Rosenfeldt MT, Ryan KM (2009) The role of autophagy in tumour development and cancer therapy. Expert Rev Mol Med 11:e36

Rubinstein AD, Eisenstein M, Ber Y, Bialik S, Kimchi A (2011) The autophagy protein Atg12 associates with antiapoptotic Bcl-2 family members to promote mitochondrial apoptosis. Mol Cell 44:698–709

Rubinsztein DC, Gestwicki JE, Murphy LO, Klionsky DJ (2007) Potential therapeutic applications of autophagy. Nat Rev Drug Discov 6:304–312

Sadoshima J (2008) The role of autophagy during ischemia/reperfusion. Autophagy 4:402–403

Schweichel JU, Merker HJ (1973) The morphology of various types of cell death in prenatal tissues. Teratology 7:253–266

Shen HM, Codogno P (2011) Autophagic cell death: Loch Ness monster or endangered species? Autophagy 7

Shen HM, Codogno P (2012) Autophagy is a survival force via suppression of necrotic cell death. Exp Cell Res 318:1304–1308

Shen S, Kepp O, Michaud M, Martins I, Minoux H, Metivier D, Maiuri MC, Kroemer RT, Kroemer G (2011) Association and dissociation of autophagy, apoptosis and necrosis by systematic chemical study. Oncogene 30:4544–4556

Shen S, Kepp O, Kroemer G (2012) The end of autophagic cell death? Autophagy 8

Shimizu S, Kanaseki T, Mizushima N, Mizuta T, Arakawa-Kobayashi S, Thompson CB, Tsujimoto Y (2004) Role of Bcl-2 family proteins in a non-apoptotic programmed cell death dependent on autophagy genes. Nat Cell Biol 6:1221–1228

Shimizu S, Konishi A, Nishida Y, Mizuta T, Nishina H, Yamamoto A, Tsujimoto Y (2010) Involvement of JNK in the regulation of autophagic cell death. Oncogene 29:2070–2082
Shingu T, Chumbalkar VC, Gwak HS, Fujiwara K, Kondo S, Farrell NP, Bogler O (2010) The polynuclear platinum BBR3610 induces G2/M arrest and autophagy early and apoptosis late in glioma cells. Neuro Oncol 12:1269–1277
Sims NR, Muyderman H (2010) Mitochondria, oxidative metabolism and cell death in stroke. Biochim Biophys Acta 1802:80–91
Sodeoka M, Dodo K (2010) Development of selective inhibitors of necrosis. Chem Rec 10: 308–314
Speirs CK, Hwang M, Kim S, Li W, Chang S, Varki V, Mitchell L, Schleicher S, Lu B (2011) Harnessing the cell death pathway for targeted cancer treatment. Am J Cancer Res 1:43–61
Sun L, Wang H, Wang Z, He S, Chen S, Liao D, Wang L, Yan J, Liu W, Lei X, Wang X (2012) Mixed lineage kinase domain-like protein mediates necrosis signaling downstream of RIP3 kinase. Cell 148:213–227
Tan SH, Shui G, Zhou J, Li JJ, Bay BH, Wenk MR, Shen HM (2012) Induction of autophagy by palmitic acid via protein kinase C-mediated signaling pathway independent of mTOR (mammalian target of rapamycin). J Biol Chem 287:14364–14376
Tang D, Kang R, Cheh CW, Livesey KM, Liang X, Schapiro NE, Benschop R, Sparvero LJ, Amoscato AA, Tracey KJ, Zeh HJ, Lotze MT (2010a) HMGB1 release and redox regulates autophagy and apoptosis in cancer cells. Oncogene 29:5299–5310
Tang D, Kang R, Livesey KM, Cheh CW, Farkas A, Loughran P, Hoppe G, Bianchi ME, Tracey KJ, Zeh HJ 3rd, Lotze MT (2010b) Endogenous HMGB1 regulates autophagy. J Cell Biol 190:881–892
Tavernarakis N (2007) Cardiomyocyte necrosis: alternative mechanisms, effective interventions. Biochim Biophys Acta 1773:480–482
Thorburn J, Horita H, Redzic J, Hansen K, Frankel AE, Thorburn A (2009) Autophagy regulates selective HMGB1 release in tumor cells that are destined to die. Cell Death Differ 16:175–183
Tristan C, Shahani N, Sedlak TW, Sawa A (2011) The diverse functions of GAPDH: views from different subcellular compartments. Cell Signal 23:317–323
Vakifahmetoglu H, Olsson M, Zhivotovsky B (2008) Death through a tragedy: mitotic catastrophe. Cell Death Differ 15:1153–1162
van Wijk SJ, Hageman GJ (2005) Poly(ADP-ribose) polymerase-1 mediated caspase-independent cell death after ischemia/reperfusion. Free Radic Biol Med 39:81–90
Vercammen D, Beyaert R, Denecker G, Goossens V, Van Loo G, Declercq W, Grooten J, Fiers W, Vandenabeele P (1998) Inhibition of caspases increases the sensitivity of L929 cells to necrosis mediated by tumor necrosis factor. J Exp Med 187:1477–1485
Vitale I, Galluzzi L, Castedo M, Kroemer G (2011) Mitotic catastrophe: a mechanism for avoiding genomic instability. Nat Rev Mol Cell Biol 12:385–392
Wang Z, Jiang H, Chen S, Du F, Wang X (2012) The mitochondrial phosphatase PGAM5 functions at the convergence point of multiple necrotic death pathways. Cell 148:228–243
White E, DiPaola RS (2009) The double-edged sword of autophagy modulation in cancer. Clin Cancer Res 15:5308–5316
White E, Karp C, Strohecker AM, Guo Y, Mathew R (2010) Role of autophagy in suppression of inflammation and cancer. Curr Opin Cell Biol 22:212–217
Wirawan E, Vande Walle L, Kersse K, Cornelis S, Claerhout S, Vanoverberghe I, Roelandt R, De Rycke R, Verspurten J, Declercq W, Agostinis P, Vanden Berghe T, Lippens S, Vandenabeele P (2010) Caspase-mediated cleavage of Beclin-1 inactivates Beclin-1-induced autophagy and enhances apoptosis by promoting the release of proapoptotic factors from mitochondria. Cell Death Dis 1:e18
Wu YT, Tan HL, Huang Q, Kim YS, Pan N, Ong WY, Liu ZG, Ong CN, Shen HM (2008) Autophagy plays a protective role during zVAD-induced necrotic cell death. Autophagy 4:457–466
Wu YT, Tan HL, Huang Q, Ong CN, Shen HM (2009) Activation of the PI3K-Akt-mTOR signaling pathway promotes necrotic cell death via suppression of autophagy. Autophagy 5:824–834

Wu YT, Tan HL, Huang Q, Sun XJ, Zhu X, Ong CN, Shen HM (2011) zVAD-induced necroptosis in L929 cells depends on autocrine production of TNFα mediated via the PKC-MAPKs-AP-1 pathway. Cell Death Differ 18(1):26–37

Wu W, Liu P, Li J (2012) Necroptosis: an emerging form of programmed cell death. Crit Rev Oncol Hematol 82(3):249–258

Yang Z, Klionsky DJ (2009) An overview of the molecular mechanism of autophagy. Curr Top Microbiol Immunol 335:1–32

Yang Z, Klionsky DJ (2010) Mammalian autophagy: core molecular machinery and signaling regulation. Curr Opin Cell Biol 22:124–131

Yang JC, Lu MC, Lee CL, Chen GY, Lin YY, Chang FR, Wu YC (2011) Selective targeting of breast cancer cells through ROS-mediated mechanisms potentiates the lethality of paclitaxel by a novel diterpene, gelomulide K. Free Radic Biol Med 51:641–657

Yousefi S, Perozzo R, Schmid I, Ziemiecki A, Schaffner T, Scapozza L, Brunner T, Simon HU (2006) Calpain-mediated cleavage of Atg5 switches autophagy to apoptosis. Nat Cell Biol 8:1124–1132

Yu L, Alva A, Su H, Dutt P, Freundt E, Welsh S, Baehrecke EH, Lenardo MJ (2004) Regulation of an ATG7-beclin 1 program of autophagic cell death by caspase-8. Science 304:1500–1502

Zhang N, Qi Y, Wadham C, Wang L, Warren A, Di W, Xia P (2010) FTY720 induces necrotic cell death and autophagy in ovarian cancer cells: a protective role of autophagy. Autophagy 6: 1157–1167

Zhang H, Zhou X, McQuade T, Li J, Chan FK, Zhang J (2011) Functional complementation between FADD and RIP1 in embryos and lymphocytes. Nature 471:373–376

Zhao H (2007) The protective effect of ischemic postconditioning against ischemic injury: from the heart to the brain. J Neuroimmune Pharmacol 2:313–318

Zhou JN, Ng S, Huang Q, Li Z, Yao SQ, Shen HM (2013) AMPK mediates a pro-survival autophagy downstream of PARP-1 activation in response to DNA alkylating agents. FEBS Lett 587(2):170–177

Zong WX, Thompson CB (2006) Necrotic death as a cell fate. Genes Dev 20:1–15

Zong WX, Ditsworth D, Bauer DE, Wang ZQ, Thompson CB (2004) Alkylating DNA damage stimulates a regulated form of necrotic cell death. Genes Dev 18:1272–1282

Chapter 14
Microbial Programmed Necrosis: The Cost of Conflicts Between Stress and Metabolism

Joris Winderickx and Paula Ludovico

14.1 Introduction

It is well established that programmed cell death (PCD) is a critical process in organogenesis and normal tissue homeostasis, in the pathophysiology of various diseases as well as in the development and function of the immune system in metazoans. Besides apoptosis, a process first described almost 40 years ago (Kerr et al. 1972) and often used as synonym of PCD, other forms of PCD have been described. These include programmed necrosis and autophagic cell death (Kroemer et al. 2009; Galluzzi et al. 2012). Indeed, although necrosis is often regarded as an accidental form of cell death that involves plasma membrane rupture and cell lysis, accumulating evidence demonstrates that cells can alter their mode of cell death from apoptosis to necrosis. This necrotic program, also known as necroptosis, is triggered by a variety of stimuli, including the ligation of cell death receptors, the stimulation of Toll-like receptors, and genotoxic stress (Imre et al. 2011; Dickens et al. 2012; Vanlangenakker et al. 2012). It requires the assembly of either one of two multiprotein signaling platforms, the necrosome or the ripoptosome, which among other components both contain caspase-8 and the receptor-interacting protein kinase RIP1. When caspase-8 is fully active, it will cleave RIP1 in the complex and dictate apoptotic cell death. However, when caspase-8 activity is blocked, RIP1

J. Winderickx (✉)
Functional Biology, University of Leuven, Kasteelpark Arenberg,
Box 2344, 3001 Heverlee, Belgium
e-mail: joris.winderickx@bio.kuleuven.be

P. Ludovico (✉)
Life and Health Sciences Research Institute (ICVS), School of Health Sciences,
University of Minho, Campus de Gualtar, 4710-057 Braga, Portugal

ICVS/3B's – PT Government Associate Laboratory, Braga/Guimarães, Portugal
e-mail: pludovico@ecsaude.uminho.pt

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engages its homolog RIP3 to activate downstream necrotic signaling (Imre et al. 2011; Dickens et al. 2012). Autophagic cell death is accompanied by a massive cytoplasmic vacuolization and occurs in the absence of chromatin condensation (Berry and Baehrecke 2008; Kroemer et al. 2009; Galluzzi et al. 2012). Besides the involvement of specific autophagy proteins, such as ATG1, ATG7, or Beclin1, a role was also ascribed to caspase-8-mediated cleavage of the RIP kinases (Yu et al. 2004), indicative that this type of cell death converges on the necroptotic program. Consistently, it was observed that autophagy promotes necrosis in apoptosis-deficient cells in response to, or via, endoplasmic reticulum (ER) stress (Ullman et al. 2008; Claerhout et al. 2012). Moreover, in most physiological conditions, autophagy has mainly a cytoprotective, pro-survival role with significant cross talk to, and even negative modulation of, apoptosis (Gordy and He 2012). Hence, to date, the position hold by autophagy in the cell death scenery is still a matter of debate and it was proposed that autophagy, rather than acting as distinguished form of PCD, is instead contributing to the determination of cell fate by differentially affecting apoptosis and necrosis (Ullman et al. 2008; Loos et al. 2013; Nikoletopoulou et al. 2013). From the above, it is immediately clear that the different forms of PCD depend on highly interconnected subroutines and thus that in many cases PCD is actually manifested as a mixed cell death phenotype (Kroemer et al. 2009; Galluzzi et al. 2012).

Traditionally, PCD was thought to be relevant only for multicellular organisms, but this perception has been challenged by several reports during the past decades describing genetically encoded mechanisms for the regulation of cell death in unicellular eukaryotes, including yeast (Low and Yang 2008; Carmona-Gutierrez et al. 2010a), unicellular algae (Yordanova et al. 2010), protists (Roisin-Bouffay et al. 2004; Luciani et al. 2011), protozoan parasites (Deponte 2008; Kaczanowski et al. 2011; Reece et al. 2011; Taylor-Brown and Hurd 2013), and even in bacteria (Lewis 2000; Engelberg-Kulka et al. 2004; Dwyer et al. 2012). PCD in microbes may represent a form of altruistic behavior directed to preserve the population or colony by removal of damaged cells and by delivery of digested cellular content as nutrients for the sustainment of persisting cells or, in case of parasites, directed to prevent killing of the host (Lewis 2000; Longo et al. 2005; Pollitt et al. 2010; Nedelcu et al. 2011). In addition, rudimentary PCD events have also been proposed to sustain the release of endospores in certain bacteria (Lewis 2000), to support the formation of fruiting bodies in *Dictyostelium* (Roisin-Bouffay et al. 2004; Luciani et al. 2011), and to operate during postmeiotic programmed nuclear degradation in ciliates (Madireddi et al. 1996; Coyne et al. 1999; Maercker et al. 1999) and gametogenesis in yeast (Eastwood et al. 2012).

Given the similarities in diagnostic features of cell death in multicellular and unicellular organisms, it is conceivable that mechanisms for PCD may have originated early in evolution. This interpretation, however, is all too often subject for discussions because for many microbes the underlying regulatory machinery of PCD is only poorly elucidated and because the microbial genomes do not encode for canonical caspases, RIP kinase orthologues, or bona fide cell death receptors. However, as is often the case for matters dealing with evolutionary aspects, it may

be better to look at the overall concept rather than to focus on definitions that would restrict PCD programs to the action of a particular class of regulatory proteins. Indeed, evolution teaches us that regulatory proteins gradually adopted their domain architecture, allowing them to gain or lose functional specificity, which may even result in the relay of entire signaling cascade modules in order to acquire more adequate or altered signaling control. Nonetheless, comprehensive studies dealing with the phylogenetic conservation of protein domains typifying players involved in metazoan apoptosis revealed, for instance, the existence of proteins in *Dictyostelium* with the same RING, CART, and MATH domain architecture as the TNFR-associated factor, TRAF, as well as several proteins in bacteria and even one archaeon harboring an AP-ATPase domain, which characterizes the apoptosis peptidase APAF-1 (Aravind et al. 1999; Koonin and Aravind 2002). While the functional relevance still needs to be demonstrated for the two examples given, other predictions of in silico analyses have meanwhile been validated. Studies performed by several groups identified a growing list of functionally relevant apoptotic regulators in different single-celled species. In *Escherichia coli*, a very recent study showed that the multifunctional DNA damage sensing protein RecA shares peptide substrate specificity with metazoan caspases specifically under conditions of antibiotic-induced cell death (Dwyer et al. 2012). The same study also demonstrated that a treatment with bactericidal antibiotics induces phosphatidylserine (PS) exposure and membrane depolarization, chromosome condensation, DNA fragmentation, and the generation of reactive oxygen species (ROS), which to our knowledge is the first report of typical hallmarks of apoptosis in bacteria. In unicellular eukaryotes, isolated examples of apoptotic regulators include the apoptosis-inducing factors (AIF) in *Dictyostelium discoideum* (Arnoult et al. 2001) and *Tetrahymena thermophila* (Akematsu and Endoh 2010) and endonuclease G (EndoG) in *Trypanosoma brucei* (Gannavaram et al. 2008), *Leishmania major* (Gannavaram et al. 2008), and *Leishmania infantum* (Rico et al. 2009). A more compelling list, however, was generated for the budding yeast *Saccharomyces cerevisiae* and the fission yeast *Schizosaccharomyces pombe*. Besides confirmation for the involvement of, respectively, the *S. cerevisiae* and *S. pombe* metacaspases Yca1 and Pca1 (Madeo et al. 2002; Low and Yang 2008; Carmona-Gutierrez et al. 2010b), the EndoG homologs Nuc1 and spNuc1 (Büttner et al. 2007; Oda et al. 2007), or the BH3-only proteins Ybh3 and SpRad9 (Low and Yang 2008; Büttner et al. 2011), a role in apoptosis was documented for the budding yeast AIF homolog, Aif1 (Wissing et al. 2004), the IAP homolog Bir1 (Walter et al. 2006), and the HtrA2-like serine protease, Nma111/Ynm3 (Fahrenkrog et al. 2004; Fahrenkrog 2011). In addition, the use of the yeast models allowed to identify several factors that linked apoptosis to other cellular processes. Examples of these are Bre1 (Walter et al. 2010), an E3 ubiquitin protein ligase required for the ubiquitylation of histone H2B, and Cdc48 (Madeo et al. 1997; Heo et al. 2010; Taylor and Rutter 2011), which is a crucial component for protein quality control via the ER-associated degradation process (ERAD) and mitochondria-associated degradation process (MAD). The close mechanistic resemblance between apoptosis in yeast and metazoans is further emphasized when taking into account the various apoptogenic stimuli, which in

both cases include not only exogenous triggers, like H_2O_2, acetic acid, viral killer toxins, and antitumor drugs, but also several endogenous triggers, such as defective DNA replication and aging (Weinberger et al. 2005, 2007; Carmona-Gutierrez et al. 2010a). Finally, as for the discussion on the action of caspases and metacaspases, it is now established that they do cleave common death-related substrates as nicely documented for the TSN nuclease, which in humans is a target of caspase-3 and in the Norway spruce a target of metacaspase (Sundstrom et al. 2009). Furthermore, the metabolic enzyme glyceraldehyde-3-phosphate dehydrogenase (GAPDH) specifically cleaved by caspase-1 in higher eukaryotic cells (Shao et al. 2007) was also described as a specific target of yeast metacaspase (Silva et al. 2011). Thus, although mammalian caspases and yeast metacaspase apparently have distinct target cleavage specificities, there is sufficient evidence supporting that intrinsic apoptotic mechanisms found their roots early in evolution.

14.2 Setting the Scene: Defining "Programmed Necrosis" in Microbiology

Before going into detail whether also other forms of PCD have an early evolutionary origin, we first need some conceptual clarification, especially in case of programmed necrosis. The reason for this is that "apoptosis" and "necrosis" are not standard vocabulary in microbiology and these terms only entered the field fairly recently, once researchers appreciated the appearance of apoptosis-like cell death phenomena in microbial organisms. More common are the terms "autolysis" and "autolytic activity," which refer to endogenously controlled self-destructive processes that are part of developmental microbial programs and, therefore, should not be confused with the process of autolysis in medical pathology that denotes the enzymatic digestion and removal of death tissue and cells. Thus, if the term "programmed necrosis" is solely meant to indicate that necrosis is not merely the consequence of brutal cell rupture, but rather the result of a molecular program unrolled under certain conditions, then let it be clear that there are probably more examples to be found in the microbial world than in metazoans. For instance, the release of endospores, the development of fruiting bodies and sporulation, the formation and stabilization of biofilms, and the genetic exchange in some species by phenomenon called natural transformation all rely on controlled autolytic programs (Lewis 2000; Montanaro et al. 2011). However, when "programmed necrosis" is used in terms of defining a controlled switch between apoptotic and necrotic cell death, then the task to find arguments for an early evolutionary origin becomes more difficult, not to say almost impossible when it would be confined to the interplay of caspases and RIP kinases, simply because there are at present no indications for the existence of microbial functional counterparts of the latter. Even so, some studies in yeast suggest that also microbial cells have the capacity to make an adaptive switch from apoptosis to necrosis, whereby pathways similar to those found in metazoans appear to trigger the execution of necrosis.

14.3 Programmed Necrosis: The Solution for an Insolvable Problem?

In budding yeast, similar to metazoans, cell death programs can be executed in a metacaspase-dependent and metacaspase-independent manner. In fact, only about 40 % of the described yeast cell death scenarios appear to be clearly mediated by its metacaspase (Madeo et al. 2009), meaning that cell survival is significantly ameliorated by the deletion of the Yca1-encoding gene. Examples of such metacaspase-dependent scenarios are those induced by H_2O_2 (Madeo et al. 1999, 2002), acetic acid (Ludovico et al. 2001, 2002; Guaragnella et al. 2006), hyperosmotic stress (Silva et al. 2005), and the exposure of yeast cells to arsenic (Du et al. 2007) or viral toxins (Reiter et al. 2005; Schmitt and Reiter 2008). Also the decline in yeast cell viability during starvation and postmitotic chronological aging is Yca1 dependent (Herker et al. 2004). However, it should be noted that in most of these cases, Yca1 is just one of the contributing factors and thus that other death-regulatory subroutines play an equally important role. This is nicely illustrated for the two best-known yeast cell death inducers, H_2O_2 and acetic acid, where besides Yca1 (Madeo et al. 2002; Guaragnella et al. 2006, 2010), PCD is also independently orchestrated by the yeast apoptosis-inducing factor, Aif1 (Wissing et al. 2004; Büttner et al. 2007); the EndoG orthologue, Nuc1 (Büttner et al. 2007); the HtrA2/Omi homolog, Nma111 (Fahrenkrog et al. 2004; Fahrenkrog 2011); and the yeast BH3-only protein, Ybh3 (Büttner et al. 2011). Furthermore, both H_2O_2 and acetic acid were initially reported to trigger apoptosis when applied in low concentrations, but necrosis when higher concentrations are used (Madeo et al. 1999; Ludovico et al. 2001). Since a similar switch in the mode of cell death is observed for other cell death-inducing agents as well, like viral toxins (Reiter et al. 2005; Schmitt and Reiter 2008), copper, and manganese (Liang and Zhou 2007), it is possible that with increasing dosage, these agents caused merely an accidental and nonregulated form of necrosis. However, as summarized in Fig. 14.1 and illustrated in more detail in Fig. 14.2, there are several observations that suggest this switch to be adaptive and highly regulated. For instance, with acetic acid, the decision to commit apoptotic or necrotic cell death appears to be dependent on vacuolar activity (Schauer et al. 2009). Indeed, cells with deletions of class C VPS genes display drastically enhanced sensitivity to treatment with acetic acid as these mutant cells already show a necrotic phenotype with acetic acid concentrations that normally induce apoptosis. The increased sensitivity correlates with a strong acetic acid-induced cytoplasmic drop in pH due to the inability of class C VPS mutants to maintain pH homeostasis because of the lack of functional vacuoles (the yeast counterpart of lysosomes). As such, these data are in accordance with results obtained in nematodes, where acidic intracellular conditions are also required for necrotic cell death (Syntichaki et al. 2005). However, when the intracellular pH of wild-type yeast cells is forced to an acidity comparable to that of the class C VPS mutants, the survival rates of the wild-type and mutant cells are similarly low, as expected, but still the mode of cell death of the wild-type cells remains predominantly apoptotic. This indicates that in addition to preventing

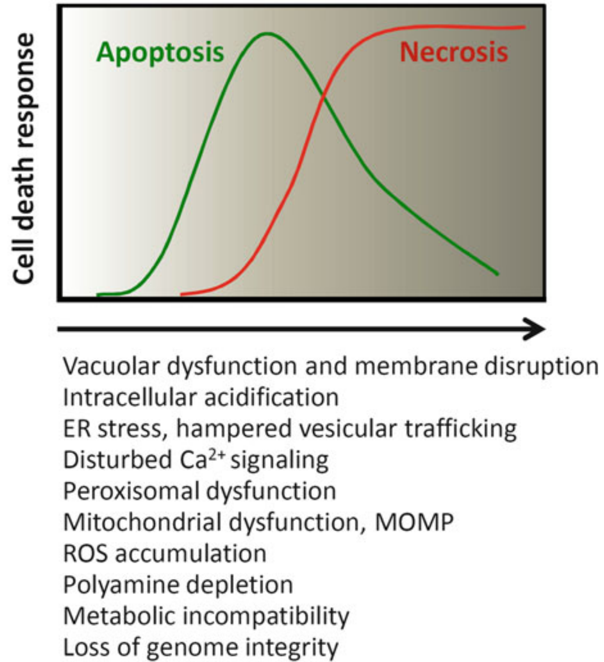

Fig. 14.1 Cell death responses of yeast cells. Schematic representation of the contribution of apoptosis and necrosis to the overall cell death response of yeast cells in function of the strength of an insult, the accumulation of intracellular damage, or the changes in metabolic activity. See main text for details

acetic acid-induced cell death by buffering the cytosolic pH, the vacuole has a specific proapoptotic function (Schauer et al. 2009).

Also the lack of Pex6, a protein involved in peroxisomal protein import, sensitizes cells for acetic acid-induced cell death, and again this is mainly ascribed to an increase in the number of necrotic cells, especially when cells enter stationary phase (Jungwirth et al. 2008). The necrotic cell death in the *pex6Δ* mutant is accompanied by a significant enhanced generation of ROS, and while being independent of Yca1 or Aif1, the effect triggered by loss of Pex6 is entirely associated with the presence of functional mitochondria. This favors the hypothesis that peroxisomal functions are connected to mitochondrial processes. Consistently, it was reported that Pex6 acts as a multicopy suppressor of an *atp2* age asymmetry mutant, whereby extra copies of *PEX6* corrected the deficit in Atp2 in mitochondria by improving its import kinetics in the mutant, resulting in almost normal mitochondrial inheritance by daughter cells (Seo et al. 2007).

Another study connected the switch between acetic acid-induced apoptotic and necrotic cell death to a differential role of HSP90 isoforms in cell survival and death. This study showed Hsc82 to promote survival and prevent necrotic cell death, while Hsc82 was found to act as a pro-death molecule involved in acetic acid-induced apoptosis (Silva et al. 2013).

It has also been suggested that the switch in the mode of cell death triggered by acetic acid may also have a metabolic basis, since the activity of the glycolytic enzymes, glyceraldehyde-3-phosphate dehydrogenase, pyruvate kinase, and

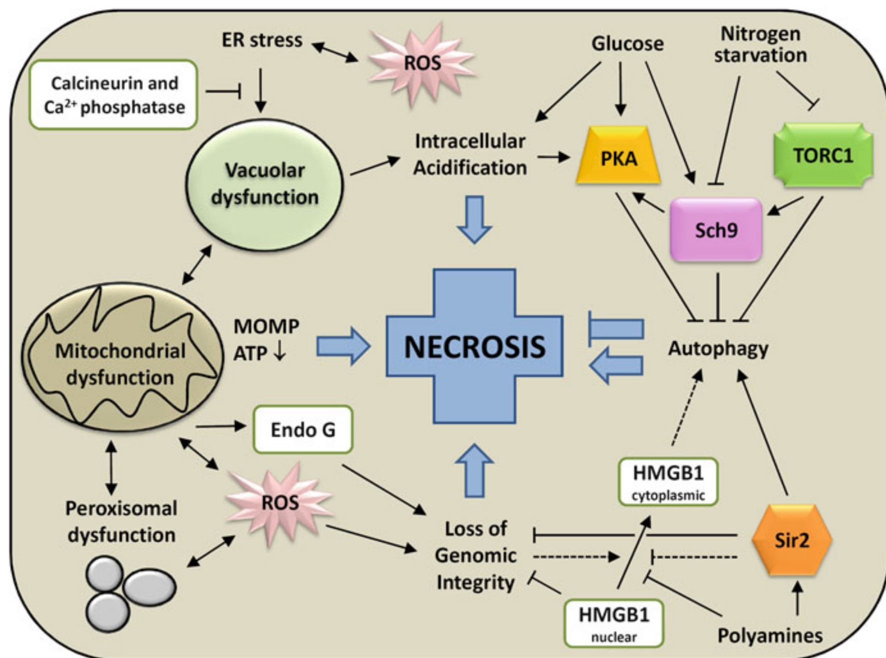

Fig. 14.2 Factors contributing to yeast necrosis. Shown are the most prominent stimuli and (dysfunctional) cellular processes known to integrate into necrotic death of yeast cells. Importantly, the regulation of fermentative/respiratory metabolism, the overall stress resistance, and lifespan by nutrient-dependent pathways are not shown for the sake of clarity and neither is a distinction made between different autophagic processes that either stimulate or prevent necrosis dependent on the physiological context. Given the strong interconnectivity of the contributing factors, it is the overall impact that tilts the balance towards necrosis. Dashed arrows and lines represent connections that remain to be confirmed in yeast cells but are known to occur in mammalian systems. See main text for more details

glucose-6-phosphate dehydrogenase, is increased with high acetic acid concentrations but significantly inhibited with lower acetic acid concentrations (Kitanovic et al. 2012). In addition, toxic acetic acid concentrations inhibit the uptake and consumption of glucose, what results in the depletion of intracellular ATP pools and in turn the disruption of the intracellular pH balance. Also, intracellular amino acid pools are depleted upon treatment with toxic acetic acid concentrations (Almeida et al. 2009), and at the same time, mitochondrial respiration is inhibited through respiratory chain uncoupling and loss of membrane potential (Ludovico et al. 2002). In consequence, cells enter a pseudo-starvation state and initiate the synthesis of reserve carbohydrates while also upregulating gluconeogenesis (Kitanovic et al. 2012). Based on these observations, it was postulated that acetic acid instigates toxicity because yeast cells receive conflicting signals. On the one hand, acetic acid-mediated inhibition of glucose uptake and amino acid depletion forces the cell to prepare to enter the stationary phase, and on the other hand,

the intracellular acidification would signal for growth due to constitutive activation of the growth promoting Ras-cAMP-PKA pathway (Kitanovic et al. 2012). In addition, the latter would also prevent the initiation of the so-called general stress response required for cells to properly enter stationary phase and lead to the accumulation of damaged and ROS-producing mitochondria (Leadsham and Gourlay 2010; Muller and Reichert 2011). Most recently, acetic acid treatment was shown to trigger the translocation of active Ras from the nucleus and the plasma membrane to mitochondria, an effect that is associated with a marked accumulation of ROS and, dependent on the acetic acid concentration, a pronounced increase in apoptotic or necrotic cell death. Intriguingly, this study also revealed a pro-survival role for Hxk2, since active Ras is constitutively localized at the mitochondria in cells lacking the hexokinase and since these mutant cells displayed enhanced acetic acid-induced cell death (Amigoni et al. 2013).

The mode of cell death of yeast cells is also determined by the nature of the missing nutrient(s) upon starvation. While deprivation of an auxotrophic requirement, such as leucine, elicits a metacaspase-mediated apoptosis, the starvation for a nutrient-like nitrogen causes the vast majority of cells to become membrane permeable to propidium iodide, a feature associated with primary necrosis (Boer et al. 2008; Klosinska et al. 2011; Dziedzic and Caplan 2012). Interestingly, this switch seems to be closely linked to autophagic processes since cells deleted for *ATG8* are more susceptible to nitrogen starvation but more resistant to leucine starvation, while cells lacking *ATG11* display the opposite phenotype. This led the authors to suggest that autophagy could concurrently facilitate either apoptotic or necrotic cell death (Dziedzic and Caplan 2012), a situation reminiscent to the control of cell fate in higher eukaryotes (Ullman et al. 2008). In yeast, the nutrient-dependent regulation of autophagy involves the interplay of different, but interconnected, nutrient-responsive pathways, including the abovementioned Ras-cAMP-PKA pathway and the pathways controlled by the rapamycin-sensitive Tor kinase complex 1 (TORC1) and the PKB/AKT and S6K orthologue Sch9 (Yorimitsu et al. 2007; Stephan et al. 2009; Smets et al. 2010; Sampaio-Marques et al. 2011).

Further evidence for a role of autophagy in the determination of cell death comes from studies on chronological aging, which defines the length of time a yeast cell can survive in a nondividing state. During chronological aging, yeast cells die exhibiting biochemical and morphological markers of apoptosis as well as necrosis (Herker et al. 2004; Eisenberg et al. 2009). Here, necrosis is not only associated with the excessive production of ROS (Herker et al. 2004; Farrugia and Balzan 2012) and the accumulation of acetic acid (Burtner et al. 2009; Murakami et al. 2012) but also with the decline of natural polyamines, like spermidine (Eisenberg et al. 2009). Consistently, the supplementation of spermidine to chronologically aging yeast suppresses necrosis and extends the lifespan by enhancing autophagy, whereas the deletion of *ATG7* sensitizes cells to necrosis during chronological aging (Eisenberg et al. 2009). Importantly, several reports indicated that spermidine regulates autophagy and necrosis at an epigenetic level via modulation of histone acetyltransferases and histone acetylation (Eisenberg et al. 2009; Madeo et al. 2010; Morselli et al. 2011). This seems to be a highly conserved mechanism because spermidine-mediated

induction of autophagy and lifespan extension is a phenomenon not limited to yeast but observed in flies, nematodes, and human cells as well (Eisenberg et al. 2009). At present, it is not clear whether changes in polyamine levels also impact on the aforementioned nutrient-responsive PKA, TOR, and Sch9 pathways in yeast, but in glioblastoma cells, the pharmacologic intervention with N(1), N(11)-diethylnorspermine, a spermine analog and prototype anticancer drug that depletes cellular polyamine, suggests this to be the case for AKT and mTOR (Jiang et al. 2007). In connection to pH homeostasis, a treatment with exogenous spermidine indeed reverses intracellular acidification both in yeast and mammalian cells (Poulin and Pegg 1995; Eisenberg et al. 2009). However, at least in yeast, this is apparently not essential for the ability of spermidine to prolong lifespan as this is mainly a pH-independent process (Eisenberg et al. 2009).

In addition, spermidine treatment of aged yeast inhibits the nuclear release of the chromatin-associated high-mobility group Box 1 protein (HMGB1) (Eisenberg et al. 2009, 2010). HMGB1 serves as the prototype DAMP or damage-associated molecular patterns in mammalians. These are substances with an immunostimulatory activity that are released as cell permeability increases during death (Raucci et al. 2007; Pisetsky 2011). Recent data identified HMGB1 as a mitophagy, autophagy, and apoptosis regulator whereby nuclear HMGB1 has an essential role in mitochondrial quality control and mitophagy through expression regulation of HSPB1, while cytosolic and extracellular HMGB1 can either stimulate autophagy by interaction with Beclin1, the orthologue of yeast Atg6, or induce apoptosis via the caspase intrinsic pathway, depending on their redox state (Tang et al. 2010a, b, c, 2011; Kang et al. 2011). Furthermore, inhibition of the NAD-dependent deacetylase SIRT1 was shown to attenuate HMGB1-mediated transcriptional effects in human periodontal ligament cells (Kim et al. 2010). Hence, it would be interesting to investigate if HMGB1 fulfills comparable regulatory roles on yeast autophagy in a Sir2-dependent manner. Similar to mammalian HMGB1, yeast HMGB1 facilitates maintenance of genome stability. Deletion of *NHP6A/B*, the genes that redundantly encode for yeast HMGB1, leads to increased genomic instability, hypersensitivity to DNA-damaging agents, levels of extrachromosomal rDNA circles, and a shorter replicative lifespan that refers to the number of daughter cells produced by a mother cell (Giavara et al. 2005). Hence, it is well possible that HMGB1 have a role in genotoxicity-induced necrosis, which in mammalian cells is mediated by the ripoptosome (Imre et al. 2011).

Another crucial player that is connected to polyamines and the regulation of necrosis during chronological aging is Pep4, a vacuolar aspartyl protease and orthologue of human cathepsin D that is important for protein turnover after oxidative damage. While the deletion of *PEP4* exacerbates both apoptotic and necrotic cell death during chronological aging, its overexpression promotes survival by specifically inhibiting necrosis. This anti-necrotic effect is triggered by histone H3 hypoacetylation and mediated by polyamines, the biosynthesis of which is enhanced by Pep4 overexpression (Carmona-Gutierrez et al. 2011). Note that Pep4 was initially identified as yeast cell death regulator in studies dealing with apoptosis induced by H_2O_2, acetic acid, and actin cytoskeleton stabilization. Here, the protease was

shown to translocate from the vacuole into the cytosol prior to the loss of plasma membrane integrity under impulse of PKA signaling (Gourlay and Ayscough 2006). Once released in the cytosol, Pep4 not only facilitates mitochondrial degradation (Pereira et al. 2010) but it also induces proteolysis of nucleoporins (Mason et al. 2005). Whether the latter precedes the abovementioned nuclear release of HMGB1 remains to be studied.

Yet, another prominent regulator of necrosis in yeast is the mitochondrial EndoG homolog Nuc1, which regulates cell death independently of Yca1 and Aif1 (Büttner et al. 2007). In actively growing cells exposed to various damaging insults, the deletion of *NUC1* clearly reduces apoptosis, while its overexpression enhances cell death, which initially is predominantly apoptotic but at later stages becomes mainly necrotic. During chronological aging, however, the situation is far more complex and the data point to dichotomous roles for Nuc1 on cell life and death. Indeed, the deletion of *NUC1* diminishes apoptotic death of cells on glycerol-containing medium, where mitochondrial respiration is induced, but it enhances necrotic death of cells on glucose-containing medium, where respiration is largely repressed (Büttner et al. 2007). This indicates that metabolic preadaptation has a dramatic impact on aging as noticed before for the determination of lifespan (Piper et al. 2006; Piper 2012). The switch from a proapoptotic to an anti-necrotic function is fascinating but not unique. There are other examples of key cell death regulators with dual death/survival functions both in yeast and mammalians, for instance, (meta)caspase(s) and the apoptosis-inducing factor, Aif1 (Galluzzi et al. 2008; Lee et al. 2010). What is equally intriguing is the switch towards necrosis upon reduced cellular respiratory conditions, certainly if one considers that respiration is largely repressed and that mitochondria are even not essential during fermentative growth. Again, the Nuc1 deletion is not the sole example. Cells lacking the mitochondrial manganese superoxide dismutase (*SOD2*) and challenged with the salicylate drug aspirin undergo apoptosis when grown on ethanol as carbon source but necrosis when grown on glucose while they survive happily when grown on glycerol or acetate. For wild-type cells or mutants lacking the Cu,ZnSOD, *SOD1*, a phenotype is only seen under glucose-repression conditions (Balzan et al. 2004). This example demonstrates nicely that not only the induction of cell death but also the determination of its mode is depending on the overall integration of the metabolic state, the genetic context, and the processes targeted by the insult. In this particular case, the aspirin-mediated induction of apoptosis on ethanol-containing medium in cells lacking MnSOD is associated to the release of most of the mitochondrial cytochrome c, a marked drop in the mitochondrial membrane potential, the accumulation of mitochondrial superoxide radicals, and mitochondrial NAD(P)H oxidation, and these effect cannot be compensated for by enhanced Cu,ZnSOD activity, which implies a specific protective effect of MnSOD on mitochondria (Sapienza et al. 2008; Farrugia et al. 2013). That aspirin induces necrotic cell death in all three strains when fermenting probably relates to the drug affecting essential enzymes and signaling components allowing for growth on glucose. For instance, in colorectal cancer cells, aspirin inhibits mTOR, activates AMPK, and induces autophagy (Din et al. 2012). In fermenting yeast, such an action would lead to conflicting

growth signals and metabolic futile cycles. Indeed, while being on glucose, the cells receive signals to sustain optimal fermentation, like enhanced PKA activity, but through the inhibition of TOR and activation of the AMPK-orthologue Snf1, aspirin would dictate to start the diauxic shift transition and to reprogram for respiratory growth, which of course is incompatible (Smets et al. 2010). In addition, the attempts to induce autophagic processes as a consequence of aspirin treatment may also contribute to the disturbance of cellular homeostasis during fermentative growth where this process is normally strictly controlled and kept within limits (Yorimitsu et al. 2007; Stephan et al. 2009; Smets et al. 2010; Sampaio-Marques et al. 2011).

Similar to aspirin, it was observed that tunicamycin, as well as other ER stress agents such as dithiothreitol and azole-class antifungal drugs, induces non-apoptotic cell death in fermenting yeast cells that is accompanied by the loss of membrane integrity. Here, necrotic cell death is linked to Ca^{2+} homeostasis, since activation of the Ca^{2+} channel, calmodulin, calcineurin, and other factors is necessary for long-term survival of cells undergoing ER stress (Bonilla et al. 2002; Bonilla and Cunningham 2003). It is also linked to the action of Hsp90, since the massive cell death instigated by tunicamycin treatment under "calcineurin-less" conditions, either by deletion of CNB1, the regulatory subunit of calcineurin, or by calcineurin inhibition, can be prevented by the additional disruption of Hsp90 using the inhibitor radicicol (Dudgeon et al. 2008). The gathered evidence raises the hypothesis that calcineurin and Hsp90 have opposing functions in the regulation of necrotic cell death in yeast. Hsp90 has a well-known pro-necrotic signaling function in mammalian cells as it is a molecular chaperone for many kinases, including RIP1 (Lewis et al. 2000). However, the lack of a RIP1 homolog in yeast cells obscures the understanding of the exact molecular function of Hsp90 during yeast necrosis. A very recent study has provided evidence that tunicamycin leads to non-apoptotic cell death in yeast cells through the permeabilization of the vacuolar membranes, which involves the V-ATPase, but seems to be independent of autophagy (Kim et al. 2012). These findings suggest that vacuole membrane permeabilization is a lethal event mediating a mechanism of necrotic cell death in yeast cells, thereby resembling the lysosomal membrane permeabilization occurring during necrosis in mammalian cells (Vandenabeele et al. 2010). Importantly, the tunicamycin-induced necrotic cell death involving vacuole membrane permeabilization could be blocked by calmodulin (Kim et al. 2012). Calmodulin, calcineurin, and the high-affinity Ca^{2+} influx channel are important components of the evolutionary conserved calcium signaling pathway that maintains cytoplasmic-free Ca^{2+} concentration within levels compatible with cell viability (Bonilla and Cunningham 2002). A variety of environmental insults can lead to the disruption of Ca^{2+} homeostasis, including osmotic shock, alkaline pH, cell wall damage, ER stress, and exposure to high levels of mating pheromones. Notably, exposure to high concentrations of mating pheromones in the absence of mating partners results in a rapid necrotic death that is seriously aggravated by deficiencies in the calcium signaling pathway (Severin and Hyman 2002; Zhang et al. 2006).

The connection between ER stress and cell death brings us to the heterologous expression of human proteins. Actually, that yeast had the potential to induce cell

death programs was first suggested by the early studies dealing with heterologous expression of key proapoptotic Bcl-2 family proteins, Bax or Bak, in *S. cerevisiae* and *S. pombe*, which induce lethal phenotypes suppressible by co-expression of the antiapoptotic members Bcl-xL, Bcl-2, Mcl-1, or A1 (Sato et al. 1994; Bodrug et al. 1995; Hanada et al. 1995; Ink et al. 1997; Jurgensmeier et al. 1997; Tao et al. 1997, 1998). Although some studies then reported that the expression of proapoptotic human proteins triggered morphological features suggesting apoptotic cell death (Ink et al. 1997; Ligr et al. 1998), later studies demonstrated that Bax-induced cell death was not apoptotic but associated with a massive cytoplasmic vacuolization, indicative for the induction of autophagy (Jurgensmeier et al. 1997; Kissova et al. 2006). In the budding yeast, these studies also pointed towards links with mitophagy (Kissova et al. 2006). Similar to mammalian cells, Bax forms homodimers at the yeast mitochondrial membrane and thereby mediates the loss of mitochondrial membrane potential and cytochrome c (cyt-c) release (Greenhalf et al. 1996; Zha et al. 1996; Jurgensmeier et al. 1997; Manon et al. 1997). As such, Bax induces similar threats as the yeast BH3-only protein Ybh3 (Büttner et al. 2011). Interestingly, Ybh3 was first described as the putative homolog of human Bax inhibitor-1 (BI-1), named Bxi1, based on sequence comparison and its ability to rescue yeast cells from Bax-induced lethality when overexpressed or to protect them against heat shock, ethanol stress, or oxidative stress. Consistent with a role as BI-1 homolog, Bxi1 resides in the ER and functions in the unfolded protein response (UPR) (Cebulski et al. 2011). This Ybh3/Bxi1 protein deserves further investigation, as it may very well represent yet another protein that exerts a pro-death or pro-survival function depending on the growth condition.

Another example of a human protein that is studied rigorously in yeast is α-synuclein, a protein associated with Parkinson's disease. When expressed in yeast, α-synuclein triggers apoptosis as well as necrosis. Its toxicity is related to α-synuclein causing an ER-to-Golgi block and a general collapse of vesicular trafficking (Cooper et al. 2006; Zabrocki et al. 2008; Sancenon et al. 2012). This is accompanied by the accumulation of ROS due to ER stress and further intensified by mitochondrial dysfunction since, indeed, the toxicity of α-synuclein requires functional mitochondria (Büttner et al. 2008). Probably as an attempt to deal with α-synuclein-instigated toxicity during aging, the yeast cells also induce autophagy and mitophagy through transcriptional activation of the *ATG* genes via the sirtuin Sir2. Interestingly, however, while enhanced autophagy and mitophagy were found to be beneficial for control cells, in that they display a longer chronological lifespan, it apparently is detrimental for cells expressing α-synuclein because their lifespan was ameliorated upon disruption of the process (Sampaio-Marques et al. 2012). This suggests that upon α-synuclein expression, the induction of autophagy and mitophagy leads to an additional burden in cells that already suffer dramatically from vesicular trafficking problems. Consistent with a close connection between ER stress and Ca^{2+} signaling, a most recent study showed α-synuclein to elevate cytosolic Ca^{2+} levels and revealed that specifically the disruption of the Golgi resident Ca^{2+}/Mn^{2+} P-type ATPase Pmr1, and not other proteins known to influence Ca^{2+} transport or sensing, inhibits both the increased cytosolic Ca^{2+} levels and the

apoptotic and necrotic cellular demise (Büttner et al. 2013a). Most recently, the EndoG homolog Nuc1 was identified as a critical executor of α-synuclein-induced toxicity and cell death in yeast (Büttner et al. 2013b). Thus, it is clear that different processes causally link to instigate and intensify α-synuclein toxicity in yeast and this eventually integrates into a mixed cell death phenotype. Note that most of the α-synuclein toxicity mechanisms identified in yeast were recapitulated in other model systems, including mammalian cell lines, nematodes, flies, or rodents, thereby not only strengthening the relevance of these findings for the pathology associated with Parkinson's disease in humans but further validating the use of yeast as a relevant toolbox to study the α-synuclein pathobiology, besides cell death and aging processes.

14.4 Concluding Remarks

To summarize, the examples given above clearly indicate that yeast can decide between an apoptotic mode of cell death and a necrotic one in function of the strength of a damaging insult and its targeted processes, and this is in relation to the metabolic status. That this is an adaptive decision, and thus dependent on a series of molecular events, is further supported by the fact that the choice between both modes of cell death can be influenced dramatically by specific genetic alterations. Also in yeast, apoptosis and necrosis appear to involve, at least in part, the same players as depicted in Fig. 14.2. Some of these players have opposing and therefore crucial discriminative roles due to their dual death/survival functions, the exposure of which is dependent on the prevailing physiological status. Furthermore, the fact that necrosis can occur without typical hallmarks of apoptosis is a strong indication that the choice for this cell death mode is not, or not always, based on a transition from apoptosis to necrosis, but that necrosis can be induced as stand-alone program. As to the question what triggers this primary necrotic program in yeast, the gathered evidence points to insults that affect processes or enforce metabolic changes incompatible with the prevailing physiological status, which prevent cells to integrate their growth and reproduction potential and, as such, leads to an insolvable deleterious conflict. During aging, a similar situation appears to occur. Here, the data indicate that induction of necrosis is not only dependent on the accumulated intracellular damage but determined by preadaptation conditions defining the respiratory potential and maintenance. Hence, it would be interesting to find out whether metabolic and signaling conflicts are indeed the reason why yeast cells induce necrosis and not apoptosis.

Being one of the oldest domesticated organisms, it may not come as a surprise that the ability of *S. cerevisiae* to undergo a self-destructing program has been exploited already a long time ago. Indeed, after the second fermentation and aging step of sparkling wines and wines aged on lees, yeast cells finally die and undergo autolysis. This autolysis is highly desired in the production of sparkling wines as it results in the release of cellular compounds with a positive effect on the wine

quality and the characteristic *méthode champenoise* bouquet (Alexandre and Guilloux-Benatier 2006). Curiously, only few studies have been performed to understand the mechanisms underlying the autolytic capacity of yeast and it is only recently that a correlation was found between autolysis and deregulated autophagy (Cebollero et al. 2005, 2008; Cebollero and Gonzalez 2006). A recent study described in detail the morphological changes in the ultrastructure of yeast cells during long-term aging in real enological conditions. This study not only confirmed the role of autophagy in the autolytic process but surprisingly also found evidence suggesting that plasma membrane disintegration starts with the production of small fragments that remain and are probably degraded inside the cell (Tudela et al. 2012). Hence, these data strongly suggest that autolysis is a well-orchestrated process representing actually autophagy-associated programmed necrosis.

Although much research remains to be done to fully unravel the underlying molecular mechanisms for apoptotic and necrotic cell death, autophagic processes certainly play a central role because similar to higher eukaryotes, these processes appear to concurrently facilitate either form of cell death in yeast (Dziedzic and Caplan 2012). Being essential for cellular homeostasis and for efficient cellular responses to stress, the autophagic processes are tightly controlled by signaling pathways that also impact on metabolism and stress resistance (Yorimitsu et al. 2007; Stephan et al. 2009; Smets et al. 2010) as well as the determination of chronological and replicative lifespan (Sampaio-Marques et al. 2011; Longo et al. 2012). Furthermore, recent studies indicated that stationary phase yeast cultures contain both quiescent and non-quiescent cells, whereby especially the latter show signs of apoptotic and necrotic cell death. In contrast to quiescent cells, which consist mainly of daughter cells well synchronized to cell cycle reentry, non-quiescent cells lost the potential to properly respond to environmental stimuli and the ability to reproduce because of genomic instability. These studies also identified more than 1,300 mRNAs that differentiate quiescent and non-quiescent cell types. As could be expected, many of those mRNAs encode proteins involved in vesicular trafficking; proteins involved in signal transduction pathways, including those controlled by TORC1, PKA, and the AMPK-kinase Snf1; or proteins required for Ty-element transposition and DNA recombination (Aragon et al. 2008; Werner-Washburne et al. 2012). Although this approach holds great promise, it will still be difficult to determine what exactly drives aging yeast cells to decide to commit necrosis instead of apoptosis, as this is likely to be dependent on the physiological context and may well be different between individual cells, certainly if one considers a yeast colony where this context is characterized by gradients of nutrient availability and signaling molecules, leading metabolic diversification between individual cells of the colony (Vachova et al. 2009, 2012; Vachova and Palkova 2011). Nonetheless, it is rather amazing that nature apparently foresaw scenarios where yeast cells that are no longer able to integrate their growth and reproduction potential, or that simply lost their ability to reproduce, commit an altruistic suicide as to remove themselves from the community thereby providing food, and possibly release danger signals aiming to increase the chances for survival of their species under adverse conditions.

References

Akematsu T, Endoh H (2010) Role of apoptosis-inducing factor (AIF) in programmed nuclear death during conjugation in Tetrahymena thermophila. BMC Cell Biol 11:13

Alexandre H, Guilloux-Benatier M (2006) Yeast autolysis in sparkling wine – a review. Aust J Grape Wine Res 12:119–127

Almeida B, Ohlmeier S, Almeida AJ, Madeo F, Leao C, Rodrigues F, Ludovico P (2009) Yeast protein expression profile during acetic acid-induced apoptosis indicates causal involvement of the TOR pathway. Proteomics 9:720–732

Amigoni L, Martegani E, Colombo S (2013) Lack of HXK2 induces localization of active Ras in mitochondria and triggers apoptosis in the yeast Saccharomyces cerevisiae. Oxid Med Cell Longev 2013:678473

Aragon AD, Rodriguez AL, Meirelles O, Roy S, Davidson GS, Tapia PH, Allen C, Joe R, Benn D, Werner-Washburne M (2008) Characterization of differentiated quiescent and nonquiescent cells in yeast stationary-phase cultures. Mol Biol Cell 19:1271–1280

Aravind L, Dixit VM, Koonin EV (1999) The domains of death: evolution of the apoptosis machinery. Trends Biochem Sci 24:47–53

Arnoult D, Tatischeff I, Estaquier J, Girard M, Sureau F, Tissier JP, Grodet A, Dellinger M, Traincard F, Kahn A, Ameisen JC, Petit PX (2001) On the evolutionary conservation of the cell death pathway: mitochondrial release of an apoptosis-inducing factor during Dictyostelium discoideum cell death. Mol Biol Cell 12:3016–3030

Balzan R, Sapienza K, Galea DR, Vassallo N, Frey H, Bannister WH (2004) Aspirin commits yeast cells to apoptosis depending on carbon source. Microbiology 150:109–115

Berry DL, Baehrecke EH (2008) Autophagy functions in programmed cell death. Autophagy 4:359–360

Bodrug SE, Aime-Sempe C, Sato T, Krajewski S, Hanada M, Reed JC (1995) Biochemical and functional comparisons of Mcl-1 and Bcl-2 proteins: evidence for a novel mechanism of regulating Bcl-2 family protein function. Cell Death Differ 2:173–182

Boer VM, Amini S, Botstein D (2008) Influence of genotype and nutrition on survival and metabolism of starving yeast. Proc Natl Acad Sci U S A 105:6930–6935

Bonilla M, Cunningham KW (2002) Calcium release and influx in yeast: TRPC and VGCC rule another kingdom. Sci STKE 2002:pe17

Bonilla M, Cunningham KW (2003) Mitogen-activated protein kinase stimulation of Ca(2+) signaling is required for survival of endoplasmic reticulum stress in yeast. Mol Biol Cell 14:4296–4305

Bonilla M, Nastase KK, Cunningham KW (2002) Essential role of calcineurin in response to endoplasmic reticulum stress. EMBO J 21:2343–2353

Burtner CR, Murakami CJ, Kennedy BK, Kaeberlein M (2009) A molecular mechanism of chronological aging in yeast. Cell Cycle 8:1256–1270

Büttner S, Bitto A, Ring J, Augsten M, Zabrocki P, Eisenberg T, Jungwirth H, Hutter S, Carmona-Gutierrez D, Kroemer G, Winderickx J, Madeo F (2008) Functional mitochondria are required for alpha-synuclein toxicity in aging yeast. J Biol Chem 283:7554–7560

Büttner S, Eisenberg T, Carmona-Gutierrez D, Ruli D, Knauer H, Ruckenstuhl C, Sigrist C, Wissing S, Kollroser M, Frohlich KU, Sigrist S, Madeo F (2007) Endonuclease G regulates budding yeast life and death. Mol Cell 25:233–246

Büttner S, Faes L, Reichelt WN, Broeskamp F, Habernig L, Benke S, Kourtis N, Ruli D, Carmona-Gutierrez D, Eisenberg T, D'Hooge P, Ghillebert R, Franssens V, Harger A, Pieber TR, Freudenberger P, Kroemer G, Sigrist SJ, Winderickx J, Callewaert G, Tavernarakis N, Madeo F (2013a) The Ca2+/Mn2+ ion-pump PMR1 links elevation of cytosolic Ca(2+) levels to alpha-synuclein toxicity in Parkinson's disease models. Cell Death Differ 20:465–477

Büttner S, Habernig L, Broeskamp F, Ruli D, Vogtle FN, Vlachos M, Macchi F, Kuttner V, Carmona-Gutierrez D, Eisenberg T, Ring J, Markaki M, Taskin AA, Benke S, Ruckenstuhl C, Braun R, Van den Haute C, Bammens T, van der Perren A, Frohlich KU, Winderickx J,

Kroemer G, Baekelandt V, Tavernarakis N, Kovacs GG, Dengjel J, Meisinger C, Sigrist SJ, Madeo F (2013b) Endonuclease G mediates alpha-synuclein cytotoxicity during Parkinson's disease. EMBO J 32(23):3041–3054

Büttner S, Ruli D, Vogtle FN, Galluzzi L, Moitzi B, Eisenberg T, Kepp O, Habernig L, Carmona-Gutierrez D, Rockenfeller P, Laun P, Breitenbach M, Khoury C, Frohlich KU, Rechberger G, Meisinger C, Kroemer G, Madeo F (2011) A yeast BH3-only protein mediates the mitochondrial pathway of apoptosis. EMBO J 30:2779–2792

Carmona-Gutierrez D, Bauer MA, Ring J, Knauer H, Eisenberg T, Büttner S, Ruckenstuhl C, Reisenbichler A, Magnes C, Rechberger GN, Birner-Gruenberger R, Jungwirth H, Frohlich KU, Sinner F, Kroemer G, Madeo F (2011) The propeptide of yeast cathepsin D inhibits programmed necrosis. Cell Death Dis 2:e161

Carmona-Gutierrez D, Eisenberg T, Büttner S, Meisinger C, Kroemer G, Madeo F (2010a) Apoptosis in yeast: triggers, pathways, subroutines. Cell Death Differ 17:763–773

Carmona-Gutierrez D, Frohlich KU, Kroemer G, Madeo F (2010b) Metacaspases are caspases. Doubt no more. Cell Death Differ 17:377–378

Cebollero E, Carrascosa AV, Gonzalez R (2005) Evidence for yeast autophagy during simulation of sparkling wine aging: a reappraisal of the mechanism of yeast autolysis in wine. Biotechnol Prog 21:614–616

Cebollero E, Gonzalez R (2006) Induction of autophagy by second-fermentation yeasts during elaboration of sparkling wines. Appl Environ Microbiol 72:4121–4127

Cebollero E, Rejas MT, Gonzalez R (2008) Autophagy in wine making. Methods Enzymol 451:163–175

Cebulski J, Malouin J, Pinches N, Cascio V, Austriaco N (2011) Yeast Bax inhibitor, Bxi1p, is an ER-localized protein that links the unfolded protein response and programmed cell death in Saccharomyces cerevisiae. PLoS One 6:e20882

Claerhout S, Dutta B, Bossuyt W, Zhang F, Nguyen-Charles C, Dennison JB, Yu Q, Yu S, Balazsi G, Lu Y, Mills GB (2012) Abortive autophagy induces endoplasmic reticulum stress and cell death in cancer cells. PLoS One 7:e39400

Cooper AA, Gitler AD, Cashikar A, Haynes CM, Hill KJ, Bhullar B, Liu K, Xu K, Strathearn KE, Liu F, Cao S, Caldwell KA, Caldwell GA, Marsischky G, Kolodner RD, Labaer J, Rochet JC, Bonini NM, Lindquist S (2006) Alpha-synuclein blocks ER-Golgi traffic and Rab1 rescues neuron loss in Parkinson's models. Science 313:324–328

Coyne RS, Nikiforov MA, Smothers JF, Allis CD, Yao MC (1999) Parental expression of the chromodomain protein Pdd1p is required for completion of programmed DNA elimination and nuclear differentiation. Mol Cell 4:865–872

Deponte M (2008) Programmed cell death in protists. Biochim Biophys Acta 1783:1396–1405

Dickens LS, Powley IR, Hughes MA, MacFarlane M (2012) The "complexities" of life and death: death receptor signalling platforms. Exp Cell Res 318:1269–1277

Din FV, Valanciute A, Houde VP, Zibrova D, Green KA, Sakamoto K, Alessi DR, Dunlop MG (2012) Aspirin inhibits mTOR signaling, activates AMP-activated protein kinase, and induces autophagy in colorectal cancer cells. Gastroenterology 142:1504–1515, e1503

Du L, Yu Y, Chen J, Liu Y, Xia Y, Chen Q, Liu X (2007) Arsenic induces caspase- and mitochondria-mediated apoptosis in Saccharomyces cerevisiae. FEMS Yeast Res 7:860–865

Dudgeon DD, Zhang N, Ositelu OO, Kim H, Cunningham KW (2008) Nonapoptotic death of Saccharomyces cerevisiae cells that is stimulated by Hsp90 and inhibited by calcineurin and Cmk2 in response to endoplasmic reticulum stresses. Eukaryot Cell 7:2037–2051

Dwyer DJ, Camacho DM, Kohanski MA, Callura JM, Collins JJ (2012) Antibiotic-induced bacterial cell death exhibits physiological and biochemical hallmarks of apoptosis. Mol Cell 46:561–572

Dziedzic SA, Caplan AB (2012) Autophagy proteins play cytoprotective and cytocidal roles in leucine starvation-induced cell death in Saccharomyces cerevisiae. Autophagy 8:731–738

Eastwood MD, Cheung SW, Lee KY, Moffat J, Meneghini MD (2012) Developmentally programmed nuclear destruction during yeast gametogenesis. Dev Cell 23:35–44

Eisenberg T, Carmona-Gutierrez D, Büttner S, Tavernarakis N, Madeo F (2010) Necrosis in yeast. Apoptosis 15:257–268

Eisenberg T, Knauer H, Schauer A, Büttner S, Ruckenstuhl C, Carmona-Gutierrez D, Ring J, Schroeder S, Magnes C, Antonacci L, Fussi H, Deszcz L, Hartl R, Schraml E, Criollo A, Megalou E, Weiskopf D, Laun P, Heeren G, Breitenbach M, Grubeck-Loebenstein B, Herker E, Fahrenkrog B, Frohlich KU, Sinner F, Tavernarakis N, Minois N, Kroemer G, Madeo F (2009) Induction of autophagy by spermidine promotes longevity. Nat Cell Biol 11:1305–1314

Engelberg-Kulka H, Sat B, Reches M, Amitai S, Hazan R (2004) Bacterial programmed cell death systems as targets for antibiotics. Trends Microbiol 12:66–71

Fahrenkrog B (2011) Nma111p, the pro-apoptotic HtrA-like nuclear serine protease in Saccharomyces cerevisiae: a short survey. Biochem Soc Trans 39:1499–1501

Fahrenkrog B, Sauder U, Aebi U (2004) The S. cerevisiae HtrA-like protein Nma111p is a nuclear serine protease that mediates yeast apoptosis. J Cell Sci 117:115–126

Farrugia G, Balzan R (2012) Oxidative stress and programmed cell death in yeast. Front Oncol 2:64

Farrugia G, Bannister WH, Vassallo N, Balzan R (2013) Aspirin-induced apoptosis of yeast cells is associated with mitochondrial superoxide radical accumulation and NAD(P)H oxidation. FEMS Yeast Res 13(8):755–768

Galluzzi L, Joza N, Tasdemir E, Maiuri MC, Hengartner M, Abrams JM, Tavernarakis N, Penninger J, Madeo F, Kroemer G (2008) No death without life: vital functions of apoptotic effectors. Cell Death Differ 15:1113–1123

Galluzzi L, Vitale I, Abrams JM, Alnemri ES, Baehrecke EH, Blagosklonny MV, Dawson TM, Dawson VL, El-Deiry WS, Fulda S, Gottlieb E, Green DR, Hengartner MO, Kepp O, Knight RA, Kumar S, Lipton SA, Lu X, Madeo F, Malorni W, Mehlen P, Nunez G, Peter ME, Piacentini M, Rubinsztein DC, Shi Y, Simon HU, Vandenabeele P, White E, Yuan J, Zhivotovsky B, Melino G, Kroemer G (2012) Molecular definitions of cell death subroutines: recommendations of the Nomenclature Committee on Cell Death 2012. Cell Death Differ 19:107–120

Gannavaram S, Vedvyas C, Debrabant A (2008) Conservation of the pro-apoptotic nuclease activity of endonuclease G in unicellular trypanosomatid parasites. J Cell Sci 121:99–109

Giavara S, Kosmidou E, Hande MP, Bianchi ME, Morgan A, d'Adda di Fagagna F, Jackson SP (2005) Yeast Nhp6A/B and mammalian Hmgb1 facilitate the maintenance of genome stability. Curr Biol 15:68–72

Gordy C, He YW (2012) The crosstalk between autophagy and apoptosis: where does this lead? Protein Cell 3:17–27

Gourlay CW, Ayscough KR (2006) Actin-induced hyperactivation of the Ras signaling pathway leads to apoptosis in Saccharomyces cerevisiae. Mol Cell Biol 26:6487–6501

Greenhalf W, Stephan C, Chaudhuri B (1996) Role of mitochondria and C-terminal membrane anchor of Bcl-2 in Bax induced growth arrest and mortality in Saccharomyces cerevisiae. FEBS Lett 380:169–175

Guaragnella N, Bobba A, Passarella S, Marra E, Giannattasio S (2010) Yeast acetic acid-induced programmed cell death can occur without cytochrome c release which requires metacaspase YCA1. FEBS Lett 584:224–228

Guaragnella N, Pereira C, Sousa MJ, Antonacci L, Passarella S, Corte-Real M, Marra E, Giannattasio S (2006) YCA1 participates in the acetic acid induced yeast programmed cell death also in a manner unrelated to its caspase-like activity. FEBS Lett 580:6880–6884

Hanada M, Aime-Sempe C, Sato T, Reed JC (1995) Structure-function analysis of Bcl-2 protein. Identification of conserved domains important for homodimerization with Bcl-2 and heterodimerization with Bax. J Biol Chem 270:11962–11969

Heo JM, Livnat-Levanon N, Taylor EB, Jones KT, Dephoure N, Ring J, Xie J, Brodsky JL, Madeo F, Gygi SP, Ashrafi K, Glickman MH, Rutter J (2010) A stress-responsive system for mitochondrial protein degradation. Mol Cell 40:465–480

Herker E, Jungwirth H, Lehmann KA, Maldener C, Frohlich KU, Wissing S, Buttner S, Fehr M, Sigrist S, Madeo F (2004) Chronological aging leads to apoptosis in yeast. J Cell Biol 164:501–507

Imre G, Larisch S, Rajalingam K (2011) Ripoptosome: a novel IAP-regulated cell death-signalling platform. J Mol Cell Biol 3:324–326

Ink B, Zornig M, Baum B, Hajibagheri N, James C, Chittenden T, Evan G (1997) Human Bak induces cell death in Schizosaccharomyces pombe with morphological changes similar to those with apoptosis in mammalian cells. Mol Cell Biol 17:2468–2474

Jiang R, Choi W, Hu L, Gerner EW, Hamilton SR, Zhang W (2007) Activation of polyamine catabolism by N1, N11-diethylnorspermine alters the cellular localization of mTOR and down-regulates mTOR protein level in glioblastoma cells. Cancer Biol Ther 6:1644–1648

Jungwirth H, Ring J, Mayer T, Schauer A, Buttner S, Eisenberg T, Carmona-Gutierrez D, Kuchler K, Madeo F (2008) Loss of peroxisome function triggers necrosis. FEBS Lett 582:2882–2886

Jurgensmeier JM, Krajewski S, Armstrong RC, Wilson GM, Oltersdorf T, Fritz LC, Reed JC, Ottilie S (1997) Bax- and Bak-induced cell death in the fission yeast Schizosaccharomyces pombe. Mol Biol Cell 8:325–339

Kaczanowski S, Sajid M, Reece SE (2011) Evolution of apoptosis-like programmed cell death in unicellular protozoan parasites. Parasit Vectors 4:44

Kang R, Livesey KM, Zeh HJ 3rd, Lotze MT, Tang D (2011) Metabolic regulation by HMGB1-mediated autophagy and mitophagy. Autophagy 7:1256–1258

Kerr JF, Wyllie AH, Currie AR (1972) Apoptosis: a basic biological phenomenon with wide-ranging implications in tissue kinetics. Br J Cancer 26:239–257

Kim H, Kim A, Cunningham KW (2012) Vacuolar H+-ATPase (V-ATPase) promotes vacuolar membrane permeabilization and nonapoptotic death in stressed yeast. J Biol Chem 287:19029–19039

Kim YS, Lee YM, Park JS, Lee SK, Kim EC (2010) SIRT1 modulates high-mobility group box 1-induced osteoclastogenic cytokines in human periodontal ligament cells. J Cell Biochem 111:1310–1320

Kissova I, Plamondon LT, Brisson L, Priault M, Renouf V, Schaeffer J, Camougrand N, Manon S (2006) Evaluation of the roles of apoptosis, autophagy, and mitophagy in the loss of plating efficiency induced by Bax expression in yeast. J Biol Chem 281:36187–36197

Kitanovic A, Bonowski F, Heigwer F, Ruoff P, Kitanovic I, Ungewiss C, Wolfl S (2012) Acetic acid treatment in S. cerevisiae creates significant energy deficiency and nutrient starvation that is dependent on the activity of the mitochondrial transcriptional complex Hap2-3-4-5. Front Oncol 2:118

Klosinska MM, Crutchfield CA, Bradley PH, Rabinowitz JD, Broach JR (2011) Yeast cells can access distinct quiescent states. Genes Dev 25:336–349

Koonin EV, Aravind L (2002) Origin and evolution of eukaryotic apoptosis: the bacterial connection. Cell Death Differ 9:394–404

Kroemer G, Galluzzi L, Vandenabeele P, Abrams J, Alnemri ES, Baehrecke EH, Blagosklonny MV, El-Deiry WS, Golstein P, Green DR, Hengartner M, Knight RA, Kumar S, Lipton SA, Malorni W, Nunez G, Peter ME, Tschopp J, Yuan J, Piacentini M, Zhivotovsky B, Melino G (2009) Classification of cell death: recommendations of the Nomenclature Committee on Cell Death 2009. Cell Death Differ 16:3–11

Leadsham JE, Gourlay CW (2010) cAMP/PKA signaling balances respiratory activity with mitochondria dependent apoptosis via transcriptional regulation. BMC Cell Biol 11:92

Lee RE, Brunette S, Puente LG, Megeney LA (2010) Metacaspase Yca1 is required for clearance of insoluble protein aggregates. Proc Natl Acad Sci U S A 107:13348–13353

Lewis J, Devin A, Miller A, Lin Y, Rodriguez Y, Neckers L, Liu ZG (2000) Disruption of hsp90 function results in degradation of the death domain kinase, receptor-interacting protein (RIP), and blockage of tumor necrosis factor-induced nuclear factor-kappaB activation. J Biol Chem 275:10519–10526

Lewis K (2000) Programmed death in bacteria. Microbiol Mol Biol Rev 64:503–514

Liang Q, Zhou B (2007) Copper and manganese induce yeast apoptosis via different pathways. Mol Biol Cell 18:4741–4749

Ligr M, Madeo F, Frohlich E, Hilt W, Frohlich KU, Wolf DH (1998) Mammalian Bax triggers apoptotic changes in yeast. FEBS Lett 438:61–65

Longo VD, Mitteldorf J, Skulachev VP (2005) Programmed and altruistic ageing. Nat Rev Genet 6:866–872

Longo VD, Shadel GS, Kaeberlein M, Kennedy B (2012) Replicative and chronological aging in Saccharomyces cerevisiae. Cell Metab 16:18–31

Loos B, Engelbrecht AM, Lockshin RA, Klionsky DJ, Zakeri Z (2013) The variability of autophagy and cell death susceptibility: unanswered questions. Autophagy 9:1270–1285

Low CP, Yang H (2008) Programmed cell death in fission yeast Schizosaccharomyces pombe. Biochim Biophys Acta 1783:1335–1349

Luciani MF, Giusti C, Harms B, Oshima Y, Kikuchi H, Kubohara Y, Golstein P (2011) Atg1 allows second-signaled autophagic cell death in Dictyostelium. Autophagy 7:501–508

Ludovico P, Rodrigues F, Almeida A, Silva MT, Barrientos A, Corte-Real M (2002) Cytochrome c release and mitochondria involvement in programmed cell death induced by acetic acid in Saccharomyces cerevisiae. Mol Biol Cell 13:2598–2606

Ludovico P, Sousa MJ, Silva MT, Leao C, Corte-Real M (2001) Saccharomyces cerevisiae commits to a programmed cell death process in response to acetic acid. Microbiology 147: 2409–2415

Madeo F, Carmona-Gutierrez D, Ring J, Büttner S, Eisenberg T, Kroemer G (2009) Caspase-dependent and caspase-independent cell death pathways in yeast. Biochem Biophys Res Commun 382:227–231

Madeo F, Frohlich E, Frohlich KU (1997) A yeast mutant showing diagnostic markers of early and late apoptosis. J Cell Biol 139:729–734

Madeo F, Frohlich E, Ligr M, Grey M, Sigrist SJ, Wolf DH, Frohlich KU (1999) Oxygen stress: a regulator of apoptosis in yeast. J Cell Biol 145:757–767

Madeo F, Herker E, Maldener C, Wissing S, Lachelt S, Herlan M, Fehr M, Lauber K, Sigrist SJ, Wesselborg S, Frohlich KU (2002) A caspase-related protease regulates apoptosis in yeast. Mol Cell 9:911–917

Madeo F, Tavernarakis N, Kroemer G (2010) Can autophagy promote longevity? Nat Cell Biol 12:842–846

Madireddi MT, Coyne RS, Smothers JF, Mickey KM, Yao MC, Allis CD (1996) Pdd1p, a novel chromodomain-containing protein, links heterochromatin assembly and DNA elimination in Tetrahymena. Cell 87:75–84

Maercker C, Kortwig H, Nikiforov MA, Allis CD, Lipps HJ (1999) A nuclear protein involved in apoptotic-like DNA degradation in Stylonychia: implications for similar mechanisms in differentiating and starved cells. Mol Biol Cell 10:3003–3014

Manon S, Chaudhuri B, Guerin M (1997) Release of cytochrome c and decrease of cytochrome c oxidase in Bax-expressing yeast cells, and prevention of these effects by coexpression of Bcl-xL. FEBS Lett 415:29–32

Mason DA, Shulga N, Undavai S, Ferrando-May E, Rexach MF, Goldfarb DS (2005) Increased nuclear envelope permeability and Pep4p-dependent degradation of nucleoporins during hydrogen peroxide-induced cell death. FEMS Yeast Res 5:1237–1251

Montanaro L, Poggi A, Visai L, Ravaioli S, Campoccia D, Speziale P, Arciola CR (2011) Extracellular DNA in biofilms. Int J Artif Organs 34:824–831

Morselli E, Marino G, Bennetzen MV, Eisenberg T, Megalou E, Schroeder S, Cabrera S, Benit P, Rustin P, Criollo A, Kepp O, Galluzzi L, Shen S, Malik SA, Maiuri MC, Horio Y, Lopez-Otin C, Andersen JS, Tavernarakis N, Madeo F, Kroemer G (2011) Spermidine and resveratrol induce autophagy by distinct pathways converging on the acetylproteome. J Cell Biol 192:615–629

Muller M, Reichert AS (2011) Mitophagy, mitochondrial dynamics and the general stress response in yeast. Biochem Soc Trans 39:1514–1519

Murakami C, Delaney JR, Chou A, Carr D, Schleit J, Sutphin GL, An EH, Castanza AS, Fletcher M, Goswami S, Higgins S, Holmberg M, Hui J, Jelic M, Jeong KS, Kim JR, Klum S, Liao E,

Lin MS, Lo W, Miller H, Moller R, Peng ZJ, Pollard T, Pradeep P, Pruett D, Rai D, Ros V, Schuster A, Singh M, Spector BL, Vander Wende H, Wang AM, Wasko BM, Olsen B, Kaeberlein M (2012) pH neutralization protects against reduction in replicative lifespan following chronological aging in yeast. Cell Cycle 11:3087–3096

Nedelcu AM, Driscoll WW, Durand PM, Herron MD, Rashidi A (2011) On the paradigm of altruistic suicide in the unicellular world. Evolution 65:3–20

Nikoletopoulou V, Markaki M, Palikaras K, Tavernarakis N (2013) Crosstalk between apoptosis, necrosis and autophagy. Biochim Biophys Acta 1833(12):3448–3459

Oda K, Kawasaki N, Fukuyama M, Ikeda S (2007) Ectopic expression of mitochondria endonuclease Pnu1p from Schizosaccharomyces pombe induces cell death of the yeast. J Biochem Mol Biol 40:1095–1099

Pereira C, Chaves S, Alves S, Salin B, Camougrand N, Manon S, Sousa MJ, Corte-Real M (2010) Mitochondrial degradation in acetic acid-induced yeast apoptosis: the role of Pep4 and the ADP/ATP carrier. Mol Microbiol 76:1398–1410

Piper PW (2012) Maximising the yeast chronological lifespan. Subcell Biochem 57:145–159

Piper PW, Harris NL, MacLean M (2006) Preadaptation to efficient respiratory maintenance is essential both for maximal longevity and the retention of replicative potential in chronologically ageing yeast. Mech Ageing Dev 127:733–740

Pisetsky D (2011) Cell death in the pathogenesis of immune-mediated diseases: the role of HMGB1 and DAMP-PAMP complexes. Swiss Med Wkly 141:w13256

Pollitt LC, Colegrave N, Khan SM, Sajid M, Reece SE (2010) Investigating the evolution of apoptosis in malaria parasites: the importance of ecology. Parasit Vectors 3:105

Poulin R, Pegg AE (1995) Stable intracellular acidification upon polyamine depletion induced by alpha-difluoromethylornithine or N1, N12-bis(ethyl)spermine in L1210 leukaemia cells. Biochem J 312(Pt 3):749–756

Raucci A, Palumbo R, Bianchi ME (2007) HMGB1: a signal of necrosis. Autoimmunity 40: 285–289

Reece SE, Pollitt LC, Colegrave N, Gardner A (2011) The meaning of death: evolution and ecology of apoptosis in protozoan parasites. PLoS Pathog 7:e1002320

Reiter J, Herker E, Madeo F, Schmitt MJ (2005) Viral killer toxins induce caspase-mediated apoptosis in yeast. J Cell Biol 168:353–358

Rico E, Alzate JF, Arias AA, Moreno D, Clos J, Gago F, Moreno I, Dominguez M, Jimenez-Ruiz A (2009) *Leishmania infantum* expresses a mitochondrial nuclease homologous to EndoG that migrates to the nucleus in response to an apoptotic stimulus. Mol Biochem Parasitol 163: 28–38

Roisin-Bouffay C, Luciani MF, Klein G, Levraud JP, Adam M, Golstein P (2004) Developmental cell death in dictyostelium does not require paracaspase. J Biol Chem 279:11489–11494

Sampaio-Marques B, Felgueiras C, Silva A, Rodrigues F, Ludovico P (2011) Yeast chronological lifespan and proteotoxic stress: is autophagy good or bad? Biochem Soc Trans 39:1466–1470

Sampaio-Marques B, Felgueiras C, Silva A, Rodrigues M, Tenreiro S, Franssens V, Reichert AS, Outeiro TF, Winderickx J, Ludovico P (2012) SNCA (alpha-synuclein)-induced toxicity in yeast cells is dependent on sirtuin 2 (Sir2)-mediated mitophagy. Autophagy 8:1494–1509

Sancenon V, Lee SA, Patrick C, Griffith J, Paulino A, Outeiro TF, Reggiori F, Masliah E, Muchowski PJ (2012) Suppression of alpha-synuclein toxicity and vesicle trafficking defects by phosphorylation at S129 in yeast depends on genetic context. Hum Mol Genet 21: 2432–2449

Sapienza K, Bannister W, Balzan R (2008) Mitochondrial involvement in aspirin-induced apoptosis in yeast. Microbiology 154:2740–2747

Sato T, Hanada M, Bodrug S, Irie S, Iwama N, Boise LH, Thompson CB, Golemis E, Fong L, Wang HG et al (1994) Interactions among members of the Bcl-2 protein family analyzed with a yeast two-hybrid system. Proc Natl Acad Sci U S A 91:9238–9242

Schauer A, Knauer H, Ruckenstuhl C, Fussi H, Durchschlag M, Potocnik U, Frohlich KU (2009) Vacuolar functions determine the mode of cell death. Biochim Biophys Acta 1793:540–545

Schmitt MJ, Reiter J (2008) Viral induced yeast apoptosis. Biochim Biophys Acta 1783:1413–1417

Seo JG, Lai CY, Miceli MV, Jazwinski SM (2007) A novel role of peroxin PEX6: suppression of aging defects in mitochondria. Aging Cell 6:405–413

Severin FF, Hyman AA (2002) Pheromone induces programmed cell death in *S. cerevisiae*. Curr Biol 12:R233–R235

Shao W, Yeretssian G, Doiron K, Hussain SN, Saleh M (2007) The caspase-1 digestome identifies the glycolysis pathway as a target during infection and septic shock. J Biol Chem 282: 36321–36329

Silva A, Almeida B, Sampaio-Marques B, Reis MI, Ohlmeier S, Rodrigues F, Vale A, Ludovico P (2011) Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) is a specific substrate of yeast metacaspase. Biochim Biophys Acta 1813:2044–2049

Silva A, Sampaio-Marques B, Fernandes A, Carreto L, Rodrigues F, Holcik M, Santos MA, Ludovico P (2013) Involvement of yeast HSP90 isoforms in response to stress and cell death induced by acetic acid. PLoS One 8:e71294

Silva RD, Sotoca R, Johansson B, Ludovico P, Sansonetty F, Silva MT, Peinado JM, Corte-Real M (2005) Hyperosmotic stress induces metacaspase- and mitochondria-dependent apoptosis in *Saccharomyces cerevisiae*. Mol Microbiol 58:824–834

Smets B, Ghillebert R, De Snijder P, Binda M, Swinnen E, De Virgilio C, Winderickx J (2010) Life in the midst of scarcity: adaptations to nutrient availability in *Saccharomyces cerevisiae*. Curr Genet 56:1–32

Stephan JS, Yeh YY, Ramachandran V, Deminoff SJ, Herman PK (2009) The Tor and PKA signaling pathways independently target the Atg1/Atg13 protein kinase complex to control autophagy. Proc Natl Acad Sci U S A 106:17049–17054

Sundstrom JF, Vaculova A, Smertenko AP, Savenkov EI, Golovko A, Minina E, Tiwari BS, Rodriguez-Nieto S, Zamyatnin AA Jr, Valineva T, Saarikettu J, Frilander MJ, Suarez MF, Zavialov A, Stahl U, Hussey PJ, Silvennoinen O, Sundberg E, Zhivotovsky B, Bozhkov PV (2009) Tudor staphylococcal nuclease is an evolutionarily conserved component of the programmed cell death degradome. Nat Cell Biol 11:1347–1354

Syntichaki P, Samara C, Tavernarakis N (2005) The vacuolar H+−ATPase mediates intracellular acidification required for neurodegeneration in *C. elegans*. Curr Biol 15:1249–1254

Tang D, Kang R, Cheh CW, Livesey KM, Liang X, Schapiro NE, Benschop R, Sparvero LJ, Amoscato AA, Tracey KJ, Zeh HJ, Lotze MT (2010a) HMGB1 release and redox regulates autophagy and apoptosis in cancer cells. Oncogene 29:5299–5310

Tang D, Kang R, Livesey KM, Cheh CW, Farkas A, Loughran P, Hoppe G, Bianchi ME, Tracey KJ, Zeh HJ 3rd, Lotze MT (2010b) Endogenous HMGB1 regulates autophagy. J Cell Biol 190:881–892

Tang D, Kang R, Livesey KM, Kroemer G, Billiar TR, Van Houten B, Zeh HJ 3rd, Lotze MT (2011) High-mobility group box 1 is essential for mitochondrial quality control. Cell Metab 13:701–711

Tang D, Loze MT, Zeh HJ, Kang R (2010c) The redox protein HMGB1 regulates cell death and survival in cancer treatment. Autophagy 6:1181–1183

Tao W, Kurschner C, Morgan JI (1997) Modulation of cell death in yeast by the Bcl-2 family of proteins. J Biol Chem 272:15547–15552

Tao W, Kurschner C, Morgan JI (1998) Bcl-xS and Bad potentiate the death suppressing activities of Bcl-xL, Bcl-2, and A1 in yeast. J Biol Chem 273:23704–23708

Taylor-Brown E, Hurd H (2013) The first suicides: a legacy inherited by parasitic protozoans from prokaryote ancestors. Parasit Vectors 6:108

Taylor EB, Rutter J (2011) Mitochondrial quality control by the ubiquitin-proteasome system. Biochem Soc Trans 39:1509–1513

Tudela R, Gallardo-Chacon JJ, Rius N, Lopez-Tamames E, Buxaderas S (2012) Ultrastructural changes of sparkling wine lees during long-term aging in real enological conditions. FEMS Yeast Res 12:466–476

Ullman E, Fan Y, Stawowczyk M, Chen HM, Yue Z, Zong WX (2008) Autophagy promotes necrosis in apoptosis-deficient cells in response to ER stress. Cell Death Differ 15:422–425

Vachova L, Cap M, Palkova Z (2012) Yeast colonies: a model for studies of aging, environmental adaptation, and longevity. Oxid Med Cell Longev 2012:601836

Vachova L, Kucerova H, Devaux F, Ulehlova M, Palkova Z (2009) Metabolic diversification of cells during the development of yeast colonies. Environ Microbiol 11:494–504

Vachova L, Palkova Z (2011) Aging and longevity of yeast colony populations: metabolic adaptation and differentiation. Biochem Soc Trans 39:1471–1475

Vandenabeele P, Galluzzi L, Vanden Berghe T, Kroemer G (2010) Molecular mechanisms of necroptosis: an ordered cellular explosion. Nat Rev Mol Cell Biol 11:700–714

Vanlangenakker N, Vanden Berghe T, Vandenabeele P (2012) Many stimuli pull the necrotic trigger, an overview. Cell Death Differ 19:75–86

Walter D, Matter A, Fahrenkrog B (2010) Bre1p-mediated histone H2B ubiquitylation regulates apoptosis in *Saccharomyces cerevisiae*. J Cell Sci 123:1931–1939

Walter D, Wissing S, Madeo F, Fahrenkrog B (2006) The inhibitor-of-apoptosis protein Bir1p protects against apoptosis in *S. cerevisiae* and is a substrate for the yeast homologue of Omi/HtrA2. J Cell Sci 119:1843–1851

Weinberger M, Feng L, Paul A, Smith DL Jr, Hontz RD, Smith JS, Vujcic M, Singh KK, Huberman JA, Burhans WC (2007) DNA replication stress is a determinant of chronological lifespan in budding yeast. PLoS One 2:e748

Weinberger M, Ramachandran L, Feng L, Sharma K, Sun X, Marchetti M, Huberman JA, Burhans WC (2005) Apoptosis in budding yeast caused by defects in initiation of DNA replication. J Cell Sci 118:3543–3553

Werner-Washburne M, Roy S, Davidson GS (2012) Aging and the survival of quiescent and non-quiescent cells in yeast stationary-phase cultures. Subcell Biochem 57:123–143

Wissing S, Ludovico P, Herker E, Buttner S, Engelhardt SM, Decker T, Link A, Proksch A, Rodrigues F, Corte-Real M, Frohlich KU, Manns J, Cande C, Sigrist SJ, Kroemer G, Madeo F (2004) An AIF orthologue regulates apoptosis in yeast. J Cell Biol 166:969–974

Yordanova ZP, Iakimova ET, Cristescu SM, Harren FJ, Kapchina-Toteva VM, Woltering EJ (2010) Involvement of ethylene and nitric oxide in cell death in mastoparan-treated unicellular alga *Chlamydomonas reinhardtii*. Cell Biol Int 34:301–308

Yorimitsu T, Zaman S, Broach JR, Klionsky DJ (2007) Protein kinase A and Sch9 cooperatively regulate induction of autophagy in *Saccharomyces cerevisiae*. Mol Biol Cell 18:4180–4189

Yu L, Lenardo MJ, Baehrecke EH (2004) Autophagy and caspases: a new cell death program. Cell Cycle 3:1124–1126

Zabrocki P, Bastiaens I, Delay C, Bammens T, Ghillebert R, Pellens K, De Virgilio C, Van Leuven F, Winderickx J (2008) Phosphorylation, lipid raft interaction and traffic of alpha-synuclein in a yeast model for Parkinson. Biochim Biophys Acta 1783:1767–1780

Zha H, Fisk HA, Yaffe MP, Mahajan N, Herman B, Reed JC (1996) Structure-function comparisons of the proapoptotic protein Bax in yeast and mammalian cells. Mol Cell Biol 16:6494–6508

Zhang NN, Dudgeon DD, Paliwal S, Levchenko A, Grote E, Cunningham KW (2006) Multiple signaling pathways regulate yeast cell death during the response to mating pheromones. Mol Biol Cell 17:3409–3422

Chapter 15
Necrotic Cell Death in *Caenorhabditis elegans*

Vassiliki Nikoletopoulou and Nektarios Tavernarakis

15.1 Introduction

Early studies in the field of cell death described two major forms of cellular demise, apoptosis and necrosis, and contrasted them as being diametrically different in every aspect examined (Walker et al. 1988). In 1972, Kerr, Wyllie, and Currie described apoptosis as a controlled cell death process and proposed that it functions as a tissue homeostatic mechanism that is complementary and opposite to cell division (Kerr et al. 1972). Necrosis was classically contrasted to apoptosis, also referred to as caspase-dependent programmed cell death, not only on grounds of context and mechanistic regulation or lack thereof but also based on notable morphological differences. The apoptotic cell profile is characterized by cell rounding, detachment from the basal membrane or cell culture substrate, chromatin condensation and nuclear fragmentation, blebbing of the plasma membrane, and shedding of vacuoles known as apoptotic bodies (Galluzzi et al. 2007). Necrotic cells were initially characterized in a negative fashion, exhibiting neither an apoptotic morphological profile nor an extensive vacuolization characteristic of autophagic cell death. However, specific morphological features were soon attributed to necrotic cells. These included in particular an increasingly translucent cytoplasm, osmotic swelling of most organelles, increased cell volume, and finally rupture of the plasma membrane (Fig. 15.1). Notably, unlike apoptosis, necrosis does not feature major nuclear modifications but only minor ultrastructural changes. Moreover, necrotic cells do not fragment into distinct corpses as their apoptotic counterparts do (Galluzzi et al. 2007).

V. Nikoletopoulou • N. Tavernarakis (✉)
Institute of Molecular Biology and Biotechnology, Foundation for Research and Technology, N. Plastira 100, Vassilika Vouton, PO Box 1385, Heraklion 71110, Crete, Greece
e-mail: tavernarakis@imbb.forth.gr

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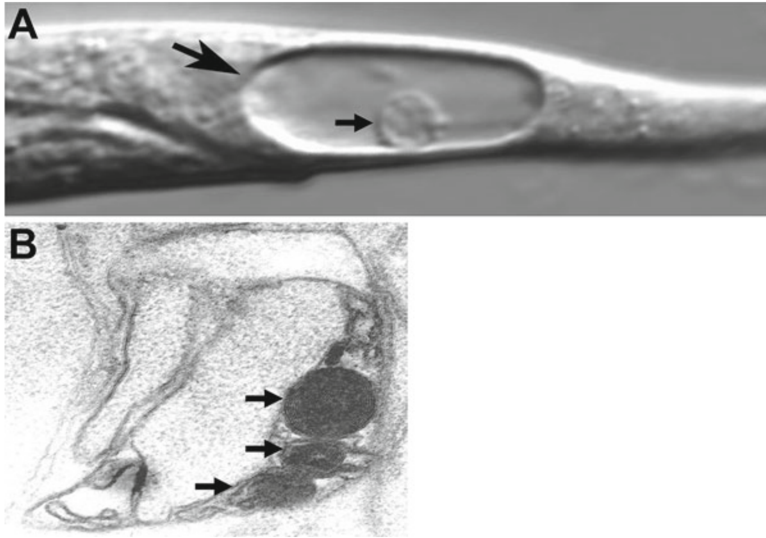

Fig. 15.1 Morphology of necrotic cell death in *Caenorhabditis elegans*. Nematode neurons undergoing necrosis as a result of degenerin ion channel hyperactivation show distinct morphological features (Kourtis and Tavernarakis 2007). (**a**) The degenerating cell (*arrow*) appears extensively swollen, while the nucleus is distended and has a distorted morphology (*arrowhead*). (**b**) Distinctive electron-dense membranous circumvolutions (*arrows*) observed under the electron microscope (Hall et al. 1997). Similar membranous inclusions are observed in rat neurons undergoing excitotoxic cell death

C. elegans has been instrumental in deciphering both apoptotic and necrotic cellular programs. This can be largely attributed to the specific characteristics and well-described developmental stages of this nematode, which make it exceptionally well suited for the study of both normal and aberrant cell death at the cellular, genetic, and molecular level. Due to its transparency, the visualization and tracking of single cells as well as of individual nuclei is readily feasible by differential interference contrast optics, enabling researchers to follow somatic cell divisions from the fertilized egg all the way to the 959 cell adult hermaphrodite (Sulston and Horvitz 1977; Sulston et al. 1983). The resulting cell lineage map indicated early on that in certain lineages, particular divisions generate cells which are destined to die at specific times and locations that remain faithfully invariant from one animal to another. Exactly 131 somatic cells die every time the fertilized egg normally develops into the adult animal, by an apoptotic programmed cell death (PCD) process.

Genetic and molecular studies performed in *C. elegans* provided a fundamental insight into the mechanisms underlying this cell death process. In the 131 cells destined to die during development, the level of EGL-1, a BH3 domain protein, is increased. EGL-1 interacts with a protein complex composed of CED-9 (similar to the mammalian B-cell lymphoma protein 2, BCL-2) and CED-4 (similar to the mammalian apoptotic protease activating factor 1, Apaf1), releasing CED-4 which in turn

activates CED-3 (similar to human caspases) (Hengartner 2000). In *C. elegans*, four caspase-related genes exist: *ced-3*, *csp-1*, *csp-2*, and *csp-3* (Yuan et al. 1993; Shaham 1998); however, only *ced-3* seems to be required for programmed cell death (Yuan et al. 1993; Abraham and Shaham 2004), and only ced-3 and *csp-1* are proteolytically active (Shaham 1998). The CSP-2 caspase lacks key active-site residues, and *csp-3* encodes only a C-terminal caspase domain, entirely lacking the active site (Shaham 1998). As it turns out, the genetic encoding for the regulation and execution of developmental apoptosis has been remarkably conserved between *C. elegans* and mammals.

15.2 Necrotic Cell Paradigms During *C. elegans* Development

The identification of the caspase CED-3 as a key regulator of apoptosis has been a key contribution of *C. elegans* to the cell death field, as caspases also play crucial roles in the execution of programmed cell death across many species. However, is all developmental cell death in *C. elegans* dependent on caspases? As it turns out, not quite all cell death events during *C. elegans* development follow the typical apoptotic pathway that involves CED-4 and CED-3.

15.2.1 Death of the Linker Cell

The *C. elegans* linker cell has been identified as such an exemption (Horvitz et al. 1983). The linker cell is born during the second larval stage (L2) in the central region of the animal and follows a stereotypical path of migration. As the cell migrates, it leads the extension of the male gonad behind it (Kimble and Hirsh 1979; Sulston et al. 1980), and upon completion of its migratory route, it is positioned between the gonad (vas deferens) and the cloacal tube, serving as an exit channel for sperm in the adult. It is generally thought that the death and removal of the linker cell around the L4/adult transition facilitates the fusion between the vas deferens and cloaca, to connect the male reproductive system to the exterior.

Following up on early observations that the programmed death of the linker cell persists even in *ced-3* mutant animals, Abraham and colleagues thoroughly studied the death of this cell by following the fate of a GFP-marked linker cell in animals harboring mutations in core genes of the apoptotic machinery, such as *ced-3* and *ced-4*, as well as in engulfment genes. They convincingly demonstrated that the linker cell dies in a cell autonomous manner that, unlike what was postulated by previous reports (Sulston et al. 1980), does not require extrinsic signals from engulfing or other cells. Moreover, they showed that this death event is independent of any known apoptotic genes, in line with the lack of apoptotic morphological features, such as chromatin condensation. Instead, there was a noted presence of swollen and

degraded mitochondria within large multilayered membrane-bound structures, as well as small electron-translucent "empty" membrane-bound cytoplasmic structures that resembled vacuoles typically seen during necrotic cell death in *C. elegans* (Hall et al. 1997) (Fig. 15.1). Although linker cell death does not satisfy all classical criteria of necrotic death, it is even further away from classical apoptotic paradigms, perhaps falling under the characteristics of more recently described programmed necrosis processes, also known as necroptosis. More experiments are however needed to further characterize the precise mode of death of the linker cell.

15.2.2 Death of Mis-specified Uterine–Vulval (uv1) Cells

Yet, a more robust example of a necrotic event during development is the demise of mis-specified uterine–vulval (uv1) cells that have an important role in egg laying. Egg laying in *C. elegans* requires a connection between the lumens of the uterus in the somatic gonad and the vulva in the extra-gonadal epithelium, facilitated by cell–cell interactions between gonadal and vulval cells. Two specialized cell types of the ventral uterine π lineage are integral components of the uterine–vulval connection. These are the syncytial uterine seam (utse) cell, which overlies the vulval lumen, and the four uterine–vulval (uv1) cells, which directly contact the most dorsal vulval cell vulF (Newman et al. 1996). The temporal and spatial specification of both these cell types largely relies on a specific signaling axis, where an inductive LIN-3 epidermal growth factor (EGF) signal derived from a single gonadal cell called the anchor cell (AC) activates the LET-23 EGF receptor on the receiving vulval precursor cells (Aroian et al. 1990; Hill and Sternberg 1992). Mutations in genes of the LIN-3/LET-23/Ras signaling pathway compromise uv1 fate specification. Work from the laboratory of Hanna-Rose (Huang and Hanna-Rose 2006) described the isolation of the *cog-3*(*ku212*) mutant, which uncouples gonadogenesis from its normal progression relative to the development of the vulva and shares phenotypes with heterochronic mutations that disturb the temporal coordination of vulval and uterine development. In *cog-3*(*ku212*) mutants, the entire uterus, including the pre-uv1 cells, is generated at a later stage of vulval development than is normal. Notably, the delayed pre-uv1 cells subsequently die by necrosis leading to the absence of uv1 cells in the adult stage. Moreover, the study investigated if a LIN-3/LET-23/Ras signaling defect underlies the necrosis of uv1 defect in *cog-3* (*ku212*) mutants, by analyzing *cog-3*(*ku212*) double mutants with a gain-of-function allele of *let-23*. The results indicated that the *let-23*(*gf*) mutation rescued the mis-specification and death phenotype of uv1 cells, suggesting that the necrotic program is recruited during development in response to uncoordinated spatiotemporal development.

A recent study revealed the involvement of the *ku212* allele in uv1 cell necrosis, which maps to the *pnc-1* gene locus, encoding a nicotinamidase (van der Horst et al. 2007; Vrablik et al. 2009). Nicotinamidases are the first enzymes of the NAD^+ salvage pathway in invertebrates, using nicotinamide (NAM) as a substrate (Magni et al. 1999). Administration of high levels of nicotinamide causes uv1 cells to die by

necrosis at high frequency in wild-type animals. Thus, instead of compromised EGF signaling, the necrotic death of uv1 cells in *pnc-1* mutants may result from accumulation of the substrate nicotinamide. In addition, the gonad-defective and uv1 cell death phenotypes are separable in *pnc-1* mutants. Constitutively active LET-23/EGF receptor prevents NAM-induced uv1 necrotic cell death, suggesting that EGF signaling may provide a survival cue that rescues uv1 cells from NAM-induced necrosis (reviewed in Vlachos and Tavernarakis 2010).

15.3 Nondevelopmental Necrotic Death

In the adult nematode, necrotic cell death can be triggered by a wide variety of both extrinsic and intrinsic signals (Walker et al. 1988). Several well-defined conditions are known to trigger necrotic cell death in *C. elegans*. The best-characterized case is that of unusual gain-of-function mutations, in several ion channel genes, which inflict a necrotic pattern of death on the neurons that express their protein products. Cell demise in these paradigms is accompanied by characteristic morphological features of necrosis, starting with the appearance of a distorted nucleus and cell body during the early phase of death. Gradually, the cell swells to several times its normal diameter, and small, tightly wrapped membrane whorls form, originating from the plasma membrane and coalescing into large, electron-dense membranous structures (Hall et al. 1997). Interestingly enough, these membranous inclusions also represent characteristic hallmarks in mammalian neurodegenerative disorders, such as in neuronal ceroid lipofuscinosis (Batten's disease, the *mnd* mouse) as well as in the wobbler mouse, a model of amyotrophic lateral sclerosis (ALS) (Cooper et al. 1999; Blondet et al. 2002).

15.3.1 Cell Death Induced by Ionic Imbalance

The most extensively characterized paradigm of nonprogrammed cell death in adult *C. elegans* animals is the necrosis of cells expressing aberrant ion channels harboring unusual gain-of-function mutations (Syntichaki and Tavernarakis 2003). For example, dominant mutations in *deg-1* [degenerin; *deg-1*(*d*)] induce death of a group of interneurons of the nematode posterior touch sensory circuit (Chalfie and Wolinsky 1990). Similarly, dominant mutations in the mec-4 gene [mechanosensory; *mec-4*(*d*)] induce degeneration of six touch receptor neurons required for the sensation of gentle touch to the body (Syntichaki and Tavernarakis 2004).

deg-1 and *mec-4* encode proteins that are very similar in sequence and were the first identified members of the *C. elegans* "degenerin" family, so named because several members can mutate to forms that induce cell degeneration (Chalfie et al. 1993). Degenerins bear sequence similarity to mammalian epithelial sodium channels (ENaCs). The time of degeneration onset correlates with the initiation of

degenerin gene expression, and the severity of cell death is analogous to the dose of the toxic allele (Hall et al. 1997). Expression of mammalian homologous proteins, carrying amino acid substitutions analogous to those of toxic degenerins, leads to degeneration of cells in a manner reminiscent of necrotic cell death in *C. elegans*. Additional members of the degenerin family are *mec-10*, which can be engineered to encode toxic degeneration-inducing substitutions; *unc-8*, which can mutate to a semidominant form that induces swelling and dysfunction of ventral nerve cord; and *unc-105*, which appears to be expressed in muscle and can mutate to a semidominant form that induces muscle hyper-contraction (Syntichaki and Tavernarakis 2004). Thus, a unifying feature of degenerin family members is that specific gain-of-function mutations have deleterious consequences for the cells in which they are expressed, which, at least in neurons, culminate into a necrotic cell death event.

C. elegans degenerins share sequence similarity with Drosophila Ripped Pocket (RPK) and Pickpocket (PPK), with subunits of the vertebrate amiloride-sensitive epithelial sodium channel (ENaC), and with other neuronally expressed ion channels. Together, these proteins define the DEG/ENaC protein superfamily (Tavernarakis and Driscoll 2001). Although mutant degenerins can kill different groups of neurons depending on their expression patterns, the morphological features of the cell death that they induce are the same and resemble those of mammalian cells undergoing necrotic cell death. The pattern of necrotic cell death inflicted by degenerins is not a peculiarity of this gene class. For example, *C. elegans deg-3*, whose product is related to the vertebrate α-7 nicotinic acetylcholine receptor and together with the related protein DES-2 forms a very efficient calcium channel, can mutate to induce necrotic cell death similar to that induced by degenerins (Treinin et al. 1998). In addition, mutant-activated forms of the heterotrimeric G protein α-subunit ($G\alpha_s$ Q208L), from both *C. elegans* and rat, cause swelling and degeneration of many cell types when expressed in *C. elegans* (Korswagen et al. 1997; Berger et al. 1998).

In addition to degenerins, gain-of-function mutations in other ion channel genes, such as deg-3, lead to vacuolar degeneration of various types of *C. elegans* neurons. deg-3 encodes an acetylcholine receptor ion channel, related to the vertebrate nicotinic acetylcholine receptor (nAChr) that participates in the formation of a channel highly permeable to Ca^{2+} (Treinin and Chalfie 1995). Moreover, expression of a constitutively active form of a heterotrimeric G protein subunit $G\alpha_s$ results in degeneration of a specific subset of neurons. Genetic suppressor analysis identified an adenylyl cyclase as a downstream effector of $G\alpha_s$-induced neurodegeneration, indicating that cAMP signaling is critical for degeneration (Berger et al. 1998; Korswagen et al. 1998).

Ionic imbalance and subsequent necrotic cell death induced by aberrant ion channel function in *C. elegans* are mechanistically and morphologically similar to excitotoxicity in vertebrates. Excitotoxic cell death is prevalent during stroke, where the energy required for sustaining ionic gradients and the resting potential of neurons is lost. Because membrane potential collapses, massive amounts of the excitatory neurotransmitter glutamate are released at synaptic clefts (Kauppinen et al. 1988a, b). Energy depletion also prevents reuptake of glutamate by dedicated

transporters leading to accumulation of glutamate at synapses, hyperexcitation, and eventually necrotic death of downstream synaptic target neurons. Excitotoxicity is critically dependent on Ca^{2+} influx through glutamate-gated receptor ion channels (reviewed in Kourtis and Tavernarakis 2007).

Malfunction of glutamate transporters and the resulting accumulation of glutamate are known to trigger excitotoxicity in several neurodegenerative diseases (Cleveland and Rothstein 2001). However, the details on the cascade of events leading to neurodegeneration remain unclear. The molecular components of glutamatergic synapses assembled in *C. elegans* are highly conserved from nematodes to humans. A recent study describes a novel paradigm for nematode excitotoxicity, by investigating the in vivo effects of multiple mediators of glutamate-induced neuronal necrosis (Mano and Driscoll 2009). Combined *Δglt-3* glutamate transporter-null mutations and expression of a constitutively active form of the alpha subunit of the G protein Gs induce extensive neurodegeneration in head interneurons. *Δglt-3-dependent* neurodegeneration acts through Ca^{2+}-permeable Glu receptors of the a-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) subtype, requires calreticulin function, and is modulated by calcineurin and type-9 adenylyl cyclase (AC9). This glutamate-dependent toxicity defines a novel necrotic death paradigm in *C. elegans* that shares many basic features with excitotoxicity in mammalian neurons and may potentially be operative also in higher organisms.

15.3.2 Heat-Induced Necrotic Death

Climate change has brought about a dramatic increase in the cases of heatstroke and related pathologies in humans. With core body temperature reaching over 40 °C, heatstroke causes immediate devastating tissue damage and inflammatory response that can be fatal, as well as long-term defects. To gain insight into the molecular mechanisms of heat cytotoxicity and to circumvent the confounding influence of secondary physiological and inflammatory responses, our laboratory developed and characterized a genetically tractable model of heatstroke in *C. elegans*. Widespread cell death across several tissues could be observed in animals exposed to hyperthermia, which in the nematode was simulated by a short exposure to 39 °C (Kourtis et al. 2012). Dying cells displayed morphological features characteristic of necrosis, expressed markers of necrotic death, and became permeable to propidium iodide. Moreover, depletion of proteins required for necrosis strongly facilitated survival after heatstroke. In contrast, loss of key mediators and core components of the apoptotic or autophagic machineries did not suppress heatstroke-induced cell death. Thus, heatstroke compromises viability by triggering extensive necrotic cell death and represents a newly added necrotic cell paradigm in the nematode.

Notably, we also observed that preconditioning animals at an intermediate, nonlethal temperature markedly enhanced their capacity to withstand a subsequent heatstroke. This protective effect is in line with the previously described phenomenon of hormesis (Calabrese 2004), where preexposure to mild stress elicits increased

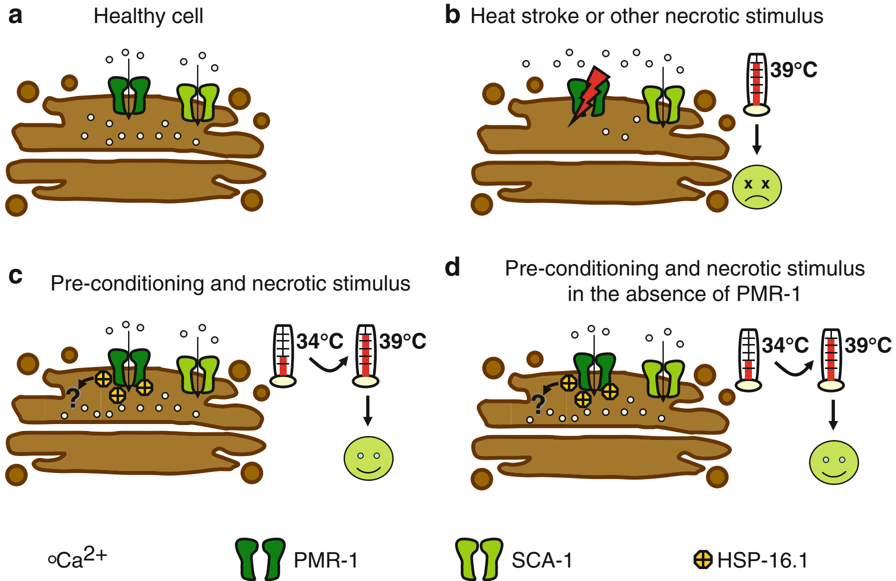

Fig. 15.2 A cytoprotective mechanism against necrotic cell death engaged by heat preconditioning. The contribution of the medial Golgi, shown here by brown cisternae, is depicted (**a**) in healthy cells under normal conditions, (**b**) upon exposure to heatstroke or other necrotic stimuli, (**c**) upon heat preconditioning and subsequent heatstroke, and (**d**) upon heat preconditioning and subsequent heatstroke in the absence of PMR-1. Under normal conditions (**a**) the Golgi P-type ATPase Ca^{2+}/Mn^{2+} pump PMR-1 and SCA-1 maintain Ca^{2+} homeostasis by sequestering Ca^{2+} inside the Golgi. (**b**) Heatstroke perturbs the normal function of PMR-1 leading to aberrant release of Ca^{2+} into the cytoplasm and causing necrosis. Heat preconditioning (**c**) increases the levels of HSP-16.1, which restores PMR-1 function upon subsequent heatstroke, therefore preventing necrosis. The effect of HSP-16.1 is entirely dependent on PMR-1; as in the absence of PMR-1 (**b**), heat preconditioning cannot confer cytoprotection against subsequent heatstroke (Adapted from Kourtis et al. 2012)

resistance to subsequent severe stress. It is also worth noting that in addition to heatstroke, heat preconditioning conferred resistance against a wide range of necrotic death insults, including in particular ionic imbalance paradigms (discussed earlier), overexpression of aggregation-prone proteins (such as α-synuclein), and hypoxic conditions. In the case of hormesis by heat preconditioning, we found that cytoprotection is orchestrated at the molecular level by the hermetic induction of a single sHSP, HSP-16.1. sHSPs assemble into oligomeric complexes and serve as molecular chaperones, efficiently binding denatured proteins and/or preventing irreversible protein aggregation and insolubilization (Van Montfort et al. 2001). HSP-16.1 localizes in the Golgi, where it functions together with the PMR-1 pump to prevent cytoplasmic Ca^{2+} overload under extreme stress. We propose that HSP-16.1 contributes to stabilize and protect the stress-labile PMR-1 pump, allowing for efficient clearance of Ca^{2+} from the cytoplasm, after necrotic insult (see Fig. 15.2).

Importantly, mammalian PMR1 is selectively impaired during ischemic or reperfusion brain injury (Lehotsky et al. 2002; Gidday 2006; Pavlikova et al. 2009). Given the strong evolutionary conservation of the proteins involved, this mechanism is probably relevant to related human pathologies. Relevant to that, we also demonstrated that heatstroke induces widespread necrotic death in mammalian neurons, which can be largely prevented by heat preconditioning. Moreover, hormesis in mammalian neurons in response to heat preconditioning also requires the function of PMR1 and is mediated by the same molecular players as in the nematode.

15.3.3 Bacterial Infection-Induced Necrosis

Infection of *C. elegans* with different bacterial pathogens has been shown to induce necrotic death of intestinal cells as part of a pathogen-shared response to infection (Wong et al. 2007). At later stages of infection, necrotic vacuoles are also observed in epidermal and gonadal cells. Mutations in genes required for necrosis ameliorate the consequences of infection, suggesting that necrosis is an integral part of host–pathogen interaction that contributes to the pathology associated with infection in *C. elegans*.

15.3.4 Hypoosmotic Shock-Induced Cell Death

Lysosomal integrity and lysosomal proteolytic mechanisms are key factors modulating necrotic cell death in the nematode. Serpins are extracellular or intracellular regulators of proteolytic pathways and inhibitors of multiple peptidases (Silverman et al. 2001). One of the functions of intracellular serpins is the inhibition of lysosomal cysteine peptidases. SRP-6 is such an intracellular serpin in *C. elegans*. *srp-6-null* mutants experiencing hypoosmotic conditions die rapidly and display marked increase of necrotic cell death of the intestinal epithelium (Luke et al. 2007). Ca^{2+} release from endoplasmic reticulum (ER) stores, together with other factors, induces calpain-mediated lysosomal rupture and massive release of lysosomal peptidases into the cytoplasm that mediate necrotic cell death. In addition to hypoosmotic conditions, *srp-6*-null mutants are susceptible to other stressors such as thermal and oxidative stress, hypoxia, and channel hyperactivity. SRP-6 appears to protect cells from lysosomal rupture and also ameliorate the deleterious consequences of lysosomal rupture triggered by various stressors. The protective function of SRP-6 may be adaptive by enhancing the degradation of misfolded proteins or by aiding cytoskeletal rearrangements through altering lysosomal membrane permeability and allowing the leakage of small amounts of peptidases. In the absence of SRP-6, the uncontrolled release of these peptidases leads to necrotic cell death.

15.4 Execution of Necrosis

Intracellular calcium overload through different sources is considered as one of the leading steps in the necrotic pathway. Calcium may enter the cell through voltage-gated channels, and the Na^+/Ca^{2+} exchanger and mutations that increase sodium influx augment calcium entry through these paths. The main intracellular compartment for calcium storage is the endoplasmic reticulum (ER) (Mattson et al. 2000; Paschen 2001; Paschen and Frandsen 2001), where calcium is sequestered by the sarco–endoplasmic reticulum Ca^{2+}-ATPase (SERCA) and is released back to the cytoplasm by ryanodine (RyR) and inositol 1,4,5-trisphosphate receptors [Ins(1,4,5) P3PR]. In *C. elegans*, extensive genetic screens for suppressors of *mec-4*(*d*)-induced necrosis have identified genes required for the execution of necrotic cell death. Two of these genes encode the calcium-binding chaperones calreticulin and calnexin, which were found to regulate intracellular calcium levels and to be required for necrotic cell death (Xu et al. 2001). Moreover, treatment of animals with thapsigargin, a drug that induces release of calcium from the ER to the cytoplasm, triggers necrotic cell death, whereas pharmacological treatments or genetic mutations that inhibit calcium release from the ER have a strong protective effect against necrotic cell death.

Genetic studies in *C. elegans* have also shown that in addition to calcium homeostasis, intracellular pH is also an important modulator of necrotic cell death. Cytoplasmic acidification occurs during necrosis, whereas the vacuolar H^+-ATPase, which is a pump that acidifies lysosomes and other intracellular organelles, is required downstream of cytoplasmic calcium overload to promote necrotic cell death (Syntichaki et al. 2005). In line with this, reduced vacuolar H^+-ATPase activity or alkalization of acidic endosomal/lysosomal compartments by weak bases has a neuroprotective role against necrosis. Acidic conditions are required for full activity of cathepsins, aspartyl proteases that are primarily confined to lysosomes and other acidic endosomal compartments (Ishidoh and Kominami 2002).

Lysosomal as well as cytoplasmic proteases have been implicated as downstream effectors of cellular destruction in necrosis. Calpains are cytoplasmic, papain-like cysteine proteases that depend on calcium for their activity. Under normal conditions, calpains function to mediate essential signaling and metabolic processes. However, during the course of necrotic cell death, these proteases localize onto lysosomal membranes and may compromise lysosomal integrity, thereby causing leakage of their acidic contents, including lysosomal proteases, into the cytoplasm (Yamashima 2004). In primates, calpains rapidly localize to lysosomal membranes after the onset of ischemic episodes (Yamashima 2000). In *C. elegans*, two specific calpains, TRA-3 and CLP-1, and two lysosomal cathepsin proteases, ASP-3 and ASP-4, are required for neurodegeneration (Syntichaki et al. 2002). It is likely that ensuing cytoplasmic acidification, activation of the lysosomal, low-pH-dependent cathepsins and hydrolases contributes to cell demise. Mutations that interfere with lysosomal biogenesis and function influence necrotic cell death. For example, necrosis is exacerbated in mutants that accumulate abnormally large lysosomes, whereas

impairment of lysosomal biogenesis protects from cell death (Artal-Sanz et al. 2006). Interestingly, lysosomes appear to coalesce around the nucleus and dramatically enlarge during early and intermediate stages of necrosis. In advanced stages of cell death, GFP-labeled lysosomal membranes fade, as lysosomes rupture.

In a recent study from our laboratory, we utilized well-characterized necrosis models in *C. elegans* to dissect the involvement of clathrin-mediated endocytosis and intracellular trafficking by kinesin motor proteins in cellular destruction during necrotic death (Troulinaki and Tavernarakis 2012a). Our findings revealed for the first time that both clathrin-mediated endocytosis and intracellular trafficking are required for the execution of necrosis in the nematode. Downregulation of endocytosis or kinesin-mediated trafficking by interfering with key proteins regulating these processes, including SNT-1, endophilin (UNC-57), AP180 (UNC-11), synaptojanin (UNC-26), heavy chain of kinesin 1 (UNC-116), and the monomeric kinesin UNC-104, significantly suppresses neurodegeneration induced by hyperactive ion channels without affecting the expression, the localization, or the function of the toxic insults.

Moreover, using the same well-defined necrotic cell paradigm, we assayed animals that were deficient for both autophagy and endocytosis and observed significant synergistic protection against degeneration. These results suggest that autophagy and endocytosis function in parallel to contribute to necrotic cell death (Troulinaki and Tavernarakis 2012a). A graphical representation of the crosstalk of the different mechanisms that cooperate in the execution of necrosis is depicted in Fig. 15.3.

15.5 *C. elegans* as a Model for Human Diseases Entailing Necrosis

Nematode genes and major signaling pathways show significant conservation during evolution, and more than 50 % of the *C. elegans* genes have counterparts in humans. In addition to its contribution in elucidating developmental processes, the worm has also served as a platform to model many human pathological conditions such as neurodegenerative disorders, cancer, aging, and associated diseases (Lee et al. 2001; Baumeister and Ge 2002; Poulin et al. 2004). Systematic mapping of gene interactions and signaling pathways implicated in human disease using *C. elegans* has provided better understanding of complex pathologies (Bussey et al. 2006). The ability to produce "humanized" worms, which express human genes not present in the *C. elegans* genome, has further enhanced the experimental value of the nematode by allowing the dissection of molecular mechanisms relevant to human disorders. In addition, the ease of drug testing coupled with the efficiency of genetic screens in worms has made *C. elegans* a favorable tool for the identification and validation of novel drugs and drug targets, aiming to battle human pathological conditions (Kaletta and Hengartner 2006). Here, we overview *C. elegans* models of human diseases that entail necrosis, focusing on hypoxia, Parkinson's disease, and tauopathies.

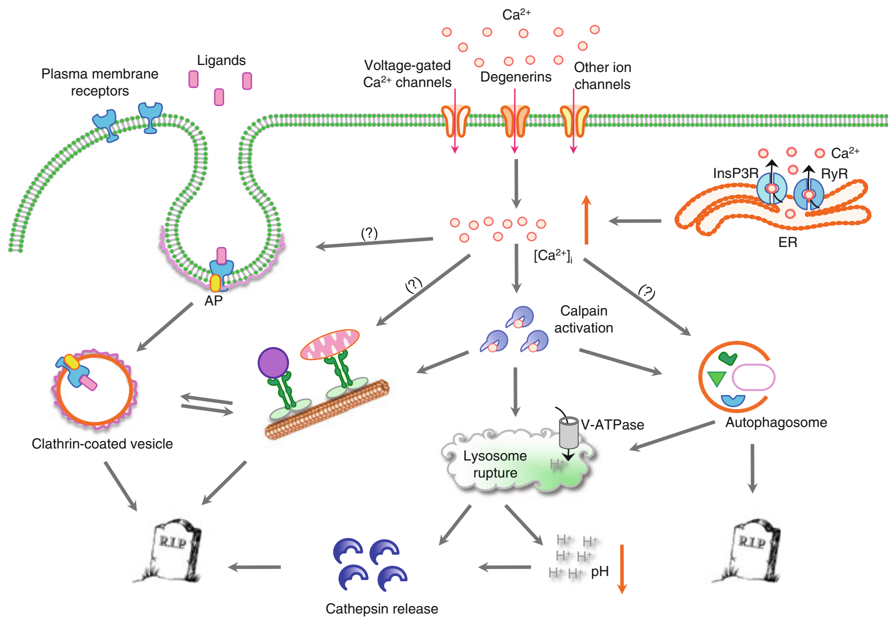

Fig. 15.3 Crosstalk between necrotic cell death mechanisms in *C. elegans*. Necrotic insults converge to increase intracellular Ca^{2+} levels by calcium influx from extracellular pools through various plasma membrane channels, such as voltage-gated receptors or sodium/calcium exchangers (NCX), or by calcium efflux from subcellular organelles with substantial Ca^{2+} stores, such as the endoplasmic reticulum via the ryanodine (RyR) and the inositol 1,4,5-trisphosphate receptors [Ins(1,4,5)P3R]. Ca^{2+} ions then activate cytoplasmic calpain proteases that attack lysosomal membrane proteins, compromising lysosomal integrity and causing the release of hydrolytic enzymes, such as cathepsin proteases. Vacuolar H^+ ATPase (V-ATPase)-mediated lysosomal acidification is important for subsequent acidification of the cytoplasm and enhancement of cathepsin activity. In addition, autophagy is induced during necrosis, either directly or through calpain activation, and synergizes with lysosomal cathepsin proteases to facilitate cellular destruction. Moreover, both clathrin-mediated endocytosis and intracellular trafficking are required for cell death and become upregulated by necrosis-triggering insults. $[Ca^{2+}]_i$ cytoplasmic calcium, *InsP3R* inositol trisphosphate receptor, *RyR* ryanodine receptor, *ER* endoplasmic reticulum, *AP* adaptor proteins for clathrin-mediated endocytosis, *V-ATPase* vacuolar H^+ ATPase (Adapted from Troulinaki and Tavernarakis 2012b)

Clearly, this list is only indicative of the applications of *C. elegans* in understanding complex human pathologies that involve necrotic death, and many more such diseases that are not mentioned here have been usefully modeled in the nematode.

15.5.1 Hypoxia

In humans oxygen deprivation induces cell death in pathological conditions such as stroke and heart attack. In *C. elegans*, hypoxia inflicts necrotic death in a variety of cell types (Scott et al. 2002). Interestingly, mutations in the *daf-2* gene, which

encodes the *C. elegans* insulin/IGF receptor tyrosine kinase, confer resistance against hypoxic cell death. DAF-2 is also known to regulate aging and dauer formation in *C. elegans* (Libina et al. 2003). Related to this, many human neurodegenerative disorders show a late-onset pathogenesis, indicating that aging may alter the vulnerability of cells to various insults. However, while hypoxia resistance in *C. elegans* appears to be modulated by insulin signaling, other *daf-2* mutations that affect longevity and stress resistance do not affect hypoxic death. Selective expression of wild-type *daf-2* in neurons and muscles restores hypoxic death in *daf-2* hypoxia-resistant mutants, demonstrating a role of the insulin/IGF receptor in the protection of myocytes and neurons from hypoxic injury. Na^+-activated potassium channels (KNa) have been identified in cardiomyocytes and neurons as mediators of the protective mechanisms against hypoxic death (Kameyama et al. 1984; Bader et al. 1985). In *C. elegans*, a KNa ion channel is encoded by the *slo-2* gene. *slo-2* mutants are hypersensitive to hypoxic death, suggesting that SLO-2 protects against hypoxia effects. Thus, molecular characterization of KNa channels may allow the development of specific agonists and antagonists, in an effort to combat hypoxia-caused pathologies (Yuan et al. 2003).

15.5.2 Parkinson's Disease

Inclusions of α-synuclein represent a hallmark feature of pathology in both sporadic and familial cases of Parkinson's disease. α-synuclein is the main component of Lewy bodies found in degenerating dopamine neurons (Spillantini et al. 1997). Mutations in the α-synuclein gene or multiplications of the α-synuclein locus have also been associated with some autosomal dominant familial cases of Parkinson's disease (Polymeropoulos et al. 1997; Singleton et al. 2003; Chartier-Harlin et al. 2004). *C. elegans* models of wild-type or mutated human α-synuclein overexpression have been established, either pan-neuronally or specifically in dopaminergic neurons (Lakso et al. 2003; Cao et al. 2005; Cooper et al. 2006; Kuwahara et al. 2006; Qiao et al. 2008), and result in significant motor deficits. No inclusion bodies or α-synuclein aggregation is observed, and intracellular inclusions are rarely observed in these transgenic animals. Overexpression of wild-type or mutant human α-synuclein specifically in worm dopaminergic neurons causes their degeneration, which becomes more pronounced as the animal ages (Cao et al. 2005; Cooper et al. 2006; Kuwahara et al. 2006).

One of the mechanisms implicated in the pathogenesis of Parkinson's disease is mitochondrial dysfunction (Schapira 2008). Autosomal dominant mutations in the leucine-rich repeat kinase 2 (LRRK2) have been associated with both familial and late-onset cases of PD, with G2019S being a prominent such mutation. *C. elegans* engineered to express the human LRRK2 (G2019S) mutant form show extensive loss of dopaminergic neurons (Saha et al. 2009), by increasing their vulnerability to mitochondrial stress. Expression of the wild-type LRRK2 has a milder effect on neuron loss. Similarly, loss-of-function mutations in the *lrk-1* gene, encoding the worm orthologue of LRRK2, also sensitize dopaminergic neurons to mitochondrial stress.

C. elegans models of α-synuclein-induced dopaminergic neurodegeneration have been used as a platform to identify suppressors of dopaminergic neuron loss with some success. For example, specific overexpression of human torsinA or the worm homologue TOR-2 protects dopamine neurons in these models (Cao et al. 2005). In addition, overexpression of the human lysosomal enzyme cathepsin D has a similar neuroprotective effect (Qiao et al. 2008). Several other molecules involved in autophagy, lysosomal function, trafficking, and G protein signaling have also been identified in RNAi suppressor screenings (Hamamichi et al. 2008).

15.5.3 Tau Toxicity

A significant number of neurodegenerative diseases (including in particular Alzheimer's disease, frontotemporal dementia and Parkinsonism linked to chromosome 17) is characterized by neurofibrillary tangles consisting of hyperphosphorylated forms of the microtubule-associated protein *Tau*, encoded by the *mapt* gene (Lee et al. 2001). Although the exact role of *tau* in the pathogenesis of these diseases is not clear, the identification of autosomal dominant mutations in the *mapt* gene indicates a crucial role for the altered *tau* protein in the neurodegenerative process (Hutton et al. 1998; Poorkaj et al. 1998; Spillantini et al. 1998).

Two studies have reported the expression of human *tau* (wild-type *tau* or *tau* carrying FTDP-17 mutations) either pan-neuronally, under the control of the *aex-3* promoter (Kraemer et al. 2003), or specifically in touch receptor neurons of *C. elegans*, under the control of the *mec-7* promoter (Miyasaka et al. 2005). In the first model, expression of the human *tau* results in reduced lifespan, behavioral abnormalities, progressive uncoordinated movement, and accumulation of insoluble phosphorylated *tau*, defective cholinergic neurotransmission, and age-dependent axonal and neuronal degeneration. Among the morphological features of neurodegeneration are axonal vacuolar clearing, collapsed membrane structure, and membranous infoldings and whorls (which are characteristic of necrotic cell death), with associated amorphous *tau* accumulations and abnormal *tau*-positive aggregates. Axonal degeneration and uncoordinated movement are more severe in lines expressing mutant *tau*. However, no *tau* filaments are observed.

15.6 Concluding Remarks

In this chapter, we have attempted to provide a comprehensive overview of the necrotic cell death paradigms that have been established in *C. elegans* (see Table 15.1) and to also convey our current understanding of the molecular mechanisms involved. The rich repertoire of necrotic cell death events that occur in *C. elegans* both during development and in the adult renders the nematode a particularly attractive platform for dissecting the mechanisms of pathological cell death in humans, which is typically mediated by necrotic processes.

Table 15.1 Triggers and paradigms of necrotic death in *C. elegans*. Necrotic stimuli and cell populations affected

Death initiator	Type of insult	Dying cells
mec-4(*u231*) referred to as *mec-4*(*d*)	Hyperactive degenerin ion channel	Touch receptor neurons
mec-10(A673V) referred to as *mec-10*(*d*)	Hyperactive degenerin ion channel	Touch receptor neurons
deg-1(*u38*) referred to as *deg-1*(*d*)	Hyperactive degenerin ion channel	Some polymodal neurons and specific interneurons
unc-8(*n491*)	Hyperactive degenerin ion channel	Motor neurons
pnc-1(*ku212*) or *cog-3*(*ku212*)	Excess nicotinamide levels	Uterine–vulval 1 cells
Nicotinamide	Excess nicotinamide levels	Uterine–vulval 1 cells
deg-3(*u662*) referred to as *deg-3*(*d*)	Hyperactive nicotinic acetylcholine receptor	Subset of sensory neurons and interneurons
gsa-1(Q208L), $G\alpha_s$(Q227L) referred to as α_s(*gf*)	Constitutively active GTP-binding protein $G\alpha_s$	Motor neurons, interneurons, head and tail ganglia neurons, and pharyngeal neurons or epithelial cells (unidentified)
Thapsigargin	Elevation of intracellular Ca^{2+} levels	Random cells (including neuronal)
Δ*glt-3*;α_s(*gf*)	Glutamate-dependent toxicity	Head neurons
Erwinia carotovora *Photorhabdus luminescens*	Pathogen infection	Intestinal, epidermal, and gonadal cells
Hypoxic treatment	Oxygen/energy limitation	Pharynx, gonad primordium, body wall muscles, unidentified cells
α-synuclein	Stress induction	Dopaminergic neurons
LRRK2, leucine-rich repeat kinase 2	Stress induction	Dopaminergic neurons
Tau protein	Stress induction	Several neurons (including motor neurons)

The similarity of necrotic cell death triggered by hyperactive ion channels in *C. elegans* to excitotoxic cell death and neurodegeneration in mammals, both in terms of morphological characteristics and mechanistic aspects, reflects the extensive evolutionary conservation of necrosis-relevant genes between *C. elegans* and mammals. Moreover, conservation of the mechanisms that protect *C. elegans* and mammalian cells from necrotic death inflicted by diverse stimuli, as exhibited, for example, by the hormetic induction of HSF16.1 upon heat preconditioning, provides new prospects for employing the nematode in the battle against degeneration. Concomitantly, modeling of human degenerative disorders, such as Parkinson's disease and others, in *C. elegans* has already accelerated the pace of the molecular dissection of the underlying mechanisms and holds promise for the development and testing of innovative intervention strategies.

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References

Abraham MC, Shaham S (2004) Death without caspases, caspases without death. Trends Cell Biol 14:184–193

Aroian RV, Koga M, Mendel JE, Ohshima Y, Sternberg PW (1990) The let-23 gene necessary for *Caenorhabditis elegans* vulval induction encodes a tyrosine kinase of the EGF receptor subfamily. Nature 348:693–699

Artal-Sanz M, Samara C, Syntichaki P, Tavernarakis N (2006) Lysosomal biogenesis and function is critical for necrotic cell death in Caenorhabditis elegans. J Cell Biol 173:231–239

Bader CR, Bernheim L, Bertrand D (1985) Sodium-activated potassium current in cultured avian neurones. Nature 317:540–542

Baumeister R, Ge L (2002) The worm in us – Caenorhabditis elegans as a model of human disease. Trends Biotechnol 20:147–148

Berger AJ, Hart AC, Kaplan JM (1998) G alphas-induced neurodegeneration in Caenorhabditis elegans. J Neurosci 18:2871–2880

Blondet B, Carpentier G, Ait-Ikhlef A, Murawsky M, Rieger F (2002) Motoneuron morphological alterations before and after the onset of the disease in the wobbler mouse. Brain Res 930:53–57

Bussey H, Andrews B, Boone C (2006) From worm genetic networks to complex human diseases. Nat Genet 38:862–863

Calabrese EJ (2004) Hormesis: a revolution in toxicology, risk assessment and medicine. EMBO Rep 5(1):S37–S40

Cao S, Gelwix CC, Caldwell KA, Caldwell GA (2005) Torsin-mediated protection from cellular stress in the dopaminergic neurons of Caenorhabditis elegans. J Neurosci 25:3801–3812

Chalfie M, Driscoll M, Huang M (1993) Degenerin similarities. Nature 361:504

Chalfie M, Wolinsky E (1990) The identification and suppression of inherited neurodegeneration in Caenorhabditis elegans. Nature 345:410–416

Chartier-Harlin MC, Kachergus J, Roumier C, Mouroux V, Douay X, Lincoln S, Levecque C, Larvor L, Andrieux J, Hulihan M, Waucquier N, Defebvre L, Amouyel P, Farrer M, Destee A (2004) Alpha-synuclein locus duplication as a cause of familial Parkinson's disease. Lancet 364:1167–1169

Cleveland DW, Rothstein JD (2001) From Charcot to Lou Gehrig: deciphering selective motor neuron death in ALS. Nat Rev Neurosci 2:806–819

Cooper AA, Gitler AD, Cashikar A, Haynes CM, Hill KJ, Bhullar B, Liu K, Xu K, Strathearn KE, Liu F, Cao S, Caldwell KA, Caldwell GA, Marsischky G, Kolodner RD, Labaer J, Rochet JC, Bonini NM, Lindquist S (2006) Alpha-synuclein blocks ER-Golgi traffic and Rab1 rescues neuron loss in Parkinson's models. Science 313:324–328

Cooper JD, Messer A, Feng AK, Chua-Couzens J, Mobley WC (1999) Apparent loss and hypertrophy of interneurons in a mouse model of neuronal ceroid lipofuscinosis: evidence for partial response to insulin-like growth factor-1 treatment. J Neurosci 19:2556–2567

Galluzzi L, Maiuri MC, Vitale I, Zischka H, Castedo M, Zitvogel L, Kroemer G (2007) Cell death modalities: classification and pathophysiological implications. Cell Death Differ 14:1237–1243

Gidday JM (2006) Cerebral preconditioning and ischaemic tolerance. Nat Rev Neurosci 7:437–448

Hall DH, Gu G, Garcia-Anoveros J, Gong L, Chalfie M, Driscoll M (1997) Neuropathology of degenerative cell death in Caenorhabditis elegans. J Neurosci 17:1033–1045

Hamamichi S, Rivas RN, Knight AL, Cao S, Caldwell KA, Caldwell GA (2008) Hypothesis-based RNAi screening identifies neuroprotective genes in a Parkinson's disease model. Proc Natl Acad Sci U S A 105:728–733

Hengartner MO (2000) The biochemistry of apoptosis. Nature 407:770–776

Hill RJ, Sternberg PW (1992) The gene lin-3 encodes an inductive signal for vulval development in *C. elegans*. Nature 358:470–476

Horvitz HR, Sternberg PW, Greenwald IS, Fixsen W, Ellis HM (1983) Mutations that affect neural cell lineages and cell fates during the development of the nematode Caenorhabditis elegans. Cold Spring Harb Symp Quant Biol 48(Pt 2):453–463

Huang L, Hanna-Rose W (2006) EGF signaling overcomes a uterine cell death associated with temporal mis-coordination of organogenesis within the *C. elegans* egg-laying apparatus. Dev Biol 300:599–611

Hutton M, Lendon CL, Rizzu P, Baker M, Froelich S, Houlden H, Pickering-Brown S, Chakraverty S, Isaacs A, Grover A, Hackett J, Adamson J, Lincoln S, Dickson D, Davies P, Petersen RC, Stevens M, de Graaff E, Wauters E, van Baren J, Hillebrand M, Joosse M, Kwon JM, Nowotny P, Che LK, Norton J, Morris JC, Reed LA, Trojanowski J, Basun H, Lannfelt L, Neystat M, Fahn S, Dark F, Tannenberg T, Dodd PR, Hayward N, Kwok JB, Schofield PR, Andreadis A, Snowden J, Craufurd D, Neary D, Owen F, Oostra BA, Hardy J, Goate A, van Swieten J, Mann D, Lynch T, Heutink P (1998) Association of missense and 5′-splice-site mutations in *tau* with the inherited dementia FTDP-17. Nature 393:702–705

Ishidoh K, Kominami E (2002) Processing and activation of lysosomal proteinases. Biol Chem 383:1827–1831

Kaletta T, Hengartner MO (2006) Finding function in novel targets: *C. elegans* as a model organism. Nat Rev Drug Discov 5:387–398

Kameyama M, Kakei M, Sato R, Shibasaki T, Matsuda H, Irisawa H (1984) Intracellular Na+ activates a K+ channel in mammalian cardiac cells. Nature 309:354–356

Kauppinen RA, Enkvist K, Holopainen I, Akerman KE (1988a) Glucose deprivation depolarizes plasma membrane of cultured astrocytes and collapses transmembrane potassium and glutamate gradients. Neuroscience 26:283–289

Kauppinen RA, McMahon HT, Nicholls DG (1988b) Ca^{2+}-dependent and Ca^{2+}-independent glutamate release, energy status and cytosolic free Ca^{2+} concentration in isolated nerve terminals following metabolic inhibition: possible relevance to hypoglycaemia and anoxia. Neuroscience 27:175–182

Kerr JF, Wyllie AH, Currie AR (1972) Apoptosis: a basic biological phenomenon with wide-ranging implications in tissue kinetics. Br J Cancer 26:239–257

Kimble J, Hirsh D (1979) The postembryonic cell lineages of the hermaphrodite and male gonads in Caenorhabditis elegans. Dev Biol 70:396–417

Korswagen HC, Park JH, Ohshima Y, Plasterk RH (1997) An activating mutation in a Caenorhabditis elegans Gs protein induces neural degeneration. Genes Dev 11:1493–1503

Korswagen HC, van der Linden AM, Plasterk RH (1998) G protein hyperactivation of the Caenorhabditis elegans adenylyl cyclase SGS-1 induces neuronal degeneration. EMBO J 17:5059–5065

Kourtis N, Nikoletopoulou V, Tavernarakis N (2012) Small heat-shock proteins protect from heat-stroke-associated neurodegeneration. Nature 490:213–218

Kourtis N, Tavernarakis N (2007) Non-developmentally programmed cell death in Caenorhabditis elegans. Semin Cancer Biol 17:122–133

Kraemer BC, Zhang B, Leverenz JB, Thomas JH, Trojanowski JQ, Schellenberg GD (2003) Neurodegeneration and defective neurotransmission in a Caenorhabditis elegans model of tauopathy. Proc Natl Acad Sci U S A 100:9980–9985

Kuwahara T, Koyama A, Gengyo-Ando K, Masuda M, Kowa H, Tsunoda M, Mitani S, Iwatsubo T (2006) Familial Parkinson mutant alpha-synuclein causes dopamine neuron dysfunction in transgenic Caenorhabditis elegans. J Biol Chem 281:334–340

Lakso M, Vartiainen S, Moilanen AM, Sirvio J, Thomas JH, Nass R, Blakely RD, Wong G (2003) Dopaminergic neuronal loss and motor deficits in Caenorhabditis elegans overexpressing human alpha-synuclein. J Neurochem 86:165–172

Lee VM, Goedert M, Trojanowski JQ (2001) Neurodegenerative tauopathies. Annu Rev Neurosci 24:1121–1159

Lehotsky J, Kaplan P, Murin R, Raeymaekers L (2002) The role of plasma membrane Ca^{2+} pumps (PMCAs) in pathologies of mammalian cells. Front Biosci 7:d53–d84

Libina N, Berman JR, Kenyon C (2003) Tissue-specific activities of *C. elegans* DAF-16 in the regulation of lifespan. Cell 115:489–502

Luke CJ, Pak SC, Askew YS, Naviglia TL, Askew DJ, Nobar SM, Vetica AC, Long OS, Watkins SC, Stolz DB, Barstead RJ, Moulder GL, Bromme D, Silverman GA (2007) An intracellular serpin regulates necrosis by inhibiting the induction and sequelae of lysosomal injury. Cell 130:1108–1119

Magni G, Amici A, Emanuelli M, Raffaelli N, Ruggieri S (1999) Enzymology of NAD+ synthesis. Adv Enzymol Relat Areas Mol Biol 73:135–182, xi

Mano I, Driscoll M (2009) Caenorhabditis elegans glutamate transporter deletion induces AMPA-receptor/adenylyl cyclase 9-dependent excitotoxicity. J Neurochem 108:1373–1384

Mattson MP, LaFerla FM, Chan SL, Leissring MA, Shepel PN, Geiger JD (2000) Calcium signaling in the ER: its role in neuronal plasticity and neurodegenerative disorders. Trends Neurosci 23:222–229

Miyasaka T, Ding Z, Gengyo-Ando K, Oue M, Yamaguchi H, Mitani S, Ihara Y (2005) Progressive neurodegeneration in *C. elegans* model of tauopathy. Neurobiol Dis 20:372–383

Newman AP, White JG, Sternberg PW (1996) Morphogenesis of the *C. elegans* hermaphrodite uterus. Development 122:3617–3626

Paschen W (2001) Dependence of vital cell function on endoplasmic reticulum calcium levels: implications for the mechanisms underlying neuronal cell injury in different pathological states. Cell Calcium 29:1–11

Paschen W, Frandsen A (2001) Endoplasmic reticulum dysfunction – a common denominator for cell injury in acute and degenerative diseases of the brain? J Neurochem 79:719–725

Pavlikova M, Tatarkova Z, Sivonova M, Kaplan P, Krizanova O, Lehotsky J (2009) Alterations induced by ischemic preconditioning on secretory pathways Ca^{2+}-ATPase (SPCA) gene expression and oxidative damage after global cerebral ischemia/reperfusion in rats. Cell Mol Neurobiol 29:909–916

Polymeropoulos MH, Lavedan C, Leroy E, Ide SE, Dehejia A, Dutra A, Pike B, Root H, Rubenstein J, Boyer R, Stenroos ES, Chandrasekharappa S, Athanassiadou A, Papapetropoulos T, Johnson WG, Lazzarini AM, Duvoisin RC, Di Iorio G, Golbe LI, Nussbaum RL (1997) Mutation in the alpha-synuclein gene identified in families with Parkinson's disease. Science 276:2045–2047

Poorkaj P, Bird TD, Wijsman E, Nemens E, Garruto RM, Anderson L, Andreadis A, Wiederholt WC, Raskind M, Schellenberg GD (1998) *Tau* is a candidate gene for chromosome 17 frontotemporal dementia. Ann Neurol 43:815–825

Poulin G, Nandakumar R, Ahringer J (2004) Genome-wide RNAi screens in Caenorhabditis elegans: impact on cancer research. Oncogene 23:8340–8345

Qiao L, Hamamichi S, Caldwell KA, Caldwell GA, Yacoubian TA, Wilson S, Xie ZL, Speake LD, Parks R, Crabtree D, Liang Q, Crimmins S, Schneider L, Uchiyama Y, Iwatsubo T, Zhou Y, Peng L, Lu Y, Standaert DG, Walls KC, Shacka JJ, Roth KA, Zhang J (2008) Lysosomal enzyme cathepsin D protects against alpha-synuclein aggregation and toxicity. Mol Brain 1:17

Saha S, Guillily MD, Ferree A, Lanceta J, Chan D, Ghosh J, Hsu CH, Segal L, Raghavan K, Matsumoto K, Hisamoto N, Kuwahara T, Iwatsubo T, Moore L, Goldstein L, Cookson M, Wolozin B (2009) LRRK2 modulates vulnerability to mitochondrial dysfunction in Caenorhabditis elegans. J Neurosci 29:9210–9218

Schapira AH (2008) Mitochondria in the aetiology and pathogenesis of Parkinson's disease. Lancet Neurol 7:97–109

Scott BA, Avidan MS, Crowder CM (2002) Regulation of hypoxic death in *C. elegans* by the insulin/IGF receptor homolog DAF-2. Science 296:2388–2391

Shaham S (1998) Identification of multiple Caenorhabditis elegans caspases and their potential roles in proteolytic cascades. J Biol Chem 273:35109–35117

Silverman GA, Bird PI, Carrell RW, Church FC, Coughlin PB, Gettins PG, Irving JA, Lomas DA, Luke CJ, Moyer RW, Pemberton PA, Remold-O'Donnell E, Salvesen GS, Travis J, Whisstock JC (2001) The serpins are an expanding superfamily of structurally similar but functionally diverse proteins. Evolution, mechanism of inhibition, novel functions, and a revised nomenclature. J Biol Chem 276:33293–33296

Singleton AB, Farrer M, Johnson J, Singleton A, Hague S, Kachergus J, Hulihan M, Peuralinna T, Dutra A, Nussbaum R, Lincoln S, Crawley A, Hanson M, Maraganore D, Adler C, Cookson MR, Muenter M, Baptista M, Miller D, Blancato J, Hardy J, Gwinn-Hardy K (2003) Alpha-Synuclein locus triplication causes Parkinson's disease. Science 302:841

Spillantini MG, Crowther RA, Kamphorst W, Heutink P, van Swieten JC (1998) *Tau* pathology in two Dutch families with mutations in the microtubule-binding region of tau. Am J Pathol 153:1359–1363

Spillantini MG, Schmidt ML, Lee VM, Trojanowski JQ, Jakes R, Goedert M (1997) Alpha-synuclein in Lewy bodies. Nature 388:839–840

Sulston JE, Albertson DG, Thomson JN (1980) The Caenorhabditis elegans male: postembryonic development of nongonadal structures. Dev Biol 78:542–576

Sulston JE, Horvitz HR (1977) Post-embryonic cell lineages of the nematode, Caenorhabditis elegans. Dev Biol 56:110–156

Sulston JE, Schierenberg E, White JG, Thomson JN (1983) The embryonic cell lineage of the nematode Caenorhabditis elegans. Dev Biol 100:64–119

Syntichaki P, Samara C, Tavernarakis N (2005) The vacuolar H+-ATPase mediates intracellular acidification required for neurodegeneration in *C. elegans*. Curr Biol 15:1249–1254

Syntichaki P, Tavernarakis N (2003) The biochemistry of neuronal necrosis: rogue biology? Nat Rev Neurosci 4:672–684

Syntichaki P, Tavernarakis N (2004) Genetic models of mechanotransduction: the nematode Caenorhabditis elegans. Physiol Rev 84:1097–1153

Syntichaki P, Xu K, Driscoll M, Tavernarakis N (2002) Specific aspartyl and calpain proteases are required for neurodegeneration in *C. elegans*. Nature 419:939–944

Tavernarakis N, Driscoll M (2001) Degenerins. At the core of the metazoan mechanotransducer? Ann N Y Acad Sci 940:28–41

Treinin M, Chalfie M (1995) A mutated acetylcholine receptor subunit causes neuronal degeneration in *C. elegans*. Neuron 14:871–877

Treinin M, Gillo B, Liebman L, Chalfie M (1998) Two functionally dependent acetylcholine subunits are encoded in a single Caenorhabditis elegans operon. Proc Natl Acad Sci U S A 95:15492–15495

Troulinaki K, Tavernarakis N (2012a) Endocytosis and intracellular trafficking contribute to necrotic neurodegeneration in *C. elegans*. EMBO J 31:654–666

Troulinaki K, Tavernarakis N (2012b) Necrotic cell death and neurodegeneration: the involvement of endocytosis and intracellular trafficking. Worm 1:176–181

van der Horst A, Schavemaker JM, Pellis-van Berkel W, Burgering BM (2007) The Caenorhabditis elegans nicotinamidase PNC-1 enhances survival. Mech Ageing Dev 128:346–349

Van Montfort R, Slingsby C, Vierling E (2001) Structure and function of the small heat shock protein/alpha-crystallin family of molecular chaperones. Adv Protein Chem 59:105–156

Vlachos M, Tavernarakis N (2010) Non-apoptotic cell death in Caenorhabditis elegans. Dev Dyn 239:1337–1351

Vrablik TL, Huang L, Lange SE, Hanna-Rose W (2009) Nicotinamidase modulation of NAD+ biosynthesis and nicotinamide levels separately affect reproductive development and cell survival in *C. elegans*. Development 136:3637–3646

Walker NI, Harmon BV, Gobe GC, Kerr JF (1988) Patterns of cell death. Methods Achiev Exp Pathol 13:18–54

Wong D, Bazopoulou D, Pujol N, Tavernarakis N, Ewbank JJ (2007) Genome-wide investigation reveals pathogen-specific and shared signatures in the response of Caenorhabditis elegans to infection. Genome Biol 8:R194

Xu K, Tavernarakis N, Driscoll M (2001) Necrotic cell death in *C. elegans* requires the function of calreticulin and regulators of Ca($^{2+}$) release from the endoplasmic reticulum. Neuron 31:957–971
Yamashima T (2000) Implication of cysteine proteases calpain, cathepsin and caspase in ischemic neuronal death of primates. Prog Neurobiol 62:273–295
Yamashima T (2004) Ca^{2+}-dependent proteases in ischemic neuronal death: a conserved 'calpain-cathepsin cascade' from nematodes to primates. Cell Calcium 36:285–293
Yuan A, Santi CM, Wei A, Wang ZW, Pollak K, Nonet M, Kaczmarek L, Crowder CM, Salkoff L (2003) The sodium-activated potassium channel is encoded by a member of the Slo gene family. Neuron 37:765–773
Yuan J, Shaham S, Ledoux S, Ellis HM, Horvitz HR (1993) The *C. elegans* cell death gene ced-3 encodes a protein similar to mammalian interleukin-1 beta-converting enzyme. Cell 75: 641–652

Chapter 16
Necrostatin-1: Its Discovery and Application in Cell Death Research

Dana E. Christofferson, Ying Li, and Junying Yuan

Pathological cell death occurs by both apoptosis and necrosis. Necrosis has traditionally been believed to be a passive form of cell death caused by overwhelming stress. However, the observation that death receptor stimulation could induce necrosis when apoptosis was inhibited led to the idea that at least a subset of necrosis might be the result of a programmed cell death pathway (Kawahara et al. 1998; Vercammen et al. 1998a; Holler et al. 2000). This form of caspase-independent, programmed necrotic cell death is now known as necroptosis (Degterev et al. 2005). Receptor-interacting protein 1 (RIP1) was the first identified key component of this pathway, and the kinase activity of RIP1 is essential to the pathway's execution (Holler et al. 2000; Degterev et al. 2005, 2008).

16.1 Receptor-Interacting Protein 1 is a Critical Regulator of Cell Death

Receptor-interacting protein 1 (RIP1) was first identified as a 74 kDa Fas-interacting protein but is also recruited to other death receptors, including the TNFα receptor (Stanger et al. 1995; Hsu et al. 1996a). RIP1 contains a C-terminal death domain (DD), an intermediate domain, and an N-terminal kinase domain with serine/threonine kinase activity (Stanger et al. 1995; Hsu et al. 1996a). Each of these domains of RIP1 is important for modulating cell fate after death receptor stimulation: the intermediate domain is responsible for NF-κB activation and cell survival, the death domain mediates apoptosis as part of the death-inducing signaling complex (DISC), and the kinase domain is required for necroptosis.

D.E. Christofferson • Y. Li • J. Yuan (✉)
Department of Cell Biology, Harvard Medical School, Boston, MA, USA
e-mail: jyuan@hms.harvard.edu

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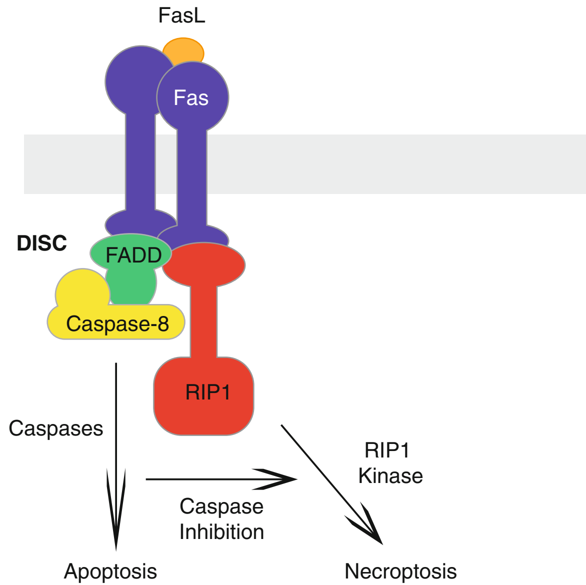

Fig. 16.1 Model of Fas receptor-stimulated pathways resulting in cell death. FasL binding induces dimerization of Fas in the cell membrane to recruit RIP1, FADD, and caspase-8 to form the DISC. Caspase-8 is activated to induce apoptosis; however, when caspases are inhibited, cell death occurs via RIP1 kinase-mediated necroptosis

16.1.1 FasL Signaling Induces Cell Death

Fas ligand (FasL/CD95L/APO1L) signals through its receptor, Fas (CD95/APO1), to directly induce formation of the death-inducing signaling complex (DISC) and cell death. FasL induces dimerization of the Fas receptor death domains (DD) which recruits the DD-containing proteins FADD and RIP1 (Stanger et al. 1995). FADD in turn recruits procaspase-8 through interaction of their death-effector domains (DEDs), thus forming the DISC at the Fas receptor (Boldin et al. 1996). The DISC promotes the activation of caspase-8, allowing it to cleave Bid and downstream caspases to induce apoptosis (Boldin et al. 1996). In some cell types, when FasL-induced apoptosis is inhibited, RIP1 kinase is activated, resulting in the induction of necroptosis and cell death (Kawahara et al. 1998; Vercammen et al. 1998a; Holler et al. 2000) (Fig. 16.1).

16.1.2 TNFα Signaling Activates Cell Survival or Cell Death

TNFα is a pleiotropic cytokine that can activate cell survival and proliferation pathways via the transcription factor NF-κB or cell death by either apoptosis or necroptosis. TNFα was first isolated as the endotoxin-induced factor that caused necrosis of tumors in mice and was found to have cytotoxic activity against some cell lines, including the mouse fibrosarcoma L929 cells (Carswell et al. 1975). Only a small subset of cells, however, are actually sensitive to TNFα alone, and in fibroblasts and other tumor cell lines, TNFα instead promotes cell growth and proliferation (Sugarman et al. 1985; Fransen et al. 1986; Vilcek et al. 1986).

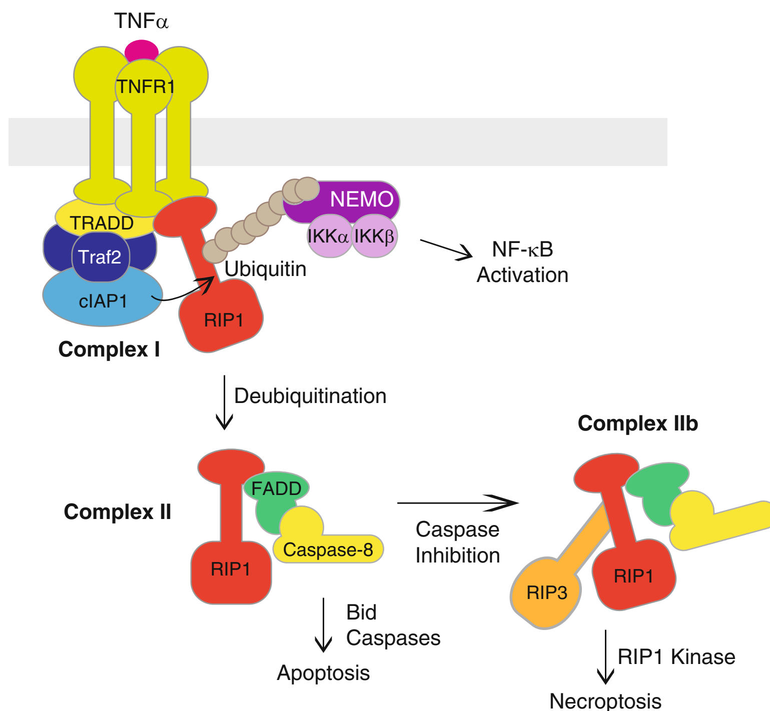

Fig. 16.2 Model of pathways activated after TNFα stimulation. TNFα trimerization of TNFR1 recruits TRADD, Traf2, cIAP1, and RIP1 to form complex I, which activates NF-κB by ubiquitination of RIP1. Deubiquitinases promote the transition to complex II containing RIP1, FADD, and caspase-8 to activate apoptosis. When apoptosis is inhibited, an alternative complex IIb forms, also containing RIP3, to activate necroptosis, dependent on the kinase activity of RIP1

Trimerization of TNFR1 triggered by TNFα brings together its cytoplasmic death domains to recruit TRADD and RIP1 (Hsu et al. 1995, 1996a). TRADD also recruits TRAF2 to TNFR1 (Hsu et al. 1996b; Ermolaeva et al. 2008; Pobezinskaya et al. 2008). TRAF2 binds to the cellular inhibitor of apoptosis proteins (cIAP1 and cIAP2) and is responsible for their recruitment to the TNFα receptor signal complex (Rothe et al. 1995; Shu et al. 1996; Vince et al. 2009; Zhang et al. 2009b). This complex of proteins at the TNFα receptor is known as “complex I” (Micheau and Tschopp 2003). The intermediate domain of RIP1 is ubiquitinated at complex I, activating the transcription factor NF-κB to promote the expression of genes involved in cell proliferation and survival, including a number of antiapoptotic proteins such as c-Flip (Lowenthal et al. 1989; Osborn et al. 1989; Wang et al. 1998; Micheau et al. 2001). In the absence of survival signaling, complex I transits to cytosolic “complex II” containing FADD, caspase-8, and RIP1, similar to the DISC in Fas signaling, to induce apoptosis (Kull and Cuatrecasas 1981). If apoptosis is blocked, some cells utilize an alternative complex II, complex IIb that requires RIP1 kinase activity, and an additional component, RIP3, to induce necroptosis (Cho et al. 2009) (Fig. 16.2).

16.1.3 The Kinase Activity of RIP1 Is Required for Necroptosis

In most cells, inhibition of caspase activation blocks apoptosis and cell death. It was with surprise that pan-caspase inhibitors such as zVAD.fmk or deletion of the upstream initiator caspase-8 were unable to inhibit FasL-induced cell death in some cell types (Kawahara et al. 1998; Vercammen et al. 1998a). L929 cells, a mouse fibrosarcoma cell line, do not express Fas and undergo necrosis when treated with TNFα (Carswell et al. 1975). Reconstitution of L929 cells with human Fas and treatment with a Fas agonist antibody induces apoptosis (Schulze-Osthoff et al. 1994; Vercammen et al. 1998a). However, studies using Fas-reconstituted L929 cells found that treatment with caspase inhibitors to block apoptosis did not inhibit cell death. Instead, the cells underwent necrotic cell death, similar to that induced by TNFα in L929 cells (Vercammen et al. 1998a), indicating that if apoptosis was blocked, the cells could activate a necrotic, caspase-independent mechanism of cell death.

The same phenomenon was seen in a model of activation-induced cell death (AICD) in Jurkat cells, a human T cell line. Activation of the Fas receptor induced apoptosis, but caspase-8-deficient cells or cells treated with the pan-caspase inhibitor zVAD.fmk were not resistant to cell death (Kawahara et al. 1998; Holler et al. 2000). As in the FasL/zVAD.fmk-stimulated L929 cells, after caspase inhibition the dying Jurkat cells exhibited a necrotic morphology (Kawahara et al. 1998; Holler et al. 2000). Jurkat cells deficient in the DISC proteins FADD, caspase-8, and RIP1 helped to establish essential components of the caspase-independent necrotic cell death pathway. Caspase-8-deficient Jurkat cells undergo necroptosis after treatment with FasL, and similarly, FADD-deficient Jurkat cells are blocked from undergoing apoptosis, but die by necroptosis after TNFα stimulation (Kawahara et al. 1998; Holler et al. 2000). FADD-deficient Jurkat cells are commonly used as a model for TNFα-induced necroptosis (Holler et al. 2000; Degterev et al. 2005; Lawrence and Chow 2005; Christofferson and Yuan 2010).

Apoptosis is blocked in FADD and caspase-8-deficient cells, resulting in sensitization to necroptosis. In contrast, RIP1-deficient Jurkat cells were found to be sensitive to apoptosis, but completely resistant to necroptosis, suggesting the importance of RIP1 in mediating necroptosis (Holler et al. 2000). RIP1-deficient cells reconstituted with kinase dead RIP1 remained resistant to necroptosis, while expression of wild-type RIP1 resensitized cells to death (Holler et al. 2000). Thus, it was established that the kinase activity of RIP1 is required for necroptosis.

16.1.4 Necrostatin-1 Is an Inhibitor of RIP1 Kinase

These studies were the first to establish that necrotic cell death occurred as the result of a specific signaling pathway and is dependent on the kinase activity of RIP1 (Degterev et al. 2005). The identification of a small molecule that could specifically inhibit necroptosis supported the hypothesis that necroptosis follows a defined

Table 16.1 Cell death models inhibited by Nec-1

Death receptor stimulation		
Cell line	Stimulus	References
U937	TNFα/zVAD	(Degterev et al. 2005)
Jurkat wt	FasL/CHX/zVAD	(Degterev et al. 2005)
Jurkat FADD deficient	TNFα	
BALB/c 3T3	TNFα/zVAD	(Degterev et al. 2005)
	FasL/zVAD	
MEF	TNFα/CHX/zVAD	(Degterev et al. 2005)
Rela$^{-/-}$ MEF	Poly (I:C)	(Basagoudanavar et al. 2011)
Cpdm MEF	TNFα	(Gerlach et al. 2011)
L929	TNFα	(Degterev et al. 2005)
	zVAD	(Hitomi et al. 2008)
	poly (I:C)/IFNγ	
	TNFα/BV6 (SM)	(Vanlangenakker et al. 2011)
HaCaT	TRAIL/SM/zVAD	(Geserick et al. 2009)
HT-29	SM/zVAD	(He et al. 2009)
SVEC4-10	TNFα/zVAD	(Upton et al. 2010)
EJ	CD154	(Knox et al. 2011)
Bone marrow-derived macrophages	Anti-Fas/CHX/zVAD	(Kaiser et al. 2011)
HeLa, HT1080, MDA-MB-231	Etoposide	(Biton and Ashkenazi 2011; Tenev et al. 2011)
HT-29, MEF, HepG2	TRAIL at acidic pH 6.5	(Jouan-Lanhouet et al. 2012)

signaling pathway. A high throughput small molecule screen identified necrostatin-1 (Nec-1), a small molecule that is able to inhibit necroptosis in U937 cells (Degterev et al. 2005). Although the original Nec-1 isolated from the high throughput screen, methyl-thiohydantoin-tryptophan (MTH-Trp), can also inhibit indoleamine 2,3-dioxygenase (IDO), 7-Cl-O-Nec-1 (5-((7-chloro-1H-indol-3-yl)methyl)-3-methylimidazolidine-2,4-dione), an improved analog of Nec-1, has no IDO inhibitory activity (Takahashi et al. 2012; Degterev et al. 2012).

Nec-1 potently inhibits necroptosis in many cell types, including TNFα-induced necroptosis in FADD-deficient Jurkat cells (Degterev et al. 2005) (Table 16.1). After the discovery of this compound as an inhibitor of necroptosis, Nec-1 was found to specifically target RIP1 kinase, validating the important role of RIP1 kinase in necroptosis (Degterev et al. 2008). Nec-1 has no effect on the kinase activity of the closely related kinases, RIP2 or RIP3, and is very specific to RIP1 when tested against a panel of kinases (Degterev et al. 2008; Biton and Ashkenazi 2011). The specificity and activity of Nec-1 have allowed this compound to be used as a chemical tool to study the roles of RIP1 kinase and necroptosis in cellular pathways and pathological conditions.

Nec-1 acts as an ATP competitive inhibitor, and structural studies show that it binds an inactive conformation of RIP1 (Degterev et al. 2008; Xie et al. 2013). RIP1 is autophosphorylated on Ser161 within the activation loop or T-loop of the kinase (Degterev et al. 2008). This loop, modeled after the closely related kinase B-RAF,

has two conformations: open and closed. In the closed form, the loop blocks access to the catalytic cleft but when open allows the kinase access to its substrates. Autophosphorylation on Ser161 is predicted to move the loop into its open form and allow kinase activation; accordingly, Nec-1 is unable to inhibit necroptosis in cells expressing the phosphomimetic S161E RIP1 (Degterev et al. 2008). Nec-1 binds to a hydrophobic pocket between the N- and C-lobes of RIP1's kinase domain, stabilizing the inactive conformation of RIP1 in part through interactions with the activation loop (Xie et al. 2013).

16.2 Importance of Necrostatin-1 as a Tool to Understand Necroptosis and RIP1 Activity

Nec-1 has been proven to be a critical tool in understanding the molecular pathways involved in necroptosis as well as the cellular roles of RIP1 kinase. Genetic screens have identified genes that play critical roles in necroptosis (Hitomi et al. 2008; Wang et al. 2008). Chemical biological approaches have also yielded important information about the necroptotic cell death pathway: the use of Nec-1 as a chemical inhibitor of RIP1 kinase has allowed the investigation of pathways activated by RIP1 kinase independent from altering other RIP1 activities, such as RIP1 activation of NF-κB signaling.

16.2.1 Nec-1 Demonstrates Role of cIAP1/2 in Activating RIP1 Kinase

Canonical apoptosis induced by TNFα and CHX does not require RIP1 kinase activity; loss of RIP1 in many cases does not block apoptosis (Holler et al. 2000; Wang et al. 2008). However, RIP1 knockdown completely blocks apoptosis when cIAP1/2 are inhibited during death receptor stimulation (Gaither et al. 2007; Petersen et al. 2007; Bertrand et al. 2008; Wang et al. 2008; Geserick et al. 2009; Wong et al. 2010). Loss of cI AP1/2 promotes the formation of complex II containing RIP1, caspase-8, and FADD (Bertrand et al. 2008; Wang et al. 2008; Geserick et al. 2009). In contrast to other inducers of apoptosis, apoptosis induced by Smac mimetics(IAP antagonists) is blocked by Nec-1 inhibition of RIP1 kinase, suggesting that RIP1 kinase activity is activated upon Smac mimetic treatment to induce cell death (Wang et al. 2008). cIAP1/2 therefore regulate the activation of RIP1 kinase and formation of complex II after TNFα stimulation.

As further evidence that cIAP1/2 regulate the formation of complex II, loss of cIAP1/2 with Smac mimetic or etoposide treatment can spontaneously induce association of complex II containing RIP1, FADD, and caspase-8 and RIP1

kinase-dependent cell death that can be inhibited by Nec-1 (Feoktistova et al. 2011; Tenev et al. 2011). This complex II, which forms independent of death receptor signaling, has been termed the "ripoptosome." Together, these findings suggest that the cIAPs normally inhibit formation of a RIP1-containing complex II. Nec-1 as an inhibitor of RIP1 kinase was used to demonstrate a novel role for RIP1 kinase in inducing apoptosis in the absence of cIAP1/2.

16.2.2 Mechanism of RIP1 and RIP3 Kinase Activation in Necroptosis Is Explored with Nec-1

RIP3 plays an important role in caspase-independent necroptotic cell death. Knockdown of RIP3 protects against multiple inducers of necroptosis, and it is thought that expression of RIP3 sensitizes cells to necroptosis over apoptosis (Cho et al. 2009; He et al. 2009; Zhang et al. 2009a). The kinase activity of RIP3 is also critical for necroptosis: cells expressing kinase dead RIP3 are resistant to necroptosis (Cho et al. 2009; He et al. 2009). RIP3 and RIP1 are both required for the formation of complex IIb in necroptosis (Cho et al. 2009; He et al. 2009; Zhang et al. 2009a).

After death receptor stimulation, Nec-1 blocks RIP1 interaction with caspase-8 and FADD (Wang et al. 2008; Geserick et al. 2009), as well as RIP3 recruitment to complex IIb (Cho et al. 2009; He et al. 2009). This suggests that the Nec-1 stabilized inactive conformation of RIP1 is unable to form complex IIb (Xie et al. 2013). The active conformation of RIP1 itself, however, is not sufficient to induce necroptosis: autophosphorylation on Ser161 is predicted to move the loop into its open form and allow kinase activation; however, a phosphomimetic RIP1 mutant, S161E, does not induce necroptosis by itself, indicating that autophosphorylation and an open conformation of the activation loop are not sufficient for RIP1 kinase activation (Degterev et al. 2008). RIP3 is also required for complex IIb formation, as RIP1 does not bind FADD after necroptosis stimulation in *Rip3*$^{-/-}$ cells (Cho et al. 2009).

RIP1 and RIP3 kinases cross-phosphorylate and activate each other: after necroptosis stimulation, RIP1 kinase activates RIP3 autophosphorylation, and RIP3, either directly or indirectly, also increases RIP1 phosphorylation (Cho et al. 2009; He et al. 2009). The exact mechanism of activation of both kinases is unclear; however, the use of Nec-1 demonstrates that activated RIP1 kinase is required both for assembly of complex IIb and for necroptosis to occur.

Nec-1 does not show any activity against RIP3 kinase (Cho et al. 2009). To date, none of the identified necrostatin compounds have demonstrated any inhibition of RIP3 kinase, which is interesting in its own right, as one would expect that RIP3 kinase inhibitors should have been recovered from cell-based screens in addition to the RIP1 kinase inhibitors. Identification of a RIP3-specific inhibitor would allow further study to better define the role of RIP3 kinase activity in cell death.

16.2.3 The Complex II Components FADD and Caspase-8 Also Regulate Necroptosis

Although essential for the activation of apoptosis, FADD and caspase-8 have an important role in the inhibition of necroptosis and promotion of cell survival in embryonic development and in T cell activation. FADD or caspase-8 knockout mice are embryonic lethal, but viability can be restored by crossing with either RIP1 knockout or RIP3 knockout mice, suggesting that FADD and caspase-8 negatively regulate necroptosis during embryonic development (Varfolomeev et al. 1998; Yeh et al. 1998; Zhang et al. 1998; Kaiser et al. 2011; Oberst et al. 2011).

The pro-survival role of FADD and caspase-8 has been shown more specifically in studies of T cell activation. After antigen stimulation, T cells undergo clonal expansion to combat infection (Green et al. 2003). After activation, T cells deficient in FADD or caspase-8 do not proliferate and accumulate like WT cells do, due to an increase in necroptosis (Newton et al. 1998; Zhang et al. 1998; Bell et al. 2008; Ch'en et al. 2008; Osborn et al. 2010; Ch'en et al. 2011). The overactivation of necroptosis in FADD- and caspase-8-deficient cells suggests a pro-survival role for FADD and caspase-8 during T cell activation. Nec-1, the RIP1 kinase inhibitor, or loss of RIP1 restores the normal T cell activation-induced proliferative response in FADD-deficient cells (Bell et al. 2008; Ch'en et al. 2008; Osborn et al. 2010; Zhang et al. 2011). Correspondingly, T cell activation-induced necroptosis in caspase-8-deficient cells is reversed by the co-deletion of *Rip3* (Ch'en et al. 2011; Kaiser et al. 2011; Oberst et al. 2011). Thus, FADD and caspase-8 negatively regulate RIP1 and RIP3 activation and necroptosis during T cell activation.

16.3 Nec-1 Has Been Used to Identify Downstream Pathways That Mediate Necroptosis

16.3.1 Cyclophilin D Mediates Oxidative Stress-Induced Necroptosis

Cyclophilin D (CypD) is a peptidyl-prolyl *cis-trans* isomerase found in the mitochondrial matrix as a component of the mitochondrial permeability transition (MPT) pore and is specifically involved in regulating necrotic cell death. RIP1 and RIP3 are reported to mediate a cellular model of nitric oxide (NO)-induced death (Li et al. 2004b; Davis et al. 2010b). CypD overexpression blocks apoptosis, but sensitizes cells to nitric oxide (NO)-induced necrosis, suggesting CypD might act in the same pathway as RIP1 and RIP3 (Lin and Lechleiter 2002; Schubert and Grimm 2004; Li et al. 2004b). CypD knockout cells are resistant to Ca^{2+} or hydrogen peroxide-induced necrotic cell death, but still sensitive to apoptosis (Baines et al. 2005; Nakagawa et al. 2005; Schinzel et al. 2005). Altogether, the data suggests that

CypD functions specifically in necrosis. There have, however, been conflicting reports as to the role of CypD in necroptosis. CypD overexpression blocked necroptosis induced by TNFα and zVAD.fmk, suggesting it does not play a role in mediating necroptosis (Temkin et al. 2006). Defects in mitochondrial function after TNFα/CHX/zVAD.fmk stimulation in MEF cells and the resulting necroptosis could not be blocked by cyclosporin A, a CypD inhibitor (Irrinki et al. 2011). These studies suggest that although CypD is important in oxidative stress-induced necrosis, CypD may not play a role in death receptor-induced necroptosis.

Animal models, however, confirm an important role for CypD in necroptosis. CypD knockout mice are protected against ischemia-reperfusion injury (Baines et al. 2005; Nakagawa et al. 2005; Schinzel et al. 2005; Devalaraja-Narashimha et al. 2009). Inhibition of RIP1 kinase with Nec-1 also reduces infarct size in ischemia-reperfusion injury (Degterev et al. 2005). Although both CypD knockout mice and those treated with Nec-1 have reduced infarct size after cardiac ischemia compared to controls, Nec-1 was not able to further reduce infarct size in CypD-deficient mice after cardiac ischemia, suggesting that RIP1 kinase acts upstream of CypD in ischemia-induced necroptosis (Lim et al. 2007). CypD function in the MPT might cause mitochondrial dysfunction in necroptosis.

16.3.2 Reactive Oxygen Species in Necroptosis

RIP3 binds to several metabolic enzymes, both cytosolic and mitochondria associated, after the stimulation of necroptosis (Zhang et al. 2009a). RIP3 may directly modulate cellular metabolic activity to promote necroptosis; RIP3 kinase promotes the activity of the metabolic enzymes glycogen phosphorylase (PYGL), glutamate-ammonia ligase (GLUL), and glutamate dehydrogenase 1 (GLUD1) (Zhang et al. 2009a). The activation of RIP3 and increased metabolic activity promotes production of reactive oxygen species (ROS), which are involved in the execution of necroptotic cell death (Cho et al. 2009; Zhang et al. 2009a).

The generation of free radicals or ROS is involved in TNFα-induced cytotoxicity in some cell types. TNFα-induced necroptosis in L929 and MEF cells can be inhibited by antioxidants such as BHA (butylated hydroxyanisole), which blocks ROS accumulation and cell death (Matthews et al. 1987; Yamauchi et al. 1989; Zimmerman et al. 1989; Goossens et al. 1995; Vercammen et al. 1998b; Sakon et al. 2003; Lin et al. 2004; Degterev et al. 2005). ROS specifically mediates TNFα-induced necroptosis, as BHA is unable to block TNFα-induced apoptosis (Sakon et al. 2003). RIP1 is required for TNFα-induced ROS, potentially by recruiting the NADPH oxidase Nox1 and its regulatory subunits NOXO1 and NOXA1 to TNFR1 (Lin et al. 2004; Ventura et al. 2004; Kim et al. 2007). Recruitment of the NADPH oxidase complex to TNFR1 is required for TNFα to induce ROS production in L929 cells (Kim et al. 2007).

While ROS generation in L929 and MEF cells is important for the execution of necroptosis, it is not required for necroptosis in all cell types (Degterev et al. 2005).

Jurkat cells, for example, undergo TNFα-induced necroptosis even with NADPH oxidase inhibitors or BHA (Matsumura et al. 2000; Degterev et al. 2005, 2008). The amoeba *N. fowleri*, however, induces ROS-dependent necroptosis in infected Jurkat cells that can be inhibited by pretreatment with Nec-1 (Song et al. 2011). These conflicting reports on the role of ROS in necroptosis suggest that the requirement of ROS in mediating necroptosis is cell type and stimulus dependent.

16.3.3 Role of Autophagy in Necroptosis

Autophagy is a cellular mechanism activated in response to cell stress that breaks down cellular organelles and other components and recycles them into their basic parts to maintain cellular energy levels and homeostasis (Levine and Klionsky 2004). Most notably, autophagy is activated under starvation conditions and required for cellular survival in response to limiting nutrients, but is also involved in mediating cell death (Levine and Klionsky 2004; Degterev et al. 2008).

Autophagy is activated downstream of RIP1 kinase in necroptotic cell death. Necroptosis stimulation increased autophagy downstream of RIP1 kinase activation (Degterev et al. 2005). Inhibition of cell death with Nec-1 reduced the accumulation of LC3-II, a marker of autophagy, in several cell types. In vivo, autophagy induced in response to tissue injury in a rat model of retinal ischemia was also reduced by treatment with Nec-1, indicating that autophagy is activated downstream of RIP1 kinase (Rosenbaum et al. 2010). However, the importance of autophagy in mediating necroptosis is cell type dependent. Autophagy is reportedly required for cell death in zVAD.fmk-stimulated L929 cells, U937 cells, and macrophages; however, although autophagy is activated in TNFα-stimulated FADD-deficient Jurkat cells, or TNFα- and zVAD.fmk-treated BALB/c 3T3 and MEF cells, these cell types do not require autophagy to undergo cell death; for example, Nec-1 was still able to inhibit necroptosis in *Atg5*$^{-/-}$ MEFs (Yu et al. 2004; Degterev et al. 2005; Xu et al. 2006).

The in vivo requirement of autophagy in necroptosis was examined using a model of T cell activation. T cell activation-induced necroptosis occurs in FADD-deficient and caspase-8-deficient T cells, independent of autophagy (Ch'en et al. 2008, 2011; Osborn et al. 2010). However, in a FADDdd transgenic mouse model, expressing dominant negative FADD, T cell activation induces cell death dependent on both RIP1 kinase and autophagy (Bell et al. 2008). While the differential role of autophagy in activation-induced necroptosis in FADD-deficient and FADDdd-expressing T cells is not clear, it might be due to a requirement of FADD for the activation of autophagy. A tandem affinity purification of FADD indicates that FADD, RIP1 kinase, and caspase-8 are in a complex with components of the autophagosome, Atg5, Atg12, and Atg16L, suggesting that FADD and caspase-8 might target RIP1 kinase to directly activate the autophagosome (Bell et al. 2008).

16.4 Nec-1 Has Been Used to Identify Novel Functions of RIP1 Kinase

zVAD.fmk treatment activates necroptosis that is inhibited by Nec-1 in select cell types, such as L929 cells and macrophages (Yu et al. 2004; Hitomi et al. 2008; Christofferson et al. 2012). zVAD.fmk is a pan-caspase inhibitor, and an initial report showed that knockdown of caspase-8 could recapitulate the effects of zVAD.fmk on inducing cell death in L929 cells (Yu et al. 2004). This result has not been repeated, and furthermore, specific caspase-8 inhibitors are insufficient to replicate the effect of zVAD.fmk and induce necroptosis (Madden et al. 2007; Wu et al. 2011). Toxicity of fluoromethylketone (fmk)-conjugated caspase inhibitors and the lack of specificity of pan-caspase inhibitors, like zVAD.fmk, have been suggested as possible mechanisms for the induction of cell death (Chauvier et al. 2007). The effect of zVAD.fmk in L929 cells has been attributed to nonspecific inhibition of cathepsins and calpains; however, there are conflicting reports as to the ability of zVAD.fmk to inhibit these enzymes (Chauvier et al. 2007; Madden et al. 2007; Chen et al. 2011). Use of a specific pan-caspase inhibitor lacking the fmk group, QVD.oph, is unable to replicate the results of zVAD.fmk on L929 cells; however, other compounds conjugated to the fmk group were not toxic to cells, suggesting that fmk toxicity is not the primary cause of cell death in response to zVAD.fmk (Wu et al. 2011). IDN 6556, a more specific pan-caspase inhibitor than zVAD.fmk that lacks the fmk group, completely replicates the effects of zVAD.fmk on L929 cells (D. Christofferson, unpublished data). Together, this data suggests that zVAD.fmk does not induce nonspecific toxicity but, instead, inhibits caspase activity to induce necroptosis, although the specific caspase requirements and their role remain unknown.

ROS and autophagy are activated by zVAD.fmk treatment and reported to be required for necroptosis and are blocked by Nec-1 (Yu et al. 2004; Degterev et al. 2005; Hitomi et al. 2008; Chen et al. 2011; Wu et al. 2011). Although activated downstream of RIP1 kinase, a genome-wide siRNA screen for genes involved in mediating zVAD.fmk-induced necroptosis did not identify any autophagy-related genes as essential for zVAD.fmk-induced cell death (Hitomi et al. 2008). Instead, this siRNA screen suggested that autocrine TNFα production is involved in mediating zVAD.fmk-induced necroptosis (Hitomi et al. 2008).

A chemical biological approach uncovered the role of RIP1 kinase in mediating autocrine TNFα production. In response to zVAD.fmk treatment, mouse L929 fibrosarcoma and J774 macrophages activated TNFα production that was blocked by Nec-1 treatment (Christofferson et al. 2012). L929 cells and human MDA-MB-231 breast cancer cells treated with Smac mimetic also induced TNFα production inhibited by Nec-1, altogether suggesting that activation of RIP1 kinase by inhibition of caspases or loss of cIAP1/2 activated TNFα production. This study identified a novel role for RIP1 kinase in activating transcription of TNFα, through a pathway dependent on EDD, JNK signaling, and the transcription factor Sp1 (Christofferson et al. 2012).

16.5 Use of Nec-1 in Determining the Physiological Role of Necroptosis

Nec-1 has been instrumental in demonstrating the physiological and pathological roles of necroptosis. From the initial discovery of Nec-1, which used Nec-1 to block ischemia-reperfusion (I/R) injury in a mouse model of stroke, to other I/R models, models of oxidative stress, and various disease models, Nec-1 has been used to demonstrate the role of necroptosis in pathological cell death (Table 16.2).

16.5.1 Necroptosis Occurs in Response to Infection

Necroptosis was proposed to be an alternative "backup" cell death pathway for when apoptosis is blocked. In many ways, its function is similar to apoptosis. Necroptosis and apoptosis both are important in the elimination of damaged or infected cells. A viral gene essential for viral replication and pathogenesis in the murine cytomegalovirus (MCMV), M45, encodes an inhibitor of necroptosis, M45-encoded viral inhibitor of RIP activation (vIRA) (Lembo et al. 2004; Mack et al. 2008). vIRA is essential for inhibiting necroptosis upon infection by directly interacting with RIP1 and RIP3 (Mack et al. 2008; Upton et al. 2010). Infected virus expressing vIRA with a mutated RIP-binding motif induces necroptosis, which is blocked in *Rip3*$^{-/-}$ cells but not in Nec-1-treated cells (Upton et al. 2010). The inability of Nec-1 to inhibit cell death indicated that while MCMV infection induces necroptosis that is dependent on RIP3, this pathway is independent of RIP1 kinase. The preferential increased sensitivity of *Rip3*$^{-/-}$-deficient cells, but not that of Nec-1-treated cells, to MCMV infection suggests the differential involvement of RIP1 and RIP3 kinases in the antiviral response and the possible consequence of increased sensitivity to certain viral infection upon blocking of RIP3 kinase but not RIP1 kinase.

Vaccinia virus infection also induces cell death via necroptosis. RIP1-deficient or *Rip3*$^{-/-}$ cells infected with vaccinia virus have enhanced viability after TNFα treatment compared to WT cells (Chan et al. 2003; Cho et al. 2009). This enhanced cellular viability after viral infection translates into reduced survival of *Rip3*$^{-/-}$ mice infected with vaccinia virus, supporting a role for necroptotic cell death during viral infection to promote clearance of the virus and organism survival (Cho et al. 2009).

Infection with pathogens aside from viruses can also induce necroptosis. The amoeba *N. fowleri* can cause primary amoebic meningoencephalitis in mammals, characterized by a severe inflammatory response and necrotic cell death. In order to understand the mechanism of cell death induced by this pathogen, researchers incubated Jurkat T cells with *N. fowleri* trophozoites (Song et al. 2011). *N. fowleri* infection caused ROS generation and necroptotic cell death in Jurkat cells, which was markedly reduced with Nec-1 treatment, suggesting that ROS production and cell death are dependent on the kinase activity of RIP1 (Song et al. 2011).

Table 16.2 Disease models inhibited by Nec-1

Ischemia-reperfusion (I/R)

Treatment	*Model*	*References*
Oxygen-glucose deprivation	Mouse, primary cortical neurons	Xu et al. (2010)
Middle cerebral artery occlusion (MCAO)	Rat, neuronal I/R	Degterev et al. (2005)
Myocardial I/R	Mouse, heart I/R	Lim et al. (2007)
Langendorff perfused heart I/R	Mouse, heart	Smith et al. (2007)
Left anterior descending coronary artery I/R	Mouse, heart	Smith et al. (2007); Oerlemans et al. (2012)
High intraocular pressure	Rat, retinal I/R	Rosenbaum et al. (2010)
Retinal detachment	Mouse, retina	Trichonas et al. (2010)
Neonatal hypoxia-ischemia	Mouse, Vannucci model	Northington et al. (2011); Chavez-Valdez et al. (2012)
Bilateral renal pedicle clipping	Mouse, renal I/R	Linkermann et al. (2012)

Oxidative stress/NO donor-induced death

Cell line	*Stimulus*	*References*
C2C12	t-BuOOH	Smith et al. (2007)
H9c2		
Premyelinated oligodendrocytes (preOLs, rat)	Arachidonic acid	Kim et al. (2010)
	Cystine deprivation	
	BSO (buthionine sulfoximine)	
Cardiomyocyte progenitor cells (CMPC, human)	H_2O_2	Liu et al. (2011)
HPMVEC	DeaNONOate	Davis et al. (2010a)
Pancreatic islet cells, βTC-6, INS-1, Jurkat	GSNO, SNAP	Tamura et al. (2011)[a]

(continued)

Table 16.2 (continued)

Neuronal cell injury		
Treatment	*Model*	*References*
NMDA, excitotoxicity	Primary cortical neurons, rat	Li et al. (2008)
Glutamate, excitotoxicity	HT-22	Xu et al. (2007)
Aluminum chloride	SH-SY5Y	Zhang et al. (2010)
Light insult	RGC-5, retina cell line	Ji et al. (2011)
AD model, 24S-OHC	SH-SY5Y	Yamanaka et al. (2011)
HD model, zVAD	Striatal cell line (ST-14A 8plx)	Zhu et al. (2011)
	R6/2 HD mouse model	
Controlled cortical impact (CCI)	Mouse	You et al. (2008)
Weight drop	Traumatic brain injury	Wang et al. (2012)

Chemotherapy/cancer treatments		
Treatment	*Cell line/model*	*References*
Dexamethasone/obatoclax	CEM-C1, 697, Jurkat/ALL	Bonapace et al. (2010)
AraC/SM	Reh/ALL	Löder et al. (2012)
Obatoclax (GX15-070)	TE671, RMS13/rhabdomyosarcoma cells	Basit et al. (2013)
DT-GMCSF, diphtheria toxin	U937/AML	Horita et al. (2008)
D-galactose	Malignant cancer cells (Neuro2a, SH-SY5Y, PC-3, HepG2)	Li et al. (2011)
Radiation	TPC-1, 8505c, SW13, human papillary thyroid carcinoma/anaplastic thyroid and adrenocortical cancers	Nehs et al. (2011)
Shikonin	MCF-7/NIH3T3/leukemia	Han et al. (2007, 2012); Park et al. (2012)
Cisplatin/zVAD	Human proximal tubule cells	Tristão et al. (2012)

Activation-induced cell death

Cell line	*Stimulus*	*References*
Mouse T cells	Anti-CD3/zVAD	Cho et al. (2009)
FADD*dd* mouse T cells	OVA activated	Bell et al. (2008)
Casp8$^{-/-}$ T cells	Anti-CD3/anti-CD28, OVA activated	Bell et al. (2008); Ch'en et al. (2008, 2011)
tFADD$^{-/-}$ T cells	Anti-CD3/anti-CD28	Osborn et al. (2010)

Other

Treatment	*Cell line/model*	*References*
N. fowleri infection	Jurkat	Song et al. (2011)
Acetyl-LDL lipoprotein particles (rAcL-SO)	Mouse peritoneal macrophages	Bao et al. (2006)
Cadmium	CHO K1, RTgill-W1 (rainbow trout gill cell line)	Hsu et al. (2009); Krumschnabel et al. (2010)
mTNFα-induced systemic inflammatory response	Mouse	Duprez et al. (2011)
Germanium nanoparticle	CHO K1	Ma et al. (2011)
Staurosporine (1 μM)	Rat astrocyte	Simenc and Lipnik-Stangelj (2012)
Cyclosporin A	Renal tubular epithelia cell	Ouyang et al. (2012)
Concanavalin A	Hepatitis	Jouan-Lanhouet et al. (2012)

[a]Tamura et al. 2011 shows a protective effect of Nec-1, but Nec-1 is still protective in siRIP1 cells, making it unclear if the effect of Nec-1 is due to inhibition of RIP1 kinase. They use 100 μM Nec-1, which is high enough to have nonspecific effects but however does not show the efficiency of RIP1 knockdown by siRNA

Together, these studies suggest that necroptotic cell death occurs in response to infection with various pathogens, likely to contribute to the removal of infected and damaged cells, similar to apoptosis. Further study is necessary to determine under what conditions RIP1 kinase is involved and the importance of the inflammatory response that can occur as a result of necroptotic cell death to clearance of the infection and overall survival of the organism.

16.5.2 Necroptosis Occurs in Response to Acute Pathologies

Apoptosis plays a major role in physiological cell death; however, under pathological conditions, necrosis is very common (Artal-Sanz and Tavernarakis 2005). Necroptosis occurs physiologically in acute neuronal pathologies such as stroke or ischemia. Nec-1 can reduce infarct size and cell loss in mouse models of focal cerebral ischemia, cardiac ischemia, neonatal hypoxia-ischemia, and retinal ischemia (Degterev et al. 2005; Lim et al. 2007; Smith et al. 2007; Rosenbaum et al. 2010; Northington et al. 2011). Neuronal excitotoxicity can induce necroptosis and is involved in acute neurodegeneration such as that resulting from stroke, but also in pathologies associated with chronic neurodegeneration, such as Alzheimer's disease and Parkinson's disease (Artal-Sanz and Tavernarakis 2005; Xu et al. 2007; Li et al. 2008). Additionally Nec-1 significantly reduces tissue damage and improves functional outcome after traumatic brain injury (TBI), indicating that RIP1 kinase activation and necroptosis contribute to the pathology of TBI (You et al. 2008).

16.5.3 Activation of Necroptosis May Circumvent Chemotherapeutic Resistance in Tumor Cells

Many tumor cells are resistant to apoptosis and research is underway for therapies that can increase sensitivity of these cells to apoptosis, such as Smac mimetics (IAP antagonists) (Li et al. 2004a). An alternative is to induce necrotic cell death (Hu and Xuan 2008). Shikonin is a compound that can induce necroptosis and works in MCF-7 xenografts in vivo to significantly reduce tumor weight (Han et al. 2007).

Some cytotoxic compounds and proteins used for cancer therapies have also been shown to induce necroptosis (Horita et al. 2008; Bonapace et al. 2010), suggesting that induction of necroptosis is a viable alternative to activate cancer cell death in apoptosis-resistant cells. Glucocorticoid (GC) resistance is a clinical predictor of poor outcome in childhood acute lymphoblastic leukemia (ALL). Obatoclax (GX15-070), a small molecule inhibitor of Bcl-2 proteins, has been shown to resensitize GC-resistant ALL cells to cell death in a manner dependent on autophagy and RIP1 kinase activity (Bonapace et al. 2010). Obatoclax induces autophagy and the assembly of the necroptosis-inducing complex of RIP1 and RIP3 on the autophagosome membranes to induce necroptosis in rhabdomyosarcoma cells. Cell death induced by obatoclax requires RIP1 kinase activity as it can be inhibited by Nec-1.

Activation of necroptosis shows promise for the treatment of tumors that are resistant to traditional chemotherapies. Further investigation of specific activators of necroptosis may provide novel clinical treatments for resistant tumors.

Nec-1 has been used in vitro, in cellular models and in animal models to investigate the function and role of RIP1 kinase. Use of Nec-1 has enabled the characterization of novel roles for RIP1 kinase in apoptosis, in the induction of TNFα, and in mediating necroptosis. Understanding the role of RIP1 kinase and necroptosis after infection, acute injury, and other pathological conditions will facilitate the targeting and development of clinical strategies to treat a variety of diseases. The use of Nec-1 as a tool has advanced scientific understanding of a number of conditions and may one day itself be developed as a clinical treatment for disease.

References

Artal-Sanz M, Tavernarakis N (2005) Proteolytic mechanisms in necrotic cell death and neurodegeneration. FEBS Lett 579:3287–3296

Baines CP, Kaiser RA, Purcell NH, Blair NS, Osinska H, Hambleton MA, Brunskill EW, Sayen MR, Gottlieb RA, Dorn GW et al (2005) Loss of cyclophilin D reveals a critical role for mitochondrial permeability transition in cell death. Nature 434:658–662

Bao L, Li Y, Deng S-X, Landry D, Tabas I (2006) Sitosterol-containing lipoproteins trigger free sterol-induced caspase-independent death in ACAT-competent macrophages. J Biol Chem 281:33635–33649

Basagoudanavar SH, Thapa RJ, Nogusa S, Wang J, Beg AA, Balachandran S (2011) Distinct roles for the NF-kappa B RelA subunit during antiviral innate immune responses. J Virol 85: 2599–2610

Basit F, Cristofanon S, Fulda S (2013) Obatoclax (GX15-070) triggers necroptosis by promoting the assembly of the necrosome on autophagosomal membranes. Cell Death Differ 20:1161

Bell BD, Leverrier S, Weist BM, Newton RH, Arechiga AF, Luhrs KA, Morrissette NS, Walsh CM (2008) FADD and caspase-8 control the outcome of autophagic signaling in proliferating T cells. Proc Natl Acad Sci U S A 105:16677–16682

Bertrand MJM, Milutinovic S, Dickson KM, Ho WC, Boudreault A, Durkin J, Gillard JW, Jaquith JB, Morris SJ, Barker PA (2008) cIAP1 and cIAP2 facilitate cancer cell survival by functioning as E3 ligases that promote RIP1 ubiquitination. Mol Cell 30:689–700

Biton S, Ashkenazi A (2011) NEMO and RIP1 control cell fate in response to extensive DNA damage via TNF-α feedforward signaling. Cell 145:92–103

Boldin MP, Goncharov TM, Goltsev YV, Wallach D (1996) Involvement of MACH, a novel MORT1/FADD-interacting protease, in Fas/APO-1- and TNF receptor-induced cell death. Cell 85:803–815

Bonapace L, Bornhauser BC, Schmitz M, Cario G, Ziegler U, Niggli FK, Schäfer BW, Schrappe M, Stanulla M, Bourquin J-P (2010) Induction of autophagy-dependent necroptosis is required for childhood acute lymphoblastic leukemia cells to overcome glucocorticoid resistance. J Clin Invest 120:1310–1323

Carswell EA, Old LJ, Kassel RL, Green S, Fiore N, Williamson B (1975) An endotoxin-induced serum factor that causes necrosis of tumors. Proc Natl Acad Sci U S A 72:3666–3670

Ch'en IL, Beisner DR, Degterev A, Lynch C, Yuan J, Hoffmann A, Hedrick SM (2008) Antigen-mediated T cell expansion regulated by parallel pathways of death. Proc Natl Acad Sci U S A 105:17463–17468

Ch'en IL, Tsau JS, Molkentin JD, Komatsu M, Hedrick SM (2011) Mechanisms of necroptosis in T cells. J Exp Med 208:633–641

Chan FK, Shisler J, Bixby JG, Felices M, Zheng L, Appel M, Orenstein J, Moss B, Lenardo MJ (2003) A role for tumor necrosis factor receptor-2 and receptor-interacting protein in programmed necrosis and antiviral responses. J Biol Chem 278:51613–51621

Chauvier D, Ankri S, Charriaut-Marlangue C, Casimir R, Jacotot E (2007) Broad-spectrum caspase inhibitors: from myth to reality? Cell Death Differ 14:387–391

Chavez-Valdez R, Martin LJ, Flock DL, Northington FJ (2012) Necrostatin-1 attenuates mitochondrial dysfunction in neurons and astrocytes following neonatal hypoxia-ischemia. Neuroscience 219:192–203

Chen S-Y, Chiu L-Y, Maa M-C, Wang J-S, Chien C-L, Lin W-W (2011) zVAD-induced autophagic cell death requires c-Src-dependent ERK and JNK activation and reactive oxygen species generation. Autophagy 7:217–228

Cho YS, Challa S, Moquin D, Genga R, Ray TD, Guildford M, Chan FK-M (2009) Phosphorylation-driven assembly of the RIP1-RIP3 complex regulates programmed necrosis and virus-induced inflammation. Cell 137:1112–1123

Christofferson DE, Yuan J (2010) Necroptosis as an alternative form of programmed cell death. Curr Opin Cell Biol 22:263–268

Christofferson DE, Li Y, Hitomi J, Zhou W, Upperman C, Zhu H, Gerber SA, Gygi S, Yuan J (2012) A novel role for RIP1 kinase in mediating TNFα production. Cell Death Dis 3:e320

Davis CW, Hawkins BJ, Ramasamy S, Irrinki KM, Cameron BA, Islam K, Daswani VP, Doonan PJ, Manevich Y, Madesh M (2010a) Nitration of the mitochondrial complex I subunit NDUFB8 elicits RIP1- and RIP3-mediated necrosis. Free Radic Biol Med 48:306–317

Davis TL, Walker JR, Campagna-Slater V, Finerty PJ, Paramanathan R, Bernstein G, MacKenzie F, Tempel W, Ouyang H, Lee WH et al (2010b) Structural and biochemical characterization of the human cyclophilin family of peptidyl-prolyl isomerases. PLoS Biol 8:e1000439

Degterev A, Hitomi J, Germscheid M, Ch'en IL, Korkina O, Teng X, Abbott D, Cuny GD, Yuan C, Wagner G et al (2008) Identification of RIP1 kinase as a specific cellular target of necrostatins. Nat Chem Biol 4:313–321

Degterev A, Huang Z, Boyce M, Li Y, Jagtap P, Mizushima N, Cuny GD, Mitchison TJ, Moskowitz MA, Yuan J (2005) Chemical inhibitor of nonapoptotic cell death with therapeutic potential for ischemic brain injury. Nat Chem Biol 1:112–119

Degterev A, Maki JL, Yuan J (2012) Activity and specificity of necrostatin-1, small-molecule inhibitor of RIP1 kinase. Cell Death Differ 20:366

Devalaraja-Narashimha K, Diener AM, Padanilam BJ (2009) Cyclophilin D gene ablation protects mice from ischemic renal injury. Am J Physiol Renal Physiol 297:F749–F759

Duprez L, Takahashi N, Van Hauwermeiren F, Vandendriessche B, Goossens V, Vanden Berghe T, Declercq W, Libert C, Cauwels A, Vandenabeele P (2011) RIP kinase-dependent necrosis drives lethal systemic inflammatory response syndrome. Immunity 35:908–918

Ermolaeva MA, Michallet M-C, Papadopoulou N, Utermöhlen O, Kranidioti K, Kollias G, Tschopp J, Pasparakis M (2008) Function of TRADD in tumor necrosis factor receptor 1 signaling and in TRIF-dependent inflammatory responses. Nat Immunol 9:1037–1046

Feoktistova M, Geserick P, Kellert B, Dimitrova DP, Langlais C, Hupe M, Cain K, Macfarlane M, Häcker G, Leverkus M (2011) cIAPs block ripoptosome formation, a RIP1/caspase-8 containing intracellular cell death complex differentially regulated by cFLIP isoforms. Mol Cell 43:449

Fransen L, Ruysschaert MR, Van der Heyden J, Fiers W (1986) Recombinant tumor necrosis factor: species specificity for a variety of human and murine transformed cell lines. Cell Immunol 100:260–267

Gaither A, Porter D, Yao Y, Borawski J, Yang G, Donovan J, Sage D, Slisz J, Tran M, Straub C et al (2007) A Smac mimetic rescue screen reveals roles for inhibitor of apoptosis proteins in tumor necrosis factor-alpha signaling. Cancer Res 67:11493–11498

Gerlach B, Cordier SM, Schmukle AC, Emmerich CH, Rieser E, Haas TL, Webb AI, Rickard JA, Anderton H, Wong WW-L et al (2011) Linear ubiquitination prevents inflammation and regulates immune signalling. Nature 471:591–596

Geserick P, Hupe M, Moulin M, Wong WW-L, Feoktistova M, Kellert B, Gollnick H, Silke J, Leverkus M (2009) Cellular IAPs inhibit a cryptic CD95-induced cell death by limiting RIP1 kinase recruitment. J Cell Biol 187:1037–1054

Goossens V, Grooten J, De Vos K, Fiers W (1995) Direct evidence for tumor necrosis factor-induced mitochondrial reactive oxygen intermediates and their involvement in cytotoxicity. Proc Natl Acad Sci U S A 92:8115–8119

Green DR, Droin N, Pinkoski M (2003) Activation-induced cell death in T cells. Immunol Rev 193:70–81

Han W, Li L, Qiu S, Lu Q, Pan Q, Gu Y, Luo J, Hu X (2007) Shikonin circumvents cancer drug resistance by induction of a necroptotic death. Mol Cancer Ther 6:1641–1649

Han W, Xie J, Fang Y, Wang Z, Pan H (2012) Nec-1 enhances shikonin-induced apoptosis in leukemia cells by inhibition of RIP-1 and ERK1/2. Int J Mol Sci 13:7212–7225

He S, Wang L, Miao L, Wang T, Du F, Zhao L, Wang X (2009) Receptor interacting protein kinase-3 determines cellular necrotic response to TNF-alpha. Cell 137:1100–1111

Hitomi J, Christofferson DE, Ng A, Yao J, Degterev A, Xavier RJ, Yuan J (2008) Identification of a molecular signaling network that regulates a cellular necrotic cell death pathway. Cell 135:1311–1323

Holler N, Zaru R, Micheau O, Thome M, Attinger A, Valitutti S, Bodmer JL, Schneider P, Seed B, Tschopp J (2000) Fas triggers an alternative, caspase-8-independent cell death pathway using the kinase RIP as effector molecule. Nat Immunol 1:489–495

Horita H, Frankel AE, Thorburn A (2008) Acute myeloid leukemia-targeted toxin activates both apoptotic and necroptotic death mechanisms. PLoS One 3:e3909

Hsu H, Huang J, Shu HB, Baichwal V, Goeddel DV (1996a) TNF-dependent recruitment of the protein kinase RIP to the TNF receptor-1 signaling complex. Immunity 4:387–396

Hsu H, Shu HB, Pan MG, Goeddel DV (1996b) TRADD-TRAF2 and TRADD-FADD interactions define two distinct TNF receptor 1 signal transduction pathways. Cell 84:299–308

Hsu H, Xiong J, Goeddel DV (1995) The TNF receptor 1-associated protein TRADD signals cell death and NF-kappa B activation. Cell 81:495–504

Hsu T-S, Yang P-M, Tsai J-S, Lin L-Y (2009) Attenuation of cadmium-induced necrotic cell death by necrostatin-1: potential necrostatin-1 acting sites. Toxicol Appl Pharmacol 235:153–162

Hu X, Xuan Y (2008) Bypassing cancer drug resistance by activating multiple death pathways – a proposal from the study of circumventing cancer drug resistance by induction of necroptosis. Cancer Lett 259:127–137

Irrinki KM, Mallilankaraman K, Thapa RJ, Chandramoorthy HC, Smith FJ, Jog NR, Gandhirajan RK, Kelsen SG, Houser SR, May MJ et al (2011) Requirement of FADD, NEMO, and BAX/BAK for aberrant mitochondrial function in tumor necrosis factor alpha-induced necrosis. Mol Cell Biol 31:3745–3758

Ji D, Kamalden TA, del Olmo-Aguado S, Osborne NN (2011) Light- and sodium azide-induced death of RGC-5 cells in culture occurs via different mechanisms. Apoptosis 16:425–437

Jouan-Lanhouet S, Arshad MI, Piquet-Pellorce C, Martin-Chouly C, Le Moigne-Muller G, Van Herreweghe F, Takahashi N, Sergent O, Lagadic-Gossmann D, Vandenabeele P et al (2012) TRAIL induces necroptosis involving RIPK1/RIPK3-dependent PARP-1 activation. Cell Death Differ 19:2003–2014

Kaiser WJ, Upton JW, Long AB, Livingston-Rosanoff D, Daley-Bauer LP, Hakem R, Caspary T, Mocarski ES (2011) RIP3 mediates the embryonic lethality of caspase-8-deficient mice. Nature 471:368–372

Kawahara A, Ohsawa Y, Matsumura H, Uchiyama Y, Nagata S (1998) Caspase-independent cell killing by Fas-associated protein with death domain. J Cell Biol 143:1353–1360

Kim S, Dayani L, Rosenberg PA, Li J (2010) RIP1 kinase mediates arachidonic acid-induced oxidative death of oligodendrocyte precursors. Int J Physiol Pathophysiol Pharmacol 2:137–147

Kim YS, Morgan MJ, Choksi S, Liu ZG (2007) TNF-induced activation of the Nox1 NADPH oxidase and its role in the induction of necrotic cell death. Mol Cell 26:675–687

Knox PG, Davies CC, Ioannou M, Eliopoulos AG (2011) The death domain kinase RIP1 links the immunoregulatory CD40 receptor to apoptotic signaling in carcinomas. J Cell Biol 192: 391–399

Krumschnabel G, Ebner HL, Hess MW, Villunger A (2010) Apoptosis and necroptosis are induced in rainbow trout cell lines exposed to cadmium. Aquat Toxicol 99:73–85

Kull FC, Cuatrecasas P (1981) Possible requirement of internalization in the mechanism of in vitro cytotoxicity in tumor necrosis serum. Cancer Res 41:4885–4890

Lawrence CP, Chow SC (2005) FADD deficiency sensitises Jurkat T cells to TNF-alpha-dependent necrosis during activation-induced cell death. FEBS Lett 579:6465–6472

Lembo D, Donalisio M, Hofer A, Cornaglia M, Brune W, Koszinowski U, Thelander L, Landolfo S (2004) The ribonucleotide reductase R1 homolog of murine cytomegalovirus is not a functional enzyme subunit but is required for pathogenesis. J Virol 78:4278–4288

Levine B, Klionsky DJ (2004) Development by self-digestion: molecular mechanisms and biological functions of autophagy. Dev Cell 6:463–477

Li L, Thomas RM, Suzuki H, De Brabander JK, Wang X, Harran PG (2004a) A small molecule Smac mimic potentiates TRAIL- and TNFalpha-mediated cell death. Science 305:1471–1474

Li N, He Y, Wang L, Mo C, Zhang J, Zhang W, Li J, Liao Z, Tang X, Xiao H (2011) D-galactose induces necroptotic cell death in neuroblastoma cell lines. J Cell Biochem 112:3834–3844

Li Y, Johnson N, Capano M, Edwards M, Crompton M (2004b) Cyclophilin-D promotes the mitochondrial permeability transition but has opposite effects on apoptosis and necrosis. Biochem J 383:101–109

Li Y, Yang X, Ma C, Qiao J, Zhang C (2008) Necroptosis contributes to the NMDA-induced excitotoxicity in rat's cultured cortical neurons. Neurosci Lett 447:120–123

Lim SY, Davidson SM, Mocanu MM, Yellon DM, Smith CCT (2007) The cardioprotective effect of necrostatin requires the cyclophilin-D component of the mitochondrial permeability transition pore. Cardiovasc Drugs Ther 21:467–469

Lin D-T, Lechleiter JD (2002) Mitochondrial targeted cyclophilin D protects cells from cell death by peptidyl prolyl isomerization. J Biol Chem 277:31134–31141

Lin Y, Choksi S, Shen HM, Yang QF, Hur GM, Kim YS, Tran JH, Nedospasov SA, Liu ZG (2004) Tumor necrosis factor-induced nonapoptotic cell death requires receptor-interacting protein-mediated cellular reactive oxygen species accumulation. J Biol Chem 279:10822–10828

Linkermann A, Bräsen JH, Himmerkus N, Liu S, Huber TB, Kunzendorf U, Krautwald S (2012) Rip1 (receptor-interacting protein kinase 1) mediates necroptosis and contributes to renal ischemia/reperfusion injury. Kidney Int 81:751–761

Liu J, van Mil A, Vrijsen K, Zhao J, Gao L, Metz CHG, Goumans M-J, Doevendans PA, Sluijter JPG (2011) MicroRNA-155 prevents necrotic cell death in human cardiomyocyte progenitor cells via targeting RIP1. J Cell Mol Med 15:1474–1482

Lowenthal JW, Ballard DW, Böhnlein E, Greene WC (1989) Tumor necrosis factor alpha induces proteins that bind specifically to kappa B-like enhancer elements and regulate interleukin 2 receptor alpha-chain gene expression in primary human T lymphocytes. Proc Natl Acad Sci U S A 86:2331–2335

Löder S, Fakler M, Schoeneberger H, Cristofanon S, Leibacher J, Vanlangenakker N, Bertrand MJM, Vandenabeele P, Jeremias I, Debatin K-M et al (2012) RIP1 is required for IAP inhibitor-mediated sensitization of childhood acute leukemia cells to chemotherapy-induced apoptosis. Leukemia 26:1020–1029

Ma Y-H, Huang C-P, Tsai J-S, Shen M-Y, Li Y-K, Lin L-Y (2011) Water-soluble germanium nanoparticles cause necrotic cell death and the damage can be attenuated by blocking the transduction of necrotic signaling pathway. Toxicol Lett 207:258–269

Mack C, Sickmann A, Lembo D, Brune W (2008) Inhibition of proinflammatory and innate immune signaling pathways by a cytomegalovirus RIP1-interacting protein. Proc Natl Acad Sci U S A 105:3094–3099

Madden DT, Egger L, Bredesen DE (2007) A calpain-like protease inhibits autophagic cell death. Autophagy 3:519–522

Matsumura H, Shimizu Y, Ohsawa Y, Kawahara A, Uchiyama Y, Nagata S (2000) Necrotic death pathway in Fas receptor signaling. J Cell Biol 151:1247–1256
Matthews N, Neale ML, Jackson SK, Stark JM (1987) Tumour cell killing by tumour necrosis factor: inhibition by anaerobic conditions, free-radical scavengers and inhibitors of arachidonate metabolism. Immunology 62:153–155
Micheau O, Tschopp J (2003) Induction of TNF receptor I-mediated apoptosis via two sequential signaling complexes. Cell 114:181–190
Micheau O, Lens S, Gaide O, Alevizopoulos K, Tschopp J (2001) NF-kappaB signals induce the expression of c-FLIP. Mol Cell Biol 21:5299–5305
Nakagawa T, Shimizu S, Watanabe T, Yamaguchi O, Otsu K, Yamagata H, Inohara H, Kubo T, Tsujimoto Y (2005) Cyclophilin D-dependent mitochondrial permeability transition regulates some necrotic but not apoptotic cell death. Nature 434:652–658
Nehs MA, Lin CI, Kozono DE, Whang EE, Cho NL, Zhu K, Moalem J, Moore FD Jr, Ruan DT (2011) Necroptosis is a novel mechanism of radiation-induced cell death in anaplastic thyroid and adrenocortical cancers. Surgery 150:1032–1039
Newton K, Harris AW, Bath ML, Smith KG, Strasser A (1998) A dominant interfering mutant of FADD/MORT1 enhances deletion of autoreactive thymocytes and inhibits proliferation of mature T lymphocytes. EMBO J 17:706–718
Northington FJ, Chavez-Valdez R, Graham EM, Razdan S, Gauda EB, Martin LJ (2011) Necrostatin decreases oxidative damage, inflammation, and injury after neonatal HI. J Cereb Blood Flow Metab 31:178–189
Oberst A, Dillon CP, Weinlich R, McCormick LL, Fitzgerald P, Pop C, Hakem R, Salvesen GS, Green DR (2011) Catalytic activity of the caspase-8-FLIP(L) complex inhibits RIPK3-dependent necrosis. Nature 471:363–367
Oerlemans MIFJ, Liu J, Arslan F, den Ouden K, van Middelaar BJ, Doevendans PA, Sluijter JPG (2012) Inhibition of RIP1-dependent necrosis prevents adverse cardiac remodeling after myocardial ischemia-reperfusion in vivo. Basic Res Cardiol 107:270
Osborn L, Kunkel S, Nabel GJ (1989) Tumor necrosis factor alpha and interleukin 1 stimulate the human immunodeficiency virus enhancer by activation of the nuclear factor kappa B. Proc Natl Acad Sci U S A 86:2336–2340
Osborn SL, Diehl G, Han S-J, Xue L, Kurd N, Hsieh K, Cado D, Robey EA, Winoto A (2010) Fas-associated death domain (FADD) is a negative regulator of T-cell receptor-mediated necroptosis. Proc Natl Acad Sci U S A 107:13034–13039
Ouyang Z, Zhu S, Jin J, Li J, Qiu Y, Huang M, Huang Z (2012) Necroptosis contributes to the cyclosporin A-induced cytotoxicity in NRK-52E cells. Pharmazie 67:725–732
Park S, Shin H, Cho Y (2012) Shikonin induces programmed necrosis-like cell death through the formation of receptor interacting protein 1 and 3 complex. Food Chem Toxicol 55C:36–41
Petersen SL, Wang L, Yalcin-Chin A, Li L, Peyton M, Minna J, Harran P, Wang X (2007) Autocrine TNFalpha signaling renders human cancer cells susceptible to Smac-mimetic-induced apoptosis. Cancer Cell 12:445–456
Pobezinskaya YL, Kim Y-S, Choksi S, Morgan MJ, Li T, Liu C, Liu Z (2008) The function of TRADD in signaling through tumor necrosis factor receptor 1 and TRIF-dependent Toll-like receptors. Nat Immunol 9:1047–1054
Rosenbaum DM, Degterev A, David J, Rosenbaum PS, Roth S, Grotta JC, Cuny GD, Yuan J, Savitz SI (2010) Necroptosis, a novel form of caspase-independent cell death, contributes to neuronal damage in a retinal ischemia-reperfusion injury model. J Neurosci Res 88:1569–1576
Rothe M, Pan MG, Henzel WJ, Ayres TM, Goeddel DV (1995) The TNFR2-TRAF signaling complex contains two novel proteins related to baculoviral inhibitor of apoptosis proteins. Cell 83:1243–1252
Sakon S, Xue X, Takekawa M, Sasazuki T, Okazaki T, Kojima Y, Piao J-H, Yagita H, Okumura K, Doi T et al (2003) NF-kappaB inhibits TNF-induced accumulation of ROS that mediate prolonged MAPK activation and necrotic cell death. EMBO J 22:3898–3909
Schinzel AC, Takeuchi O, Huang Z, Fisher JK, Zhou Z, Rubens J, Hetz C, Danial NN, Moskowitz MA, Korsmeyer SJ (2005) Cyclophilin D is a component of mitochondrial permeability transi-

tion and mediates neuronal cell death after focal cerebral ischemia. Proc Natl Acad Sci U S A 102:12005–12010
Schubert A, Grimm S (2004) Cyclophilin D, a component of the permeability transition-pore, is an apoptosis repressor. Cancer Res 64:85–93
Schulze-Osthoff K, Krammer PH, Dröge W (1994) Divergent signalling via APO-1/Fas and the TNF receptor, two homologous molecules involved in physiological cell death. EMBO J 13:4587–4596
Shu HB, Takeuchi M, Goeddel DV (1996) The tumor necrosis factor receptor 2 signal transducers TRAF2 and c-IAP1 are components of the tumor necrosis factor receptor 1 signaling complex. Proc Natl Acad Sci U S A 93:13973–13978
Simenc J, Lipnik-Stangelj M (2012) Staurosporine induces different cell death forms in cultured rat astrocytes. Radiol Oncol 46:312–320
Smith CCT, Davidson SM, Lim SY, Simpkin JC, Hothersall JS, Yellon DM (2007) Necrostatin: a potentially novel cardioprotective agent? Cardiovasc Drugs Ther 21:227–233
Song K-J, Jang YS, Lee YA, Kim KA, Lee SK, Shin MH (2011) Reactive oxygen species-dependent necroptosis in Jurkat T cells induced by pathogenic free-living Naegleria fowleri. Parasite Immunol 33:390–400
Stanger BZ, Leder P, Lee TH, Kim E, Seed B (1995) RIP: a novel protein containing a death domain that interacts with Fas/APO-1 (CD95) in yeast and causes cell death. Cell 81: 513–523
Sugarman BJ, Aggarwal BB, Hass PE, Figari IS, Palladino MA, Shepard HM (1985) Recombinant human tumor necrosis factor-alpha: effects on proliferation of normal and transformed cells in vitro. Science 230:943–945
Takahashi N, Duprez L, Grootjans S, Cauwels A, Nerinckx W, DuHadaway JB, Goossens V, Roelandt R, Van Hauwermeiren F, Libert C et al (2012) Necrostatin-1 analogues: critical issues on the specificity, activity and in vivo use in experimental disease models. Cell Death Dis 3:e437
Tamura Y, Chiba Y, Tanioka T, Shimizu N, Shinozaki S, Yamada M, Kaneki K, Mori S, Araki A, Ito H et al (2011) NO donor induces Nec-1-inhibitable, but RIP1-independent, necrotic cell death in pancreatic β-cells. FEBS Lett 585:3058–3064
Temkin V, Huang Q, Liu H, Osada H, Pope RM (2006) Inhibition of ADP/ATP exchange in receptor-interacting protein-mediated necrosis. Mol Cell Biol 26:2215–2225
Tenev T, Bianchi K, Darding M, Broemer M, Langlais C, Wallberg F, Zachariou A, Lopez J, Macfarlane M, Cain K et al (2011) The ripoptosome, a signaling platform that assembles in response to genotoxic stress and loss of IAPs. Mol Cell 43:432
Trichonas G, Murakami Y, Thanos A, Morizane Y, Kayama M, Debouck CM, Hisatomi T, Miller JW, Vavvas DG (2010) Receptor interacting protein kinases mediate retinal detachment-induced photoreceptor necrosis and compensate for inhibition of apoptosis. Proc Natl Acad Sci U S A 107:21695–21700
Tristão VR, Gonçalves PF, Dalboni MA, Batista MC, Durão Mde S, Monte JC (2012) Nec-1 protects against nonapoptotic cell death in cisplatin-induced kidney injury. Ren Fail 34:373–377
Upton JW, Kaiser WJ, Mocarski ES (2010) Virus inhibition of RIP3-dependent necrosis. Cell Host Microbe 7:302–313
Vanlangenakker N, Vanden Berghe T, Bogaert P, Laukens B, Zobel K, Deshayes K, Vucic D, Fulda S, Vandenabeele P, Bertrand MJM (2011) cIAP1 and TAK1 protect cells from TNF-induced necrosis by preventing RIP1/RIP3-dependent reactive oxygen species production. Cell Death Differ 18:656–665
Varfolomeev EE, Schuchmann M, Luria V, Chiannilkulchai N, Beckmann JS, Mett IL, Rebrikov D, Brodianski VM, Kemper OC, Kollet O et al (1998) Targeted disruption of the mouse Caspase 8 gene ablates cell death induction by the TNF receptors, Fas/Apo1, and DR3 and is lethal prenatally. Immunity 9:267–276
Ventura J-J, Cogswell P, Flavell RA, Baldwin AS, Davis RJ (2004) JNK potentiates TNF-stimulated necrosis by increasing the production of cytotoxic reactive oxygen species. Genes Dev 18: 2905–2915

Vercammen D, Beyaert R, Denecker G, Goossens V, Van Loo G, Declercq W, Grooten J, Fiers W, Vandenabeele P (1998a) Inhibition of caspases increases the sensitivity of L929 cells to necrosis mediated by tumor necrosis factor. J Exp Med 187:1477–1485

Vercammen D, Brouckaert G, Denecker G, Van de Craen M, Declercq W, Fiers W, Vandenabeele P (1998b) Dual signaling of the Fas receptor: initiation of both apoptotic and necrotic cell death pathways. J Exp Med 188:919–930

Vilcek J, Palombella VJ, Henriksen-DeStefano D, Swenson C, Feinman R, Hirai M, Tsujimoto M (1986) Fibroblast growth enhancing activity of tumor necrosis factor and its relationship to other polypeptide growth factors. J Exp Med 163:632–643

Vince JE, Pantaki D, Feltham R, Mace PD, Cordier SM, Schmukle AC, Davidson AJ, Callus BA, Wong WW-L, Gentle IE et al (2009) TRAF2 must bind to cellular inhibitors of apoptosis for tumor necrosis factor (tnf) to efficiently activate nf-{kappa}b and to prevent tnf-induced apoptosis. J Biol Chem 284:35906–35915

Wang CY, Mayo MW, Korneluk RG, Goeddel DV, Baldwin AS (1998) NF-kappaB antiapoptosis: induction of TRAF1 and TRAF2 and c-IAP1 and c-IAP2 to suppress caspase-8 activation. Science 281:1680–1683

Wang L, Du F, Wang X (2008) TNF-alpha induces two distinct caspase-8 activation pathways. Cell 133:693–703

Wang Y-Q, Wang L, Zhang M-Y, Wang T, Bao H-J, Liu W-L, Dai D-K, Zhang L, Chang P, Dong W-W et al (2012) Necrostatin-1 suppresses autophagy and apoptosis in mice traumatic brain injury model. Neurochem Res 37:1849–1858

Wong WW-L, Gentle IE, Nachbur U, Anderton H, Vaux DL, Silke J (2010) RIPK1 is not essential for TNFR1-induced activation of NF-kappaB. Cell Death Differ 17:482–487

Wu Y-T, Tan H-L, Huang Q, Sun X-J, Zhu X, Shen HM (2011) zVAD-induced necroptosis in L929 cells depends on autocrine production of TNFα mediated by the PKC-MAPKs-AP-1 pathway. Cell Death Differ 18:26–37

Xie T, Peng W, Liu Y, Yan C, Maki J, Degterev A, Yuan J, Shi Y (2013) Structural basis of RIP1 inhibition by necrostatins. Structure 21:493–499

Xu X, Chua CC, Kong J, Kostrzewa RM, Kumaraguru U, Hamdy RC, Chua BH (2007) Necrostatin-1 protects against glutamate-induced glutathione depletion and caspase-independent cell death in HT-22 cells. J Neurochem 103:2004–2014

Xu X, Chua K-W, Chua CC, Liu C-F, Hamdy RC, Chua BHL (2010) Synergistic protective effects of humanin and necrostatin-1 on hypoxia and ischemia/reperfusion injury. Brain Res 1355:189–194

Xu Y, Kim SO, Li Y, Han J (2006) Autophagy contributes to caspase-independent macrophage cell death. J Biol Chem 281:19179–19187

Yamanaka K, Saito Y, Yamamori T, Urano Y, Noguchi N (2011) 24(S)-Hydroxycholesterol induces neuronal cell death through necroptosis, a form of programmed necrosis. J Biol Chem 286:24666–24673

Yamauchi N, Kuriyama H, Watanabe N, Neda H, Maeda M, Niitsu Y (1989) Intracellular hydroxyl radical production induced by recombinant human tumor necrosis factor and its implication in the killing of tumor cells in vitro. Cancer Res 49:1671–1675

Yeh WC, Pompa JL, McCurrach ME, Shu HB, Elia AJ, Shahinian A, Ng M, Wakeham A, Khoo W, Mitchell K et al (1998) FADD: essential for embryo development and signaling from some, but not all, inducers of apoptosis. Science 279:1954–1958

You Z, Savitz SI, Yang J, Degterev A, Yuan J, Cuny GD, Moskowitz MA, Whalen MJ (2008) Necrostatin-1 reduces histopathology and improves functional outcome after controlled cortical impact in mice. J Cereb Blood Flow Metab 28:1564–1573

Yu L, Alva A, Su H, Dutt P, Freundt E, Welsh S, Baehrecke EH, Lenardo MJ (2004) Regulation of an ATG7-beclin 1 program of autophagic cell death by caspase-8. Science 304:1500–1502

Zhang D-W, Shao J, Lin J, Zhang N, Lu B-J, Lin S-C, Dong M-Q, Han J (2009a) RIP3, an energy metabolism regulator that switches TNF-induced cell death from apoptosis to necrosis. Science 325:332–336

Zhang H, Zhou X, McQuade T, Li J, Chan FK-M, Zhang J (2011) Functional complementation between FADD and RIP1 in embryos and lymphocytes. Nature 471:373–376

Zhang J, Cado D, Chen A, Kabra NH, Winoto A (1998) Fas-mediated apoptosis and activation-induced T-cell proliferation are defective in mice lacking FADD/Mort1. Nature 392:296–300

Zhang L, Blackwell K, Thomas GS, Sun S, Yeh WC, Habelhah H (2009b) TRAF2 suppresses basal IKK activity in resting cells and TNFalpha can activate IKK in TRAF2 and TRAF5 double knockout cells. J Mol Biol 389:495–510

Zhang QL, Niu Q, Niu PY, Ji XL, Zhang C, Wang L (2010) Novel interventions targeting on apoptosis and necrosis induced by aluminum chloride in neuroblastoma cells. J Biol Regul Homeost Agents 24:137–148

Zhu S, Zhang Y, Bai G, Li H (2011) Necrostatin-1 ameliorates symptoms in R6/2 transgenic mouse model of Huntington's disease. Cell Death Dis 2:e115

Zimmerman RJ, Chan A, Leadon SA (1989) Oxidative damage in murine tumor cells treated in vitro by recombinant human tumor necrosis factor. Cancer Res 49:1644–1648

Chapter 17
Small-Molecule Inhibitors of Necroptosis

Colleen R. McNamara and Alexei Degterev

17.1 Introduction

Necroptosis is a form of programmed cell death that is characterized by cellular swelling, breakage of the plasma membrane, and release of intracellular contents into the extracellular space, i.e., major hallmarks typically associated with unregulated, pathologic necrosis (Vandenabeele et al. 2010). Although hints of an existence of regulated form of necrosis have been around for the last quarter of a century (Laster et al. 1988), necroptosis has begun to be more thoroughly understood just in the last decade. A number of findings promoted the understanding of necroptotic cell death. First, several cellular models of TNF family-induced necrosis have been developed (Fiers et al. 1995; Holler et al. 2000; Kawahara et al. 1998), laying groundwork for further analysis. Subsequently, Holler et al. reported a specific role for receptor-interacting-protein 1 (RIP1) kinase activity in this process (Holler et al. 2000). In 2005 we identified a first selective small-molecule inhibitor of necroptosis, called necrostatin-1 (Nec-1) (Degterev et al. 2005). It was later found that Nec-1 inhibits RIP1 kinase (Degterev et al. 2008). In 2009 three laboratories independently discovered RIP3 as another kinase that plays an important role in necroptosis (Cho et al. 2009; He et al. 2009; Zhang et al. 2009), followed by identification of mixed-lineage kinase domain-like (MLKL) and PGAM5 proteins as key downstream mediators of RIP3 (Sun et al. 2012; Wang et al. 2012). All of these findings

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C.R. McNamara
Graduate Program in Biochemistry, Sackler School of Graduate Biomedical Sciences, Tufts University, Boston, MA 02111, USA

A. Degterev (✉)
Department of Biochemistry, Sackler School of Graduate Biomedical Sciences, Tufts University, Boston, MA 02111, USA
e-mail: alexei.degterev@tufts.edu

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set off a chain of discoveries that promoted a better understanding of the signaling pathways involved in necroptosis and its pathophysiological significance.

Necroptosis can be viewed as a process involving initiation, execution, and pathophysiologic response to necrotic cell death, and each of these phases can be potentially modulated in order to treat disease. While there are many known initiators of necroptosis, TNFα activation of the TNF receptor 1 (TNFR1) is one of the best defined mechanisms. Depending on the cellular context, TNFR1 activation can lead to three possible cellular outcomes including nuclear factor kappa-B (NF-κB) activation and cell survival, apoptosis, or necroptosis. During pro-death signaling, TNFR1 activation by TNFα leads to the formation of an intracellular membrane-associated complex called TNFR1 complex I (Micheau and Tschopp 2003). This complex contains TNFR-associated death domain (TRADD) protein, TNFR-associated factor 2 (TRAF2), TRAF5, cellular inhibitor of apoptosis proteins 1 and 2 (cIAP1/2), and RIP1. Endocytosis of the TNFR1 leads to the rearrangement of complex I and formation of complex II or death-inducing signaling complex (DISC). The makeup of complex II determines whether the cell will die from apoptosis or necroptosis. Apoptotic complex II contains RIP1, TRADD, FAS-associated protein with death domain (FADD), and caspase-8. Activated caspase-8 in complex II leads to the execution of caspase-dependent apoptosis and the cleavage and inactivation of both RIP1 and RIP3 as well as other substrates, such as CYLD, a de-ubiquitinase critical for necroptosis (Hitomi et al. 2008; O'Donnell et al. 2011). Caspase-independent necroptosis and formation of the RIP1- and RIP3-containing necrosome (also known as complex IIb) occur when caspase-8 is inhibited in either a genetic or a pharmacologic fashion. Both the formation and the activity of necrosome are key possible targets for modulation of necroptosis.

In addition to the initiation of necroptosis that we briefly described above, there are several known mechanisms that contribute to the execution of TNFR1-activated necroptosis (reviewed in Christofferson and Yuan 2010; Vandenabeele et al. 2010 and in other chapters of this book). Unlike during apoptosis, bioenergetic processes such the consumption of ATP through translation (Saelens et al. 2005), proteasome-dependent protein degradation (Sun et al. 2004), and poly (ADP-ribose) polymerase 1 (PARP1) (Soldani and Scovassi 2002) continue to occur during necroptosis. Activation of glycolysis and PARP1 have been linked to some instances of necroptosis (Jouan-Lanhouet et al. 2012; Xu et al. 2010; Zhang et al. 2009). Reactive oxygen species (ROS) generation both at the mitochondria and at the plasma membrane by NADPH oxidase (Nox1) has also been shown to be important (Kim et al. 2007; Schulze-Osthoff et al. 1992). It has been shown that ROS can lead to lipid peroxidation and lysosome membrane permeabilization (LMP) (Benedetti et al. 1980; Vandenabeele et al. 2010). Many of these downstream signals could also be modulated in order to inhibit necroptosis.

Massive lysis of necrotic cells can lead to hyperacute inflammation and multiple organ failure, which are hallmarks of many acute necrotizing pathologies. Multiple approaches are under development to control these processes and are not discussed in this chapter.

As our understanding of the signal transduction pathways involved in necroptosis has increased, the role that necroptosis plays in disease has also begun to be clarified. Necroptosis may be a significant contributor to many disease paradigms (reviewed in Galluzzi et al. 2011) and has been shown to occur in a number of experimental models: ischemia–reperfusion injury, cerulein-induced acute pancreatitis, viral infection, atherosclerosis, traumatic brain injury, retinal detachment-induced photoreceptor necrosis, and skin and intestinal inflammation and lethal systemic inflammatory response syndrome (SIRS) (Bonnet et al. 2011; Degterev et al. 2005; Duprez et al. 2011; Gunther et al. 2011; He et al. 2009; Lin et al. 2013; Linkermann et al. 2012; Oerlemans et al. 2012; Rosenbaum et al. 2010; Trichonas et al. 2010; Upton et al. 2012; Welz et al. 2011; You et al. 2008). In order to fully take advantage of the treatment opportunities associated with necroptosis, both a thorough understanding of the signaling pathways involved and the ability to generate small-molecule inhibitors of necroptosis are critical. In this review we discuss inhibitors of necroptosis that are currently being developed, including molecules targeting RIP1 kinase, MLKL, and ROS, as well as additional possible targets for manipulation.

17.2 RIP1 Kinase and Necrostatins

The formation of the necrosome, containing RIP1 and RIP3, which occurs when activity of caspase-8 is lacking, leads to activation of necroptosis. Even though RIP1 and RIP3 interact with each other through their RHIM domains (He et al. 2009; Li et al. 2012), trans-phosphorylation mediated by their kinase domains is critical for complex formation and necroptosis (Cho et al. 2009). It should be noted that it is currently unknown whether other targets of RIP1 kinase exist beyond RIP3. In 2005, we reported a cell-based screen of necroptotic U937 monocytes treated with TNFα and zVAD.fmk and identified the first small molecule, Nec-1 (Fig. 17.1a), that could selectively and efficiently inhibit necroptosis (Degterev et al. 2005). Furthermore, Nec-1 significantly reduced brain ischemia–reperfusion injury. Subsequently, Nec-1 has been shown to be effective in a variety of additional ischemia–reperfusion injuries (brain, heart, kidney, retina), intestinal necrosis, traumatic brain injury, bacterial infections, SIRS, retinal detachment, Huntington's disease, and a number of other pathologic conditions (Degterev et al. 2005; Duprez et al. 2011; Gunther et al. 2011; Linkermann et al. 2012; Oerlemans et al. 2012; Roca and Ramakrishnan 2013; Rosenbaum et al. 2010; Trichonas et al. 2010; You et al. 2008; Zhu et al. 2011). This gives additional fuel to the concept that inhibition of necroptosis could display a broad therapeutic benefit.

It was discovered that Nec-1 is a selective sub-micromolar inhibitor of RIP1 kinase (Degterev et al. 2008). A structure–activity relationship (SAR) analysis was performed surrounding the original Nec-1, and a more potent and metabolically stable version called 7-Cl-O-Nec-1 (Fig. 17.1b) was found, with R-isomer representing an active moiety (Degterev et al. 2008; Teng et al. 2005). Recently, the

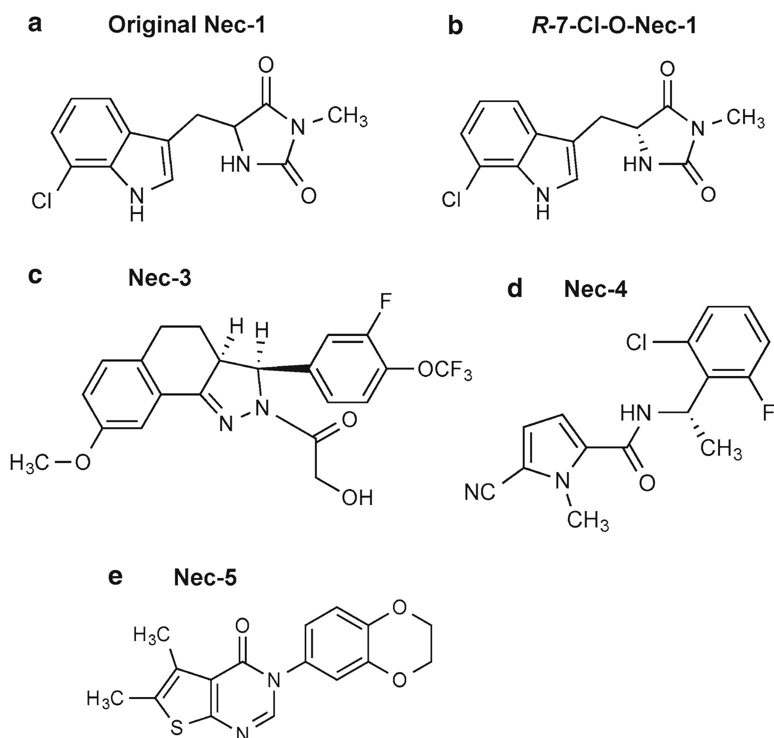

Fig. 17.1 Chemical structures of necrostatins. (**a**) Original Nec-1 identified in a cell-based screen, (**b**) optimized Nec-1 analog 7-Cl-O-Nec-1, (**c**–**e**) optimized analogs of Nec-3, Nec-4, and Nec-5

exclusive selectivity of *R*-7-Cl-O-Nec-1 towards RIP1 kinase was further validated in a screen against a panel of more than 400 human kinases (Christofferson et al. 2012). It should be noted that the original Nec-1 (Fig. 17.1a) also inhibits indoleamine-2,3-dioxygenase (IDO) (Muller et al. 2005) and this activity is eliminated in the optimized 7-Cl-O-Nec-1 (Fig. 17.1b) (Degterev et al. 2013; Takahashi et al. 2012). Therefore, caution should be used when interpreting data that has used the original Nec-1 compound due to possible off-target effects. In addition, original Nec-1 has been shown to display very limited metabolic stability and some toxicity in vivo, which are also improved or eliminated, respectively, with 7-Cl-O-Nec-1 (Degterev et al. 2013; Takahashi et al. 2012), which makes this analog a much preferred choice for in vivo experiments.

In addition to Nec-1, three more sub-micromolar necrostatins, Nec-3 (Jagtap et al. 2007) (Fig. 17.1c), Nec-4 (Teng et al. 2007) (Fig. 17.1d), and Nec-5 (Wang et al. 2007) (Fig. 17.1e), were identified in the original U-937 cell screen and optimized. Remarkably, all of these molecules also target RIP1 kinase either directly (Nec-3 and Nec-4) or indirectly (Nec-5) (Degterev et al. 2008). Discovery that these structurally dissimilar inhibitors, identified in an unbiased cell-based screen, all target RIP1 kinase further highlighted the significance of this protein as a critical necroptotic target amenable to pharmacological inhibition.

Recently, the crystal structure of the RIP1 kinase domain bound to three different necrostatins (Nec-1, Nec-3, Nec-4) was solved (Xie et al. 2013). Again remarkably, given the lack of structural similarity between necrostatins, all three molecules stabilize the inactive conformation of the kinase by binding to the hydrophobic DLG-out pocket, located between the N- and the C-lobes of the kinase, rather than the ATP-binding pocket. The binding of all three molecules is driven by van der Waals interactions with the same hydrophobic residues located in the activation loop and surrounding segments and is further stabilized by hydrogen bonds, of which the one formed by the amide nitrogen of Asp156 is shared by all three molecules. The generation of a necrostatin-bound crystal structure for RIP1 gave a structural basis for the inhibition of RIP1 by necrostatins and will provide a wealth of information to allow further drug discovery efforts. It should be noted that while the identified DLG-out binding pocket is also highly conserved in RIP3, this kinase is not inhibited by any of the necrostatins. Therefore, additional determinants of necrostatins' selectivity are yet to be defined.

Inhibition of the RIP1 partner in the necrosome, RIP3, could also represent an attractive means for the inhibition of necroptosis. For example, RIP1-independent RIP3-dependent necroptosis can occur following murine cytomegalovirus (MCMV) infection, suggesting that the critical role of RIP1 in RIP3 activation may be bypassed under some circumstances by other molecules, i.e., virus-sensing adaptor DAI in the case of MCMV (Upton et al. 2012). Therefore, inhibition of RIP3 may in some cases provide additional benefits. However, it should be noted that despite the important role of RIP3 in necroptosis and its homology to RIP1, neither our cell-based screen nor a screen reported by Sun et al. (Sun et al. 2012) resulted in the selection of direct RIP3 kinase inhibitors. An RIP3 kinase inhibitor, developed in an in vitro screen by GlaxoSmithKline, has been reported at a recent conference (Hildebrand et al. 2013); however, further discussion will have to await full publication of its properties.

17.3 MLKL and Necrosulfonamide

A new protein, the pseudo-kinase MLKL, has recently been discovered to be part of the necrosome complex and to be involved in necroptosis. Xiaodong Wang's group screened a chemical library and found that one hit, number 14 (Fig. 17.2a), inhibited necroptosis in both HT-29 adenocarcinoma and FADD null Jurkat lymphocyte cells (Sun et al. 2012). They went on to develop a more efficacious derivative of this compound, which was named necrosulfonamide (NSA) (Fig. 17.2b). The authors observed that NSA did not interfere with but rather enhanced the interaction between RIP1 and RIP3, unlike Nec-1, suggesting that it may target a more downstream step in a pathway. Using analysis of RIP3-binding factors in NSA-treated cells, MLKL was found to be a new RIP3 partner. MLKL was subsequently established to be a direct target of NSA using biotin–NSA pulldown.

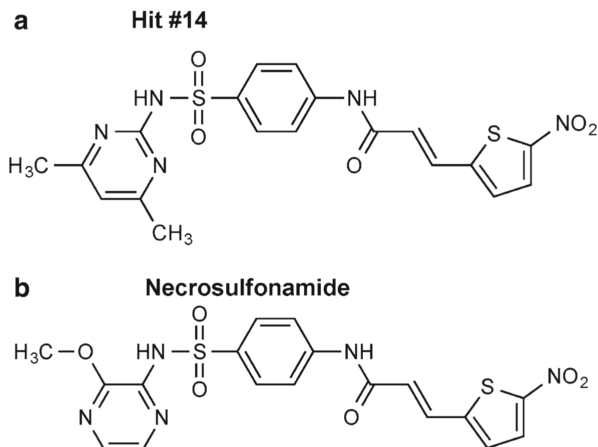

Fig. 17.2 Chemical structures of MLKL inhibitors (Hit#14 and necrosulfonamide)

The role of MLKL in necroptosis was confirmed by Sun et al. using siRNA against MLKL in several cell types. Independently, Zhao et al. used an shRNA library to also identify MLKL as a critical molecule in necroptosis (Zhao et al. 2012). The RIP3/MLKL interaction requires phosphorylation of RIP3 on Ser227 and results in the phosphorylation of MLKL on Thr357 and Thr358 by RIP3 (Sun et al. 2012). The RIP3/MLKL interaction is required for necroptosis in both human and mouse cell types. Interestingly, the RIP3/MLKL interaction is species specific (i.e., hRIP3 cannot bind mMLKL and vice versa) (Chen et al. 2013). Importantly, NSA is only able to bind and inhibit hMLKL and is unable to inhibit mMLKL. This is due to NSA forming a covalent adduct with the Cys86 residue of hMLKL through Michael addition. This residue is missing in mMLKL.

Sun et al. (2012) completed SAR studies of NSA and found that NSA activity required an α,β-unsaturated enone moiety in order to be active. Further SAR studies are needed in order to fully elucidate the most efficacious MLKL inhibitor compound. Importantly, a version of NSA that is able to inhibit mMLKL will be very useful for in vivo studies. Although inhibition of MLKL in the prevention of necroptosis has a lot of potential, it remains to be determined how inhibitors of a step downstream of RIP1 (or RIP3) kinase activity will perform in animal models of necroptosis. Furthermore, while inhibitors of MLKL could potentially be useful in death that occurs in an RIP1-independent fashion, it also remains to be formally tested whether MLKL is involved in death under these circumstances.

17.4 ROS and Butylated Hydroxyanisole

ROS were first shown to be an important part of regulated necrotic cell death in 1992 when it was reported that ROS generation is a requirement for TNFα-induced killing of L929 cells (Schulze-Osthoff et al. 1992). Multiple consequences of

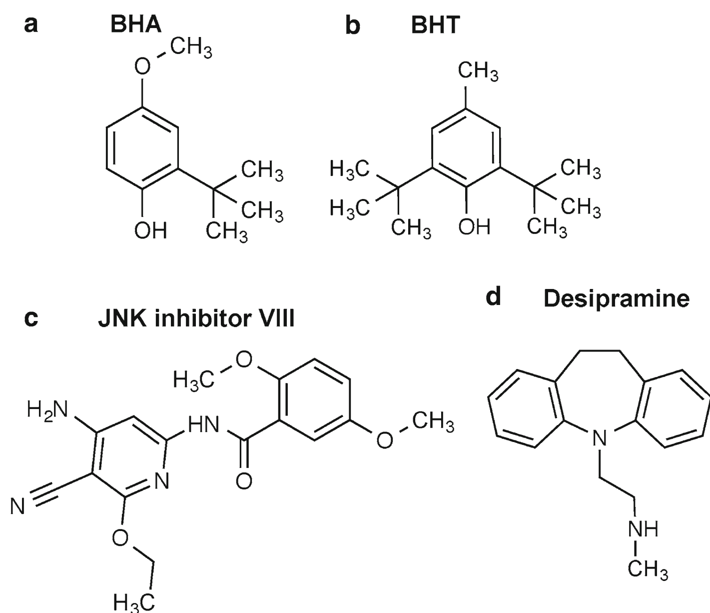

Fig. 17.3 Chemical structures of necroptosis-related inhibitors. (**a** and **b**) ROS (BHA, BHT), (**c**) JNK kinase (JNK inhibitor VIII), (**d**) ASM (desipramine)

increased ROS levels have been described in the literature, including lipid peroxidation and LMP (Benedetti et al. 1980), which are likely to contribute to cellular demise. Mitochondrial electron transport chain complexes I and II were identified as the principal mediators of ROS in necroptotic L929 cells, and inhibition of these complexes using amytal or thenoyltrifluoroacetone, respectively, efficiently attenuated L929 cell death. Furthermore, a pathway involving RIP1 kinase-dependent phosphorylation on Ser727 of Stat3 followed by its translocation to mitochondria through binding to the complex I component GRIM-19 has been linked to ROS production (Shulga and Pastorino 2012). Recent evidence also suggested that RIP3 kinase activity links TNFR signaling to mitochondrial bioenergetics and ROS production. RIP3 physically interacts with several metabolic enzymes including glycogen phosphorylase (PYGL), glutamate-ammonia ligase (GLUL), and glutamate dehydrogenase 1 (GLUD1) leading to their activation (Zhang et al. 2009). Knockdown of these enzymes protects cells from necroptosis and inhibits ROS production. In addition to generation of ROS at the mitochondria, ROS generation at the plasma membrane by NADPH oxidase (Nox1) has also been found to be important (Kim et al. 2007). Multiple NADPH oxidase inhibitors have been developed (see Cifuentes-Pagano et al. 2012 for a recent review) but are yet to be tested in necroptosis.

Several groups have shown that the radical scavenging antioxidant butylated hydroxyanisole (BHA) (Fig. 17.3a) is an efficient inhibitor of necroptotic death in multiple cellular models including L929 and NIH3T3 cells (Chen et al. 2009;

Goossens et al. 1995; Luschen et al. 2000; Thon et al. 2005; Vercammen et al. 1998). Furthermore, BHA as well as several additional antioxidants and mitochondrial ETC/NADPH oxidase inhibitors attenuated the hyperacute toxicity of TNFα and zVAD.fmk in vivo (Cauwels et al. 2003). The role of ROS as a downstream mediator of RIP1-dependent necroptosis has been reiterated in a recent study (Shindo et al. 2013). The authors reported that while Nec-1 inhibits RIP1 phosphorylation, BHA does not, even though it is able to protect cells from death. Importantly, the authors performed SAR analysis of BHA and found that 4-hydroxy-anisole, where the *tert*-butyl group is removed, is not able to prevent accumulation of ROS or cell death. It also revealed that a hydroxy group at the R_3 position is required for the anti-necroptotic and antioxidant activities of these small molecules.

Given that ROS have been shown to contribute to many disease states in which necroptosis has also been implicated including neurodegenerative diseases (Hernandes and Britto 2012), ischemia–reperfusion injuries (Madamanchi and Runge 2013), and gastrointestinal tract diseases such as colitis (Kim et al. 2012), it will be interesting to investigate whether the use of antioxidants such as BHA or related compounds can protect from necroptosis in animal models of these diseases. At the same time, questions remain regarding the role of ROS and the exact mechanism of BHA action. For example, while Shindo et al. also observed inhibition of necroptosis by butylated hydroxytoluene (BHT) (Fig. 17.3b), an antioxidant highly similar to BHA, several groups have not observed this, suggesting that additional factors rather than general antioxidant properties may contribute to protection by BHA (Luschen et al. 2000; Thon et al. 2005). Furthermore, neither the ROS increase nor the protection by BHA was observed in necroptotic Jurkat cells, suggesting that the role of ROS could be cell type specific (Degterev et al. 2005; Thon et al. 2005).

17.5 Akt, JNK, and c-Jun

Recent studies by Han-Ming Shen and our groups have demonstrated an unexpected importance of Akt kinase in necroptosis in L929 cells and raised interesting new possibilities about the known role of c-Jun/JNK (McNamara et al. 2013; Wu et al. 2009). Akt, or protein kinase B, is a serine/threonine kinase that can be activated by the PI3K signaling pathway in response to growth factor stimulation of various receptor tyrosine kinases (Stephens et al. 1993). Akt is an important signaling hub that plays a role in the regulation of many cellular processes including protein synthesis, cell metabolism, cell proliferation, and cell death and survival (Chan et al. 1999; Manning and Cantley 2007). Wu et al. proposed that this pathway contributes to necroptosis through suppression of pro-survival autophagy (Wu et al. 2009). Our work showed that Akt is activated through selective RIP1 kinase-dependent phosphorylation of residue Thr308, which leads to the induction of multiple components of Akt signaling network and contributes to cell death and TNFα production through JNK/c-Jun activation. This is particularly intriguing because while Akt is typically considered a pro-survival protein, these and other recent data (Andrabi et al. 2007;

Jin et al. 2007) begin to show that Akt can act in a pro-death fashion under various circumstances. It is important to note that in several cell types other than L929, Akt mediated production of TNFα rather than cell death itself. It will be interesting to determine the activation of Akt in animal models of necroptosis and whether inhibitors of Akt reduce injury in these models. Discovery and development of Akt inhibitors is currently a very active area of research (McNamara and Degterev 2011; Welker and Kulik 2013).

Many studies have implicated JNK activation in necroptosis (Chen et al. 2011; Christofferson et al. 2012; Hitomi et al. 2008; Kim et al. 2007; Wu et al. 2011; Yu et al. 2004). Our data using small-molecule inhibitor of JNK, JNK inhibitor VIII (Fig. 17.3c), and JNK knockdown demonstrated that while JNK is indeed activated during necroptosis, it is either functionally redundant or dispensable in the production of TNFα and necroptosis in L929 cells (McNamara et al. 2013). On the other hand, it has also been shown that c-Jun acts to mediate TNFα production during necroptosis (Christofferson et al. 2012; Wu et al. 2011; Yu et al. 2004). Using c-Jun knockdown, we confirmed that c-Jun is critical for both TNFα production and death by necroptosis in L929 cells. These data are important because they indicate that c-Jun rather than JNK could be a critical molecule for inhibition and therapeutic protection from necroptosis-related injuries. Small-molecule inhibition of JNK is currently an active area of research (Bogoyevitch et al. 2010). However, to our knowledge, direct small-molecule inhibitors of c-Jun or AP-1 (Fanjul et al. 1994) are much less of a focus but may represent an important direction for future work. It is also important to note that the relative roles of JNK and c-Jun remain to be examined in other cellular and animal models of necroptosis.

Curiously, in another recent work transcription factor IRF3 has been found to act in a transcription-independent fashion to mediate *Listeria monocytogenes*-induced necrosis through a pathway, which appears to be separate from necroptosis (Di Paolo et al. 2013). These data raise a more general possibility that previously unappreciated connections may exist between transcriptional machinery and various mechanisms of necrosis.

17.6 Other Potential Targets

There are multiple additional components of the necroptosis signaling pathway that could potentially be targeted for therapeutic development, although in most cases exact details of molecular regulation remain to be fully elucidated.

Overactivation of PARP1, an enzyme that mediates the poly-ADP ribosylation of many cellular factors, has been previously linked to necrotic cell death through the acute depletion of cellular ATP and by promoting nuclear translocation of apoptosis-inducing factor (AIF) (Andrabi et al. 2008). To our knowledge, there are two specific instances where activation of PARP1 downstream from RIP1 kinase was found to contribute to necroptosis (Jouan-Lanhouet et al. 2012; Xu et al. 2010). However,

more generally, RIP1 and PARP1 appear to control distinct pathways of cell death (Degterev et al. 2005; Sosna et al. 2013).

Phorbol 12-myristate 13-acetate (PMA), a phorbol ester, activates protein kinase C (PKC). It was recently found that PMA-induced activation of PKC protected cells from TNFα-induced necroptosis by disrupting formation of the TNFR signaling complex and inhibiting the recruitment of RIP1 and TRADD (Byun et al. 2006). On the other hand, PMA was found to promote autocrine production of TNFα and necroptosis in L929 cells (Wu et al. 2011), suggesting a context- and/or cell type-specific roles for PKC. The availability of a large number of PKC inhibitors, targeting different family members, will undoubtedly facilitate investigation of the role of this kinase family in necroptosis.

Lysosomal membrane permeabilization is an important step in both oxidative stress-induced necrosis and necroptosis triggered by TNFα (Vanden Berghe et al. 2010). Dendritic cells undergo low levels of necroptosis in response to treatment with polyinosinic-polycytidylic acid (poly(I:C)), a surrogate of virus-derived dsRNA. In these cells RIG-I-like receptors (RLRs) are activated by poly(I:C) leading to destabilization of lysosomes and release of cathepsin D, a lysosomal hydrolase, into the cytosol where it promotes cleavage and inhibition of caspase-8, activates NFκB, and induces production of cytokines (Zou et al. 2013). Knockdown of cathepsin D or treatment with the aspartyl protease inhibitor pepstatin A protects cells from necroptosis. It will be important to analyze the role of lysosomal cathepsins in animal models of necroptosis.

Cytosolic phospholipase A2 (cPLA2) and cyclooxygenases, critical mediators of arachidonic acid release and eicosanoid synthesis, have also been shown to contribute to necroptosis both in L929 cells and in mice injected with TNFα and zVAD.fmk (Cauwels et al. 2003; Hayakawa et al. 1993). Because oxidative reactions are involved, the role of cPLA2 was proposed to be connected to ROS overproduction, although it remains to be fully tested. These data may also point to an intriguing new connection between necrotic death and hyperacute inflammation in vivo.

Finally, accumulation of ceramide, a sphingolipid, has been implicated in necroptosis. Acid sphingomyelinase (ASM) is the member of the sphingomyelinase family responsible for converting sphingomyelin into ceramide. It is best known for its role in the lysosomal storage disorder Niemann–Pick disease. However, more recently the role of this enzyme and ceramide in multiple forms of cell death began to emerge (Smith and Schuchman 2008). Necroptosis in Jurkat, L929, and fibroblast cells was found to be associated with RIP1 kinase-dependent accumulation of ceramide (Thon et al. 2005). Furthermore, genetic deletion, knockdown, or pharmacologic inhibition of ASM using small-molecule inhibitors D609 (inhibitor of an upstream step of lysosomal ASM activation) and desipramine (Fig. 17.3d) protected cells from death. ASM was also found to act together with cyclophilin D (CypD) in mediating TNFα-dependent necroptosis in macrophages and zebra fish infected with mycobacteria (Roca and Ramakrishnan 2013). Dual administration of the CypD inhibitor alisporivir and desipramine promoted bacterial clearance by blocking macrophage cell death while preserving macrophage-mediated innate immune responses. It remains to be determined whether similar inhibition of necroptosis in higher

species may promote or hamper clearance of pathogens in vivo. It is also worth noting that ceramide has been shown to directly bind and activate cathepsin D as well as to serve as a precursor for sphingosine-1-phosphate, a prominent activator of pro-inflammatory gene expression (Heinrich et al. 1999; Pyne and Pyne 2000).

17.7 Conclusion

Activation of necroptotic cell death has now been demonstrated in multiple animal models, and its inhibition has been shown to protect from multiple forms of injury. In order to take full advantage of the treatment opportunity associated with necroptosis, a thorough understanding of the signaling pathways involved is required. Multiple mediators of necroptosis have been described, although the exact wiring of necroptosis execution, especially in vivo, remains to be uncovered. The increased availability of small-molecule inhibitors of key steps in the pathway is also critical. Multiple inhibitors of both upstream initiators of necroptosis, such as RIP1 and MLKL, as well as more downstream execution steps have been identified. These molecules represent excellent tools for furthering the understanding of the mechanisms of necroptosis. Targeting upstream necrosome components, RIP1, RIP3, and MLKL, currently seems to offer the best direction for therapeutic drug development. RIP1 kinase inhibitors, necrostatins, represent a well-characterized method to inhibit necroptosis. The use of Nec-1 in animal models has given not only a clearer understanding of the contribution that necroptosis plays in multiple pathologies but also a sound motive for the continued study of small-molecule inhibitors of RIP1 and for the development of inhibitors of other important components in the execution of necroptosis. For example, the first inhibitor of MLKL has been reported, and, although its use is currently limited to human cells, it is a promising start for finding additional tools targeting necrosome. As inhibitors of necrosome components continue to evolve, it will be important to understand whether particular disease conditions would benefit more from targeting particular complex components, i.e., RIP1 vs. RIP3 vs. MLKL. Additionally, given the homology of the RIP1 and RIP3 DLG-out pockets, the development of dual-RIP1/RIP3 inhibitors may represent an intriguing opportunity. It is also worth noting that necroptosis mediators have been directly linked to not only cell death but also pro-inflammatory signaling (Christofferson et al. 2012; Kang et al. 2013; Lukens et al. 2013; McNamara et al. 2013; Vince et al. 2012; Wallach et al. 2011). Further study of the specific molecular mechanisms involved in both processes offers an opportunity to fine-tune future therapies. Other targets, described in this chapter, such as ROS, Akt, JNK, and cathepsin D, already have relatively well-developed inhibitors in the literature, and additional work needs to be done to either demonstrate or further study their effectiveness in inhibiting necroptosis in vivo. Overall, the outlook for the development of small-molecule inhibitors of necroptosis is extremely promising.

References

Andrabi S, Gjoerup OV, Kean JA, Roberts TM, Schaffhausen B (2007) Protein phosphatase 2A regulates life and death decisions via Akt in a context-dependent manner. Proc Natl Acad Sci U S A 104:19011–19016

Andrabi SA, Dawson TM, Dawson VL (2008) Mitochondrial and nuclear cross talk in cell death: parthanatos. Ann N Y Acad Sci 1147:233–241

Benedetti A, Comporti M, Esterbauer H (1980) Identification of 4-hydroxynonenal as a cytotoxic product originating from the peroxidation of liver microsomal lipids. Biochim Biophys Acta 620:281–296

Bogoyevitch MA, Ngoei KR, Zhao TT, Yeap YY, Ng DC (2010) c-Jun N-terminal kinase (JNK) signaling: recent advances and challenges. Biochim Biophys Acta 1804:463–475

Bonnet MC, Preukschat D, Welz PS, van Loo G, Ermolaeva MA, Bloch W, Haase I, Pasparakis M (2011) The adaptor protein FADD protects epidermal keratinocytes from necroptosis in vivo and prevents skin inflammation. Immunity 35:572–582

Byun HS, Park KA, Won M, Yang KJ, Shin S, Piao L, Kwak JY, Lee ZW, Park J, Seok JH et al (2006) Phorbol 12-myristate 13-acetate protects against tumor necrosis factor (TNF)-induced necrotic cell death by modulating the recruitment of TNF receptor 1-associated death domain and receptor-interacting protein into the TNF receptor 1 signaling complex: Implication for the regulatory role of protein kinase C. Mol Pharmacol 70:1099–1108

Cauwels A, Janssen B, Waeytens A, Cuvelier C, Brouckaert P (2003) Caspase inhibition causes hyperacute tumor necrosis factor-induced shock via oxidative stress and phospholipase A2. Nat Immunol 4:387–393

Chan TO, Rittenhouse SE, Tsichlis PN (1999) AKT/PKB and other D3 phosphoinositide-regulated kinases: kinase activation by phosphoinositide-dependent phosphorylation. Annu Rev Biochem 68:965–1014

Chen TY, Chi KH, Wang JS, Chien CL, Lin WW (2009) Reactive oxygen species are involved in FasL-induced caspase-independent cell death and inflammatory responses. Free Radic Biol Med 46:643–655

Chen SY, Chiu LY, Maa MC, Wang JS, Chien CL, Lin WW (2011) zVAD-induced autophagic cell death requires c-Src-dependent ERK and JNK activation and reactive oxygen species generation. Autophagy 7:217–228

Chen W, Zhou Z, Li L, Zhong CQ, Zheng X, Wu X, Zhang Y, Ma H, Huang D, Li W et al (2013) Diverse sequence determinants control human and mouse receptor interacting protein 3 (RIP3) and mixed lineage kinase domain-like (MLKL) interaction in necroptotic signaling. J Biol Chem 288:16247–16261

Cho YS, Challa S, Moquin D, Genga R, Ray TD, Guildford M, Chan FK (2009) Phosphorylation-driven assembly of the RIP1-RIP3 complex regulates programmed necrosis and virus-induced inflammation. Cell 137:1112–1123

Christofferson DE, Yuan J (2010) Necroptosis as an alternative form of programmed cell death. Curr Opin Cell Biol 22:263–268

Christofferson DE, Li Y, Hitomi J, Zhou W, Upperman C, Zhu H, Gerber SA, Gygi S, Yuan J (2012) A novel role for RIP1 kinase in mediating TNFalpha production. Cell Death Dis 3:e320

Cifuentes-Pagano E, Csanyi G, Pagano PJ (2012) NADPH oxidase inhibitors: a decade of discovery from Nox2ds to HTS. Cell Mol Life Sci 69:2315–2325

Degterev A, Huang Z, Boyce M, Li Y, Jagtap P, Mizushima N, Cuny GD, Mitchison TJ, Moskowitz MA, Yuan J (2005) Chemical inhibitor of nonapoptotic cell death with therapeutic potential for ischemic brain injury. Nat Chem Biol 1:112–119

Degterev A, Hitomi J, Germscheid M, Ch'en IL, Korkina O, Teng X, Abbott D, Cuny GD, Yuan C, Wagner G et al (2008) Identification of RIP1 kinase as a specific cellular target of necrostatins. Nat Chem Biol 4:313–321

Degterev A, Maki JL, Yuan J (2013) Activity and specificity of necrostatin-1, small-molecule inhibitor of RIP1 kinase. Cell Death Differ 20:366

Di Paolo NC, Doronin K, Baldwin LK, Papayannopoulou T, Shayakhmetov DM (2013) The transcription factor IRF3 triggers "Defensive Suicide" necrosis in response to viral and bacterial pathogens. Cell Rep 3(6):1840–1846

Duprez L, Takahashi N, Van Hauwermeiren F, Vandendriessche B, Goossens V, Vanden Berghe T, Declercq W, Libert C, Cauwels A, Vandenabeele P (2011) RIP kinase-dependent necrosis drives lethal systemic inflammatory response syndrome. Immunity 35:908–918

Fanjul A, Dawson MI, Hobbs PD, Jong L, Cameron JF, Harlev E, Graupner G, Lu XP, Pfahl M (1994) A new class of retinoids with selective inhibition of AP-1 inhibits proliferation. Nature 372:107–111

Fiers W, Beyaert R, Boone E, Cornelis S, Declercq W, Decoster E, Denecker G, Depuydt B, De Valck D, De Wilde G et al (1995) TNF-induced intracellular signaling leading to gene induction or to cytotoxicity by necrosis or by apoptosis. J Inflamm 47:67–75

Galluzzi L, Vanden Berghe T, Vanlangenakker N, Buettner S, Eisenberg T, Vandenabeele P, Madeo F, Kroemer G (2011) Programmed necrosis from molecules to health and disease. Int Rev Cell Mol Biol 289:1–35

Goossens V, Grooten J, De Vos K, Fiers W (1995) Direct evidence for tumor necrosis factor-induced mitochondrial reactive oxygen intermediates and their involvement in cytotoxicity. Proc Natl Acad Sci U S A 92:8115–8119

Gunther C, Martini E, Wittkopf N, Amann K, Weigmann B, Neumann H, Waldner MJ, Hedrick SM, Tenzer S, Neurath MF et al (2011) Caspase-8 regulates TNF-alpha-induced epithelial necroptosis and terminal ileitis. Nature 477:335–339

Hayakawa M, Ishida N, Takeuchi K, Shibamoto S, Hori T, Oku N, Ito F, Tsujimoto M (1993) Arachidonic acid-selective cytosolic phospholipase A2 is crucial in the cytotoxic action of tumor necrosis factor. J Biol Chem 268:11290–11295

He S, Wang L, Miao L, Wang T, Du F, Zhao L, Wang X (2009) Receptor interacting protein kinase-3 determines cellular necrotic response to TNF-alpha. Cell 137:1100–1111

Heinrich M, Wickel M, Schneider-Brachert W, Sandberg C, Gahr J, Schwandner R, Weber T, Saftig P, Peters C, Brunner J et al (1999) Cathepsin D targeted by acid sphingomyelinase-derived ceramide. EMBO J 18:5252–5263

Hernandes MS, Britto LR (2012) NADPH oxidase and neurodegeneration. Curr Neuropharmacol 10:321–327

Hildebrand JM, Sun L, Silke J (2013) An appointment with death, 生死有时: 2013 Cold Spring Harbor Asia meeting 'Mechanisms and Functions of Non-Apoptotic Cell Death'. Cell Death Differ 20:1593–1594

Hitomi J, Christofferson DE, Ng A, Yao J, Degterev A, Xavier RJ, Yuan J (2008) Identification of a molecular signaling network that regulates a cellular necrotic cell death pathway. Cell 135:1311–1323

Holler N, Zaru R, Micheau O, Thome M, Attinger A, Valitutti S, Bodmer JL, Schneider P, Seed B, Tschopp J (2000) Fas triggers an alternative, caspase-8-independent cell death pathway using the kinase RIP as effector molecule. Nat Immunol 1:489–495

Jagtap PG, Degterev A, Choi S, Keys H, Yuan J, Cuny GD (2007) Structure-activity relationship study of tricyclic necroptosis inhibitors. J Med Chem 50:1886–1895

Jin S, DiPaola RS, Mathew R, White E (2007) Metabolic catastrophe as a means to cancer cell death. J Cell Sci 120:379–383

Jouan-Lanhouet S, Arshad MI, Piquet-Pellorce C, Martin-Chouly C, Le Moigne-Muller G, Van Herreweghe F, Takahashi N, Sergent O, Lagadic-Gossmann D, Vandenabeele P et al (2012) TRAIL induces necroptosis involving RIPK1/RIPK3-dependent PARP-1 activation. Cell Death Differ 19:2003–2014

Kang TB, Yang SH, Toth B, Kovalenko A, Wallach D (2013) Caspase-8 blocks kinase RIPK3-mediated activation of the NLRP3 inflammasome. Immunity 38:27–40

Kawahara A, Ohsawa Y, Matsumura H, Uchiyama Y, Nagata S (1998) Caspase-independent cell killing by Fas-associated protein with death domain. J Cell Biol 143:1353–1360

Kim YS, Morgan MJ, Choksi S, Liu ZG (2007) TNF-induced activation of the Nox1 NADPH oxidase and its role in the induction of necrotic cell death. Mol Cell 26:675–687

Kim YJ, Kim EH, Hahm KB (2012) Oxidative stress in inflammation-based gastrointestinal tract diseases: challenges and opportunities. J Gastroenterol Hepatol 27:1004–1010

Laster SM, Wood JG, Gooding LR (1988) Tumor necrosis factor can induce both apoptotic and necrotic forms of cell lysis. J Immunol 141:2629–2634

Li J, McQuade T, Siemer AB, Napetschnig J, Moriwaki K, Hsiao YS, Damko E, Moquin D, Walz T, McDermott A et al (2012) The RIP1/RIP3 necrosome forms a functional amyloid signaling complex required for programmed necrosis. Cell 150:339–350

Lin J, Li H, Yang M, Ren J, Huang Z, Han F, Huang J, Ma J, Zhang D, Zhang Z et al (2013) A role of RIP3-mediated macrophage necrosis in atherosclerosis development. Cell Rep 3:200–210

Linkermann A, Brasen JH, Himmerkus N, Liu S, Huber TB, Kunzendorf U, Krautwald S (2012) Rip1 (receptor-interacting protein kinase 1) mediates necroptosis and contributes to renal ischemia/reperfusion injury. Kidney Int 81:751–761

Lukens JR, Vogel P, Johnson GR, Kelliher MA, Iwakura Y, Lamkanfi M, Kanneganti TD (2013) RIP1-driven autoinflammation targets IL-1alpha independently of inflammasomes and RIP3. Nature 498:224–227

Luschen S, Ussat S, Scherer G, Kabelitz D, Adam-Klages S (2000) Sensitization to death receptor cytotoxicity by inhibition of fas-associated death domain protein (FADD)/caspase signaling. Requirement of cell cycle progression. J Biol Chem 275:24670–24678

Madamanchi NR, Runge MS (2013) Redox signaling in cardiovascular health and disease. Free Radic Biol Med 61C:473–501

Manning BD, Cantley LC (2007) AKT/PKB signaling: navigating downstream. Cell 129: 1261–1274

McNamara CR, Degterev A (2011) Small-molecule inhibitors of the PI3K signaling network. Future Med Chem 3:549–565

McNamara CR, Ahuja R, Osafo-Addo AD, Barrows D, Kettenbach A, Skidan I, Teng X, Cuny GD, Gerber S, Degterev A (2013) Akt regulates TNFalpha synthesis downstream of RIP1 kinase activation during necroptosis. PLoS One 8:e56576

Micheau O, Tschopp J (2003) Induction of TNF receptor I-mediated apoptosis via two sequential signaling complexes. Cell 114:181–190

Muller AJ, DuHadaway JB, Donover PS, Sutanto-Ward E, Prendergast GC (2005) Inhibition of indoleamine 2,3-dioxygenase, an immunoregulatory target of the cancer suppression gene Bin1, potentiates cancer chemotherapy. Nat Med 11:312–319

O'Donnell MA, Perez-Jimenez E, Oberst A, Ng A, Massoumi R, Xavier R, Green DR, Ting AT (2011) Caspase 8 inhibits programmed necrosis by processing CYLD. Nat Cell Biol 13:1437–1442

Oerlemans MI, Liu J, Arslan F, den Ouden K, van Middelaar BJ, Doevendans PA, Sluijter JP (2012) Inhibition of RIP1-dependent necrosis prevents adverse cardiac remodeling after myocardial ischemia-reperfusion in vivo. Basic Res Cardiol 107:270

Pyne S, Pyne NJ (2000) Sphingosine 1-phosphate signalling in mammalian cells. Biochem J 349:385–402

Roca FJ, Ramakrishnan L (2013) TNF dually mediates resistance and susceptibility to mycobacteria via mitochondrial reactive oxygen species. Cell 153:521–534

Rosenbaum DM, Degterev A, David J, Rosenbaum PS, Roth S, Grotta JC, Cuny GD, Yuan J, Savitz SI (2010) Necroptosis, a novel form of caspase-independent cell death, contributes to neuronal damage in a retinal ischemia-reperfusion injury model. J Neurosci Res 88:1569–1576

Saelens X, Festjens N, Parthoens E, Vanoverberghe I, Kalai M, van Kuppeveld F, Vandenabeele P (2005) Protein synthesis persists during necrotic cell death. J Cell Biol 168:545–551

Schulze-Osthoff K, Bakker AC, Vanhaesebroeck B, Beyaert R, Jacob WA, Fiers W (1992) Cytotoxic activity of tumor necrosis factor is mediated by early damage of mitochondrial functions. Evidence for the involvement of mitochondrial radical generation. J Biol Chem 267:5317–5323

Shindo R, Kakehashi H, Okumura K, Kumagai Y, Nakano H (2013) Critical contribution of oxidative stress to TNFalpha-induced necroptosis downstream of RIPK1 activation. Biochem Biophys Res Commun 436(2):212–216

Shulga N, Pastorino JG (2012) GRIM-19-mediated translocation of STAT3 to mitochondria is necessary for TNF-induced necroptosis. J Cell Sci 125:2995–3003

Smith EL, Schuchman EH (2008) The unexpected role of acid sphingomyelinase in cell death and the pathophysiology of common diseases. FASEB J 22:3419–3431

Soldani C, Scovassi AI (2002) Poly(ADP-ribose) polymerase-1 cleavage during apoptosis: an update. Apoptosis 7:321–328

Sosna J, Voigt S, Mathieu S, Lange A, Thon L, Davarnia P, Herdegen T, Linkermann A, Rittger A, Chan FK et al (2013) TNF-induced necroptosis and PARP-1-mediated necrosis represent distinct routes to programmed necrotic cell death. Cell Mol Life Sci 71:331–348

Stephens LR, Jackson TR, Hawkins PT (1993) Agonist-stimulated synthesis of phosphatidylinositol(3,4,5)-trisphosphate: a new intracellular signalling system? Biochim Biophys Acta 1179:27–75

Sun XM, Butterworth M, MacFarlane M, Dubiel W, Ciechanover A, Cohen GM (2004) Caspase activation inhibits proteasome function during apoptosis. Mol Cell 14:81–93

Sun L, Wang H, Wang Z, He S, Chen S, Liao D, Wang L, Yan J, Liu W, Lei X et al (2012) Mixed lineage kinase domain-like protein mediates necrosis signaling downstream of RIP3 kinase. Cell 148:213–227

Takahashi N, Duprez L, Grootjans S, Cauwels A, Nerinckx W, DuHadaway JB, Goossens V, Roelandt R, Van Hauwermeiren F, Libert C et al (2012) Necrostatin-1 analogues: critical issues on the specificity, activity and in vivo use in experimental disease models. Cell Death Dis 3:e437

Teng X, Degterev A, Jagtap P, Xing X, Choi S, Denu R, Yuan J, Cuny GD (2005) Structure-activity relationship study of novel necroptosis inhibitors. Bioorg Med Chem Lett 15:5039–5044

Teng X, Keys H, Jeevanandam A, Porco JA Jr, Degterev A, Yuan J, Cuny GD (2007) Structure-activity relationship study of [1,2,3]thiadiazole necroptosis inhibitors. Bioorg Med Chem Lett 17:6836–6840

Thon L, Mohlig H, Mathieu S, Lange A, Bulanova E, Winoto-Morbach S, Schutze S, Bulfone-Paus S, Adam D (2005) Ceramide mediates caspase-independent programmed cell death. FASEB J 19:1945–1956

Trichonas G, Murakami Y, Thanos A, Morizane Y, Kayama M, Debouck CM, Hisatomi T, Miller JW, Vavvas DG (2010) Receptor interacting protein kinases mediate retinal detachment-induced photoreceptor necrosis and compensate for inhibition of apoptosis. Proc Natl Acad Sci U S A 107:21695–21700

Upton JW, Kaiser WJ, Mocarski ES (2012) DAI/ZBP1/DLM-1 complexes with RIP3 to mediate virus-induced programmed necrosis that is targeted by murine cytomegalovirus vIRA. Cell Host Microbe 11:290–297

Vanden Berghe T, Vanlangenakker N, Parthoens E, Deckers W, Devos M, Festjens N, Guerin CJ, Brunk UT, Declercq W, Vandenabeele P (2010) Necroptosis, necrosis and secondary necrosis converge on similar cellular disintegration features. Cell Death Differ 17:922–930

Vandenabeele P, Galluzzi L, Vanden Berghe T, Kroemer G (2010) Molecular mechanisms of necroptosis: an ordered cellular explosion. Nat Rev Mol Cell Biol 11:700–714

Vercammen D, Beyaert R, Denecker G, Goossens V, Van Loo G, Declercq W, Grooten J, Fiers W, Vandenabeele P (1998) Inhibition of caspases increases the sensitivity of L929 cells to necrosis mediated by tumor necrosis factor. J Exp Med 187:1477–1485

Vince JE, Wong WW, Gentle I, Lawlor KE, Allam R, O'Reilly L, Mason K, Gross O, Ma S, Guarda G et al (2012) Inhibitor of apoptosis proteins limit RIP3 kinase-dependent interleukin-1 activation. Immunity 36:215–227

Wallach D, Kovalenko A, Kang TB (2011) 'Necrosome'-induced inflammation: must cells die for it? Trends Immunol 32:505–509

Wang K, Li J, Degterev A, Hsu E, Yuan J, Yuan C (2007) Structure–activity relationship analysis of a novel necroptosis inhibitor, Necrostatin-5. Bioorg Med Chem Lett 17:1455–1465

Wang Z, Jiang H, Chen S, Du F, Wang X (2012) The mitochondrial phosphatase PGAM5 functions at the convergence point of multiple necrotic death pathways. Cell 148:228–243

Welker ME, Kulik G (2013) Recent syntheses of PI3K/Akt/mTOR signaling pathway inhibitors. Bioorg Med Chem 21:4063–4091

Welz PS, Wullaert A, Vlantis K, Kondylis V, Fernandez-Majada V, Ermolaeva M, Kirsch P, Sterner-Kock A, van Loo G, Pasparakis M (2011) FADD prevents RIP3-mediated epithelial cell necrosis and chronic intestinal inflammation. Nature 477:330–334

Wu YT, Tan HL, Huang Q, Ong CN, Shen HM (2009) Activation of the PI3K-Akt-mTOR signaling pathway promotes necrotic cell death via suppression of autophagy. Autophagy 5:824–834

Wu YT, Tan HL, Huang Q, Sun XJ, Zhu X, Shen HM (2011) zVAD-induced necroptosis in L929 cells depends on autocrine production of TNFalpha mediated by the PKC-MAPKs-AP-1 pathway. Cell Death Differ 18:26–37

Xie T, Peng W, Liu Y, Yan C, Maki J, Degterev A, Yuan J, Shi Y (2013) Structural basis of RIP1 inhibition by necrostatins. Structure 21:493–499

Xu X, Chua CC, Zhang M, Geng D, Liu CF, Hamdy RC, Chua BH (2010) The role of PARP activation in glutamate-induced necroptosis in HT-22 cells. Brain Res 1343:206–212

You Z, Savitz SI, Yang J, Degterev A, Yuan J, Cuny GD, Moskowitz MA, Whalen MJ (2008) Necrostatin-1 reduces histopathology and improves functional outcome after controlled cortical impact in mice. J Cereb Blood Flow Metab 28:1564–1573

Yu L, Alva A, Su H, Dutt P, Freundt E, Welsh S, Baehrecke EH, Lenardo MJ (2004) Regulation of an ATG7-beclin 1 program of autophagic cell death by caspase-8. Science 304:1500–1502

Zhang DW, Shao J, Lin J, Zhang N, Lu BJ, Lin SC, Dong MQ, Han J (2009) RIP3, an energy metabolism regulator that switches TNF-induced cell death from apoptosis to necrosis. Science 325:332–336

Zhao J, Jitkaew S, Cai Z, Choksi S, Li Q, Luo J, Liu ZG (2012) Mixed lineage kinase domain-like is a key receptor interacting protein 3 downstream component of TNF-induced necrosis. Proc Natl Acad Sci U S A 109:5322–5327

Zhu S, Zhang Y, Bai G, Li H (2011) Necrostatin-1 ameliorates symptoms in R6/2 transgenic mouse model of Huntington's disease. Cell Death Dis 2:e115

Zou J, Kawai T, Tsuchida T, Kozaki T, Tanaka H, Shin KS, Kumar H, Akira S (2013) Poly IC triggers a cathepsin D- and IPS-1-dependent pathway to enhance cytokine production and mediate dendritic cell necroptosis. Immunity 38:717–728

Chapter 18
Methods to Study and Distinguish Necroptosis

Sasker Grootjans, Vera Goossens, Peter Vandenabeele, and Tom Vanden Berghe

18.1 Introduction

Initially, it was assumed that apoptosis was the only regulated mode of cell death (Kerr et al. 1972), while necrosis according to standard textbooks was considered to be accidental following physicochemical injury and was essentially unregulated (Dave et al. 2012). Hence it was largely disregarded as a phenomenon that could be specifically targeted, until the first evidence of its molecular regulation arose (Laster et al. 1988; Schulze-Osthoff et al. 1993; Vercammen et al. 1998a, b). Later on, the discovery of receptor-interacting protein kinase-1 (RIPK1) and RIPK3 as key mediators of necrotic cell death (Holler et al. 2000; Cho et al. 2009; Zhang et al. 2009), the identification of chemical inhibitors of necrotic cell death and its targets (Degterev et al. 2005, 2008; Wang et al. 2007; You et al. 2008), and the finding of pathologies associated with regulated necrosis (Degterev et al. 2005; You et al. 2008; Takahashi et al. 2012) resulted in a general acceptance of necrosis being also the result of a molecularly regulated process (Vandenabeele et al. 2010). One of the best studied forms of regulated necrosis (RN) is initiated by the kinase activity of RIPK1 and -3 and has been coined necroptosis. However, the discovery of the role of RIPK1 and RIPK3 in necroptosis also allowed the distinction of other types of RN that could not be inhibited by the RIPK1 inhibitor necrostatin-1 (Nec-1), such as H_2O_2-induced necrosis (Vanden Berghe et al. 2010), MNNG-induced

S. Grootjans • V. Goossens • P. Vandenabeele (✉) • T. Vanden Berghe
Molecular Signaling and Cell Death Unit, Inflammation Research Center, VIB, 9052 Ghent, Belgium

Department of Biomedical Molecular Biology (UGent), University of Ghent, Technologiepark 927, 9052 Ghent, Belgium
e-mail: Peter.Vandenabeele@irc.vib-UGent.be

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PARP-hyperactivation (parthanatos) (Xu et al. 2010; Sosna et al. 2013), and ferroptosis (Dixon et al. 2012). Due to the complexity of cell death signaling and the existence of many deviations, switches, and overlapping mechanisms, there is currently not a single method that at once discriminates between apoptosis and RN. To determine how cells die, we propose to follow a flow chart (Fig. 18.1), which covers three subsequent steps: (I) determining cell death rate and sensitivity, (II) observing the morphological features, and (III) analyzing the molecular signaling events involved. We discuss different approaches for each of these three main steps in further detail in this chapter. For detailed protocols on these methods, we refer to a more technical methodology paper (Vanden Berghe et al. 2013).

18.2 Three Steps to Determine Cell Death Modality

18.2.1 Detection of Cell Death (Step I)

The first step in analyzing cell death processes is to determine the cell death rate and sensitivity (Fig. 18.1, part I). Traditionally, cell survival, clonogenic, and membrane permeability assays are used to assess the cell death rate. Cell survival assays measure metabolic activity such as mitochondrial succinate dehydrogenase activity using a colorimetric substrate, while clonogenic assays typically stain colonies of cells that expanded from cells that are resistant to or recovered from a cell death challenge and retained the proliferation potential. Therefore, senescent cells or cells arrested in G_1 or G_2 will not score in a clonogenicity assay, although the cells did not undergo cell death. Regarding metabolic measurements, some drugs or targeted genes may affect metabolism or proliferation without inducing cell death. Also these conditions may lead to misinterpretation using metabolic readouts without really reflecting cell death induction. Therefore, membrane permeability assays are more reliable, as they measure the end stage of the cell death process itself, viz., cell membrane rupture. Generally, the available equipment, cell type, scientific question, and scale of the experiment all contribute to the final choice of cell death assay (Table 18.1).

18.2.1.1 Reduction of Tetrazolium Salts Measured by Spectrophotometry

This assay measures mitochondrial succinate dehydrogenase activity in cells, which reflects cell viability. Succinate dehydrogenase is part of the Krebs cycle and complex II at the inner mitochondrial membrane. 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) is a pale yellow substrate that is reduced by succinate dehydrogenase in living cells and reverted to a dark blue formazan product. Alternative substrates, such as (2,3-bis-(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide) (XTT), 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) and water-soluble tetrazolium

salts (WSTs), are also often used. They have advantages over the non-soluble MTT in that they can be directly quantified and do not need dissolving in 10 % SDS/HCl. The major disadvantage of these viability assays, especially at the early stages of apoptotic cell death, is that dead cells often still have partially intact succinate dehydrogenase activity, which is erroneously scored as survival. Some conditions and some compounds may also modulate mitochondrial respiration or the Krebs cycle, resulting in increased or decreased succinate dehydrogenase activity, misleadingly appearing to be cell death-modulating conditions. For instance, resveratrol enhances the MTT-reducing ability of cells, although it induces growth arrest (Bernhard et al. 2003). MTT conversion is also affected by cell density and exhaustion of medium, which can result in underestimation of cell viability. Media containing phenol red also absorb light in the same wavelengths as the MTT/MTS/WST products, at ~750 nm (Pick and Keisari 1980). This can elevate background signals and hence reduce the dynamic range of the assay. The latter issue can be circumvented by using media free of phenol red or by subtracting background absorbance measured at 630–690 nm.

18.2.1.2 Dye Exclusion Methods

Trypan blue is a naphthalene sulfonate, impermeable to intact cell membranes and named after its ability to kill trypanosomes in animal models (Wainwright 2010). The negatively charged dye stains dead cells blue, which can be visualized under a light microscope and counted with a hemocytometer. Note that viable cells may stain light blue after more than 5-min exposure to the dye and may even die after 30 min of exposure to trypan blue (Awad et al. 2011). Although this method is most commonly used as a cell viability check during routine cell culture maintenance, devices for (semi)automated measurements of cell death using trypan blue are currently available (Louis and Siegel 2011). A major advantage of this method is its accessibility: a light microscope is all that is required. Unfortunately, the time-dependent toxicity of the dye may result in an overestimation of cell death.

Several types of fluorescent dyes exist that are excluded by an intact cell membrane. Such dyes can intercalate with DNA, can covalently bind to cellular proteins (amine-reactive dyes), or are trapped in viable cells after loading the dye ester and subsequent enzymatic processing (e.g., acetomethoxy-calcein). Although most DNA-intercalating dyes are non-fixable (except ethidium monoazide), they typically display an increase in intensity upon binding to DNA (up to 1,000-fold). As such, they do not require sequential washing steps and can even be added directly to the medium in multiwell-based assays. DNA-intercalating dyes include Sytox® probes, propidium iodide (PI), and 7-amino actinomycin D (7-AAD) and are all ±1 kDa in size. Fixation of PI-stained cells has been reported (Shen et al. 2011) but requires intensive washing before fixation. The amine-reactive dyes, such as LIVE/DEAD® stains, are routinely used with fixation procedures but also require washing steps before fixation and have a lower signal-to-noise ratio (50- to 80-fold increase

(I) Cell death
(II) Morphology
(III) Molecular signaling events
Observation of cell death
Morphologic analysis in funtion of time
re-evaluation
NO
re-evaluation
YES
Apoptotic[a,#]
Necrotic[b]
CASP3 dependent?
YES
CASP3 dependent?
NO
CASP8 dependent?
YES
CASP1/11 dependent?
NO
RIPK1/3 dependent?
RIPK1/3 dependent?
NO
YES
NO
CASP2/9 dependent?
YES
MLKL dependent?
NO
Dependent on other mediator?
NO
YES
YES
APOPTOSIS
dependent on CASP8
APOPTOSIS
dependent on CASP8 & RIPK1 or -3
APOPTOSIS
dependent on CASP2 or -9
NECROPTOSIS
dependent on MLKL & RIPK1 or -3
ACCIDENTAL NECROSIS
REGULATED NECROSIS
dependent on: see Table 2
PYROPTOSIS
dependent on CASP1 or -11
• WB/IHC anti-active CASP8
• IETD-amc*
• FRET reporter*
• CASP8 depletion
• WB/IHC anti-active CASP2 or -9
• FRET reporter*
• CASP2/9 depletion
• Necrosulfonamide
• MLKL depletion
• Metabolic activity
• Clonogenicity
• Membrane permeability
• Light and electron microscopy
• Live cell imaging
• Quantitative imaging
• WB/IHC anti-active CASP3
• DEVD-amc*
• FRET reporter*
• WB/IHC anti-active CASP1
• YVAD-amc*
• FRET reporter*
• Necrostatin-1
• RIPK3 depletion
• Pharmacological or genetic targeting (e.g. see Table 3)

Fig. 18.1 Determination of cell death type following a decision tree. (I) Determine the cell death rate and sensitivity by measuring metabolic activity or by a colorimetric or a fluorometric dye exclusion method (Sect. 2). (II) Analyze the morphology of the dying cells by light or electron microscopy in function of time (Sect. 3.1), and characterize other biochemical events by multiplex methods. [a]Apoptotic morphology is described in Fig. 18.2d–f. [b]Necrotic morphology is described in Fig. 18.2a–c. (III) Dependent on the activation of CASP3 two different (but partially overlapping) decision trees can be followed (Sects. 3 and 4). The decision tree for apoptosis determines which initiator caspase is involved and whether the apoptosis is dependent on RIPK1 or -3. The decision tree for necrosis analyzes first the involvement of caspase-1 or -11, which could be indicative for pyroptosis. This is followed by determining the involvement of RIPK1, RIPK3, and MLKL, which is indicative for necroptosis. If cell death occurs independent of caspase-1, caspase-11, RIPK1, RIPK3, and MLKL, other mediators of RN (mentioned in Table 18.3) should be analyzed using pharmacological and genetic approaches. [#]The observation of nuclear condensation and fragmentation during apoptosis should be extended by an approach to analyze DNA fragmentation (Sect. 3.2). *Fluorometric caspase activation assays and FRET/luciferase reporter systems should be interpreted with care, because the catalytic activity of caspases may be more influential in a cellular context than their supposed substrate preference. As such, specificity of the fluorometric activation assay or FRET/luciferase reporter systems should be validated after knockdown of the target caspase in the same cellular model and with the same stimulus

Table 18.1 Main characteristics, advantages, and disadvantages of cell death assays

	Light microscopy	Plate reader	Flow cytometer	HCI microscopy
Cell type	Suspension or adherent	Suspension or adherent	Suspension or trypsinized adherent	Adherent or suspension (coated surface)
Exp. size	Small	Medium to large	Medium to large	Small to medium
Dye	Trypan blue	Fluorescent exclusion	Fluorescent exclusion	Fluorescent exclusion
Advantage	Cheap	Fast	Multiparameter	Multiparameter/ morphology
Disadvantage	Time consuming, dye toxicity	Quenching/ autofluorescence	Quenching/ autofluores-cence	Data processing labor intensive
Measures	Single cell	Population only	Single cell and population	Single cell and population
Application	Viability check, quality control	Inhibitor study determination IC50	Analysis of blood cells or suspension cells	Qualitative and quantitative analysis

of signal in dead cells versus living cells) as compared to unfixed DNA-intercalating dyes (Perfetto et al. 2006).

Fluorescent exclusion dyes can be analyzed by image- or flow-based cytometers or fluorescent plate readers. The latter approach may involve risks: certain chemical compounds may quench the fluorescent exclusion dyes or they may be autofluorescent, resulting in, respectively, under- or overestimation of the actual cell death. It is therefore advisable to visually inspect fluorescent cell death assays by light/fluorescent microscopy as well or to complement them by a cell survival assay. Image- or flow-based cytometers have the advantage that they allow to monitor other parameters simultaneously, but they are more complicated to use and to extract data. Cytometric methods will be discussed in more detail in the following sections.

18.2.1.3 Determining Cell Death In Vivo

Few studies address the direct visualization of necrotic cell death in vivo using fluorescent techniques (Cordeiro et al. 2010), because fluorescent light penetrates poorly through tissues of a living animal and because most fluorescent membrane exclusion dyes are not fixable. Therefore, most reports on direct measurement of necrotic cell death in vivo are based on radiolabeling (Table 18.2). These markers have been developed for a clinical setting, so often it is not clear whether they detect cell death in general or specifically apoptosis or necrosis. However, some probes exist that accumulate in regions that correspond with caspase activation in postmortem examination. These either bind to activated caspases (Smith et al. 2008; Cohen

Table 18.2 Methods for the detection of necrosis and cell death in general in vivo

	Probe/principle	*IV* / *PM*	Preferred application	Refs.
Fluorescent	Propidium iodide, Annexin V PI enters dead cells, fluorescent Annexin V binds exposed PS	*IV*	Detect cell death modality in the eye	Cordeiro et al. (2010)
Radio-active tracers	18F-ML-10, 3H-ML-9, 18F-ICMT-11, 18F-isatin sulfonamides Radiolabeled synthetic caspase substrate	*IV*	Detect caspase activation in living animals	Smith et al., (2008), Cohen et al., (2009), Grimberg et al. (2009), Nguyen et al. (2009)
	99mTc-HYNIC Annexin V Radiolabeled Annexin V binds exposed PS	*IV*	Detects cell death in living animals	Blankenberg et al. (1999)
	9mTc-glucarate Radiolabeled compound accumulates in necrotic tissue	*IV*	Identifies necrotic tissue less than 9 h after insult	Mariani et al. (1999)
	123I-labeled hypericin Radiolabeled compound accumulates in necrotic tissue	*IV*	Identifies necrotic tissue more than 9 h after insult	Ni et al. (2006), Li et al. (2012)
	99mTc(CO)3-labeled bis-hydrazide-bis-DTPA pamoic acid Radiolabeled compound accumulates in necrotic tissue	*IV*	Identifies necrotic tissue 4–18 h after insult	Fonge et al. (2007)
Nuclear magnetic reso-nance	99mTc(CO)3-labeled 3,3′-(benzylidene)-bis-(1H-indole-2-carbohydrazide) Compound accumulates in necrotic tissue	*IV*	Specific detection of primary necrotic tissue	Prinsen et al. (2011)
	Bis-hydrazide-bis-DTPA pamoic acid Compound accumulates in necrotic tissue	*IV*	Identifies necrotic tissue 4–18 h after insult	Fonge et al. (2007)
	Paramagnetic metalloporphyrins Compound accumulates in necrotic tissue	*IV*	Detect necrotic tissue in a nonradioactive way	Ni et al. (1997)
DWMR imaging	Diffusion-weighted MR imaging measures loss of free diffusion of water (proton spins) when moving out of extracellular matrix into the cell during cell death	*IV*	Detect dying cells in a nonradioactive way	Wendland et al. (2008), Chiaradia et al. (2013)

(continued)

Table 18.2 (continued)

	Probe/principle	IV / PM	Preferred application	Refs.
Indirect markers	Released lactate dehydrogenase is measured in the blood flow	*IV* *PM*	Detect DAMPs in plasma	Kuenzler et al. (2002)
	Released hexosaminidase is measured in the blood flow	*IV* *PM*	Detect DAMPs in plasma	Kuenzler et al. (2002)
	Released mitochondrial DNA is measured in the blood flow by quantitative PCR	*IV* *PM*	Detect DAMPs in plasma	Zhang et al. (2010); Duprez et al. (2011), Krysko et al. (2011)
	Released HMGB1 is measured in the blood flow by ELISA	*IV* *PM*	Detect DAMPs in plasma	Scaffidi et al. (2002)
	Released IL-33, IL-1α, or cyclophilin A is measured in the blood flow	*IV* *PM*	Detect DAMPs in plasma	Luthi et al. (2009), Christofferson and Yuan (2010), Cohen et al. (2010)
Biochemical markers	TUNEL labeling of nicked DNA	*PM*	Extra validation of apoptosis besides caspase activation	Gavrieli et al. (1992)
	IHC active CASP3 immunohistochemistry with antibody against active CASP3	*PM*	Demonstrates caspase activation, after demonstration of cell death	Nagata (2000)

IV in vivo, *PM* postmortem

et al. 2009; Grimberg et al. 2009; Nguyen et al. 2009) or exposed phosphatidyl serine (Blankenberg et al. 1999). It is important to note that caspase activation also occurs in physiological settings without involvement of cell death, such as inflammation (Lamkanfi et al. 2007; Kuranaga 2012). As such, confirmation of cell death occurring in these regions where caspases are active is required. On the other hand, probes have been described that accumulate in necrotic regions induced by various insults (De Saint-Hubert et al. 2009) but not in cell death regions induced by apoptosis-inducing stimuli (Prinsen et al. 2011). Some of these markers, such as the metalloporphyrins can also be detected with nonradioactive nuclear magnetic resonance (NMR) (Ni et al. 1997). Diffusion-weighted magnetic resonance imaging can also detect cell death without injecting contrast markers (Chiaradia et al. 2013), but no real distinction between apoptotic and necrotic cell regions can be made (Wendland et al. 2008).

Cell death can also be measured in vivo by indirect markers, such as the release of intracellular content in the blood flow. These can be general cell death markers such as the release of cytosolic lactate dehydrogenase, lysosomal hexosaminidase (Kuenzler et al. 2002), and mitochondrial DNA (Zhang et al. 2010; Duprez et al. 2011; Krysko et al. 2011) or biomarkers supposed to be more specific for necrosis such as HMGB1 (Scaffidi et al. 2002), IL-33 (Luthi et al. 2009), IL-1α (Cohen et al. 2010), and cyclophilin A (Christofferson and Yuan 2010). Note that HMGB1 can also be released by macrophages in the absence of cell death (Andersson et al. 2000). Moreover, in the case of massive apoptosis with insufficient phagocytic capacity, apoptotic cells can lose their confinement and evolve to secondary necrosis, during which intracellular content is also released. However, in contrast to necrosis, cells that undergo secondary necrosis did undergo a wave of caspase-mediated proteolysis resulting in the cleavage of certain cellular proteins.

18.2.2 Discrimination of Apoptosis Versus Necrosis (Step II)

Next to the detection of cell death, we want to define the cell death modality. Typically, morphologic examination (Fig. 18.1, part II Morphology), followed by some biochemical approaches (Fig. 18.1, part III Molecular signaling events), will allow to pinpoint whether the cells die by apoptosis, necroptosis, or another form of RN. Generally, it is important to combine several methods from each of the main categories described below in order to reach a valid conclusion. In addition, we want to emphasize that cells should be analyzed at different time points, because these cell death features change over time: absence of apoptotic hallmarks should be confirmed over the entire course of the cell death process for a validation of necrotic cell death. Moreover, some inhibitors may block one cell death modality (necroptosis or apoptosis) and allow a switch to another cell death modality (e.g., the pan-caspase inhibitor zVAD-fmk blocks Fas-induced apoptosis in L929

cells, but allows a switch to necrosis) (Vanden Berghe et al. 2003, 2010). Many more examples of such cell death modality switches have been reported (Vanlangenakker et al. 2011a; Dondelinger et al. 2013; Remijsen et al. 2014). This illustrates that although the extent of cell death may have not been changed at the endpoint, the type of cell death clearly is.

18.2.2.1 Morphologic Analysis by Light Microscopy

Differences in morphology between apoptotic and necrotic cell death were the initial clues that different cellular processes were ongoing (Fig. 18.1, part II). Today, these morphological differences are still the most important telltale signal of necrotic cell death. The typical rounding and swelling of the cells, together with increased cytoplasmic granularity and intact nuclei, are easy to recognize even by light microscopy (Fig. 18.2a, b). These early events are followed by a rapid loss of plasma membrane integrity, which is visualized by the uptake of PI and clear nuclear staining (Fig. 18.2c). In contrast, apoptotic cells shrink, show membrane ruffling and nuclear condensation, form apoptotic bodies (membrane-bound vesicles that contain compacted organelles and nuclear components), and expose phosphatidyl serine (Fig. 18.2d–e) (Wyllie 1981). The morphological necrotic events until today still form the best distinctive marker of necrosis and can be considered as a quick and easy first checkpoint to discriminate apoptotic and necrotic cell death. Apoptotic cells in the absence of phagocytic cells can proceed to secondary necrosis, in which cells have lost plasma membrane integrity, but even at this stage clear morphologic markers of apoptosis can be appreciated such as nuclear fragmentation and presence of apoptotic bodies containing nuclear DNA (compare Fig. 18.2c and f).

18.2.2.2 Morphologic and Multiparametric Analysis by Multiplex Methods

Considering the dynamic behavior of dying cells, it is important to document the entire process by, e.g., live cell imaging or by high-content cytometry (HCC). This will avoid erroneous categorization of apoptotic cells as necrotic, because they also proceed to a secondary necrotic phase upon plasma membrane rupture in the absence of sufficient phagocytic activity. Notice that these live cell imaging and HCC techniques can be extended by using fluorescent probes to monitor cell death-related biochemical events such as plasma membrane rupture, lysosomal membrane rupture (using lysotracker®), loss of mitochondrial membrane potential (using tetramethylrosamine methyl ester, TMRM), generation of reactive oxygen species (ROS, using carboxy-H_2DCFDA or DHR123), phosphatidyl serine exposure (using fluorescently labeled Annexin V), or calcium fluxes (Fluo-3)—all probes are available at Life Technologies (Vanden Berghe et al. 2010). Secondary necrotic cells still retain some apoptotic features, such as condensed and

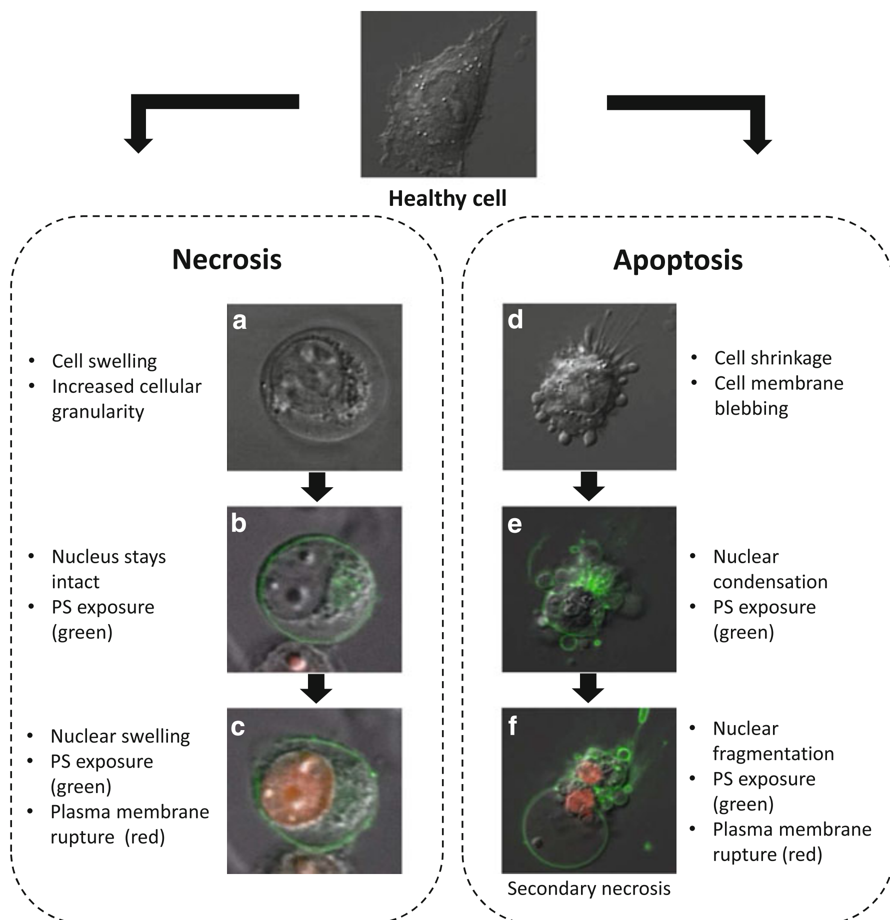

Fig. 18.2 Main morphological characteristics of necrotic and apoptotic cell death observed by (fluorescence) light microscopy, illustrated in the L929 model system. Necrosis is characterized by (**a**) cellular rounding, swelling, and an increased granularity; (**b**) minor changes to the nucleus and phosphatidyl serine (PS) exposure on the plasma membrane; and, finally, (**c**) nuclear swelling, continued PS exposure, and rupture of the plasma membrane. Apoptosis is recognized by (**d**) cellular shrinking, formation of cell membrane ruffles and blebs, (**e**) nuclear condensation, PS exposure, and (**f**) nuclear fragmentation, PS exposure, and plasma membrane rupture (secondary necrosis). Notice that secondary necrotic cells still have condensed and fragmented nuclei caused by the apoptotic process, which is in strong contrast to the normal to swollen nuclei observed during primary necrosis

fragmented nuclei (see Fig. 18.2f), loss of chromatin structure (due to caspase-mediated cleavage of lamins), and internucleosomal cleavage (due to the activity of the caspase-activated DNase). Condensation of the nuclei and loss of chromatin structure result in an intense but homogeneous staining with Hoechst or PI. In contrast,

primary necrotic cells still retain their chromatin structure and are stained less intensely by Hoechst or PI, initially featuring brightly stained nucleoli after membrane permeabilization (time-lapse movies available at http://www.youtube.com/user/dmbrUPVA?ob=0).

Cytometers can be either image based or flow based and can measure multiple parameters simultaneously when using fluorescent probes, and this on a cell-by-cell basis. This provides a high resolution at the level of the cell population and thus delivers meaningful insights into intrinsically heterogeneous responses, such as the response of individual cells in a population to a cell death stimulus. Flow cytometry requires the use of cells in suspension, possibly enhancing the risk of artifacts from manipulation when working with adherent cell cultures. However, fluorescence measurements using flow cytometry have a broad dynamic range and allow good separation of positive and negative cell populations. As cellular, nuclear, and organelle morphological aspects are key to get insight into an ongoing cell death modality, high-content imaging systems became extremely useful in the analysis of cell death. These instruments combine multiparameter, automated image acquisition in different cell plate formats and at selective optical resolutions, with automated image analysis algorithms that calculate morphological as well as fluorescence intensity parameters. Depending on the optical resolution and acquisition speed required, data on large cell populations can be processed and analyzed. Assays on high-content imaging systems allow combination of the cell death endpoint measurement with measurements of mitochondrial function, nuclear morphology, lysosomal rupture, PS exposure, autophagy, caspase activity reporters, or any other fluorescence-based parameter.

18.2.2.3 Morphologic Analysis by Electron Microscopy

Although morphologic analysis using light microscopy might be indicative of necrotic cell death, transmission electron microscopy (TEM) is still the most accurate but labor-intensive method for differentiating between apoptosis and necrosis in cell culture, because it allows the visualization of two-dimensional structures at the cellular and subcellular level. Necrotic cells are characterized by swollen organelles and irregular chromatin condensation, while in apoptotic cells, organelles are compacted in the apoptotic bodies and uniformly dense masses of chromatin are distributed against the nuclear envelope (karyopyknosis) (Cummings et al. 1997; Krysko et al. 2003). Recent evolutions in electron microscopy such as focused ion beam/scanning electron microscopy (FIB/SEM) have even rendered 3-dimensional images of tissues at the ultrastructural level (Knott et al. 2011), and the creation of electron-dense protein tags allows direct visualization of proteins of interest in both fluorescent light and EM (Shu et al. 2011). Dying cells that detach during the cell death process may represent a technical challenge for EM: centrifugation of these cells may change their ultrastructural appearance. Fortunately, a method has been developed to trap detached cells by adherent macrophages, thus tethering them to a solid substrate, suitable for fixation (Vanden Berghe et al. 2013). Although TEM presents

the most detailed view of morphological changes during cell death, it is a complex and time-consuming method, requiring expert knowledge to recognize subcellular structures, and not suitable for routine examination of cell death processes.

18.2.2.4 DNA Fragmentation Analysis by Electrophoresis, Flow Cytometry, or TUNEL Assay

DNA fragmentation is considered a hallmark of apoptotic cell death and results in fragments of more than 5 kbp and nucleosomal sized fragments of multiples of 200 bp responsible for the typical DNA ladder pattern (Nagata 2000, 2002), which normally does not occur during necrotic cell death. These DNA ladders can be detected by agarose gel electrophoresis (Walker et al. 1999), but this method does not allow a quantitative analysis on the single-cell level, a disadvantage that can be overcome by flow cytometry or terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick-end labeling (TUNEL) assay.

The flow cytometry method visualizes the DNA fragmentation that occurs during apoptosis but not during necrosis. DNA degradation in apoptotic cells causes generation of small DNA fragments (Nicoletti et al. 1991). The mean intensity of PI seems to drop due to DNA and nuclear fragmentation (referred to as "hypoploid population"). This method will only work properly after permeabilization of all cells, such as achieved by a freeze–thaw cycle, since apoptotic cells maintain membrane integrity after DNA fragmentation. Due to its increase in intensity after binding to DNA, there is no need to remove the PI after staining (Vanden Berghe et al. 2013).

The TUNEL assay is a frequently used histochemical method to demonstrate apoptosis in in vivo-derived samples. In this assay, terminal deoxynucleotidyl transferase recognizes nicks in the DNA and adds dUTPs, modified with bromine, biotin, or fluorophores, which are then directly visualized or indirectly using fluorescent or peroxidase-coupled streptavidin or antibodies (Gavrieli et al. 1992). It is noteworthy that extracellular DNAse activity (Napirei et al. 2004), active gene transcription (Kockx et al. 1998), or even inappropriate processing of samples may also result in a positive TUNEL signal. The same holds true for caspase-independent release of EndoG by ischemic cardiomyocytes (Zhang et al. 2011) and AIF-mediated DNA processing in a caspase-independent manner during hippocampal neuronal cell death (Thal et al. 2011). As such, the TUNEL assay is not sufficient as the only biochemical proof of apoptosis.

18.2.2.5 Analysis of Caspase Activation

Caspases are cysteine-containing aspartate-specific proteases that drive multiple cellular processes such as apoptosis, pyroptosis, proliferation, and differentiation depending on the caspase involved and cellular context (Lamkanfi et al. 2007; Crawford and Wells 2011). Caspases consist of a p20 and a p10 subunit that contain the residues essential for substrate recognition and catalytic activity and a

prodomain of variable length. Proteolytic activation results in the physical separation of the p10, p20, and prodomain subunits, forming active heterotrimers in an antiparallel orientation (Kersse et al. 2011). Although all caspases require an aspartate at the P1 site of their substrates, the preference for other upstream amino acid residues (P4–P2) in their substrates varies between different caspases, thus defining different substrate specificities for different caspases. Activation of the two major executioner caspases (caspase-3 and -7) in dying cells is a hallmark of apoptosis and therefore a strong argument in favor. The activity of caspases is transient, and thus analysis in function of time, covering the entire course of the cell death process, is required. Caspase activation can be monitored by fluorometry (fluorescently labeled substrates), antibody-based methods, or reporter assays.

A fluorometric caspase activation assay uses the preferred tetrapeptide substrate for the caspase of interest, where the aspartic residue at P1 is coupled to 7-amino-4-methylcoumarin (AMC) or 7-amino-4-trifluoromethylcoumarin (AFC) by a peptide bond. If the caspase under investigation is active, it hydrolyzes this peptide bond after the P1 aspartic acid, thus releasing free AMC or AFC. Free AMC or AFC can be excited to fluorescence, which can be measured in plate-based fluorometers. Notice that some caspases (such as caspase-3) have higher absolute $k_{cat}\backslash K_m$ values in general, presumably due to a higher intrinsic catalytic efficiency (Stennicke et al. 2000), which overrules the supposed specificity of tetrapeptide substrates for the different caspases (Pop et al. 2008; Pop and Salvesen 2009). Therefore researchers should be aware that the limited specificity of these small peptide substrates does not allow conclusion on the identity of caspases involved.

Fortunately, antibody-based techniques for caspase activation became available and allow the direct detection of activated caspases or the products of their proteolytic activity. Western blot is a commonly used technique requiring antibodies raised against epitopes created after proteolytic caspase activation, including both cleaved caspases and their processed substrates. ELISA-based methods require antibodies that recognize epitopes specific for proteolytic cleavage by caspases but are faster and more quantitative than western blots. Activated caspases can also be detected via histochemistry on fixed cells or tissue samples (Nagata 2000). Monitoring activation of the apoptotic executioner caspase-3 and processing of its prototype substrate PARP-1 (116 kDa), generating the characteristic 89 kDa cleavage fragment, is the most reliable approach to confirm or to rule out apoptosis.

A special and very useful case of an antibody-based assay for epithelial cell death involves cytokeratin 18 (CK18), which is cleaved during apoptosis (neo-epitope detectable with M30-Apoptosense® assay) but released in its non-cleaved form during necrosis (detectable by M65® ELISA). Both M30 and M65 are commercialized by Peviva AB (Bromma, Sweden), which also provides standard calibration material to normalize both assays. A high M30:M65 ratio corresponds to induction of apoptosis in cultured cells, while a low M30:M65 ratio is representative for necrosis induction. In vivo detection of caspase-cleaved CK18 is also possible in plasma and by immunohistochemistry (Cummings et al. 2008).

18.2.2.6 Caspase Activity Measurement Using Fluorescence- and Luminescence-Based Reporter Assays

Caspase activity can be monitored by genetically encoded reporter constructs, usually based on Förster resonance energy transfer (FRET) (Rehm et al. 2003; Laussmann et al. 2011). These reporters contain a caspase-cleavage site between both fluorescent proteins that make up the FRET pair; after caspase activation, the fluorescent proteins are physically separated and diffuse apart, thus reducing the signal of the FRET acceptor when exciting with light of the donor-excitation wavelength. Importantly, both fluorescent proteins can be excited by light of their own excitation wavelength, which allows distinguishing proteasomal degradation of the acceptor from *bona fide* caspase activation. FRET can be measured on live or fixed cells both by fluorescent (confocal) microscopy and flow cytometry. The Casper3-BG vector (FP970; Evrogen) was successfully used to demonstrate cell death switches between apoptosis and necrosis in the L929 model system (Vanlangenakker et al. 2011a). Casper3-BG consists of a blue fluorescent protein connected by a DEVD sequence with a green fluorescent protein. During apoptosis, caspase-3 processes DEVD, eliminates FRET, and increases the blue/green ratio (Fig. 18.3a). However, PI-positive cells should be excluded from the FRET analysis (Fig. 18.3b). Other similar FRET-based probes monitoring caspase-1 (Mahajan et al. 1999), caspase-6/8 (He et al. 2004), or caspase-9 (Figueroa et al. 2011; Wu et al. 2011) activity are also available. Simultaneous monitoring of caspase-8 and caspase-3 activity in single cells is also possible using a dual-FRET system (Kominami et al. 2012). Once a stable cell line has been generated, the FRET technology allows easy, fast, and reliable discrimination between apoptosis and necrosis. A disadvantage is that FRET signals can be quite weak and difficult to detect, requiring stable high expression levels. Fluorescent reporter probes based on a quenching peptide that is removed after activation of caspases have been developed for caspase-6 and -7, but these appear to have a very low signal-to-noise ratio (Wu et al. 2013).

Recently, other reporter constructs for caspase-1 and caspase-3/7 based on luciferase activity have been published, GloSensor and iGLuc (Bartok et al. 2013; Galban et al. 2013). The GloSensor system was created by inserting the caspase-3/7 recognition sequence in the middle of the *Photuris pennsylvanica* luciferase, which inactivates the luciferase. After processing by caspase-3/7, both parts of the luciferase can reconstitute the active luciferase and a 50- to 100-fold induction of luciferase activity was observed (Bartok et al. 2013). This system was successfully used in a compound screening and to monitor caspase-3/7 activation in an in vivo mouse model of pancreatitis (Galban et al. 2013). The second system, iGLuc, is a fusion between pro-IL-1β and Gaussia luciferase, which forms nonluminescent aggregates. After overexpression of caspase-1 or activation of the inflammasome, pro-IL-1β is processed, releasing the mature IL-1β fused to luciferase from the aggregates and resulting in a 571-fold increase in luminescence (Bartok et al. 2013). iGLuc was successfully used to monitor inflammasome activation in vivo in a mouse infection model with *Listeria* (Bartok et al. 2013). The system can be

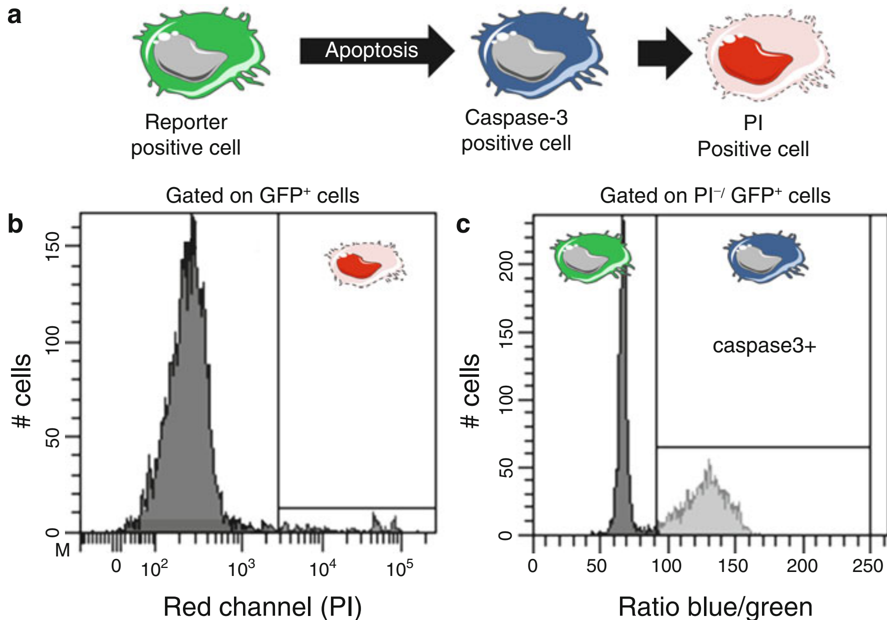

Fig. 18.3 FRET-based reporter system for the detection of caspase activation: a blue fluorescent protein is connected to a green fluorescent protein by a caspase-3 target sequence (DEVD). (**a**) During apoptosis, activated caspase-3 physically separates both fluorophores of the FRET pair, resulting in an increased blue/green ratio, and, finally, propidium iodide (PI) positivity. After a freeze–thaw cycle, PI intensity also indicates the hypoploidy status of the cell population (not shown). (**b**) Flow cytometry allows quantification of the blue/green intensity ratio on PI-negative cells

modified to report on the activity of other caspases, such as caspase-3, by replacing the caspase-1 substrate recognition site with another one, i.e., DEVDG for caspase-3 (Bartok et al. 2013). Of note, the iGLuc system has a higher signal-to-noise ratio than the GloSensor system, which is probably related to the formation of nonluminescent aggregates in the iGLuc system, effectively reducing the background signal.

The specificity of the genetically encoded reporter probes for the intended target protease versus other off-target protease remains to be evaluated for most reporters. As they are based on the short peptide substrates, the specificity might be an issue especially in conditions of massive caspase-3 activation which has a high catalytic turnover (Pop et al. 2008; Pop and Salvesen 2009). Indeed, a FRET reporter construct with the preferred in vitro substrate recognition sequence of caspase-2 (VDVAD) appears to be cleaved mainly by effector caspases and caspase-8 when transfected in cells (Delgado et al. 2013).

18.2.2.7 Potential New Markers of Necrosis

In contrast to the proteolytic caspase cascade in apoptosis, the necroptotic signaling cascade is a kinase cascade. This kinase cascade is initiated by RIPK1 kinase activity (Degterev et al. 2005, 2008), involves the formation of an RIPK1–RIPK3 containing necrosis complex ("necrosome") in an RIPK1 activity-dependent manner (Cho et al. 2009), and proceeds with the phosphorylation and activation of MLKL at Thr357 and Ser358 (Sun et al. 2012). Phosphorylation of RIPK1 and RIPK3 is visualized on western blot by the appearance of a second band running slightly above the steady-state band (Cho et al. 2009; He et al. 2009; Zhang et al. 2009). Another as yet to be validated approach of detection of necroptosis-specific phosphorylation events will be the use of phospho-specific antibodies directed against necroptosis-specific phosphorylation events such as the RIPK3 phosphorylation site on MLKL (Thr357 and Ser358) (Sun et al. 2012), the autophosphorylation sites of RIPK1 (Ser14/15, Ser20, Ser161, and Ser166) (Degterev et al. 2008), or RIPK3 (Ser227) (Sun et al. 2012) to probe for RIPK1, RIPK3, or MLKL phosphorylation in multiple applications, such as immunohistochemistry, flow cytometry, or total cell extracts by ELISA, multiplex bead arrays, or western blot. These phospho-specific markers of necroptosis may form the longed-for parameter of necroptosis. However, in view of the role of RIPK3 and MLKL in regulating inflammasome activation in particular conditions of IAP inhibition or absence of caspase-8, rigorous controls may be required again (Vince et al. Immunity 2012; Kang et al. Immunity 2013). Similar to the FRET-based protease reporters, kinase activity reporters (KARs) for RIPK1- or RIPK3-mediated phosphorylation events could be developed (Morris 2013). These KARs are based on a FRET pair flanking a phosphorylation target sequence and a phosphoamino acid-binding domain (PAABD). When kinases phosphorylate the target sequence, the PAABD binding brings the FRET pair in each other's neighborhood and increased fluorescence is detected (Sipieter et al 2013, Sipieter et al 2014). Similar KARs based on split luciferase have been described as well. These luciferase-based KARs have the advantage that they can be used for in vivo reporting as well (Herbst et al. 2011; Williams et al. 2013).

18.2.2.8 In Vivo Markers of Necrosis

Massive apoptosis saturates the phagocytic system, and secondary necrosis will result in the release of the same marker molecules; confirming the absence of apoptosis by immunohistochemistry of particular tissues or cells is thus necessary to confirm necrotic plasma markers. *Postmortem* analyses for necrotic cell death is possible by pathologists using hematoxylin- and eosin-stained sections (Kaiser et al. 1995; Gukovskaya et al. 1996). A low M30:M65 ratio of CK18 in plasma (as discussed above), but also in sections of tissue (www.peviva.se and Duan et al. 2003), may also be indicative of necrotic cell death. Although in vivo injection of PI has been applied (You et al. 2008), this is not yet a standardized easily applicable

technique. Recently, a fusion of Hoechst with the infrared dye IR-786 (Hoechst-IR) was shown to be cell membrane impermeable and specifically bind to released extracellular DNA (Dasari et al. 2010). Unfortunately, this dye probably also recognizes secondary necrotic tissues in an LPS-GalN-induced hepatitis model (Dasari et al. 2010), underscoring the need for confirming absence of active caspases by immunohistochemistry. The most conclusive postmortem evidence for necrosis can still be obtained by TEM, since organelle swelling, patch-like irregular chromatin condensation, and ruptured plasma membranes can directly be observed. Moreover, in vivo necroptosis is often associated with accumulation of necrotic nuclei in the affected tissue or organs (Gunther et al. 2011).

Real-time and direct in vivo imaging of necrotic cell death is perhaps the most interesting application in a clinical setting. Contrast agents which accumulate in necrotic tissue (necrosis-avid contrast agents) may offer a unique combination of identifying specifically primary necrotic tissue and this in real time (Prinsen et al. 2011). The main disadvantage for this technique is that the equipment and technical staff required for these methods are usually not accessible for small animals in an academic setting.

18.2.3 Identifying Signaling Components of Regulated Necrosis (Step III)

The molecular unraveling of RN has revealed that, similar to apoptosis, it can be activated by many different triggers, which activate partly overlapping pathways, involving RIPK1/RIPK3, PARP-1, ROS, and intracellular Ca^{2+} release (Table 18.3). Finally, these processes converge on a similar cellular disintegration process, characterized by cellular swelling, lysosomal membrane permeabilization, and cell rupture (Vanden Berghe et al. 2010; Vandenabeele et al. 2010; Vanlangenakker et al. 2012).

18.2.3.1 Pharmacological Inhibition

Currently, the best characterized type of RN is necroptosis. According to its most recent definition (Galluzzi et al. 2012), any cell death that can be inhibited by genetic ablation or knockdown of either RIPK1 or RIPK3 or by chemical inhibition by necrostatin-1 (Nec-1 or 5-(1H-indol-3-ylmethyl)-3-methyl-2-thioxo-4-imidazolidinone) is defined as necroptosis; however, this is not exclusive. Recently, RIPK3-dependent (Dondelinger et al. 2013) and RIPK1 kinase activity-dependent (Wang et al. 2008; Duprez et al. 2012) apoptosis have also been reported in certain cellular settings. This illustrates again the need of using several independent methods, most importantly by checking morphological appearance and confirming absence of caspase activity, *cf.* the decision tree in Fig. 18.1. More recently, necrosulfonamide (NSA) was identified as a new inhibitor of necroptosis in human cells,

Table 18.3 Potential alternative forms of regulated necrosis

Genetic depletion	Pharmacological depletion	Necrotic model	Refs.
RPL8 IREB2 ATP5G3 ACSF2 CS TTC35	Ferrostatin-1 (Fer-1)	Ferroptosis	Dixon et al. (2012)
GPX4		Excitotoxicity	Seiler et al. (2008)
Nox1	Diphenylen iodonium (DPI)	NADPH oxidase-induced necrosis	Yamashima (2000), Kim et al. (2007), Yazdanpanah et al. (2009), Kim et al. (2010)
P22phox	GKT137831	Netosis	
NDUFB8	Rotenone	Mito CI-induced ROS	Goossens et al. (1999), Vanlangenakker et al. (2011b)
PLA_2 PLD	Bromoenol lactone (BEL) Methyl-arachidonyl fluorophosphonate (MAFP)	Phospholipase-induced necrosis	Suffys et al. (1991), De Valck et al. (1993), Shinzawa and Tsujimoto (2003), Festjens et al. (2006)
CypD	Cyclosporine A Sanglifehrin	MPT-induced necrosis	Baines et al. (2005), Nakagawa et al. (2005), Linkermann (2013)
PARP1	3-Aminobenzamide	Parthanatos	Jouan-Lanhouet et al. (2012), Sosna et al. (2013)
No protein target	Desferrioxamine (DFO)	Fenton-type-mediated necrosis	Smith (1987), Vanden Berghe et al. (2010)
No protein target	Butylated hydroxyl anisole (BHA) *N*-Acetyl-cysteine (NAC)	ROS-induced necrosis	Goossens et al. (1995), Festjens et al. (2006)
No protein target	Calcium chelators Dantrolene	Calcium-induced necrosis	Yoshioka et al. (2000), Kourtis et al. (2012)

RPL8 ribosomal protein 8, *IREB2* iron response element binding 2, *ATP5G3* ATP synthase complex subunit C3, *ACSF2* acyl-CoA synthetase family member 2, *CS* citrate synthase, *TTC35* tetratricopeptide repeat domain 35, *GPX4* glutathione peroxidase 4, *NDUFB8* NADH dehydrogenase (ubiquinone) 1 beta subcomplex 8, *NOX1* NADPH oxidase 1, PLA_2 phospholipase A_2, *CypD* cyclophilin D, *PARP*-1 poly [ADP-ribose] polymerase 1

which could also be used to confirm the necroptosis typing. This compound covalently binds to Cys86 of MLKL, a crucial substrate of RIPK3 (Sun et al. 2012). In vivo, injection of Nec-1 has been used a lot to prevent cell death in several disease models (Degterev et al. 2005; Smith et al. 2007; You et al. 2008; Duprez et al. 2011). Recently, a more stable and specific variant of Nec-1 was discovered (no off-target effect on immunomodulatory enzyme indoleamine 2,3-dioxygenase), i.e., Nec-1s (Takahashi et al. 2012; Degterev et al. 2013), which is recommended to use in vivo instead of Nec-1 (Vandenabeele et al. 2013).

In addition to Nec-1 and NSA that both block necroptosis (for NSA only in human cells), several pharmacological inhibitors having other targets than RIPK1, RIPK3, or MLKL (Table 18.3) are described to interfere with some form of RN. Often, these pathways occur independent of RIPK1/RIPK3 or the link with RIPK1/RIPK3 is not known yet. In order to study which molecular pathways contribute to the studied form of RN, an approach using pharmacological inhibitors (Table 18.3) can give a first hint. One may also consider to combine inhibitors, as often redundant mechanism turns up upon interference, e.g., depletion or inhibition of both RIPK3 and CypD is required to block ischemia/reperfusion-induced kidney injury (Linkermann et al. 2013).

18.2.3.2 Transgenic Approaches

Ripk1$^{-/-}$ mice are embryonic lethal (Kelliher et al. 1998), and genetic ablation in vitro causes a shift to apoptosis (Vanlangenakker et al. 2011a), rendering this model unsuitable to demonstrate the involvement of necroptosis. The availability of RIPK1 kinase-dead knock-in mice will probably circumvent these issues. *Ripk3*$^{-/-}$ mice develop normally and do not show any obvious spontaneous phenotypes, which allowed phenotyping in a variety of pathological models involving necroptosis (Cho et al. 2009; He et al. 2009; Zhang et al. 2009; Duprez et al. 2011). In addition to RIPK1, RIPK3, and MLKL, several other genes (Table 18.3) contribute to some forms of RN, either independent of necrosome formation or with an as yet unknown link to necroptosis. Typically, a genetic depletion strategy following a pharmacological approach is used to confirm which mechanisms are involved in the studied form of RN. Although this combined pharmacological/genetic approach is an easy and quick method to indicate potential involved mechanisms, one has to be cautious because cell death modality may shift upon interference. For example, blocking apoptosis can shift to necroptosis (Vercammen et al. 1998b), or vice versa (Vanden Berghe et al. 2003; Vanlangenakker et al. 2011a). To detect these possible cell death modality shifts, it is recommended to perform consistently a kinetic analysis to monitor potential transient caspase activation, as mentioned previously. Generally, three major genetic depletion strategies are followed: (1) RNA interference-mediated knockdown, (2) deficient cells derived from transgenic mice, and (3) deficient cells generated by genome editing technologies such as zinc finger nuclease technology (Sigma-Aldrich), TALENs (Transposagen Biopharmaceuticals, Cellectis, and others), or CRISPR (Hsu et al. 2013).

18.3 Conclusions

Research on RN, and in particular of necroptosis, is hampered by a lack of positive markers. Although some markers based on phospho-specific antibodies could have been developed years ago, none have been reported in the literature. Hence, the

detection of RN still depends on the combination of morphological observations, biochemical exclusion of caspase activation, and determination of the involved subroutines (Table 18.3). One should examine these elements over the entire course of the cell death process. It is important to combine several independent techniques to identify RN, since several genes (including RIPK1 and RIPK3) are involved in both apoptosis and necrosis. As a consequence, inhibition, knockdown, or genetic ablation of these genes may create a shift in cell death type rather than cell death inhibition. By following the decision tree (Fig. 18.1), using the approaches and pharmacological/genetic targets (Table 18.3) described, researchers should be able to pinpoint more easily the cell death modalities and the major mechanistic pathways involved.

References

Andersson U, Wang H, Palmblad K, Aveberger AC, Bloom O, Erlandsson-Harris H, Janson A, Kokkola R, Zhang M, Yang H, Tracey KJ (2000) High mobility group 1 protein (HMG-1) stimulates proinflammatory cytokine synthesis in human monocytes. J Exp Med 192:565–570

Awad D, Schrader I, Bartok M, Mohr A, Gabel D (2011) Comparative toxicology of trypan blue, brilliant blue G, and their combination together with polyethylene glycol on human pigment epithelial cells. Invest Ophthalmol Vis Sci 52:4085–4090

Baines C, Kaiser R, Purcell N, Blair N, Osinska H, Hambleton M, Brunskill E, Sayen M, Gottlieb R, Dorn G, Robbins J, Molkentin J (2005) Loss of cyclophilin D reveals a critical role for mitochondrial permeability transition in cell death. Nature 434:658–662

Bartok E, Bauernfeind F, Khaminets MG, Jakobs C, Monks B, Fitzgerald KA, Latz E, Hornung V (2013) iGLuc: a luciferase-based inflammasome and protease activity reporter. Nat Methods 10:147–154

Bernhard D, Schwaiger W, Crazzolara R, Tinhofer I, Kofler R, Csordas A (2003) Enhanced MTT-reducing activity under growth inhibition by resveratrol in CEM-C7H2 lymphocytic leukemia cells. Cancer Lett 195:193–199

Blankenberg FG, Katsikis PD, Tait JF, Davis RE, Naumovski L, Ohtsuki K, Kopiwoda S, Abrams MJ, Strauss HW (1999) Imaging of apoptosis (programmed cell death) with 99mTc annexin V. J Nucl Med 40:184–191

Chiaradia M, Baranes L, Van Nhieu JT, Vignaud A, Laurent A, Decaens T, Charles-Nelson A, Brugières P, Katsahian S, Djabbari M, Deux JF, Sobhani I, Karoui M, Rahmouni A, Luciani A (2014) Intravoxel incoherent motion (IVIM) MR imaging of colorectal liver metastases: Are we only looking at tumor necrosis? J Magn Reson Imaging 38:27-40

Cho YS, Challa S, Moquin D, Genga R, Ray TD, Guildford M, Chan FK (2009) Phosphorylation-driven assembly of the RIP1-RIP3 complex regulates programmed necrosis and virus-induced inflammation. Cell 137:1112–1123

Christofferson DE, Yuan J (2010) Cyclophilin A release as a biomarker of necrotic cell death. Cell Death Differ 17:1942–1943

Cohen A, Shirvan A, Levin G, Grimberg H, Reshef A, Ziv I (2009) From the Gla domain to a novel small-molecule detector of apoptosis. Cell Res 19:625–637

Cohen I, Rider P, Carmi Y, Braiman A, Dotan S, White MR, Voronov E, Martin MU, Dinarello CA, Apte RN (2010) Differential release of chromatin-bound IL-1alpha discriminates between necrotic and apoptotic cell death by the ability to induce sterile inflammation. Proc Natl Acad Sci U S A 107:2574–2579

Cordeiro MF, Guo L, Coxon KM, Duggan J, Nizari S, Normando EM, Sensi SL, Sillito AM, Fitzke FW, Salt TE, Moss SE (2010) Imaging multiple phases of neurodegeneration: a novel approach to assessing cell death in vivo. Cell Death Dis 1:e3

Crawford ED, Wells JA (2011) Caspase substrates and cellular remodeling. Annu Rev Biochem 80:1055–1087

Cummings J, Ward TH, Greystoke A, Ranson M, Dive C (2008) Biomarker method validation in anticancer drug development. Br J Pharmacol 153:646–656

Cummings MC, Winterford CM, Walker NI (1997) Apoptosis. Am J Surg Pathol 21:88–101

Dasari M, Lee S, Sy J, Kim D, Brown M, Davis M, Murthy N (2010) Hoechst-IR: an imaging agent that detects necrotic tissue in vivo by binding extracellular DNA. Org Lett 12:3300–3303

Dave SR, White CC, Gao X, Kavanagh TJ (2012) Luminescent quantum dots for molecular toxicology. Adv Exp Med Biol 745:117–137

De Saint-Hubert M, Prinsen K, Mortelmans L, Verbruggen A, Mottaghy FM (2009) Molecular imaging of cell death. Methods 48:178–187

De Valck D, Beyaert R, Van Roy F, Fiers W (1993) Tumor necrosis factor cytotoxicity is associated with phospholipase D activation. Eur J Biochem/FEBS 212:491–497

Degterev A, Hitomi J, Germscheid M, Ch'en IL, Korkina O, Teng X, Abbott D, Cuny GD, Yuan C, Wagner G, Hedrick SM, Gerber SA, Lugovskoy A, Yuan J (2008) Identification of RIP1 kinase as a specific cellular target of necrostatins. Nat Chem Biol 4:313–321

Degterev A, Huang Z, Boyce M, Li Y, Jagtap P, Mizushima N, Cuny GD, Mitchison TJ, Moskowitz MA, Yuan J (2005) Chemical inhibitor of nonapoptotic cell death with therapeutic potential for ischemic brain injury. Nat Chem Biol 1:112–119

Degterev A, Maki J, Yuan J (2013) Activity and specificity of necrostatin-1, small-molecule inhibitor of RIP1 kinase. Cell Death Differ 20:366

Delgado ME, Olsson M, Lincoln FA, Zhivotovsky B, Rehm M (2013) Determining the contributions of caspase-2, caspase-8 and effector caspases to intracellular VDVADase activities during apoptosis initiation and execution. Biochim Biophys Acta 1833:2279–2292

Dixon SJ, Lemberg KM, Lamprecht MR, Skouta R, Zaitsev EM, Gleason CE, Patel DN, Bauer AJ, Cantley AM, Yang WS, Morrison B 3rd, Stockwell BR (2012) Ferroptosis: an iron-dependent form of nonapoptotic cell death. Cell 149:1060–1072

Dondelinger Y, Aguileta MA, Goossens V, Dubuisson C, Grootjans S, Dejardin E, Vandenabeele P, Bertrand MJ (2013) RIPK3 contributes to TNFR1-mediated RIPK1 kinase-dependent apoptosis in conditions of cIAP1/2 depletion or TAK1 kinase inhibition. Cell Death Differ 20:1381–1392

Duan WR, Garner DS, Williams SD, Funckes-Shippy CL, Spath IS, Blomme EA (2003) Comparison of immunohistochemistry for activated caspase-3 and cleaved cytokeratin 18 with the TUNEL method for quantification of apoptosis in histological sections of PC-3 subcutaneous xenografts. J Pathol 199:221–228

Duprez L, Bertrand MJ, Vanden Berghe T, Dondelinger Y, Festjens N, Vandenabeele P (2012) Intermediate domain of receptor-interacting protein kinase 1 (RIPK1) determines switch between necroptosis and RIPK1 kinase-dependent apoptosis. J Biol Chem 287:14863–14872

Duprez L, Takahashi N, Van Hauwermeiren F, Vandendriessche B, Goossens V, Vanden Berghe T, Declercq W, Libert C, Cauwels A, Vandenabeele P (2011) RIP kinase-dependent necrosis drives lethal systemic inflammatory response syndrome. Immunity 35:908–918

Festjens N, Kalai M, Smet J, Meeus A, Van Coster R, Saelens X, Vandenabeele P (2006) Butylated hydroxyanisole is more than a reactive oxygen species scavenger. Cell Death Differ 13:166–169

Figueroa RA, Ramberg V, Gatsinzi T, Samuelsson M, Zhang M, Iverfeldt K, Hallberg E (2011) Anchored FRET sensors detect local caspase activation prior to neuronal degeneration. Mol Neurodegener 6:35

Fonge H, Chitneni SK, Lixin J, Vunckx K, Prinsen K, Nuyts J, Mortelmans L, Bormans G, Ni Y, Verbruggen A (2007) Necrosis avidity of (99m)Tc(CO)3-labeled pamoic acid derivatives: synthesis and preliminary biological evaluation in animal models of necrosis. Bioconjug Chem 18:1924–1934

Galban S, Jeon YH, Bowman BM, Stevenson J, Sebolt KA, Sharkey LM, Lafferty M, Hoff BA, Butler BL, Wigdal SS, Binkowski BF, Otto P, Zimmerman K, Vidugiris G, Encell LP, Fan F,

Wood KV, Galban CJ, Ross BD, Rehemtulla A (2013) Imaging proteolytic activity in live cells and animal models. PLoS One 8:e66248
Galluzzi L, Vitale I, Abrams JM, Alnemri ES, Baehrecke EH, Blagosklonny MV, Dawson TM, Dawson VL, El-Deiry WS, Fulda S, Gottlieb E, Green DR, Hengartner MO, Kepp O, Knight RA, Kumar S, Lipton SA, Lu X, Madeo F, Malorni W, Mehlen P, Nunez G, Peter ME, Piacentini M, Rubinsztein DC, Shi Y, Simon HU, Vandenabeele P, White E, Yuan J, Zhivotovsky B, Melino G, Kroemer G (2012) Molecular definitions of cell death subroutines: recommendations of the Nomenclature Committee on Cell Death 2012. Cell Death Differ 19:107–120
Gavrieli Y, Sherman Y, Ben-Sasson SA (1992) Identification of programmed cell death in situ via specific labeling of nuclear DNA fragmentation. J Cell Biol 119:493–501
Goossens V, Grooten J, De Vos K, Fiers W (1995) Direct evidence for tumor necrosis factor-induced mitochondrial reactive oxygen intermediates and their involvement in cytotoxicity. Proc Natl Acad Sci U S A 92:8115–8119
Goossens V, Stange G, Moens K, Pipeleers D, Grooten J (1999) Regulation of tumor necrosis factor-induced, mitochondria- and reactive oxygen species-dependent cell death by the electron flux through the electron transport chain complex I. Antioxid Redox Signal 1:285–295
Grimberg H, Levin G, Shirvan A, Cohen A, Yogev-Falach M, Reshef A, Ziv I (2009) Monitoring of tumor response to chemotherapy in vivo by a novel small-molecule detector of apoptosis. Apoptosis 14:257–267
Gukovskaya AS, Perkins P, Zaninovic V, Sandoval D, Rutherford R, Fitzsimmons T, Pandol SJ, Poucell-Hatton S (1996) Mechanisms of cell death after pancreatic duct obstruction in the opossum and the rat. Gastroenterology 110:875–884
Gunther C, Martini E, Wittkopf N, Amann K, Weigmann B, Neumann H, Waldner MJ, Hedrick SM, Tenzer S, Neurath MF, Becker C (2011) Caspase-8 regulates TNF-alpha-induced epithelial necroptosis and terminal ileitis. Nature 477:335–339
He L, Wu X, Meylan F, Olson DP, Simone J, Hewgill D, Siegel R, Lipsky PE (2004) Monitoring caspase activity in living cells using fluorescent proteins and flow cytometry. Am J Pathol 164:1901–1913
He S, Wang L, Miao L, Wang T, Du F, Zhao L, Wang X (2009) Receptor interacting protein kinase-3 determines cellular necrotic response to TNF-alpha. Cell 137:1100–1111
Herbst KJ, Allen MD, Zhang J (2011) Luminescent kinase activity biosensors based on a versatile bimolecular switch. J Am Chem Soc 133:5676–5679
Holler N, Zaru R, Micheau O, Thome M, Attinger A, Valitutti S, Bodmer JL, Schneider P, Seed B, Tschopp J (2000) Fas triggers an alternative, caspase-8-independent cell death pathway using the kinase RIP as effector molecule. Nat Immunol 1:489–495
Hsu PD, Scott DA, Weinstein J, Ran F, Konermann S, Agarwala V, Li Y, Fine E, Wu X, Shalem O, Cradick T, Marraffini L, Bao G, Zhang F (2013) DNA targeting specificity of RNA-guided Cas9 nucleases. Nat. Biotechnol 31:827–832
Jouan-Lanhouet S, Arshad MI, Piquet-Pellorce C, Martin-Chouly C, Le Moigne-Muller G, Van Herreweghe F, Takahashi N, Sergent O, Lagadic-Gossmann D, Vandenabeele P, Samson M, Dimanche-Boitrel MT (2012) TRAIL induces necroptosis involving RIPK1/RIPK3-dependent PARP-1 activation. Cell Death Differ 19:2003–2014
Kaiser AM, Saluja AK, Sengupta A, Saluja M, Steer ML (1995) Relationship between severity, necrosis, and apoptosis in five models of experimental acute pancreatitis. Am J Physiol 269:C1295–C1304
Kang TB, Yang SH, Toth B, Kovalenko A, Wallach D (2013) Caspase-8 Blocks Kinase RIPK3-Mediated Activation of the NLRP3 Inflammasome. Immunity 38:27–40
Kelliher MA, Grimm S, Ishida Y, Kuo F, Stanger BZ, Leder P (1998) The death domain kinase RIP mediates the TNF-induced NF-kappaB signal. Immunity 8:297–303
Kerr JF, Wyllie AH, Currie AR (1972) Apoptosis: a basic biological phenomenon with wide-ranging implications in tissue kinetics. Br J Cancer 26:239–257
Kersse K, Verspurten J, Vanden Berghe T, Vandenabeele P (2011) The death-fold superfamily of homotypic interaction motifs. Trends Biochem Sci 36:541–552

Kim HJ, Koo SY, Ahn BH, Park O, Park DH, Seo DO, Won JH, Yim HJ, Kwak HS, Park HS, Chung CW, Oh YL, Kim SH (2010) NecroX as a novel class of mitochondrial reactive oxygen species and ONOO(−) scavenger. Arch Pharm Res 33:1813–1823

Kim YS, Morgan MJ, Choksi S, Liu ZG (2007) TNF-induced activation of the Nox1 NADPH oxidase and its role in the induction of necrotic cell death. Mol Cell 26:675–687

Knott G, Rosset S, Cantoni M (2011) Focussed ion beam milling and scanning electron microscopy of brain tissue. J Vis Exp (53):e2588

Kockx MM, Muhring J, Knaapen MW, de Meyer GR (1998) RNA synthesis and splicing interferes with DNA in situ end labeling techniques used to detect apoptosis. Am J Pathol 152:885–888

Kominami K, Nagai T, Sawasaki T, Tsujimura Y, Yashima K, Sunaga Y, Tsuchimochi M, Nishimura J, Chiba K, Nakabayashi J, Koyamada K, Endo Y, Yokota H, Miyawaki A, Manabe N, Sakamaki K (2012) In vivo imaging of hierarchical spatiotemporal activation of caspase-8 during apoptosis. PLoS One 7:e50218

Kourtis N, Nikoletopoulou V, Tavernarakis N (2012) Small heat-shock proteins protect from heatstroke-associated neurodegeneration. Nature 490:213–218

Krysko DV, Agostinis P, Krysko O, Garg AD, Bachert C, Lambrecht BN, Vandenabeele P (2011) Emerging role of damage-associated molecular patterns derived from mitochondria in inflammation. Trends Immunol 32:157–164

Krysko DV, Brouckaert G, Kalai M, Vandenabeele P, D'Herde K (2003) Mechanisms of internalization of apoptotic and necrotic L929 cells by a macrophage cell line studied by electron microscopy. J Morphol 258:336–345

Kuenzler KA, Pearson PY, Schwartz MZ (2002) IL-11 pretreatment reduces cell death after intestinal ischemia-reperfusion. J Surg Res 108:268–272

Kuranaga E (2012) Beyond apoptosis: caspase regulatory mechanisms and functions in vivo. Genes Cells 17:83–97

Lamkanfi M, Festjens N, Declercq W, Vanden Berghe T, Vandenabeele P (2007) Caspases in cell survival, proliferation and differentiation. Cell Death Differ 14:44–55

Laster SM, Wood JG, Gooding LR (1988) Tumor necrosis factor can induce both apoptotic and necrotic forms of cell lysis. J Immunol 141:2629–2634

Laussmann MA, Passante E, Dussmann H, Rauen JA, Wurstle ML, Delgado ME, Devocelle M, Prehn JH, Rehm M (2011) Proteasome inhibition can induce an autophagy-dependent apical activation of caspase-8. Cell Death Differ 18:1584–1597

Li J, Cona MM, Chen F, Feng Y, Zhou L, Yu J, Nuyts J, de Witte P, Zhang J, Himmelreich U, Verbruggen A, Ni Y (2012) Exploring theranostic potentials of radioiodinated hypericin in rodent necrosis models. Theranostics 2:1010–1019

Linkermann A, Bräsen JH, Darding M, Jin MK, Sanz AB, Heller JO, De Zen F, Weinlich R, Ortiz A, Walczak H, Weinberg JM, Green DR, Kunzendorf U, Krautwald S (2013) Two independent pathways of regulated necrosis mediate ischemia-reperfusion injury. Proc Natl Acad Sci U S A 110:12024–12029

Louis KS, Siegel AC (2011) Cell viability analysis using trypan blue: manual and automated methods. Methods Mol Biol 740:7–12

Luthi AU, Cullen SP, McNeela EA, Duriez PJ, Afonina IS, Sheridan C, Brumatti G, Taylor RC, Kersse K, Vandenabeele P, Lavelle EC, Martin SJ (2009) Suppression of interleukin-33 bioactivity through proteolysis by apoptotic caspases. Immunity 31:84–98

Mahajan NP, Harrison-Shostak DC, Michaux J, Herman B (1999) Novel mutant green fluorescent protein protease substrates reveal the activation of specific caspases during apoptosis. Chem Biol 6:401–409

Mariani G, Villa G, Rossettin PF, Spallarossa P, Bezante GP, Brunelli C, Pak KY, Khaw BA, Strauss HW (1999) Detection of acute myocardial infarction by 99mTc-labeled D-glucaric acid imaging in patients with acute chest pain. J Nucl Med 40:1832–1839

Morris MC (2013) Fluorescent biosensors—probing protein kinase function in cancer and drug discovery. Biochim Biophys Acta 1834:1387–1395

Nagata S (2000) Apoptotic DNA fragmentation. Exp Cell Res 256:12–18

Nagata S (2002) Breakdown of chromosomal DNA. Cornea 21:S2–S6

Nakagawa T, Shimizu S, Watanabe T, Yamaguchi O, Otsu K, Yamagata H, Inohara H, Kubo T, Tsujimoto Y (2005) Cyclophilin D-dependent mitochondrial permeability transition regulates some necrotic but not apoptotic cell death. Nature 434:652–658

Napirei M, Wulf S, Mannherz HG (2004) Chromatin breakdown during necrosis by serum Dnase1 and the plasminogen system. Arthritis Rheum 50:1873–1883

Nguyen QD, Smith G, Glaser M, Perumal M, Arstad E, Aboagye EO (2009) Positron emission tomography imaging of drug-induced tumor apoptosis with a caspase-3/7 specific [18F]-labeled isatin sulfonamide. Proc Natl Acad Sci U S A 106:16375–16380

Ni Y, Huyghe D, Verbeke K, de Witte PA, Nuyts J, Mortelmans L, Chen F, Marchal G, Verbruggen AM, Bormans GM (2006) First preclinical evaluation of mono-[123I]iodohypericin as a necrosis-avid tracer agent. Eur J Nucl Med Mol Imaging 33:595–601

Ni Y, Petre C, Miao Y, Yu J, Cresens E, Adriaens P, Bosmans H, Semmler W, Baert AL, Marchal G (1997) Magnetic resonance imaging-histomorphologic correlation studies on paramagnetic metalloporphyrins in rat models of necrosis. Invest Radiol 32:770–779

Nicoletti I, Migliorati G, Pagliacci MC, Grignani F, Riccardi C (1991) A rapid and simple method for measuring thymocyte apoptosis by propidium iodide staining and flow cytometry. J Immunol Methods 139:271–279

Perfetto SP, Chattopadhyay PK, Lamoreaux L, Nguyen R, Ambrozak D, Koup RA, Roederer M (2006) Amine reactive dyes: an effective tool to discriminate live and dead cells in polychromatic flow cytometry. J Immunol Methods 313:199–208

Pick E, Keisari Y (1980) A simple colorimetric method for the measurement of hydrogen peroxide produced by cells in culture. J Immunol Methods 38:161–170

Pop C, Salvesen GS (2009) Human caspases: activation, specificity, and regulation. J Biol Chem 284:21777–21781

Pop C, Salvesen GS, Scott FL (2008) Caspase assays: identifying caspase activity and substrates in vitro and in vivo. Methods Enzymol 446:351–367

Prinsen K, Jin L, Vunckx K, De Saint-Hubert M, Zhou L, Cleynhens J, Nuyts J, Bormans G, Ni Y, Verbruggen A (2011) Radiolabeling and preliminary biological evaluation of a (99m)Tc(CO)(3) labeled 3,3′-(benzylidene)-bis-(1H-indole-2-carbohydrazide) derivative as a potential SPECT tracer for in vivo visualization of necrosis. Bioorg Med Chem Lett 21:502–505

Rehm M, Dussmann H, Prehn JH (2003) Real-time single cell analysis of Smac/DIABLO release during apoptosis. J Cell Biol 162:1031–1043

Remijsen Q, Goossens V, Grootjans S, Van den Haute C, Vanlangenakker N, Dondelinger Y, Roelandt R, Bruggeman I, Goncalves A, Bertrand MJ, Baekelandt V, Takahashi N, Berghe TV, Vandenabeele P (2014) Depletion of RIPK3 or MLKL blocks TNF-driven necroptosis and switches towards a delayed RIPK1 kinase-dependent apoptosis. Cell Death Dis 5:e1004. doi:10.1038/cddis.2013.531. PMID: 24434512

Scaffidi P, Misteli T, Bianchi ME (2002) Release of chromatin protein HMGB1 by necrotic cells triggers inflammation. Nature 418:191–195

Schulze-Osthoff K, Beyaert R, Vandevoorde V, Haegeman G, Fiers W (1993) Depletion of the mitochondrial electron transport abrogates the cytotoxic and gene-inductive effects of TNF. EMBO J 12:3095–3104

Seiler A, Schneider M, Forster H, Roth S, Wirth EK, Culmsee C, Plesnila N, Kremmer E, Radmark O, Wurst W, Bornkamm GW, Schweizer U, Conrad M (2008) Glutathione peroxidase 4 senses and translates oxidative stress into 12/15-lipoxygenase dependent- and AIF-mediated cell death. Cell Metab 8:237–248

Shen S, Kepp O, Michaud M, Martins I, Minoux H, Metivier D, Maiuri MC, Kroemer RT, Kroemer G (2011) Association and dissociation of autophagy, apoptosis and necrosis by systematic chemical study. Oncogene 30:4544–4556

Shinzawa K, Tsujimoto Y (2003) PLA2 activity is required for nuclear shrinkage in caspase-independent cell death. J Cell Biol 163:1219–1230

Shu X, Lev-Ram V, Deerinck TJ, Qi Y, Ramko EB, Davidson MW, Jin Y, Ellisman MH, Tsien RY (2011) A genetically encoded tag for correlated light and electron microscopy of intact cells, tissues, and organisms. PLoS Biol 9:e1001041

Sipieter F, Vandame P, Spriet C, Leray A, Vincent P, Trinel D, Bodart JF, Riquet FB, Héliot L (2013) From FRET imaging to practical methodology for kinase activity sensing in living cells. Prog Mol Biol Transl Sci 113:145–216. doi:10.1016/B978-0-12-386932-6.00005-3. PMID:23244791

Sipieter F, Ladik M, Vandenabeele P, Riquet F (2014) Shining light on cell death processes - a novel biosensor for necroptosis, a newly described cell death program. Biotechnol J. PMID:24390900

Smith CC, Davidson SM, Lim SY, Simpkin JC, Hothersall JS, Yellon DM (2007) Necrostatin: a potentially novel cardioprotective agent? Cardiovasc Drugs Ther 21:227–233

Smith CV (1987) Evidence for participation of lipid peroxidation and iron in diquat-induced hepatic necrosis in vivo. Mol Pharmacol 32:417–422

Smith G, Glaser M, Perumal M, Nguyen QD, Shan B, Arstad E, Aboagye EO (2008) Design, synthesis, and biological characterization of a caspase 3/7 selective isatin labeled with 2-[18F] fluoroethylazide. J Med Chem 51:8057–8067

Sosna J, Voigt S, Mathieu S, Lange A, Thon L, Davarnia P, Herdegen T, Linkermann A, Rittger A, Chan FK, Kabelitz D, Schütze S, Adam D (2014) TNF-induced necroptosis and PARP-1-mediated necrosis represent distinct routes to programmed necrotic cell death. Cell Mol Life Sci 71:331–348

Stennicke H, Renatus M, Meldal M, Salvesen G (2000) Internally quenched fluorescent peptide substrates disclose the subsite preferences of human caspases 1, 3, 6, 7 and 8. Biochem J 350(Pt 2):563–568

Suffys P, Beyaert R, De Valck D, Vanhaesebroeck B, Van Roy F, Fiers W (1991) Tumour-necrosis-factor-mediated cytotoxicity is correlated with phospholipase-A2 activity, but not with arachidonic acid release per se. Eur J Biochem/FEBS 195:465–475

Sun L, Wang H, Wang Z, He S, Chen S, Liao D, Wang L, Yan J, Liu W, Lei X, Wang X (2012) Mixed lineage kinase domain-like protein mediates necrosis signaling downstream of RIP3 kinase. Cell 148:213–227

Takahashi N, Duprez L, Grootjans S, Cauwels A, Nerinckx W, DuHadaway JB, Goossens V, Roelandt R, Van Hauwermeiren F, Libert C, Declercq W, Callewaert N, Prendergast GC, Degterev A, Yuan J, Vandenabeele P (2012) Necrostatin-1 analogues: critical issues on the specificity, activity and in vivo use in experimental disease models. Cell Death Dis 3:e437

Thal SE, Zhu C, Thal SC, Blomgren K, Plesnila N (2011) Role of apoptosis inducing factor (AIF) for hippocampal neuronal cell death following global cerebral ischemia in mice. Neurosci Lett 499:1–3

Vanden Berghe T, Grootjans S, Goossens V, Dondelinger Y, Krysko DV, Takahashi N, Vandenabeele P (2013) Determination of apoptotic and necrotic cell death in vitro and in vivo. Methods 61:117–129

Vanden Berghe T, Kalai M, van Loo G, Declercq W, Vandenabeele P (2003) Disruption of HSP90 function reverts tumor necrosis factor-induced necrosis to apoptosis. J Biol Chem 278:5622–5629

Vanden Berghe T, Vanlangenakker N, Parthoens E, Deckers W, Devos M, Festjens N, Guerin CJ, Brunk UT, Declercq W, Vandenabeele P (2010) Necroptosis, necrosis and secondary necrosis converge on similar cellular disintegration features. Cell Death Differ 17:922–930

Vandenabeele P, Galluzzi L, Vanden Berghe T, Kroemer G (2010) Molecular mechanisms of necroptosis: an ordered cellular explosion. Nat Rev Mol Cell Biol 11:700–714

Vandenabeele P, Grootjans S, Callewaert N, Takahashi N (2013) Necrostatin-1 blocks both RIPK1 and IDO: consequences for the study of cell death in experimental disease models. Cell Death Differ 20:185–187

Vanlangenakker N, Bertrand MJ, Bogaert P, Vandenabeele P, Vanden Berghe T (2011a) TNF-induced necroptosis in L929 cells is tightly regulated by multiple TNFR1 complex I and II members. Cell Death Dis 2:e230

Vanlangenakker N, Vanden Berghe T, Bogaert P, Laukens B, Zobel K, Deshayes K, Vucic D, Fulda S, Vandenabeele P, Bertrand MJ (2011b) cIAP1 and TAK1 protect cells from TNF-induced necrosis by preventing RIP1/RIP3-dependent reactive oxygen species production. Cell Death Differ 18:656–665

Vanlangenakker N, Vanden Berghe T, Vandenabeele P (2012) Many stimuli pull the necrotic trigger, an overview. Cell Death Differ 19:75–86

Vercammen D, Beyaert R, Denecker G, Goossens V, Van Loo G, Declercq W, Grooten J, Fiers W, Vandenabeele P (1998a) Inhibition of caspases increases the sensitivity of L929 cells to necrosis mediated by tumor necrosis factor. J Exp Med 187:1477–1485

Vercammen D, Brouckaert G, Denecker G, Van de Craen M, Declercq W, Fiers W, Vandenabeele P (1998b) Dual signaling of the Fas receptor: initiation of both apoptotic and necrotic cell death pathways. J Exp Med 188:919–930

Vince JE, Wong WW, Gentle I, Lawlor KE, Allam R, O'Reilly L, Mason K, Gross O, Ma S, Guarda G, Anderton H, Castillo R, Häcker G, Silke J, Tschopp J (2012) Inhibitor of Apoptosis Proteins Limit RIP3 Kinase-Dependent Interleukin-1 Activation. Immunity 36:215–227

Wainwright M (2010) Dyes, trypanosomiasis and DNA: a historical and critical review. Biotech Histochem 85:341–354

Walker PR, Leblanc J, Smith B, Pandey S, Sikorska M (1999) Detection of DNA fragmentation and endonucleases in apoptosis. Methods 17:329–338

Wang K, Li J, Degterev A, Hsu E, Yuan J, Yuan C (2007) Structure-activity relationship analysis of a novel necroptosis inhibitor, Necrostatin-5. Bioorg Med Chem Lett 17:1455–1465

Wang L, Du F, Wang X (2008) TNF-alpha induces two distinct caspase-8 activation pathways. Cell 133:693–703

Wendland MF, Faustino J, West T, Manabat C, Holtzman DM, Vexler ZS (2008) Early diffusion-weighted MRI as a predictor of caspase-3 activation after hypoxic-ischemic insult in neonatal rodents. Stroke 39:1862–1868

Williams TM, Nyati S, Ross BD, Rehemtulla A (2013) Molecular Imaging of the ATM Kinase Activity. Int J Radiat Oncol Biol Phys 86:969–977

Wu P, Nicholls SB, Hardy JA (2013) A tunable, modular approach to fluorescent protease-activated reporters. Biophys J 104:1605–1614

Wu Y, Wang D, Wang X, Wang Y, Ren F, Chang D, Chang Z, Jia B (2011) Caspase 3 is activated through caspase 8 instead of caspase 9 during H_2O_2-induced apoptosis in HeLa cells. Cell Physiol Biochem 27:539–546

Wyllie A (1981) Cell death: a new classification separating apoptosis from necrosis. In: Lockshin RA (ed) Cell death in biology and pathology. Chapman & Hall, London, pp 9–34

Xu X, Chua CC, Zhang M, Geng D, Liu CF, Hamdy RC, Chua BH (2010) The role of PARP activation in glutamate-induced necroptosis in HT-22 cells. Brain Res 1343:206–212

Yamashima T (2000) Implication of cysteine proteases calpain, cathepsin and caspase in ischemic neuronal death of primates. Prog Neurobiol 62:273–295

Yazdanpanah B, Wiegmann K, Tchikov V, Krut O, Pongratz C, Schramm M, Kleinridders A, Wunderlich T, Kashkar H, Utermohlen O, Bruning JC, Schutze S, Kronke M (2009) Riboflavin kinase couples TNF receptor 1 to NADPH oxidase. Nature 460:1159–1163

Yoshioka A, Yamaya Y, Saiki S, Kanemoto M, Hirose G, Beesley J, Pleasure D (2000) Non-N-methyl-D-aspartate glutamate receptors mediate oxygen–glucose deprivation-induced oligodendroglial injury. Brain Res 854:207–215

You Z, Savitz SI, Yang J, Degterev A, Yuan J, Cuny GD, Moskowitz MA, Whalen MJ (2008) Necrostatin-1 reduces histopathology and improves functional outcome after controlled cortical impact in mice. J Cereb Blood Flow Metab 28:1564–1573

Zhang DW, Shao J, Lin J, Zhang N, Lu BJ, Lin SC, Dong MQ, Han J (2009) RIP3, an energy metabolism regulator that switches TNF-induced cell death from apoptosis to necrosis. Science 325:332–336

Zhang J, Ye J, Altafaj A, Cardona M, Bahi N, Llovera M, Canas X, Cook SA, Comella JX, Sanchis D (2011) EndoG links Bnip3-induced mitochondrial damage and caspase-independent DNA fragmentation in ischemic cardiomyocytes. PLoS One 6:e17998

Zhang Q, Raoof M, Chen Y, Sumi Y, Sursal T, Junger W, Brohi K, Itagaki K, Hauser CJ (2010) Circulating mitochondrial DAMPs cause inflammatory responses to injury. Nature 464:104–107

Appendix
Physiology and Function of the Ripoptosome: An Intracellular Signalling Platform Regulating Apoptosis and Necroptosis

Peter Geserick, Maria Feoktistova, and Martin Leverkus

1.1 Introduction

1.1.1 The Ripoptosome, a Signalling Platform That Regulates Qualitative and Quantitative Cell Death Responses

The balance between cellular proliferation and cell death is of paramount importance for cellular homeostasis and therefore for the survival of the mammalian organism. Cancer or pathogen-induced diseases can lead to deregulation of this cellular balance, which leads to disorder of organs' function and can finally promote decease of the individuals. Durable communication of a cell with its microenvironment stimulates intracellular signalling cascades, which allow cells either to survey the conditions for proliferative expansion and survival or to trigger their own cell death. Intracellular signalling protein complexes play an indispensable role for cellular homeostasis. Many signalling platforms have been identified and named in relation to their function, e.g., apoptosome for apoptosis, necrosome for necrosis (for review *see* Dickens et al. 2012). The recently described Ripoptosome operates as a regulator of many signalling processes including different cell death types. We understand and describe the Ripoptosome as a "docking station," a central protein complex formed upon cellular stress that can participate in different proliferative, inflammatory, or cell death signalling pathways. This chapter refers to the molecular requirements for Ripoptosome formation, describes its regulation and downstream signalling, and points to physiological and pathophysiological implications.

Peter Geserick and Maria Feoktistova have equally contributed to this chapter

P. Geserick • M. Feoktistova • M. Leverkus (✉)
Section for Molecular Dermatology, Department of Dermatology, Venereology, and Allergology, Medical Faculty Mannheim, University of Heidelberg, Theodor-Kutzer-Ufer 1-3, 68167 Mannheim, Germany
e-mail: Martin.Leverkus@medma.uni-heidelberg.de

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1.1.1.1 Competing Programs of Death: Apoptosis Versus Necroptosis

Apoptosis is distinguished from necrosis by the ordered activation of cysteine proteases (Caspases). Caspases can be activated by extrinsic stimuli like death receptors (DR) or by cell-intrinsic activation of mitochondria. Upon cytokine-mediated (TRAIL or CD95L) activation of the respective DRs (TRAIL-R1, CD95) the DISC (death-inducing signalling complex) containing procaspase-8, Receptor interacting protein 1 (RIP1), FLICE-like inhibitory protein (cFLIP), and Fas-associated death domain (FADD) is formed. DISC formation finally results in activation and subsequent cleavage of caspase-8. Then caspase-8 processes its substrate Bid to truncated Bid (tBid), a member of the BH3-only subgroup of the Bcl-2 family that promotes mitochondrial outer membrane permeabilization (MOMP). In turn MOMP leads to the release of a number of proapoptotic molecules including cytochrome c from the mitochondrial outer membrane space (Ashida et al. 2011). Alternatively caspase-8 directly activates caspase-3, which then leads to apoptosis execution.

In contrast to necrosis, necroptosis is another form of programmed cell death that shares features of apoptosis and necrosis (Vanlangenakker et al. 2012). Necroptosis is in general considered to be mediated by the kinase activity of RIP1 and its interacting family member RIP3. RIP1 kinase phosphorylates RIP3 (Cho et al. 2009) that promotes necroptosis presumably by activation of a number of metabolic enzymes that are involved in ROS (reactive oxygen species) production (Zhang et al. 2009). Suppression of RIP1 kinase activity by the inhibitor necrostatin-1 inhibits necroptotic responses (Degterev et al. 2008). Intriguing new evidence about the molecular interplay of apoptosis and necroptosis came from elegant in vivo experiments which showed that caspase-8 is a critical negative regulator of necroptosis (Kaiser et al. 2011; Oberst et al. 2011; Zhang et al. 2011). These data argue that RIP1 and caspase-8 may directly interact in a signalling platform required for both processes; apoptosis and necroptosis. In this context the concept of the Ripoptosome unifies both cell biological responses. The composition of the complex as well as the stoichiometric ratio of complex-associated proteins defines the cell death quality, while the kinase RIP1 being the most important constituent (Feoktistova et al. 2011; Tenev et al. 2011).

1.1.1.2 RIP1-Containing Complexes: What Distinguishes the Ripoptosome from Others?

It has been noted over the past years that diverse complexes involved in different death signalling pathways contain the same molecules and may finally promote similar pathophysiological consequences. RIP1 is found in numerous membrane-associated complexes (e.g., TNF Complex I, TRAIL or CD95 DISC) or intracellular complexes either associated with organelles (TLR3, RIG-I, mitochondria complex) or cytoplasmic (necrosome, TNF complex II, TRAIL complex II, CD95 complex II). Importantly, the components of the Ripoptosome complex include caspase-8, FADD, RIP1, and cFLIP isoforms that are known to participate in some of the various signalling platforms (compare Fig. A.1). The localization or translocation of RIP1 to the

different signalling platforms outlined below is determined by the recruitment of such other proteins to the specific protein complex.

RIP1, the core molecule of the Ripoptosome, potently interacts with adaptor molecules FADD, TRADD (Tumor necrosis factor receptor type 1-associated death domain), and FAF (Fas-associated factor) as well as with death receptors (TRAIL-R´s, CD95, TNF-R1) via the death domain (DD). RIP1 can also interact via its RIP homotypic interacting motif (RHIM) with TLR3 (Toll-like receptor 3), TRIF, RIP3, or DNA-dependent activator of interferon regulatory factors (DAI). In addition RIP1 binds to the cytomegalovirus (CMV) viral protein M45 via its RHIM. This multitude of possible RIP1 interaction partners with functions for cell death regulation potentially allows for formation of diverse RIP1-containing complexes involved in various intracellular processes (Fig. A.1 for review *see* Han et al. 2011).

Based upon biochemical interaction studies, assembly of different RIP1-containing platforms was shown after diverse stimuli. Thus extracellular stimuli, stress responses as well as interaction with pathogen-activated pathways are likely to determine formation and subcellular localization of these protein complexes. The assembly of the signalling platforms depends on diverse posttranslational modifications including phosphorylation and ubiquitination of its components. These modifications regulate intracellular localization, translocation, and potentially disassembly of the RIP1-containing complexes, likely by proteasome-dependent degradation. For example, Ripoptosome formation requires the availability (e.g., an excess) of non-ubiquitinated RIP1 (Feoktistova et al. 2011). One important function of the ubiquitin ligase activity of cIAPs is the inhibition of platform formation. In the remainder of this subchapter we will describe the different RIP1 complexes, whose formation is initiated by quite diverse triggers.

TLR3 Complex

RIP1 is critically involved in the receptor complex of TLR3, a pattern-recognition receptor known to initiate an innate immune response when stimulated by double-stranded RNA (dsRNA). Upon stimulation TLR3 is recruiting TIR-domain-containing adapter-inducing interferon-β (TRIF), which is recruiting RIP1 via its RHIM. RIP1 then recruits FADD via its DD, and FADD, in turn, recruits pro-caspase-8 via its death effector domain (DED) (Estornes et al. 2012). This complex induces apoptosis in the presence of predominantly active caspase-8 (Estornes et al. 2012; Feoktistova et al. 2011), but with the necroptotic activity of the Ripoptosome when RIP1 is active and caspase activity is blocked.

RIG-I Complex

The RIG-I complex participates in the detection of specific RNA viruses, including Newcastle disease virus, Sendai virus (SeV), vesicular stomatitis virus, influenza virus, and Japanese encephalitis virus (Roth and Ruland 2011). The retinoic acid inducible gene I (RIG-I) is a receptor which possesses a central DExD/H-box RNA

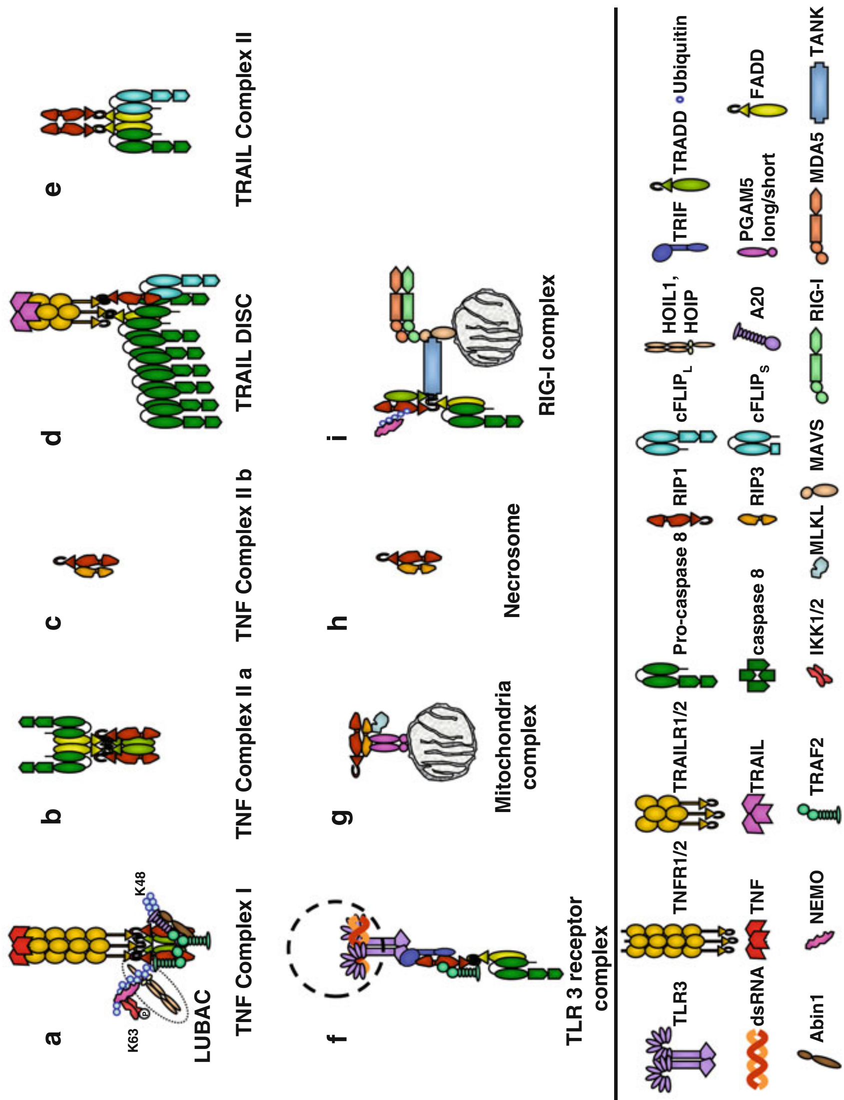
a
K63
K48
LUBAC
TNF Complex I
b
TNF Complex II a
c
TNF Complex II b
d
TRAIL DISC
e
TRAIL Complex II
f
TLR 3 receptor complex
g
Mitochondria complex
h
Necrosome
i
RIG-I complex
TLR3
TNFR1/2
TRAILR1/2
Pro-caspase 8
RIP1
cFLIP$_L$
HOIL1, HOIP
TRIF
TRADD
Ubiquitin
dsRNA
TNF
TRAIL
caspase 8
RIP3
cFLIP$_S$
A20
PGAM5 long/short
FADD
Abin1
NEMO
TRAF2
IKK1/2
MLKL
MAVS
RIG-I
MDA5
TANK

Fig. A.1 RIP1 is a constituent of cytoplasmic, membrane-, and organelle-associated complexes. RIP1 containing complexes are either membrane associated (**a**, **d**), like TNF and TRAIL DISC or cytoplasmic, such as TNF complex IIa and IIb (**b**, **c**), TRAIL complex II (**e**), or the Necrosome (**h**). Within membrane-associated complexes (TNFR1 complex I and TRAIL DISC) RIP1 is modified. RIP1 within TNF complex I is targeted by ubiquitin-editing proteins such as cIAPs, A20, Abin1, TRAF2, or CYLD. The form of ubiquitin chains defines further association with NEMO and/or IKK1/2 that leads to NF-**κ**B activation. A20-mediated deubiquitination followed by K48 ubiquitin-linking of RIP1 is required for proteasomal degradation (**a**). Translocation of RIP1 from complex I allows complex IIa formation where caspase-8, FADD, and cFLIP isoforms are recruited (**b**) that triggers apoptotic responses. Alternatively they form TNF complex IIb that promotes necrotic responses (**c**). In contrast, caspase-8 chain assembly directly occurs during activation of TRAIL-R by cytokine TRAIL and this DISC formation is FADD dependent (**d**). Translocation of the receptor-mediated signal to TRAIL complex II occurs, but is poorly understood to date (**e**). Assembly of RIP1-containing complexes also occur within organelles such as mitochondria (**g**, **i**: RIG-I complex) or endosomes (**f**: TLR3 complex). Stimulation of TLR3 receptor with respective agonists (Poly (I:C), double strand RNA) allows TRIF recruitment to the receptor and further association of RIP1, FADD, and caspase-8 (**f**). The apoptotic or necroptotic response of this complex depends on either caspase or RIP1/RIP3 activities. RIP1/RIP3 assembly is either mediated by death receptor stimulation or by self-assembly upon cellular stress. This complex promotes necrosis or necroptosis and was named necrosome (**h**). Mitochondria-associated RIP1 containing complex either depends on the direct interaction of different PGAM5 isoforms (**g**) or within the RIG-I complex from MAVS (**i**)

helicase domain and a C-terminal repressor domain for ligand binding together with two caspase recruitment domains (CARDs) for signal propagation. Upon RNA sensing RIG-I induces an intramolecular conformational change, leading to association with the mitochondrial antiviral signalling adaptor (MAVS) (Yoneyama and Fujita 2009). These events enable the formation of a large mitochondria-associated complex that contains several conserved signalling proteins, including RIP1, TRADD, FADD, and caspase-8 (Yoneyama and Fujita 2009). RIP1 and caspase-8 serve antagonistic regulatory roles: conjugation of ubiquitin chains to Lysine 377 of RIP1 facilitates assembly of the RIG-I complex, whereas caspase-8 cleaves RIP1 at D324, which inhibits further signalling (Rajput et al. 2011). Furthermore, NEMO is recruited to the activated RIG-I complex, which is essential for downstream signalling to interferon regulatory factors (IRFs) and NF-κB via IKK-related kinases (Roth and Ruland 2011). Although the RIG-I complex contains crucial molecules for cell death execution such as RIP1 and caspase-8, the function of the RIG-I complex for cell death signalling has not been elucidated thus far. However, overexpression of MDA5 was shown to induce cell death, pointing to a potential role of the RIG-I complex as a death promoter under certain conditions (Kang et al. 2004).

TNF-Induced Signalling Complexes

The most widely studied pro-necrotic RIP1-containing signalling platform is complex IIb initiated by the TNF signalling cascade in which RIP1 interacts with RIP3 (reviewed in Yuan and Kroemer 2010). Binding of TNF to TNF-R1 leads to the rapid formation of a plasma membrane-associated complex (called complex I) that contains RIP1, cIAP1 (Inhibitor of apoptosis protein 1), TRADD, TRAF2 (TNF receptor-associated factor 2), NEMO (NF-Kappa-B Essential Modulator), and A20 together with LUBAC (linear ubiquitin chain assembly complex) (Walczak et al. 2012). However, during transition from complex I only RIP1 forms-together with FADD, Caspase-8, and cFLIP-the complex IIa that triggers apoptosis whenever cFLIP proteins are absent. Alternatively, whenever the complex lacks cIAPs, RIP1 promotes necroptosis in the presence of RIP3 under conditions of limited caspase-8 activity, and this complex was referred to as complex II b or necrosome (Vanlangenakker et al. 2012). In summary, numerous reports point to the fact that TNF Complex IIa is structurally mostly related to the Ripoptosome, because its components are (thus far) highly similar.

Necrosome

Another important complex containing RIP1 is the necrosome first described for TNF signalling (for review *see* Vanlangenakker et al. 2012). The necrosome is a protein complex of RIP1 and RIP3 and was also shown to be formed during vaccinia virus infection (Cho et al. 2009). The phosphorylation of RIP1 and RIP3 stabilizes their association within the pronecrotic complex, activates the pronecrotic kinase activity, and triggers downstream production of ROS (Cho et al. 2009).

Mitochondria-Associated Complex

Recently RIP1 was also shown to form a complex with RIP3, mixed linkage kinase-like (MLKL) protein, and phosphoglycerate mutase 5 (PGAM5) (both short and long splice variants) (Wang et al. 2012). This complex is forming on the surface of mitochondria in response to TNF stimulation whenever cIAPs are absent or whenever caspase activity is blocked. The formation and function of this complex is required for necroptosis execution.

Ripoptosome

The Ripoptosome was first identified in studies investigating the role of IAP proteins for CD95-induced cell death (Geserick et al. 2009). Since RIP1 was indispensable for complex formation the term "Ripoptosome" was later proposed, to indicate a central scaffold function of RIP1, or its oligomers (Feoktistova et al. 2011; Tenev et al. 2011). The other known components of the Ripoptosome are FADD, caspase-8, and different cFLIP isoforms (Geserick et al. 2009).

At first glance there are no obvious differences in the composition of the Ripoptosome and TNF complex II a, which argues that both complexes are identical. However, the formation of both complexes is substantially different. In contrast to TNF-induced complex II, Ripoptosome formation can be triggered in the absence of TNF signalling (Feoktistova et al. 2011; Tenev et al. 2011). RIP1 in complex I is modified by CYLD and presumably promotes complex II a formation (Wang et al. 2008). In contrast cIAPs block Ripoptosome formation by ubiquitination of RIP1 by the E3 ubiquitin ligase activity of cIAPs (Vince et al. 2007). It is tempting to speculate that these differences may discriminate the Ripoptosome from TNF complex II a. Furthermore, Ripoptosome formation was detected not only upon death receptor signalling, but in other cellular signalling pathways, most prominently upon chemotherapeutic drug treatment (e.g., etoposide) (Tenev et al. 2011), or TLR3 signalling (Feoktistova et al. 2011). More recent studies demonstrate that IAP antagonists induce a conformational change in cIAP1 structure that promotes autoubiquitination and proteasomal degradation (Feltham et al. 2010; Dueber et al. 2011). In addition, spontaneous Ripoptosome formation was shown by inducible overexpression of RIP1 (Feoktistova et al. 2012). Overexpression of low levels of RIP1 solely induces spontaneous apoptotic, or in the absence of caspase activity, necroptotic cell death. This suggests that shifting the balance towards the presence of unmodified RIP1 is sufficient to promote Ripoptosome formation and cell death induction. When the size of the native Ripoptosome was investigated, it was found to be a rather large protein complex (around 2 MDa) (Feoktistova et al. 2011; Tenev et al. 2011). In line with these findings recent studies of RIP1/RIP3 interactions revealed that the RHIMs of RIP1 and RIP3 mediate the assembly of heterodimeric filamentous (amyloidal) structures in necrotic cells (Li et al. 2012). These amyloidal structures might be the core of the Ripoptosome. Because the formation of these amyloids requires RHIM-RHIM interaction, the death domains of RIP1 as well as the kinase domains of RIP1 and RIP3 are potentially freely available for

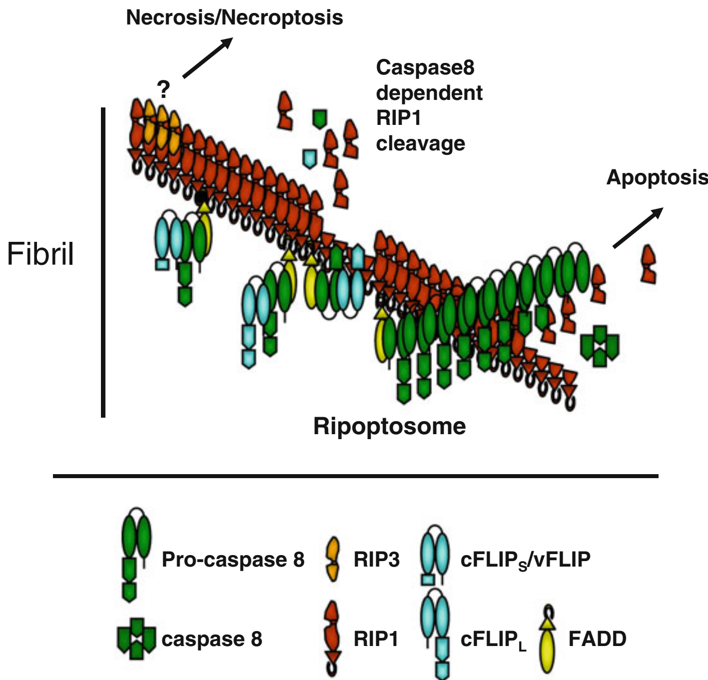

Fig. A.2 Current concept of Ripoptosome assembly and the role of caspase-8 or RIP1 activation for cell death. Fibril formation of RIP1 is proposed to represent the backbone of the Ripoptosome that is regulated by multimeric caspase-8 chains that are potentially regulated by $cFLIP_S$ or vFLIP. Active caspase-8 promotes RIP1 cleavage and finally leads to apoptotic responses. In contrast, $cFLIP_S$ or vFLIP fully suppresses caspase-8 activation and therefore RIP1 cleavage. Under those conditions necroptotic responses are promoted in the presence of RIP3

interactions and signalling (Fig. A.2). RIP1 can interact with caspase-8, which is critical for the execution of apoptotic signalling in the Ripoptosome via the adaptor molecule FADD. Recent studies of the TRAIL DISC have shown that caspase-8 is capable of forming multimeric chains by direct interaction of the DEDs (Dickens et al. 2012). Taking these studies together, it is intriguing to speculate that RIP1/RIP3 fibrillar complex and caspase-8 chains might explain the size of the Ripoptosome. To date, it is not yet clear if the necrosome is a part of Ripoptosome, or vice versa signals as an independent protein complex.

There are at least three hypotheses that derive from the concept of the Ripoptosome. First, the Ripoptosome is nothing else, but TNF complex II, which is induced by autocrine TNF, induced by various stimuli as suggested (Petersen et al. 2007; Ikner and Ashkenazi 2011). However formation of the Ripoptosome was induced by chemotherapeutic drugs or IAP antagonists independent of TNF signalling as shown by TNF-R1 knockdown or inhibition of autocrine TNF signalling (Geserick et al. 2009; Feoktistova et al. 2011; Tenev et al. 2011). During embryonic

development RIP1 needs to be controlled by cIAP1, otherwise TNF-R1-dependent cell death leads to embryonic lethality. While these embryonic data are in favor of the hypothesis, they nonetheless show (TNF-R1 deficiency does not restore viability/fertility of cIAP1/cIAP2 double knockout animals) that additional pathways are controlled by cIAPs in a RIP1-dependent manner (Moulin et al. 2012). Our data about the Ripoptosome contrast a number of other studies that figured that the loss of IAPs mainly results in autocrine TNF signalling (Biton and Ashkenazi 2011; Vince et al. 2007; Varfolomeev et al. 2007; Gaither et al. 2007; Wang et al. 2008). Taken together these data indicate cell type-specific signalling properties in response to IAP antagonists that could be employed, for example, in cancer therapy.

Second, a shift in the balance of cIAPs/RIP1 protein in the cytoplasm to lower cIAP levels may facilitate formation of RIP1 oligomers. This in turn may stimulate Ripoptosome formation, insufficient per se to kill due to caspase-8-mediated cleavage/disassembly of the Ripoptosome. Loss of complex-intrinsic caspase activity (e.g., by $cFLIP_S$, viral FLIP (vFLIP), or caspase inhibitors) then allows for complex accumulation and subsequent necroptosis while apoptosis is prevented by the different caspase inhibitors. These series of checkpoints all influence the cellular outcome of the diverse triggers. If Ripoptosome assembly overcomes a critical threshold, cell death proceeds. Alternatively caspase activity promotes disassembly of the complex and this activity promotes survival. The presence of cytoplasmic Ripoptosome may in turn facilitate membrane-bound complex formation. It is perceivable that the cytoplasmic Ripoptosome is stabilizing the membrane-bound complex by direct recruitment of the Ripoptosome to the various receptors after stimulation as indicated by our studies for CD95 signalling (Cullen et al. 2013; Geserick et al. 2009).

Third, a cytoplasmic Ripoptosome does not have cell death-inducing capabilities per se. Rather, complex I of various receptors either directly contributes activated components via internalization to the Ripoptosome or initiates signalling, leading to activation of the Ripoptosome, and inducing its cell death-promoting activity. These hypotheses await to be tested by experimental approaches in the future.

A current question of debate is if cIAPs are components of the Ripoptosome. On the one hand, loss of cIAPs was a prerequisite of Ripoptosome formation (Geserick et al. 2009; Feoktistova et al. 2011; Tenev et al. 2011). On the other hand, when the Ripoptosome was induced by modest acute RIP1 overexpression, cIAP1 was readily detected in the complex (Feoktistova et al. 2012). Thus the loss of cIAPs is not an absolute requirement, but it rather promotes Ripoptosome assembly. The cIAPs detected upon RIP1 overexpression could be considered the “last soldiers,” trying to prevent Ripoptosome formation and therefore death of the cell.

1.1.1.3 What Are the Regulators of the Ripoptosome?

Understanding the formation of the Ripoptosome implies understanding cellular regulators of RIP1 function. Ubiquitination-mediated degradation of unwanted, damaged, or misfolded proteins, regulation of protein turnover, protein

translocation, stability, and changes in protein–protein interaction are the central mechanisms that can control the enzymatic activity. Mono- and polyubiquitination are mediated by ubiquitin ligases that covalently link ubiquitin by different lysine residues (K6, K11, K27, K29, K33, K48, K63). Linear or heterotypic chains to different substrates have broad significance in immune disorders and cancer (Fulda et al. 2012). Until recently, Ubiquitin linked by lysine residue 48 (K48) was thought to promote proteasomal degradation whereby K63-linked ubiquitin was responsible for NF-κB activation and therefore for cellular survival and inflammation (Vucic et al. 2011). RIP1 is one target for many E3 ubiquitin ligases such as cIAP1/2 (cellular inhibitor of apoptosis proteins) (Park et al. 2004), TNF receptor-associated factor 2 (TRAF2) (Wajant and Scheurich 2011), as well as a target of deubiquitinase CYLD (Cylindromatosis) (Brummelkamp et al. 2003) and other ubiquitin-editing enzymes, e.g., A20, that support both K63-deubiquitination and K48-ubiquitination of RIP1 (Wertz et al. 2004).

More recently LUBAC has been described. LUBAC contains the ubiquitin ligase HOIP (HOIL-1-interacting protein), the interacting protein HOIL1 (heme-oxidized IRP2 ubiquitin ligase 1) and SHARPIN (SHANK-associated RH domain interacting protein). The activity of LUBAC triggers linear M1-linkage of ubiquitin as highly important for NF-κB-mediated inflammatory responses (Gerlach et al. 2011) and regulation of TNF-induced cell death (Walczak et al. 2012). TRAF2 is an ubiquitin ligase with central function for TNF signalling that principally mediates K63-ubiquitination of RIP1 and promotes survival during TNF-mediated NF-κB induction (O'Donnell et al. 2007). However, TRAF2 is also a target for K48- and K63-ubiquitination (Habelhah et al. 2004). Further complicating the molecular interplay, the de-ubiquitinating proteases USP2a and USP2c stabilize RIP1 by de-ubiquitination of TRAF2 and RIP1 itself (Mahul-Mellier et al. 2012). This study highlights the critical role of TRAF2 within complex II for RIP1 regulation for TNF-induced apoptosis. Since Ripoptosome formation is independent of TNF signalling, TRAF2-mediated RIP1 ubiquitination is not obviously critical for the formation of the Ripoptosome and its necroptotic activity, but merits further studies.

The ubiquitin ligases cIAPs have been reported as critical regulators of RIP1 (Moulin et al. 2012). cIAPs promote K11-ubiquitination of RIP1 and subsequently cIAP degradation (Bertrand et al. 2008; Dynek et al. 2010) suggesting that cIAPs control the turnover of RIP1 within cells to avoid spontaneous necroptotic cell death induced by high levels of RIP1 (Feoktistova et al. 2012). The critical role of cIAPs for Ripoptosome formation has been demonstrated using IAP antagonists (Geserick et al. 2009). Such compounds, identified by several groups, trigger autoubiquitination and rapid degradation of cIAPs (Bertrand et al. 2008; Gaither et al. 2007; Petersen et al. 2007; Vince et al. 2007; Varfolomeev et al. 2007) with the consequence of a strong formation of the RIP1-FADD-Caspase-8 complex (Geserick et al. 2009; Feoktistova et al. 2011). Ripoptosome assembly correlates with increased DL- or TLR3-induced cell death even in the absence of active caspase-8. Under those conditions Ripoptosome assembly promoted necroptosis in a RIP3-dependent manner (Feoktistova et al. 2011). This suggests that formation of the Ripoptosome dictates the quantity of cell death, whereas the stoichiometry directly influences the quality of cell death (Fig. A.3).

Of note, activation of caspase-8 within the Ripoptosome is required for the apoptotic response. The biochemical evidence available to date suggests that the necroptotic activity of the Ripoptosome is directly blocked by caspase-8-mediated RIP1 cleavage in the complex. However, caspase-8 activity also destabilizes/degrades the complex altogether, whereas necroptosis is unmasked whenever caspase-8 function is suppressed (Feoktistova et al. 2011; Geserick et al. 2009). cFLIP, which is homologous to caspase-8 but lacks the critical cysteine residue and therefore its active site, is not only another component of the Ripoptosome but also a strong regulator of the cell death activity within the complex. cFLIP has many different isoforms expressed in different cell types (Ozturk et al. 2012). At the protein level, cells dominantly express the long ($cFLIP_L$) and/or short ($cFLIP_S$) isoforms. Both isoforms can form heterodimers with caspase-8. Whereas $cFLIP_L$/caspase-8 heterodimers have enzymatic activity and allow for partial cleavage of caspase-8, this activity is insufficient to kill the cell. This apparent paradox is most likely caused by the ability of $cFLIP_L$ to prevent cleavage of the large subunit from the prodomain of caspase-8. This cleavage step is necessary for the release of fully active heterotetrameric caspase-8 from the different signalling platforms (Wachter et al. 2004; Scaffidi et al. 1999). Thus, a shift of the stoichiometry of caspase-8 and cFLIP in favor of cFLIP keeps the enzymatic activity within the signalling platform in which both-caspase-8 and cFLIP-are heterodimerized. This partial enzymatic activity is capable of cleaving RIP1 within the complex, leading to its dissociation and degradation (Feoktistova et al. 2011, Fig. A.3).

In contrast, the caspase-8/$cFLIP_S$ heterodimer completely lacks enzymatic activity within the DISC (Kavuri et al. 2011) or the Ripoptosome (Feoktistova et al. 2011). This may be the result of the heterodimer ability to cleave caspase-8 substrates that are closely associated within the different signalling platforms outlined above. In particular, cleavage-mediated inactivation of RIP1 may represent a critical cleavage reaction, although other substrates of localized caspase-8 activity can easily be envisioned, such as CYLD (O'Donnell et al. 2011) or others.

1.1.1.4 What Are the Functions of the Ripoptosome?

The known functions of Ripoptosome examined thus far are important to control cell death pathways. All groups that described the complex were dealing with either apoptosis which was converted to necroptosis upon caspase inhibition or with situation in which both types of cell death occurred in parallel.

Extrinsic Apoptosis

Formation of the Ripoptosome can augment extrinsic apoptotic signals, induced by stimulation of DLs or TLRs (Estornes et al. 2012). There are currently two hypotheses about the role of the Ripoptosome for apoptotic signalling. First, the Ripoptosome can be recruited to stimulated receptors, as a preformed complex, and

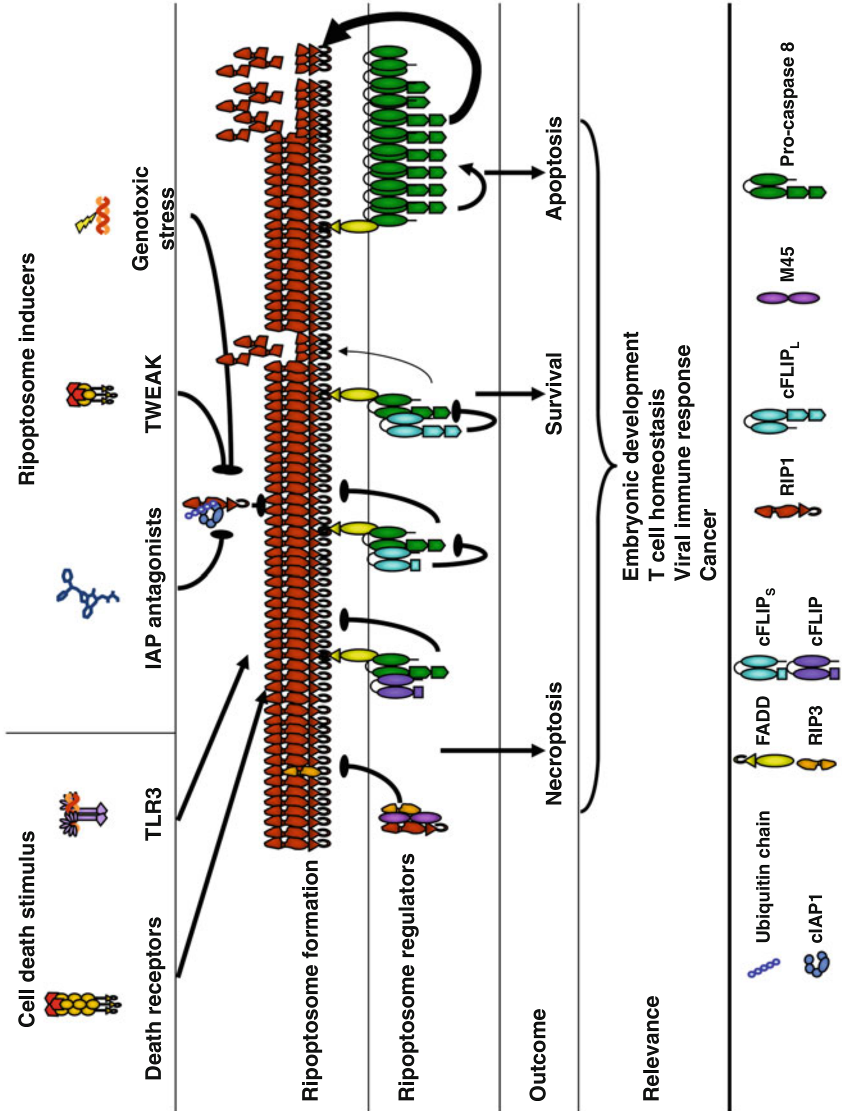
Cell death stimulus
Ripoptosome inducers
Death receptors
TLR3
IAP antagonists
TWEAK
Genotoxic stress
Ripoptosome formation
Ripoptosome regulators
Outcome
Necroptosis
Survival
Apoptosis
Relevance
Embryonic development
T cell homeostasis
Viral immune response
Cancer
Ubiquitin chain
cIAP1
FADD
RIP3
cFLIP$_S$
cFLIP
RIP1
cFLIP$_L$
M45
Pro-caspase 8

Fig. A.3 Ripoptosome regulation: from the formation and execution to physiological or pathophysiological relevance. Ripoptosome formation is blocked by IAPs. Extracellular stimuli (IAP antagonists, TWEAK or genotoxic stress) can prevent this blockade, allowing for Ripoptosome formation. Necroptotic or apoptotic signalling via death receptors or TLR3 is enhanced by the Ripoptosome. Within the Ripoptosome, active RIP1 kinase promotes phosphorylation and activation of RIP3, whose enzymatic activity is indispensable for necroptosis. Virus-derived M45 interferes with the necroptotic response of the RIP1–RIP3 complex. In contrast, full inhibition of caspase-8 activity by viral FLIP (vFLIP) or $cFLIP_S$ supports necroptotic activity of the RIP1–RIP3 complexes. Dominant caspase-8 activity promotes RIP1 cleavage and suppresses necroptosis. The cFLIP proteins heterogeneously control the cell death response of the Ripoptosome. The long form of cFLIP ($cFLIP_L$) inhibits both necroptosis and apoptosis and promotes cellular survival, whereby $cFLIP_S$ fully blocks caspase-8 activity of the Ripoptosome and thereby unmasks necroptosis. Partial suppression of caspase-8 activity by $cFLIP_L$ still allows RIP1 cleavage within the complex, therefore inducing Ripoptosome disassembly and suppression of both apoptotic and necroptotic cell death. The composition of the Ripoptosome and the regulators of the Ripoptosome activity play an important role for a number of physiological and pathophysiological conditions. These include embryonic development, immune responses against viral infection, T cell homeostasis, and cancer

therefore rapidly promote the further downstream signalling (Feoktistova et al. 2011; Geserick et al. 2009). Secondly, the Ripoptosome may serve as a cytosolic stress platform required to augment caspase-8 activation, therefore multiplying and enhancing the downstream signalling and increasing the cell death response.

Intrinsic Apoptosis

The formation of Ripoptosome was shown to be independent of the mitochondrial pathway (Tenev et al. 2011), although Ripoptosome-induced apoptosis may ultimately depend on a contribution of the intrinsic pathway. Of note, in various cells caspase-8-induced cell death requires amplification of the caspase cascade by the mitochondrial pathway (Jost et al. 2009). However, other RIP1-containing complexes are associated with the mitochondria such as the RIG-I complex (Rajput et al. 2011). However, this predominantly mitochondrial complex does not have a proapoptotic activity per se identified to date.

Necroptosis

In the past years, studies about necroptosis control are revealing more and more fascinating features of this process. Mostly this cell death occurs when extrinsic (Geserick et al. 2009) and/or intrinsic (Dunai et al. 2012) apoptotic cell death is blocked for any molecular reason. One such reason is expression of cFLIP isoforms, which can totally ($cFLIP_S$) or partially ($cFLIP_L$) block caspase-8 activity, therefore diminishing the apoptotic activity of the given protein platform.

A central regulator of necroptosis is RIP3, which is known to participate in the necrosome (Cho et al. 2009) and the pro-necrotic mitochondrial protein complexes (Lu et al. 2011a). RIP1 is phosphorylating and activating RIP3, which in turn is phosphorylating MLKL.

The Ripoptosome promotes necroptosis in caspase-compromised conditions upon death receptor (Geserick et al. 2009) and TLR3 (Feoktistova et al. 2011) triggering as well as upon genotoxic stress induced by etoposide (Tenev et al. 2011). Furthermore when $cFLIP_S$ or vFLIP (MC159) were overexpressed and cIAPs were downregulated, the Ripoptosome assembled and induced necroptosis without the need for other extrinsic stimuli (Feoktistova et al. 2011; Feoktistova et al. 2012). RIP3 was shown to be indispensable for Ripoptosome-induced/-mediated necroptosis execution (Feoktistova et al. 2011), although RIP3 was not found within the complex. It is currently an open question if this is biologically relevant or a matter of the experimental setting used. Another interesting topic is the role of MLKL in the Ripoptosome-induced signalling cascade. For instance it will be interesting to know if MLKL is found in the Ripoptosome, or activated downstream of the complex, and interrogate the precise role of the downstream targets of MLKL.

1.1.1.5 Physiological and Pathophysiological Relevance of the Ripoptosome

Role of Ripoptosome-Associated Proteins in Embryonic Development

Previously established knockout mice models and in vitro cell studies investigated the physiological relevance of Ripoptosome components and associated molecules during embryonic development, cancer, and immunological and inflammatory responses (Han et al. 2011). The lethality of FADD or Caspase 8 knockout mice (Varfolomeev et al. 1998; Yeh et al. 1998; Zhang et al. 1998), core molecules of the Ripoptosome, highlighted the critical role of this signalling platform for embryonic development. The early observation that mice lacking RIP1 died prenatally (Kelliher et al. 1998) was interpreted in the context of proinflammatory signalling, namely the ability of RIP1 to induce NF-κB. More recently deficiency of RIP1 protected FADD deficient mice from embryonic death. These data argue for a potential direct interaction of RIP1 and FADD (Zhang et al. 2011). Further mechanistic insight for the role of necroptosis was demonstrated in mouse studies using RIP3-deficient animals. Loss of RIP3 rescues the lethality of Caspase-8 deficiency (Oberst et al. 2011; Kaiser et al. 2011) or protects from skin (Bonnet et al. 2011) or gut inflammatory diseases (Welz et al. 2011; Gunther et al. 2011).

As demonstrated, the apoptotic and necroptotic activity of the Ripoptosome is strongly negative, but differentially regulated by cFLIP isoforms (Feoktistova et al. 2011; Geserick et al. 2009). Mice lacking cFLIP also died during embryogenesis at E10.5 presumably by a yolk sack defect (Yeh et al. 2000), which supports an important role of cFLIP for cell death at early stages of mouse development. As demonstrated for FADD and caspase-8, a RIP3-dependent mechanism of embryonic development failure in cFLIP deficient mice was proposed. Interestingly and in contrast to the ablation of RIP3 in caspase-8-deficient animals, loss of RIP3 in cFLIP knockout mice failed to rescue survival (Dillon et al. 2012). Presumably this suggests a parallel activation of both pathways; apoptosis and necroptosis. Moreover the caspase activity controls the execution of necroptosis, whereas it is unclear if and how necroptosis controls the extent of apoptosis. Taken together, the components of the Ripoptosome as well their potent negative regulator cFLIP are central molecules that control mouse development by regulation of both, an apoptotic and a necroptotic cell death response during embryogenesis.

Role of Ripoptosome in T-Cell Development and Antiviral Immune Responses

It has been long known that the process of necrosis also controls lymphocyte contraction and may operate as an alert signal of the immune system during pathogen invasion (Walsh et al. 1998). Also an alternative RIP1-dependent cell death in caspase-8 negative T cells was described (Ch'en et al. 2008), indicative of the crucial role of RIP1/Caspase-8 complexes for cell death regulation during T cell expansion. The adaptor molecule FADD within the RIP1/Caspase-8 complex play a pivotal role for necroptosis-dependent T-cell homeostasis, antiviral responses and T-cell

receptor-mediated necroptosis (Osborn et al. 2010; Lu et al. 2011a). Mature T cells were absent in FADD knockout mice or animals with expression of a dominant negative form of FADD consisting of the DD (FADDdd). As well their proliferative defect in early T-cell stages is either RIP3 kinase dependent (Lu et al. 2011b) or is compensated by inhibition of RIP1 kinase activity (Osborn et al. 2010). Another study describes that Caspase-8/RIP3-dependent necroptosis is required for the homeostasis and maintenance of T cell population size (Ch'en et al. 2011). In this study the lethality of caspase-8-deficient T cells was rescued by RIP3 depletion. Taken together, different studies highlight the role of RIP1, RIP3, Caspase-8, and FADD for necroptosis regulation within the T-cell compartment. Therefore the Ripoptosome could be a crucial signalling platform that controls T-cell activation and regulation of pathogen-mediated immune responses.

Disturbing the apoptotic signalling machinery of the host ensures successful virus replication and evasion. Many viral proteins potently inhibit the host-specific apoptotic machinery by inhibition of caspases (Benedict et al. 2002). Prominent examples are CrmA from poxvirus (Dobo et al. 2006), vFLIP from Karposi's sarcoma-associated herpes virus (Punj et al. 2009) and MC159 from molluscum contagiosum (Shisler and Moss 2001). Other viral proteins rather mimic anti-apoptotic Bcl-2 proteins as demonstrated for EIB-55K from adeno-virus (Querido et al. 2001) and E6 from human papilloma virus (Scheffner et al. 1990). One potential circumvention of apoptosis protection by viral proteins could be necroptosis induction (Challa and Chan 2010). More recently it was found that viruses such as cytomegalovirus (CMV) have additionally established strategies to circumvent RIP3-dependent necroptosis. M45 from the murine CMV (MCMV) inhibits necroptosis by binding to RIP1 and RIP3 via its RHIM-like motif (Mack et al. 2008; Upton et al. 2008) and thereby blocks necroptosis of the virus-infected cell. Another example is vFLIP proteins, highly resembling $cFLIP_S$ (Punj et al. 2009). In this context vFLIPs are of particular importance for cell death regulation. A study in MC159-transgenic animals showed an increased innate immune response to viral challenge (Challa and Chan 2010). Our studies demonstrated spontaneous necroptosis in cells expressing vFLIP (M159) from molluscum contagiosum in the absence of cIAPs (Feoktistova et al. 2012) and thereby demonstrated the pivotal function of cIAPs for negative regulation of the necroptotic activity. Therefore, cIAPs may prove critical to block this antiviral response by counteracting RIP1-mediated necroptosis. Suppression of IAP function by IAP antagonists could thus promote necroptotic activity of the Ripoptosome during virus infection and effectively alert innate or adaptive protective immune response. Taken together, viruses are able to block programmed cell death at different levels and protect their own "survival" by controlling both the apoptotic and necroptotic signalling machinery including Ripoptosome assembly.

The Role of the Ripoptosome in Cancer

One hallmark of cancer is a deregulation of cell death pathways. The Ripoptosome forms predominantly in cancer cells but not in primary cells (Feoktistova et al. 2011)

arguing for an indispensable role of Ripoptosome signalling in cancer. Many studies have shown a FADD-Caspase-8-RIP1 complex formation and increased sensitivity to chemotherapeutics, death ligands (DLs), TLR3 agonists in the presence of IAP antagonist or in response to genotoxic stress in, e.g., glioblastoma cells (Wagner et al. 2013), SCC cells (Geserick et al. 2009), HeLa cells (Feoktistova et al. 2011), and colorectal carcinoma cells (Tenev et al. 2011). In addition, breast and prostate cancer cells that normally resist TRAIL-induced apoptosis are sensitized in the presence of IAP antagonists (Lu et al. 2011a). All these studies provide correlative evidence of IAP antagonist-induced Ripoptosome/Necrosome formation and increased cell death responses when treatment with chemotherapeutics or DL is initiated in cancer cells. These findings highlight the potential impact of Ripoptosome in cancer and envision novel cancer therapeutics that hitchhike Ripoptosome-induced cell death. However, caspase-8 activity within the Ripoptosome is required for apoptosis and inhibits necroptosis by cleavage of RIP1 (Cho et al. 2009; Feoktistova et al. 2011). Both processes, apoptosis and necroptosis, are regulated by cFLIP proteins whereby $cFLIP_S$ and not $cFLIP_L$ promotes necroptotic Ripoptosome activity in response to IAP antagonists (Feoktistova et al. 2011). Virus-mediated carcinogenesis is associated with the upregulation of cell death regulators from virus. Of particular interest for the Ripoptosome will be viruses that utilize vFLIP proteins. They have been mainly interpreted as potent inhibitors of caspase-8 activation (Thome et al. 1997). Alternatively virus proteins may modulate the necroptotic RIP1 activity as demonstrated for M45 (Upton et al. 2008). Taken together, modulation of apoptotic and necroptotic activity of the Ripoptosome by cellular or viral FLIP isoforms is envisioned to represent a critical step during virus-mediated carcinogenesis.

References

Ashida H, Mimuro H, Ogawa M, Kobayashi T, Sanada T, Kim M, Sasakawa C (2011) Cell death and infection: a double-edged sword for host and pathogen survival. J Cell Biol 195:931–942

Benedict CA, Norris PS, Ware CF (2002) To kill or be killed: viral evasion of apoptosis. Nat Immunol 3:1013–1018

Bertrand MJ, Milutinovic S, Dickson KM, Ho WC, Boudreault A, Durkin J, Gillard JW, Jaquith JB, Morris SJ, Barker PA (2008) cIAP1 and cIAP2 facilitate cancer cell survival by functioning as E3 ligases that promote RIP1 ubiquitination. Mol Cell 30:689–700

Biton S, Ashkenazi A (2011) NEMO and RIP1 control cell fate in response to extensive DNA damage via TNF-alpha feedforward signaling. Cell 145:92–103

Bonnet MC, Preukschat D, Welz PS, van LG, Ermolaeva MA, Bloch W, Haase I, Pasparakis M (2011) The adaptor protein FADD protects epidermal keratinocytes from necroptosis in vivo and prevents skin inflammation. Immunity 35:572–582

Brummelkamp TR, Nijman SM, Dirac AM, Bernards R (2003) Loss of the cylindromatosis tumour suppressor inhibits apoptosis by activating NF-kappaB. Nature 424:797–801

Ch'en IL, Beisner DR, Degterev A, Lynch C, Yuan J, Hoffmann A, Hedrick SM (2008) Antigen-mediated T cell expansion regulated by parallel pathways of death. Proc Natl Acad Sci U S A 105:17463–17468

Ch'en IL, Tsau JS, Molkentin JD, Komatsu M, Hedrick SM (2011) Mechanisms of necroptosis in T cells. J Exp Med 208:633–641

Challa S, Chan FK (2010) Going up in flames: necrotic cell injury and inflammatory diseases. Cell Mol Life Sci 67:3241–3253

Cho YS, Challa S, Moquin D, Genga R, Ray TD, Guildford M, Chan FK (2009) Phosphorylation-driven assembly of the RIP1-RIP3 complex regulates programmed necrosis and virus-induced inflammation. Cell 137:1112–1123

Cullen SP, Henry CM, Kearney CJ, Logue SE, Feoktistova M, Tynan GA, Lavelle EC, Leverkus M, Martin SJ (2013) Fas/CD95-induced chemokines can serve as "find-me" signals for apoptotic cells. Mol Cell 49(6):1034–1048

Degterev A, Hitomi J, Germscheid M, Ch'en IL, Korkina O, Teng X, Abbott D, Cuny GD, Yuan C, Wagner G, Hedrick SM, Gerber SA, Lugovskoy A, Yuan J (2008) Identification of RIP1 kinase as a specific cellular target of necrostatins. Nat Chem Biol 4:313–321

Dickens LS, Boyd RS, Jukes-Jones R, Hughes MA, Robinson GL, Fairall L, Schwabe JW, Cain K, Macfarlane M (2012) A death effector domain chain DISC model reveals a crucial role for caspase-8 chain assembly in mediating apoptotic cell death. Mol Cell 47:291–305

Dillon CP, Oberst A, Weinlich R, Janke LJ, Kang TB, Ben-Moshe T, Mak TW, Wallach D, Green DR (2012) Survival function of the FADD-CASPASE-8-cFLIP(L) complex. Cell Rep 1:401–407

Dobo J, Swanson R, Salvesen GS, Olson ST, Gettins PG (2006) Cytokine response modifier a inhibition of initiator caspases results in covalent complex formation and dissociation of the caspase tetramer. J Biol Chem 281:38781–38790

Dueber EC, Schoeffler AJ, Lingel A, Elliott JM, Fedorova AV, Giannetti AM, Zobel K, Maurer B, Varfolomeev E, Wu P, Wallweber HJ, Hymowitz SG, Deshayes K, Vucic D, Fairbrother WJ (2011) Antagonists induce a conformational change in cIAP1 that promotes autoubiquitination. Science 334:376–380

Dunai ZA, Imre G, Barna G, Korcsmaros T, Petak I, Bauer PI, Mihalik R (2012) Staurosporine induces necroptotic cell death under caspase-compromised conditions in U937 cells. PLoS One 7:e41945

Dynek JN, Goncharov T, Dueber EC, Fedorova AV, Izrael-Tomasevic A, Phu L, Helgason E, Fairbrother WJ, Deshayes K, Kirkpatrick DS, Vucic D (2010) c-IAP1 and UbcH5 promote K11-linked polyubiquitination of RIP1 in TNF signalling. EMBO J 29:4198–4209

Estornes Y, Toscano F, Virard F, Jacquemin G, Pierrot A, Vanbervliet B, Bonnin M, Lalaoui N, Mercier-Gouy P, Pacheco Y, Salaun B, Renno T, Micheau O, Lebecque S (2012) dsRNA induces apoptosis through an atypical death complex associating TLR3 to caspase-8. Cell Death Differ 19:1482–1494

Feltham R, Moulin M, Vince JE, Mace PD, Wong WW, Anderton H, Day CL, Vaux DL, Silke J (2010) Tumor necrosis factor (TNF) signaling, but not TWEAK (TNF-like weak inducer of apoptosis)-triggered cIAP1 (cellular inhibitor of apoptosis protein 1) degradation, requires cIAP1 RING dimerization and E2 binding. J Biol Chem 285:17525–17536

Feoktistova M, Geserick P, Kellert B, Dimitrova DP, Langlais C, Hupe M, Cain K, Macfarlane M, Hacker G, Leverkus M (2011) cIAPs block Ripoptosome formation, a RIP1/caspase-8 containing intracellular cell death complex differentially regulated by cFLIP isoforms. Mol Cell 43:449–463

Feoktistova M, Geserick P, Panayotova-Dimitrova D, Leverkus M (2012) Pick your poison: the Ripoptosome, a cell death platform regulating apoptosis and necroptosis. Cell Cycle 11:460–467

Fulda S, Rajalingam K, Dikic I (2012) Ubiquitylation in immune disorders and cancer: from molecular mechanisms to therapeutic implications. EMBO Mol Med 4:545–556

Gaither A, Porter D, Yao Y, Borawski J, Yang G, Donovan J, Sage D, Slisz J, Tran M, Straub C, Ramsey T, Iourgenko V, Huang A, Chen Y, Schlegel R, Labow M, Fawell S, Sellers WR, Zawel L (2007) A Smac mimetic rescue screen reveals roles for inhibitor of apoptosis proteins in tumor necrosis factor-alpha signaling. Cancer Res 67:11493–11498

Gerlach B, Cordier SM, Schmukle AC, Emmerich CH, Rieser E, Haas TL, Webb AI, Rickard JA, Anderton H, Wong WW, Nachbur U, Gangoda L, Warnken U, Purcell AW, Silke J, Walczak H (2011) Linear ubiquitination prevents inflammation and regulates immune signalling. Nature 471:591–596

Geserick P, Hupe M, Moulin M, Wong WW, Feoktistova M, Kellert B, Gollnick H, Silke J, Leverkus M (2009) Cellular IAPs inhibit a cryptic CD95-induced cell death by limiting RIP1 kinase recruitment. J Cell Biol 187:1037–1054

Gunther C, Martini E, Wittkopf N, Amann K, Weigmann B, Neumann H, Waldner MJ, Hedrick SM, Tenzer S, Neurath MF, Becker C (2011) Caspase-8 regulates TNF-alpha-induced epithelial necroptosis and terminal ileitis. Nature 477:335–339

Habelhah H, Takahashi S, Cho SG, Kadoya T, Watanabe T, Ronai Z (2004) Ubiquitination and translocation of TRAF2 is required for activation of JNK but not of p38 or NF-kappaB. EMBO J 23:322–332

Han J, Zhong CQ, Zhang DW (2011) Programmed necrosis: backup to and competitor with apoptosis in the immune system. Nat Immunol 12:1143–1149

Ikner A, Ashkenazi A (2011) TWEAK induces apoptosis through a death-signaling complex comprising receptor-interacting protein 1 (RIP1), Fas-associated death domain (FADD), and caspase-8. J Biol Chem 286:21546–21554

Jost PJ, Grabow S, Gray D, McKenzie MD, Nachbur U, Huang DC, Bouillet P, Thomas HE, Borner C, Silke J, Strasser A, Kaufmann T (2009) XIAP discriminates between type I and type II FAS-induced apoptosis. Nature 460:1035–1039

Kaiser WJ, Upton JW, Long AB, Livingston-Rosanoff D, Daley-Bauer LP, Hakem R, Caspary T, Mocarski ES (2011) RIP3 mediates the embryonic lethality of caspase-8-deficient mice. Nature 471:368–372

Kang DC, Gopalkrishnan RV, Lin L, Randolph A, Valerie K, Pestka S, Fisher PB (2004) Expression analysis and genomic characterization of human melanoma differentiation associated gene-5, mda-5: a novel type I interferon-responsive apoptosis-inducing gene. Oncogene 23:1789–1800

Kavuri SM, Geserick P, Berg D, Dimitrova DP, Feoktistova M, Siegmund D, Gollnick H, Neumann M, Wajant H, Leverkus M (2011) Cellular FLICE-inhibitory protein (cFLIP) isoforms block. J Biol Chem 286:16631–16646

Kelliher MA, Grimm S, Ishida Y, Kuo F, Stanger BZ, Leder P (1998) The death domain kinase RIP mediates the TNF-induced NF-kappaB signal. Immunity 8:297–303

Li J, McQuade T, Siemer AB, Napetschnig J, Moriwaki K, Hsiao YS, Damko E, Moquin D, Walz T, McDermott A, Chan FK, Wu H (2012) The RIP1/RIP3 necrosome forms a functional amyloid signaling complex required for programmed necrosis. Cell 150:339–350

Lu J, McEachern D, Sun H, Bai L, Peng Y, Qiu S, Miller R, Liao J, Yi H, Liu M, Bellail A, Hao C, Sun SY, Ting AT, Wang S (2011a) Therapeutic potential and molecular mechanism of a novel, potent, nonpeptide, Smac mimetic SM-164 in combination with TRAIL for cancer treatment. Mol Cancer Ther 10:902–914

Lu JV, Weist BM, van Raam BJ, Marro BS, Nguyen LV, Srinivas P, Bell BD, Luhrs KA, Lane TE, Salvesen GS, Walsh CM (2011b) Complementary roles of Fas-associated death domain (FADD) and receptor interacting protein kinase-3 (RIPK3) in T-cell homeostasis and antiviral immunity. Proc Natl Acad Sci U S A 108:15312–15317

Mack C, Sickmann A, Lembo D, Brune W (2008) Inhibition of proinflammatory and innate immune signaling pathways by a cytomegalovirus RIP1-interacting protein. Proc Natl Acad Sci U S A 105:3094–3099

Mahul-Mellier AL, Datler C, Pazarentzos E, Lin B, Chaisaklert W, Abuali G, Grimm S (2012) De-ubiquitinating proteases USP2a and USP2c cause apoptosis by stabilising RIP1. Biochim Biophys Acta 1823:1353–1365

Moulin M, Anderton H, Voss AK, Thomas T, Wong WW, Bankovacki A, Feltham R, Chau D, Cook WD, Silke J, Vaux DL (2012) IAPs limit activation of RIP kinases by TNF receptor 1 during development. EMBO J 31:1679–1691

O'Donnell MA, Legarda-Addison D, Skountzos P, Yeh WC, Ting AT (2007) Ubiquitination of RIP1 regulates an NF-kappaB-independent cell-death switch in TNF signaling. Curr Biol 17:418–424

O'Donnell MA, Perez-Jimenez E, Oberst A, Ng A, Massoumi R, Xavier R, Green DR, Ting AT (2011) Caspase 8 inhibits programmed necrosis by processing CYLD. Nat Cell Biol 13:1437–1442

Oberst A, Dillon CP, Weinlich R, McCormick LL, Fitzgerald P, Pop C, Hakem R, Salvesen GS, Green DR (2011) Catalytic activity of the caspase-8-FLIP(L) complex inhibits RIPK3-dependent necrosis. Nature 471:363–367

Osborn SL, Diehl G, Han SJ, Xue L, Kurd N, Hsieh K, Cado D, Robey EA, Winoto A (2010) Fas-associated death domain (FADD) is a negative regulator of T-cell receptor-mediated necroptosis. Proc Natl Acad Sci U S A 107:13034–13039

Ozturk S, Schleich K, Lavrik IN (2012) Cellular FLICE-like inhibitory proteins (c-FLIPs): fine-tuners of life and death decisions. Exp Cell Res 318:1324–1331

Park SM, Yoon JB, Lee TH (2004) Receptor interacting protein is ubiquitinated by cellular inhibitor of apoptosis proteins (c-IAP1 and c-IAP2) in vitro. FEBS Lett 566:151–156

Petersen SL, Wang L, Yalcin-Chin A, Li L, Peyton M, Minna J, Harran P, Wang X (2007) Autocrine TNFalpha signaling renders human cancer cells susceptible to Smac-mimetic-induced apoptosis. Cancer Cell 12:445–456

Punj V, Matta H, Schamus S, Chaudhary PM (2009) Integrated microarray and multiplex cytokine analyses of Kaposi's Sarcoma Associated Herpesvirus viral FLICE inhibitory protein K13 affected genes and cytokines in human blood vascular endothelial cells. BMC Med Genomics 2:50

Querido E, Blanchette P, Yan Q, Kamura T, Morrison M, Boivin D, Kaelin WG, Conaway RC, Conaway JW, Branton PE (2001) Degradation of p53 by adenovirus E4orf6 and E1B55K proteins occurs via a novel mechanism involving a Cullin-containing complex. Genes Dev 15:3104–3117

Rajput A, Kovalenko A, Bogdanov K, Yang SH, Kang TB, Kim JC, Du J, Wallach D (2011) RIG-I RNA helicase activation of IRF3 transcription factor is negatively regulated by caspase-8-mediated cleavage of the RIP1 protein. Immunity 34:340–351

Roth S, Ruland J (2011) Caspase-8: clipping off RIG-I signaling. Immunity 34:283–285

Scaffidi C, Schmitz I, Krammer PH, Peter ME (1999) The role of c-FLIP in modulation of CD95-induced apoptosis. J Biol Chem 274:1541–1548

Scheffner M, Werness BA, Huibregtse JM, Levine AJ, Howley PM (1990) The E6 oncoprotein encoded by human papillomavirus types 16 and 18 promotes the degradation of p53. Cell 63:1129–1136

Shisler JL, Moss B (2001) Molluscum contagiosum virus inhibitors of apoptosis: the MC159 v-FLIP protein blocks Fas-induced activation of procaspases and degradation of the related MC160 protein. Virology 282:14–25

Tenev T, Bianchi K, Darding M, Broemer M, Langlais C, Wallberg F, Zachariou A, Lopez J, Macfarlane M, Cain K, Meier P (2011) The Ripoptosome, a signaling platform that assembles in response to genotoxic stress and loss of IAPs. Mol Cell 43:432–448

Thome M, Schneider P, Hofmann K, Fickenscher H, Meinl E, Neipel F, Mattmann C, Burns K, Bodmer JL, Schroter M, Scaffidi C, Krammer PH, Peter ME, Tschopp J (1997) Viral FLICE-inhibitory proteins (FLIPs) prevent apoptosis induced by death receptors. Nature 386: 517–521

Upton JW, Kaiser WJ, Mocarski ES (2008) Cytomegalovirus M45 cell death suppression requires receptor-interacting protein (RIP) homotypic interaction motif (RHIM)-dependent interaction with RIP1. J Biol Chem 283:16966–16970

Vanlangenakker N, Vanden Berghe T, Vandenabeele P (2012) Many stimuli pull the necrotic trigger, an overview. Cell Death Differ 19:75–86

Varfolomeev E, Blankenship JW, Wayson SM, Fedorova AV, Kayagaki N, Garg P, Zobel K, Dynek JN, Elliott LO, Wallweber HJ, Flygare JA, Fairbrother WJ, Deshayes K, Dixit VM, Vucic D (2007) IAP antagonists induce autoubiquitination of c-IAPs, NF-kappaB activation, and TNFalpha-dependent apoptosis. Cell 131:669–681

Varfolomeev EE, Schuchmann M, Luria V, Chiannilkulchai N, Beckmann JS, Mett IL, Rebrikov D, Brodianski VM, Kemper OC, Kollet O, Lapidot T, Soffer D, Sobe T, Avraham KB, Goncharov T, Holtmann H, Lonai P, Wallach D (1998) Targeted disruption of the mouse Caspase 8 gene ablates cell death induction by the TNF receptors, Fas/Apo1, and DR3 and is lethal prenatally. Immunity 9:267–276

Vince JE, Wong WW, Khan N, Feltham R, Chau D, Ahmed AU, Benetatos CA, Chunduru SK, Condon SM, McKinlay M, Brink R, Leverkus M, Tergaonkar V, Schneider P, Callus BA, Koentgen F, Vaux DL, Silke J (2007) IAP antagonists target cIAP1 to induce TNFalpha-dependent apoptosis. Cell 131:682–693

Vucic D, Dixit VM, Wertz IE (2011) Ubiquitylation in apoptosis: a post-translational modification at the edge of life and death. Nat Rev Mol Cell Biol 12:439–452

Wachter T, Sprick M, Hausmann D, Kerstan A, McPherson K, Stassi G, Brocker EB, Walczak H, Leverkus M (2004) cFLIPL inhibits tumor necrosis factor-related apoptosis-inducing ligand-mediated NF-kappaB activation at the death-inducing signaling complex in human keratinocytes. J Biol Chem 279:52824–52834

Wagner L, Marschall V, Karl S, Cristofanon S, Zobel K, Deshayes K, Vucic D, Debatin KM, Fulda S (2013) Smac mimetic sensitizes glioblastoma cells to Temozolomide-induced apoptosis in a RIP1- and NF-κB-dependent manner. Oncogene 32(8):988–997

Wajant H, Scheurich P (2011) TNFR1-induced activation of the classical NF-kappaB pathway. FEBS J 278:862–876

Walczak H, Iwai K, Dikic I (2012) Generation and physiological roles of linear ubiquitin chains. BMC Biol 10:23

Walsh CM, Wen BG, Chinnaiyan AM, O'Rourke K, Dixit VM, Hedrick SM (1998) A role for FADD in T cell activation and development. Immunity 8:439–449

Wang L, Du F, Wang X (2008) TNF-alpha induces two distinct caspase-8 activation pathways. Cell 133:693–703

Wang Z, Jiang H, Chen S, Du F, Wang X (2012) The mitochondrial phosphatase PGAM5 functions at the convergence point of multiple necrotic death pathways. Cell 148:228–243

Welz PS, Wullaert A, Vlantis K, Kondylis V, Fernandez-Majada V, Ermolaeva M, Kirsch P, Sterner-Kock A, van LG, Pasparakis M (2011) FADD prevents RIP3-mediated epithelial cell necrosis and chronic intestinal inflammation. Nature 477:330–334

Wertz IE, O'Rourke KM, Zhou H, Eby M, Aravind L, Seshagiri S, Wu P, Wiesmann C, Baker R, Boone DL, Ma A, Koonin EV, Dixit VM (2004) De-ubiquitination and ubiquitin ligase domains of A20 downregulate NF-kappaB signalling. Nature 430:694–699

Yeh JH, Hsu SC, Han SH, Lai MZ (1998) Mitogen-activated protein kinase kinase antagonized fas-associated death domain protein-mediated apoptosis by induced FLICE-inhibitory protein expression. J Exp Med 188:1795–1802

Yeh WC, Itie A, Elia AJ, Ng M, Shu HB, Wakeham A, Mirtsos C, Suzuki N, Bonnard M, Goeddel DV, Mak TW (2000) Requirement for Casper (c-FLIP) in regulation of death receptor-induced apoptosis and embryonic development. Immunity 12:633–642

Yoneyama M, Fujita T (2009) RNA recognition and signal transduction by RIG-I-like receptors. Immunol Rev 227:54–65

Yuan J, Kroemer G (2010) Alternative cell death mechanisms in development and beyond. Genes Dev 24:2592–2602

Zhang DW, Shao J, Lin J, Zhang N, Lu BJ, Lin SC, Dong MQ, Han J (2009) RIP3, an energy metabolism regulator that switches TNF-induced cell death from apoptosis to necrosis. Science 325:332–336

Zhang H, Zhou X, McQuade T, Li J, Chan FK, Zhang J (2011) Functional complementation between FADD and RIP1 in embryos and lymphocytes. Nature 471:373–376

Zhang J, Cado D, Chen A, Kabra NH, Winoto A (1998) Fas-mediated apoptosis and activation-induced T-cell proliferation are defective in mice lacking FADD/Mort1. Nature 392:296–300

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