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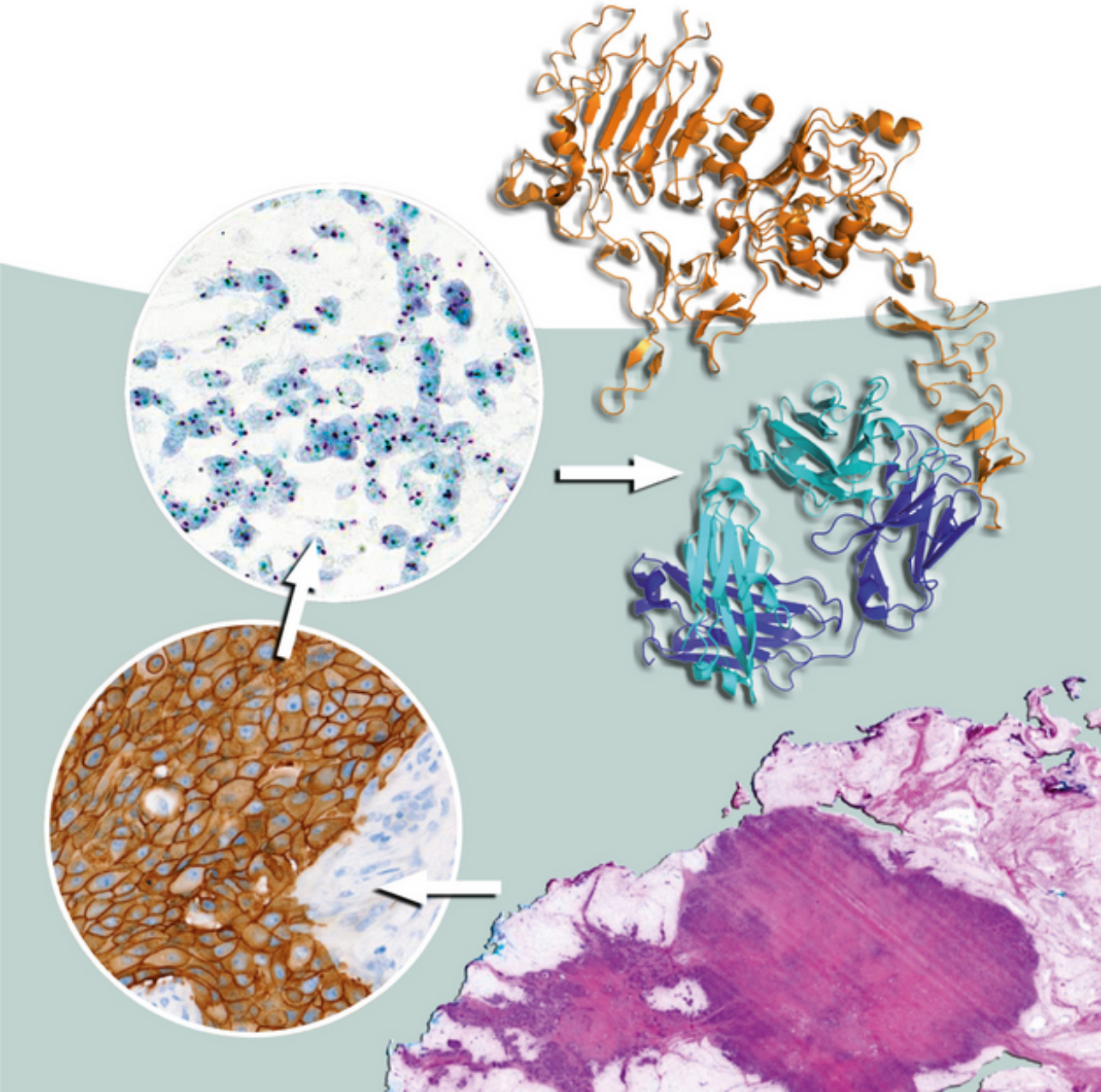
Christoph Wagener, Carol Stocking,
and Oliver Müller

Cancer Signaling

From Molecular Biology to Targeted Therapy



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From Molecular Biology to Targeted Therapy

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Authors

Professor Dr. Christoph Wagener
University Medical Center Hamburg-Eppendorf
Martinistr. 52
20251 Hamburg
Germany

Dr. Carol Stocking
Heinrich-Pette-Institute
Leibniz Institute of Experimental Virology
Martinistr. 52
20251 Hamburg
Germany

Professor Dr. Dr. Oliver Müller
University of Applied Sciences Kaiserslautern
Campus Zweibrücken
Amerikastr. 1
66482 Zweibrücken
Germany

Cover
In a breast cancer specimen (white/violet), the amplification of the *ERBB2* gene is demonstrated on the protein level by immunohistochemistry (brown membrane staining of tumor cells) and on the DNA level by in situ hybridization (dark blue dots). Based on these results, the patient is eligible for therapy by a HER2/Neu antibody. The three-dimensional structure of the antibody (blue/green) in complex to the HER2/Neu receptor (orange) is shown.

Professor Dr. Axel Niendorf, Pathologie Hamburg-West, Germany, kindly provided the breast cancer images. The structure image was designed and kindly provided by Dr. Ingrid Vetter, Max-Planck-Institut für molekulare Physiologie, Dortmund, Germany. The image is based on the entry 1N8Z in http://www.rcbs.org by Cho et al. (2003, Nature 421, 756ff).

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We dedicate our work to all young researchers and students who have chosen to face the challenges and enjoy the satisfactions of scientific and medical careers, with the intention of contributing to the fight against cancer in order to improve, prolong and save lives.

Contents

Preface

1
History of Cancer Signaling Research

Of all the various fields of biomedical science, research on intracellular signaling provides some of the best examples for the successful transfer of basic science discoveries into the clinic. Findings from signaling research, together with new techniques of prevention, diagnosis, radiation, and surgery, have helped to increase the cure rates of all cancer patients from below 10% in the 1930s to over 60% today. It has been a long road from the discovery at the beginning of the twentieth century of chromosomal aberrations in tumor genomes, providing the first molecular insight into tumorigenesis, to the modern-day targeted and personalized anticancer therapy based on over 100 years of molecular and biochemical advances.

The first successful steps in the fight against cancer were observed using broad and unspecific cytostatic drugs. In 1947, the first partial remission of pediatric leukemia in a 4-year-old girl was achieved by using the drug aminopterin. Until this time, children with acute leukemia usually died within weeks of being diagnosed. Aminopterin is a competitive analog of the vitamin folic acid that is a necessary cofactor in the synthesis of nucleobases, the building stones of DNA and RNA.

In 1949, the US Food and Drug Administration (FDA) approved nitrogen mustard, the first chemotherapeutical drug, for the treatment of Hodgkin's lymphoma. Originally developed as a weapon gas, nitrogen mustard kills (cancer) cells by modifying their DNA by alkylation. Nitrogen mustard and its derivatives paved the way for many other alkylating and nonalkylating cytostatic and cytocidal anticancer drugs.

In 1958, the first therapeutic use of a combination of different cytostatic drugs was found to prolong the survival times of leukemia patients. In 1965, it was reported that a specific combination of chemotherapeutics could cure 50% of all patients with Hodgkin's lymphoma. Combination therapy is still used today to lower the risk of side effects and resistance.

During the 1960s, scientists in a hospital in Philadelphia identified a chromosomal aberration linked to certain forms of myeloid and lymphoid leukemia. Thirty years later, a fusion protein generated by the so-called Philadelphia chromosome became the target of one of the first specific and targeted cancer treatments with the kinase inhibitor imatinib (Gleevec).

The 1970s ushered in the era of oncogenes. Researchers found that tumor-causing viruses carry mutated forms of mammalian genes. This finding led to the identification of the first proto-oncogene *KRAS* in 1979. The Ras protein was characterized as an intracellular protein that sends signals from the inner cell membrane into the nucleus. As the first protein with specific signaling activity involved in tumorigenesis, Ras opened a new chapter of cancer research, namely cancer signaling research. Over the following years, the identification of other signaling proteins led to the finding that intracellular signaling pathways control all biological processes and all cellular functions, such as proliferation, differentiation, and migration.

In the 1980s, the first tumor suppressor gene *TP53* was discovered. The corresponding protein is a paradigm for a tumor suppressing protein, acting as a regulator and an inhibitor of several important pathways of intracellular signaling that control proliferation, cell death, and DNA repair. Also during the 1980s, scientists could show that tamoxifen lowers the risk of breast cancer relapse after surgery. Tamoxifen is a steroid hormone analog that functions as a signaling molecule at important knots of the intracellular signaling network.

The 1990s can be regarded as the key decade of cancer signaling research. Many more tumor suppressor genes were discovered. Mutations in such genes, e.g., *APC* and *BRAC1*, were shown to play important roles in the development of inherited and sporadic forms of the two most common cancer types, carcinomas of the colon and breast, respectively. The function of these tumor suppressors could be linked to important intracellular signaling pathways. Also during the 1990s, many new anticancer drugs of the second generation were approved. These drugs do not inhibit tumor growth by specific killing of the cell or by attacking DNA or RNA, but rather by interfering with biological processes that are the outputs of intracellular signaling. A good example is the family of taxanes, which inhibit dynamics of microtubule and thereby block the mitotic process, and are still in use today against ovarian and breast cancer. In 1997, the FDA approved the first anticancer drug of the third generation, rituximab (Rituxan). Rituximab was not only the first drug of molecularly targeted therapy, it was also the first monoclonal antibody used in medical therapy. Rituximab targets a B-cell surface receptor that initiates important signaling cascades regulating proliferation.

During the early 2000s, Trastuzumab was introduced into the therapy of metastatic breast cancer. Trastuzumab binds to HER2/Neu, a homolog of the receptor for the epidermal growth factor (EGF), and inhibits the transfer of growth-activating signals from the cell membrane into the cell. In 2001, the FDA approved imatinib (Gleevec) after just three months of review – the fastest approval in FDA history. Imatinib is the first drug proven to counteract a

molecular defect on the so-called Philadelphia chromosome. It has since become the standard care for patients with chronic myeloid leukemia (CML). Its high efficacy and easily administered pill form enables most patients to live with CML as a chronic but manageable disease. Imatinib inhibits BCR-ABL, which is a constitutively activated kinase positioned in a central node of intracellular signaling. Other targeted drugs were also approved after the turn of the millennium, including gefitinib (Iressa), which blocks the intracellular enzymatic activity of the EGF receptor involved in driving lung cancer growth and spread.

During the late 2000s, tumor-genome sequencing projects started answering questions of how many and which genes are involved in the development of common cancers. Hundreds of the 25 000 protein-coding genes in the human genome show somatic mutations in human tumors. Today, there is strong evidence that mutations in close to 400 genes can be classified as driver mutations, that is, they causally contribute to cancer development. Nearly all cancer genes encode proteins with important functions in signaling pathways. The most prominent group is that of protein kinases, composed of both cytosolic and receptor protein kinases. Furthermore, most of the cancer genes functionally cluster within specific signaling pathways, such as the MAPK pathway, the Wnt pathway, and the PI3K/AKT pathway.

2 Outlook

Molecularly targeted tumor therapy is a rational approach based on the aberrant intracellular signaling implicated in tumorigenesis and tumor progression. However, although great successes have been reported, resistance to therapy remains a sobering phenomenon. After initial spectacular effects, tumors often recur within a year. Interconnecting and parallel signaling pathways coupled to tumor cell heterogeneity are the major reasons for therapy resistance. In order to overcome resistance to targeted therapies, the underlying molecular mechanisms need to be completely and rigorously understood. Since the spectrum of gene mutations and activated pathways can vary between tumor cells at the same location or with the same histological type, predictive diagnostic tests are essential to choose the adequate drug(s) for each individual patient. Comparable to the successful shift to combinatorial application of cytostatic and cytocidal drugs observed in the late 1950s, choosing drug combinations that target different signaling pathways is likely to be more successful than single drugs.

Resistance and ineffective response to targeted therapies have led to some disappointment in the public eye. However, one should consider that it took nearly a century from the first reports of the minor effects of chemotherapeutic drugs to the effective combination therapies applied today. Novel analytical tools, such as deep and whole-genome sequencing, allow for the first time an in-depth analysis of molecular aberrations in tumors. Using these tools, potential therapeutic targets for the rational design of clinical trials can be identified. Although we are still

waiting for the results of more recent clinical trials, it is safe to assume that it will probably not take another century until significant progress has been achieved. Combinatorial therapy has successfully been used to combat AIDS; nevertheless, cancer is much more complex than HIV.

In addition to therapy resistance, the costs of targeted therapies are a great concern to all. In order to deliver effective therapies to all patients, costs must correlate to the budgets available.

3
Intention of this Book

We wrote this book in the hope that it will serve as a useful introduction to a fascinating research field for students, physicians, and researchers. We do hope that the information provided in the book will help facilitate the transfer of results from basic research into the clinic.

4
Selection of Topics and Readers' Comments

In this book, we summarize the current knowledge of cancer signaling research. We are aware that we have not comprehensively enumerated all important findings, and we apologize for failing to cite each and every colleague who has made significant contributions to this field. Our intention was to give a short and concise overview of the important but fast-evolving field of cancer research, rather than writing an exhausting review of the scientific literature. The selection of presented topics is based on our own assessment of the relevant findings and topics discovered over the past decades.

Because nobody is perfect and some aspects in the cancer signaling field change faster than our perception, we appreciate any comments, suggestions, and corrections for this and all upcoming editions.

5
Structures of Chapters

After the introductory chapters on general aspects of intracellular signaling, we describe the molecular background of tumor relevant biological processes, such as cell birth, cell aging, and cell death. In the third part of the book, examples of important signaling pathways and their molecular players are presented. All chapters are structured similarly. After a short summary, we present general aspects of the pathway to provide a basis for the following presentation of molecular details, including key proteins and their functions. In the last part of each chapter, we introduce current therapeutic strategies and some examples

for specific drugs or drug candidates that interfere with the functions of the discussed proteins. For an up-to-date overview of clinical trials, the reader is referred to http://www.clinicaltrials.gov, a service of the U.S. National Institutes of Health. Each chapter ends with a short outlook, in which we summarize open questions and upcoming therapies.

6
Figures and Video Animations

We have set particular attention on the visual presentation to help convey complicated features and interactions. Protein functions, signaling pathways, and biological processes are presented in simplified figures within the text. In addition, several subjects are presented in short animations as part of this book. Because we want these video clips to be clear and understandable, the clips were held simple and are restricted to selected aspects. The basic message is understandable without reading the corresponding chapter in the book. Nevertheless, a viewer of a clip will not regret reading the corresponding book chapter, because he will find valuable additional information and links. Vice versa, readers of the book might use the clips as short animated summaries of the written information.

A few more video clips are found on our Onkoview channel on YouTube (https://www.youtube.com/user/Onkoview/videos) and also on the home page of our book entitled "*Molekulare Onkologie*" published in German language in 2009 (http://www.onkoview.com).

7
Nomenclature and Abbreviations

Names and abbreviations of genes and proteins are those recommended by UniProt (http://www.uniprot.org). The abbreviations deviate from the recommendations in so far as no hyphens were used in the abbreviations of proteins.

June 2016 *Hamburg and Zweibrücken*
Christoph Wagener, Carol Stocking, and Oliver Müller

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List of Abbreviations

5-FU	5-fluorouracil
ABC	activated B-cell
ACL	ATP-citrate lyase
ACTH	adrenocorticotropic hormone
ADAM	a disintegrin and metalloprotease
AFP	alpha-fetoprotein
ALL	acute lymphoblastic leukemia
AML	acute myeloid leukemia
AMPK	adenosine-monophosphate-activated kinase
APAF	apoptosis protease-activating factor
APC	adenomatous polyposis coli protein
APRIL	a proliferation-inducing ligand
Arm	armadillo repeat
ASA	acetylsalicylic acid
AT	Ataxia telangiectasia
BAF	B-cell-activating factor
BAFF	B-cell-activating factor of tumor necrosis factor family
BANGs	BMPs, activins, Nodal, GDFs
BCC	basal cell carcinoma
BCL	B-cell lymphoma
Bcl-2	B-cell lymphoma 2
BCMA	B-cell maturation antigen
BCR	breakpoint cluster region
BER	base excision repair
BH3	Bcl-2 homology domain 3
BLNK	B-cell linker protein
BrdU	bromodeoxyuridine
BTK	Bruton's kinase
BTRC	β-transducin repeat-containing protein
CADASIL	cerebral autosomal dominant arteriopathy with subcortical infarcts and leukoencephalopathy
CARD	caspase-associated recruitment domain
caspase	cysteinyl aspartate cleaving protease

CCC	cholangiocellular carcinoma
CCRCC	clear cell renal cell carcinoma
CDK	cyclin-dependent kinase
CDKI	cyclin-dependent kinase inhibiting protein
CENP-C	centromere protein-C
cIAP	cellular inhibitor of apoptosis
CIN	chromosomal instability
Cip/Kip	CDK-interacting protein/cyclin-dependent kinase inhibitor protein
CK	casein kinase
CLL	chronic lymphocytic leukemia
CML	chronic myeloid leukemia
CMML	chronic myelomonocytic leukemia
COX	cyclooxygenase
DAG	diacylglycerol
DAMPs	damage-associated molecular pattern molecules
DD	death domain
DHF	dihydrofolate
DKK	Dickkopf-related protein
DLBCL	diffuse large B-cell lymphoma
DSB	double-strand break
DUB	de-ubiquitinylating
DVL	disheveled
EBV	Epstein – Barr virus
ECM	extracellular matrix
EGF	epidermal growth factor
EGFR	epidermal growth factor receptor
eIF4	eukaryotic translation initiation factor 4
ELISA	enzyme-linked immunosorbent assay
EMT	epithelial – mesenchymal transition
EPO	erythropoietin
ERBB	avian erythroblastosis oncogene B
ERK	extracellular signal-regulated kinase
FAK	focal adhesion kinase
FAP	familial adenomatous polyposis
FAT domain	FRAP-ATM-TRAP domain
FDA	food and drug administration
FGF	fibroblast growth factor
FIH1	Factor inhibiting HIF1α
FISH	fluorescence *in situ* hybridization
FKBP12	FK-506-binding protein of 12 kD
FOXO	forkhead box O
FRB	FKBP12-rapamycin-binding
FTase	farnesyl transferase
FTI	farnesyl transferase inhibitor

FZD	Frizzled
G phase	gap phase
GAP	GTPase-activating protein
GATOR1	GAP activity toward Rag 1
GBD	GTPase-binding domain
GCB	germinal center B-cell like
GDNF	glial cell line-derived growth factor
GEF	guanine nucleotide exchange factor
GF	growth factor
GIST	gastrointestinal stroma tumor
Glut	glucose transporter
GLUT1	glucose transporter 1
GOF	gain-of-function mutation
GPCR	G-protein-coupled receptor
GRB	growth factor receptor-bound
GRB10	growth factor receptor-bound protein 10
GSI	γ-secretase inhibitor
GSK	glycogen synthase kinase
GSK-3β	glycogen synthase kinase 3β
HAT	histone acetylase
HD domain	heterodimerization domain
HDAC	histone deacetylase
HEAT	Huntington-EF3-A subunit of PP2A-TOR1
HER	human EGF receptor
HGF	hepatocyte growth factor
HIF	hypoxia-inducible factor
HIS	haploinsufficiency
HNPCC	hereditary nonpolyposis colorectal cancer
HR	homologous recombination
HRS	Hodgkin–Reed–Sternberg
HSP90	heat shock protein 90
HTLV	human T-cell leukemia virus
IDH	isocitrate dehydrogenase
IFT	intraflagellar transport
IGF	insulin-like growth factor
IGF1	insulin-like growth factor 1
IGFBP	IGF-binding protein
IKK	IkB kinases
IkB	inhibitor of kB
INK4	inhibitors of kinase CDK4
IP3	inositol-1,4,5-trisphosphate
IRS1	insulin receptor substrate 1
ITAM	immunoreceptor tyrosine-based activation motif
IWP	inhibitor of Wnt production

JAK-STAT	Janus kinase and signal transducer and activator of transcription
LAP	latency-associated protein
LDH	lactate dehydrogenase
LKB1	liver kinase B1
LNR	Lin12/Notch repeats
LOF mutation	loss-of-function mutation
LOH	loss of heterozygosity
LOX	lysyl oxidase
LRP	low density lipoprotein receptor-related protein
LTBP	latent TGFβ-binding protein
M phase	mitosis phase
M1	phase 1 of mortality
MALT	mucosa-associated lymphoid tissue
MAPK	mitogen-activated protein kinase
mar	marker chromosome
MB	medulloblastoma
MDR	multidrug resistance
MEK	MAPK/ERK kinase
MEKK	MEK Kinase
MMP2	matrix metalloproteinase 2
MMR	mismatch repair
MMTV	mouse mammary tumor virus
MNNG	*N*-methyl-*N*′-nitro-*N*-nitrosoguanidine
MOMP	mitochondrial outer membrane permeabilization
MSI	microsatellite instability
mTOR	mechanistic target of rapamycin
MTX	methotrexate
NEC fragment	Notch extracellular fragment
NEMO	NF-κB essential modulator
NES	nuclear export sequence
Neu	neural tumor
NF1	neurofibromatosis type 1
NF-κB	nuclear factor kappa B
NGF	nerve growth factor
NHEJ	nonhomologous end joining
NIC fragment	Notch intracellular fragment
NLR	nucleotide oligomerization domain-like receptor
NLS	nuclear localization sequence
NMDA	*N*-methyl-D-aspartate
NOD	nucleotide oligomerization domain
NRR	negative regulatory region
NSAID	nonsteroidal anti-inflammatory drug
NSCLC	non-small-cell lung cancer
NTMIC fragment	Notch transmembrane and intracellular fragment

PAK2	p21 activated kinase 2
PanIN	pancreatic intraepithelial neoplasia
PcG	Polycomb group
PDAC	pancreatic ductal adenocarcinoma
PDE	phosphodiesterase
PDGF	platelet-derived growth factor
PDGFR	platelet-derived growth factor receptor
PDK1	phosphoinositide-dependent kinase 1
PDPK1	3-phosphoinositide-dependent protein kinase 1
PEST domain	domain rich in proline (P), glutamic acid (E), serine (S), and threonine (T)
PH	Pleckstrin homology
PHD	prolyl hydroxylase domain
PI	phosphatidylinositol
PI3K	phosphatidylinositol-4,5-bisphosphate 3-kinase
PIP2	phosphatidylinositol-4,5-bisphosphate
PIP3	phosphatidylinositol-3,4,5-trisphosphate
PKB	protein kinase B
PKC	protein kinase C
PLC	phospholipase C
PMBL	primary mediastinal B-cell lymphoma
PP2A	protein phosphatase 2A
PPI	protein–protein interaction
PPIase	peptidyl-prolyl *cis–trans* isomerase
PPP	pentose phosphate pathway
PS	phosphatidylserine
PSA	prostate specific antigen
PTB	phosphotyrosine binding
PTEN	phosphatase and tensin homolog
pVHL	von Hippel–Lindau protein
R point	restriction point
RANK	receptor activator of NF-kB
RANKL	RANK ligand
RasGAP	RasGTPase-activating protein
Rb	retinoblastoma-associated protein
Rbx1	ring box 1
RHD	Rel homology domain
Rheb	RAS homolog enriched in brain
RICTOR	rapamycin-insensitive companion of mTOR
RIG	retinoic-acid-inducible gene
RIP	receptor-interacting protein
RKIP	Raf kinase inhibitor protein
ROCK	Rho-activated kinase
ROS	reactive oxygen species
RSV	Rous sarcoma virus

RTK	receptor protein tyrosine kinase
S phase	synthesis phase
S6K	ribosomal S6 protein kinase
SAHFs	senescence-associated heterochromatin foci
SAPK	Stress-activated protein kinase
SA-β-GAL	senescence-associated β-galactosidase
SCC	squamous cell carcinoma
SCF	stem cell factor
SCLC	small-cell lung cancer
SFRP	secreted frizzled-related protein
SH2	Src homology 2
SH3	Src homology 3
SOS	son of sevenless
SREB1/2	sterol-regulatory element-binding protein1/2
SSB	single-strand break
TACI	transmembrane activator and calcium modulator and cyclophilin ligand interactor
TAD	transactivation domain
TAK1	transforming growth factor-activated kinase-1
T-ALL	T-cell acute lymphoblastic leukemia
TCA cycle	tricarboxylic acid cycle
TCF/LEF	T-Cell Factor/Lymphoid-Enhancing Factor
TCGA	The Cancer Genome Atlas
TCR	T-cell receptor
TGF	transforming growth factor
TGFβ	transforming growth factor β
THF	tetrahydrofolate
TIAM1	T-cell lymphoma invasion and metastasis-1
TIS	therapy-induced senescence
TNF	tumor necrosis factor
TNFR	TNF receptor 1
TPA	12-*O*-tetradecanoylphorbol-13-acetate
TPR	translocated promoter region
TRADD	TNF receptor-associated protein with a death domain
TRAF	TNF receptor associated factor
TSC	tuberous sclerosis
TUNEL	TdT-mediated dUTP-biotin nick end labeling
VASP	Vasodilator-stimulated phosphoprotein
V-ATPase	vacuolar ATPase
VEGF	vascular endothelial growth factor
WIF	Wnt-inhibiting factor
WT	wild type

About the Companion Website

This book is accompanied by a companion website:

www.wiley.com/go/wagener/cancersignaling

The companion website page provides supplementary material for the majority of the chapters in this book. The content is password protected. Please see the website for further information how to access the content.

1
General Aspects of Signal Transduction and Cancer Therapy

Summary

This chapter should serve as an introduction into the field of intracellular signal transduction. The biological role of signal transduction pathways will be presented

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together with the mechanisms and the protein domains that are responsible for the direct transduction of signals between molecules. In the second part, we define and describe the major groups of anticancer drugs and their effects on different levels in a simplified model of tumorigenesis. We give examples of important classical drugs and explain their mode of action. Finally, the major mechanisms of drug resistance are described and compounds and approaches that can be used to prevent or circumvent this problem are mentioned.

1.1 General Principles of Signal Transduction

(Video: General aspects of signal transduction – enhanced ebook and closed website: signal_transduction_ebook.mp4)

1.1.1 Biological Signals have to be Processed

Levels of biological communication include communication between whole organisms, communication between organs within an organism, and communication between single cells. Mechanisms for intercellular communication are based on the transfer of signals between cells through direct contacts, by electrical signals, ions, small molecules, or macromolecules. Once a signal has reached a cell, the cell has to "decide" whether and how to react. For this reason, the cell has to process the incoming signal. Most signals are processed by intracellular signal transduction pathways. A signal transduction pathway is a biochemical cascade that connects the incoming signal with the cellular response. Such a pathway fulfills two major functions. First, it modulates the intensity of the originally extracellular signal. It can amplify, weaken, or extinguish the signal. Secondly, the pathway converts the signal into a form that allows and also prepares the cellular response. Examples for potential responses are proliferation, migration, differentiation, and apoptosis. It has to be noted that a signal can be transferred not only by the presence of a molecule but also by its absence. For example, normal cells react to the absence of growth factors by activation of signal transduction pathways that activate apoptosis.

1.1.2 What is a Signal Transduction Pathway?

A signal transduction pathway consists of factors, receptors, adapter proteins, enzymes, second messengers, and transcription factors, which together form a hierarchical sequence of signaling events. Most frequently, the signal transfer from one molecule to another is performed by direct contact and subsequent covalent or noncovalent modification resulting in conformational change of at least one of the interacting partners.

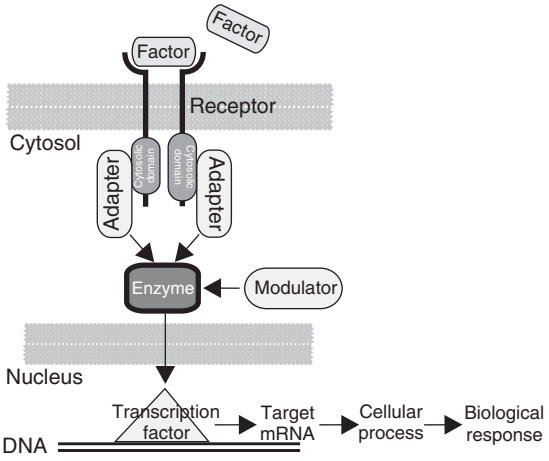


Figure 1.1 Simplified version of a general signal transduction pathway. A signal in the form of an extracellular factor binds to a membrane-bound receptor. The receptor transfers the signal through the membrane onto its cytosolic domain, which transfers the signal to an adapter protein. The adapter transfers the signal to a cytosolic protein, which is frequently an enzyme, for example, a kinase. The activity of this enzyme might be altered by a modulator. The enzyme regulates a nuclear transcription factor, which regulates the expression of target genes. The activities of the translated gene products mediate a cellular process such as proliferation or migration, which results in the biological response to the original signal. (Wagener and Müller, 2009), with permission.

A typical pathway begins with the binding of an extracellular ligand to a membrane-bound receptor. The receptor transports the signal through the plasma membrane into the cell by altering the activity of molecules on the cytosolic side (Figure 1.1). The signal is transferred through the cytoplasm via macromolecules or small molecules. In most pathways, only one or a few enzymes are necessary at this level due to their ability to amplify a signal by several orders of magnitude. Finally, the signal reaches the cell nucleus, where the activity of a transcription factor is altered. As a consequence, this factor promotes or inhibits the expression of distinct genes. The transcribed mRNA is translated and the resulting proteins mediate the biological answer to the original signal.

In this book, we describe the different pathways in the same way as this "master pathway," namely as direct and straight cascades. In this manner, we aim to clearly illustrate their important properties, their biological effects, as well as the potential sites and mechanisms of drugs interfering with them. We are aware that this approach reflects a highly simplified view, which is far from the *in vivo* processes in a living cell. Actually, every real signaling pathway consists of multifaceted parallel or antiparallel cascades, manifold branches, feedback loops, bypasses, and connections to other pathways, which are permanently or temporarily active. Thus, one has to keep in mind that despite the simplified representation, a signaling pathway has to be regarded as a complex signaling network or at least as a part of such a network, rather than as a simple and isolated linear cascade.

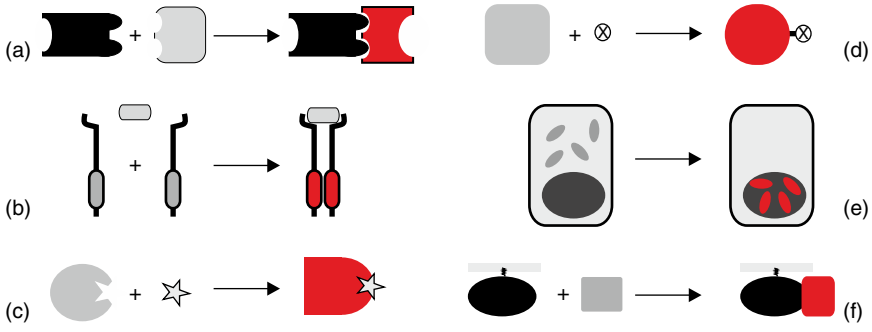


Figure 1.2 Mechanisms of signal transduction. Direct interaction of two different proteins (a), dimerization of monomers (b), interaction with a small molecule (star, c), covalent modification (d), translocation into another compartment (e), or translocation to the inner side of the cell membrane (f) might result in alteration of conformation and activity (red). (Wagener and Müller, 2009), with permission.

1.1.3 Mechanisms of Direct Signal Transduction

Signals are primarily transduced within a pathway by the direct contact of two molecules, usually proteins. There are different mechanisms for this type of signal transfer (Figure 1.2):

1) Many signals are transduced by the noncovalent interaction of two different proteins. Such a protein–protein interaction (PPI) can lead to a conformational change or to an altered activity of one or both interacting partners. An example for such a signal transfer is the interaction between the adapter protein growth factor receptor-bound protein 2 (GRB2) and the GTPase exchange factor son of sevenless-1 (SOS-1), which is part of the mitogen-activated protein kinase (MAPK) signaling pathway (Chapter 7). This binding leads to a conformational change of SOS-1 and to its ability to catalyze the nucleotide exchange of a small G-protein.
2) Secondly, the signal can be transduced by a PPI that is a homo- or a hetero-oligomerization of protein monomers. As an example, the homodimerization of the monomers of a receptor tyrosine kinase, such as the platelet-derived growth factor receptor (PDGFR), leads to the autophosphorylation of both monomers and subsequently to their activation. In the example of PDGFR, the tyrosine kinase activity of the two monomers increases by several orders of magnitude.
3) Third, the noncovalent binding of a small molecule to a protein can cause a conformational change and an altered activity of a protein. An example for such a signal transfer is the activation of the protein kinase A (PKA) by binding of the second messenger cAMP.
4) Fourth, a signal can be transduced by PPI following the covalent modification and activation of one of the interacting proteins. The most frequent covalent

protein modifications are phosphorylations of serine, threonine, and tyrosine residues by kinases. Example is the activating phosphorylation of serines/threonines of MAPK/ERK kinase (MEK) 1/2 by the kinase BRaf. Other covalent modifications leading to conformational changes and to signal transfers are acetylation and the attachment of single amino acids or peptides.

5) The fifth principle of molecular signal transfer is the change in concentration of a protein, a small molecule, or an ion in a distinct cellular compartment. In this case, the signal is not transferred by interactions and conformational changes but rather by the concentration change of a molecule in a limited cellular region. At the new concentration, the molecule can induce an effect that it could not do at the original concentration. An example is the increase in the concentration of β-catenin, a transcription-factor-activating protein, in the nucleus of cells with an activated Wnt pathway (Chapter 11). The high nuclear β-catenin concentration leads to the activation of the transcription factor TCF-4. An example for an altered ion concentration is the increase in the cytosolic calcium concentration after activation of the PI3K/AKT pathway (Chapter 8). This increase leads to the activation of calcium-dependent enzymes.
6) Finally, the altered localization of a protein within the same cellular compartment can lead to its conformational change and thus to signal transduction. Example is the recruitment of the kinase BRaf to the inner side of the plasma membrane by the active Ras protein in cells with the activated MAPK pathway (Chapter 7). At the membrane, BRaf interacts with ceramides and other membrane factors, which leads to its activation.

1.1.4 The Interactome Gives Insight into the Signaling Network

Most signals are transferred from protein to protein by their direct interaction that might be followed by covalent or noncovalent modification. By the use of a high-throughput version of the double-hybrid screen, a PPI map of nearly 6000 human proteins was generated. The screen yielded more than 3000 specific interactions between more than 1700 different proteins (Stelzl *et al.*, 2005). Because most PPIs serve to transfer signals, this map is equivalent to a view into the signaling network of a human cell.

Mathematical approximation indicated that the so-called interactome comprises more than 650 000 different PPIs between 25 000 proteins (Stumpf *et al.*, 2008). Because most PPIs lead to the transfer of a signal, the interactome is equivalent to a view into the signaling network of a living cell. The virtual display of the manifold interactions between the tumor suppressor protein cyclin-dependent kinase inhibitor 1 (CDKN1A or p21) and its interacting protein partners shows a tiny detail and the complexity of this signaling network (Figure 1.3). A simplified version of many signal transduction pathways that are important in tumor development and their interplays is shown in Figure 1.4.

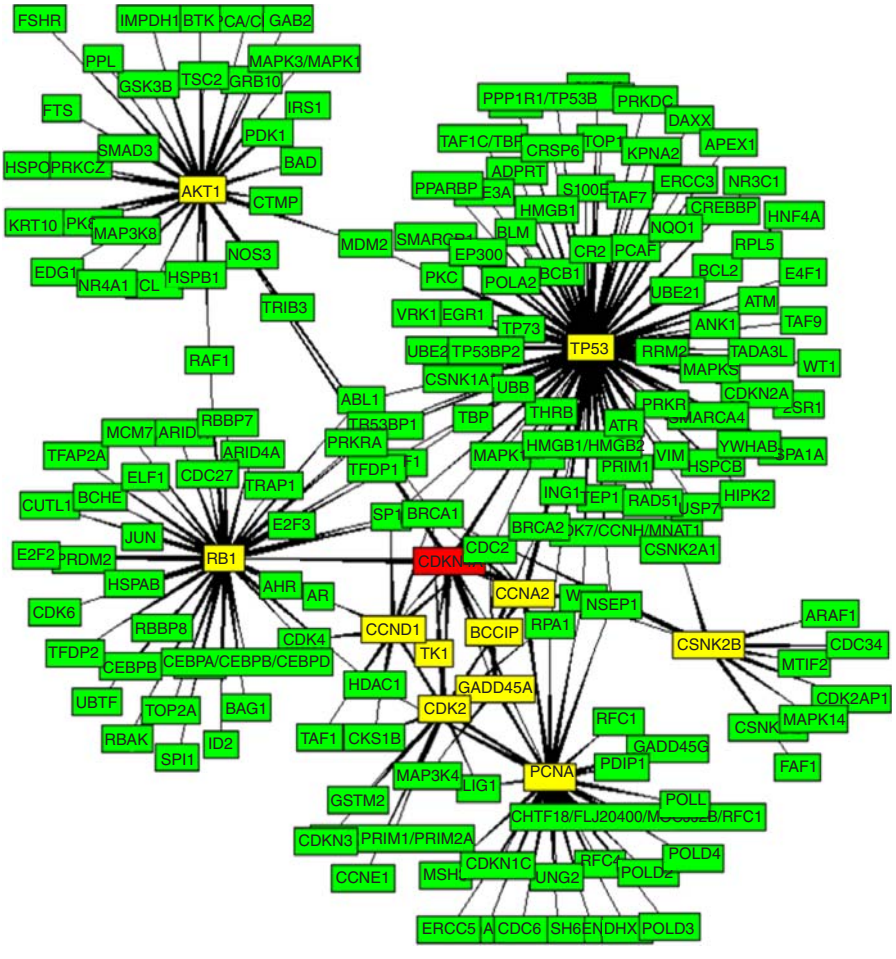


Figure 1.3 Protein interaction partners (yellow) of cyclin-dependent kinase inhibitor 1 (CDKN1A) (red) that are directly or indirectly involved in DNA repair and their protein interaction partners (green). (Wagener and Müller, 2009), with permission.

1.1.5
Protein Domains for Protein–Protein Interaction and Signal Transduction

PPIs are mediated by typical protein domains, which are sequential and structural sections of a protein. The database Gene Ontology classifies protein domains according to intracellular localization, molecular function, and the controlled cellular process (www.geneontology.org). Many signaling proteins carry conserved domains that are responsible for the interaction with a specific ligand (Pawson and Nash, 2000, 2003; Pawson, Raina, and Nash, 2002). When the sequence of a newly identified protein is compared with the database and such domains are recognized, it is possible to predict potential interacting partners and thereby its

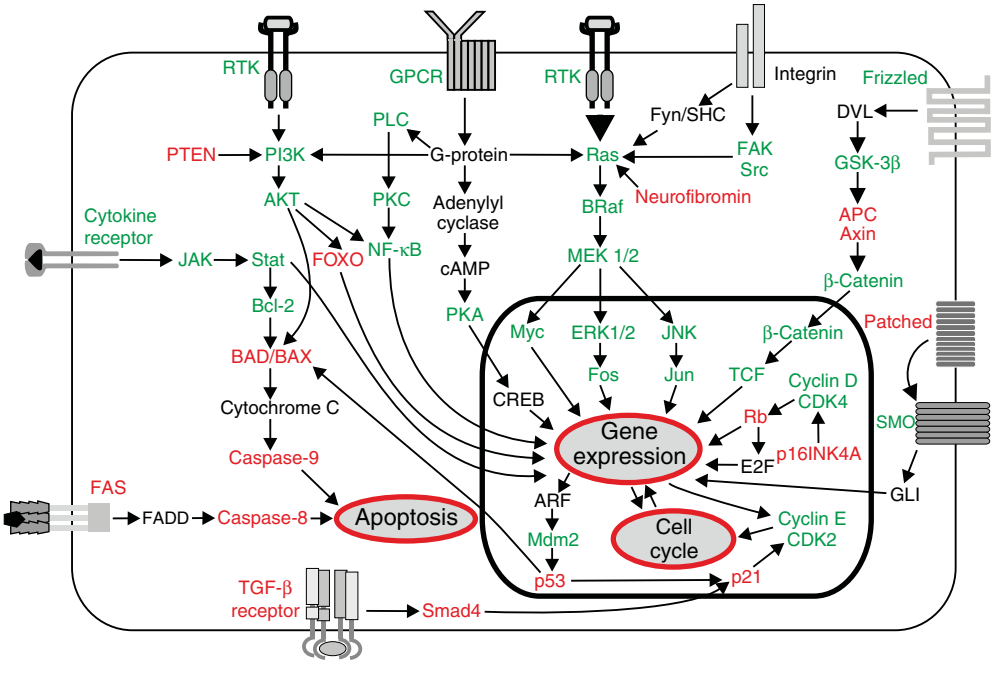


Figure 1.4 Simplified representation of important signaling pathways in tumor cells. Green, proto-oncoproteins; red, tumor suppressor proteins. Arrows indicate interaction and effects, which might be activating or inhibiting. (Wagener and Müller, 2009), with permission.

Table 1.1 Protein domains for protein–protein interaction in signal transduction.

Domain	Examples for proteins with domain	Ligand of domain
SH2	Src, GRB2, RasGAP	Phosphotyrosine
SH3	Src, GRB2, crk	Proline-rich sequences
PTB	SHC-transforming protein, IRS-1, X-11a	Phosphotyrosine
14-3-3	Cdc25, BAD, BRaf, PKC	Phosphoserine
PH	PLCδ, PKB, ATK	Phosphatidylinositol

potential functions. The domains SH2, SH3, PTB, 14-3-3, and PH mediate most of the interactions between signaling proteins (Table 1.1).

SH domains were named after their homology to the three major domains of the protein kinase Src: the catalytic SH (Src homology) 1 domain, the cytoplasmic SH2 domain, and the SH3 domain. The SH2 domain, which stretches over approximately 100 amino acids, is found in many intracellular signaling proteins. SH2 domains bind directly to other proteins with phosphorylated tyrosine residues, including phosphorylated cytoplasmic domains of receptor protein tyrosine kinases (Chapter 5). Proteins with SH2 domains can be classified in

two groups: first, proteins with enzymatic or enzyme-activating activity such as the phosphatidylinositol phosphatidylinositol-4,5-bisphosphate 3-kinase (PI3K), the phospholipase C (PLC) gamma, members of the Src family of nonreceptor tyrosine kinases, and the RasGTPase-activating protein (RasGAP); secondly, the so-called adapter molecules such as GRB2, crk, and SHC, which themselves have no enzymatic activity but serve rather as bridging proteins between proteins with phosphotyrosine domains and other signaling proteins. Structural analysis has shown that the binding pocket of an SH2 domain is necessary for its high-affinity interaction with phosphorylated tyrosines. Amino acids that are located carboxy-terminal to the tyrosines of the interacting partner determine binding specificity.

The PTB (phosphotyrosine-binding) domain is functionally related to the SH2 domain, though it has a different three-dimensional structure. Additionally, the PTB domain recognizes specific amino acids in the amino-terminal neighborhood of a phosphorylated tyrosine. PTB domains have been identified, for example, in IRS-1 (insulin receptor substrate-1) and the adapter protein Shc. The finding that Shc carries both a PTB and a SH2 domain confirms that there are functional differences between these two domains. Interactions of SH2 and PTB domains with their partners often play a role in the first steps of cytosolic signal transduction, which includes the transfer of the signal from the cytosolic receptor domain to a cytosolic protein.

Further downstream in the signaling pathway, other domains are responsible for PPIs. The best-known domain that mediates PPIs in the cytosol is the 60–70 amino-acid-long SH3 domain. SH3 domains bind preferentially to left-handed protein helices, which are rich in prolines. Besides the signaling proteins Src and GRB2, many proteins of the cytoskeleton carry SH3 domains. This finding indicates that the SH3 domain is involved not only in dynamic and fast-regulated signaling pathways but also in events that play a role in migration and interaction of cells.

Finally, many signaling proteins include the 100 amino-acid-long Pleckstrin homology (PH) domain. Although PH domains of different proteins show only low sequence homologies, their three-dimensional structures are very similar. PH domains are found, for example, in protein kinase B (PKB) and PLC and bind to the phospholipids PIP2 (phosphatidylinositol (4,5)-bisphosphate) and PIP3 (phosphatidylinositol (3,4,5)-trisphosphate).

1.1.6
Functions of Mutated Proteins in Tumor Cells

All important cellular processes and activities are regulated by signaling pathways. Tumorigenesis, metastasis, and tumor progression are caused by deregulated and dysfunctional pathways that regulate important properties of tumor cells, such as proliferation, cell adhesion, cell migration, or apoptosis. Signaling dysfunction might result from activation of proto-oncogenes or from inactivation of tumor suppressor genes. Products of these genes play key roles in signal transduction

Table 1.2 Proto-oncoproteins in signaling pathways.

Function of protein	Examples
Growth factor	Wnt, PDGF, FGF-3, FGF-4
Receptor tyrosine kinase	Egfr, CSF-1-R, Kit, Met, PDGFR, Ret, HER2 (erbB2)
Receptor without kinase activity	Mas, c-Mpl
Plasma-membrane-associated nonreceptor tyrosine kinase	Src, Fgr, Fyn, Yes
Nonreceptor tyrosine kinase	Abl, Sck, c-Fes, JAK-1, JAK-2
Kinase activator	Cyclin D1
Serine/threonine kinase	BCR, BRaf
Membrane-associated G-protein	HRas, KRas, NRas
Regulator of transcription factors	β-Catenin, Mdm2
Transcription factor	erbA, C-ets-1, c-Fos, FRA-1, FRA-2, AP-1 (c-Jun), Myc, Pax-1, c-Rel, TAL-1
Mitochondrial membrane protein	Bcl-2

Table 1.3 Tumor suppressor proteins in signaling pathways.

Function of protein	Example
Growth factor antagonist	WIF-1
Adapter protein	Axin
Kinase inhibitor	p21
GTPase activator	Neurofibromin
Transcription factor	p53
Transcriptional repressor	EVI-9 (Bcl-11A)

(Tables 1.2 and 1.3, Figure 1.4). Proto-oncogenes can be activated by mutation, amplification, or overexpression, whereas tumor suppressor genes can be inactivated by mutation, deletion, or inhibited expression.

Futreal and coworkers conducted a census of all genes that have been found to be mutated in human tumors (Futreal *et al.*, 2004). The list is updated regularly on http://cancer.sanger.ac.uk/cancergenome/projects/census/. More than 400 human genes, making 1% of all, are implicated via mutation in cancer. Of these, approximately 90% have somatic mutations in cancer, 20% bear germline mutations that predispose to cancer, and 10% show both somatic and germline mutations. Expression products include all major functions of signaling proteins, for example, growth factors and their receptors, adapter proteins, kinases, and transcription factors. Strikingly, more than half of all mutated proteins belong to only three functional groups. Nearly 10% of all tumor relevant proteins are kinases, while only 3% of all proteins in a normal cell are kinases. Secondly, mutations in transcription factors occur at an incidence 10 times higher than that predicted by their numbers. Finally, proteins that are necessary for detection and

repair of DNA mutations are significantly overrepresented among the mutated proteins.

On the other hand, some protein functions are very rarely mutated in comparison to their proportion in the proteome of a normal cell. An example is the group of G-protein-coupled membrane receptors, for example, the Rhodopsin-like seven-transmembrane domain receptors. Mutations in only 1 of 10 different members of this family have been identified. In conclusion, though mutations in many different proteins are responsible for the differences between normal and tumor cells, these mutations affect only few protein functions.

1.2 Drugs against Cancer

1.2.1 Terms and Definitions

Nearly all drugs used in anticancer therapy can be classified into five groups according to their major effects: cell-killing drugs, process-blocking drugs, molecule-interfering drugs, DNA-repairing drugs, and resistance-inhibiting drugs. Certainly, this classification is not completely strict. For example, some modifiers of methylation and acetylation bind to specific molecules and affect signal transduction and also inhibit the process of transcription.

Anticancer therapy with the cell-killing and process-blocking drugs is widely known as *chemotherapy*, abbreviated CTX or chemo. Because the term *chemotherapy* was originally used for all therapies with synthetic chemicals (chemotherapeutics) independently of the treated disease, this term is ambiguous. Instead, we use the phrase cytostatic therapy or anticancer therapy. It has to be mentioned that cytostatic describes only one aspect of a drug, namely its inhibitory effects on cell proliferation. Strictly speaking, cytostatic drugs do not include drugs with cell-killing (cytocidal) activities. Such activities may base on cytotoxic, apoptosis inducing, or starving effects. A correct, though uncommon, term covering the therapy by nearly all anticancer drugs would be "cytostatic and cytocidal therapy."

1.2.2 The Steps from a Normal Cell to a Tumor

An ideal anticancer drug should affect all tumor cells, whereas it should preserve all normal cells. Thus, the drug has to attack tumor cells at one or more distinct features that normal cells do not possess. The most important features can be summarized in a simplified cascade of tumorigenesis, which show the differences between a tumor cell and a normal cell at different levels (Figure 1.5). The ultimate cause for the transition of a normal cell into a tumor cell is mutation of its DNA. As described earlier in this chapter, mutations might lead to altered levels or altered activities of proteins that play important roles in signal transduction pathways.

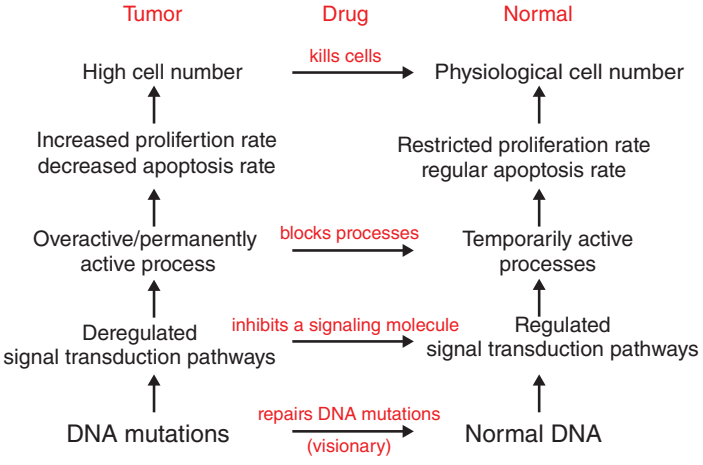


Figure 1.5 Mechanistic levels for the interference of anticancer drugs illustrated by a simplified cascade of tumorigenesis (left) and the corresponding cascade in normal cells (right). DNA mutations may cause deregulation of signaling pathways, which result in permanently active or overactive processes (e.g., transcription), in an increased proliferation rate and, at the end, in a higher cell number. Over the past decades, drug research moved from the top to the bottom of this cascade. Classical drugs kill cells or block processes underlying proliferation or apoptosis. Innovative drugs interfere with molecules involved in signaling pathways. DNA-repairing drugs are still visionary. Drugs circumventing or inhibiting drug resistance are not shown.

This results in deregulation of pathways. Signal transduction pathways control cellular processes, such as DNA replication, RNA transcription, chromosome segregation, and protein synthesis, which are the bases for proliferation, differentiation, apoptosis, and other biological functions. Deregulated signal transduction pathways might cause the permanent activation or overactivation of these processes. The resulting high proliferation rate or the low apoptosis rate might lead to an increased cell number and the formation of a tumor.

1.2.3 Interference Levels of Therapeutic Drugs

The five major groups of anticancer drugs affect different levels of this tumorigenic cascade and abolish the tumor specific features (Figure 1.5). A cell-killing drug attacks the cell in its entirety. A process-blocking drug interferes with a cellular process, such as gene transcription, proliferation, or apoptosis. Some general mechanisms and some major examples of these classical anticancer drugs are described in this chapter. More innovative drugs include molecule-interfering drugs, which bind to one specific molecule, which is involved in signal transduction, and change its activity or function. Such drugs are described in detail together with their modes of action in the chapters of the corresponding pathways.

DNA-repairing drugs, which are still visionary, might be able to convert the tumor cell back into a normal cell. The fifth group of drugs includes compounds that circumvent or inhibit drug resistance.

Over the past decades, the focus of anticancer drug research has moved from the top to the bottom of the tumorigenic cascade. Parallel to this movement, the specificity of the drugs increased from compounds attacking the cell in its entirety to those interfering with single molecules in signaling pathways.

1.2.4
Drugs Attacking the Whole Cell

During World War I, it was found that victims injured or killed by mustard gas had reduced numbers of white blood cells. This observation led to the beginning of systematic anticancer drug development during the 1940s. The mustard gas molecule sulfur mustard (bis(chloroethyl) sulfide) was chemically modified to mechlorethamine (bis(2-chloroethyl) methylamine) (Figure 1.6) and used to treat patients with Hodgkin's disease (Joensuu, 2008). Mechlorethamine (trade name Mustargen) is an alkylating agent causing irreversible DNA damage. Its two reactive ethyl groups bind covalently to the nitrogens in the rings of the nucleobases guanine and cytosine leading to permanent bonds between the two DNA strands. These bonds block the separation of the DNA double strand into single strands, which is necessary for transcription and replication, leading to cell death.

Because of its strong side effects, mechlorethamine is only used today in the treatment of few forms of leukemia and lymphoma. Nevertheless, the molecule served as the prototype structure for other therapeutics and can be regarded as the first anticancer drug of the cell-killing drug group. These first-generation drugs

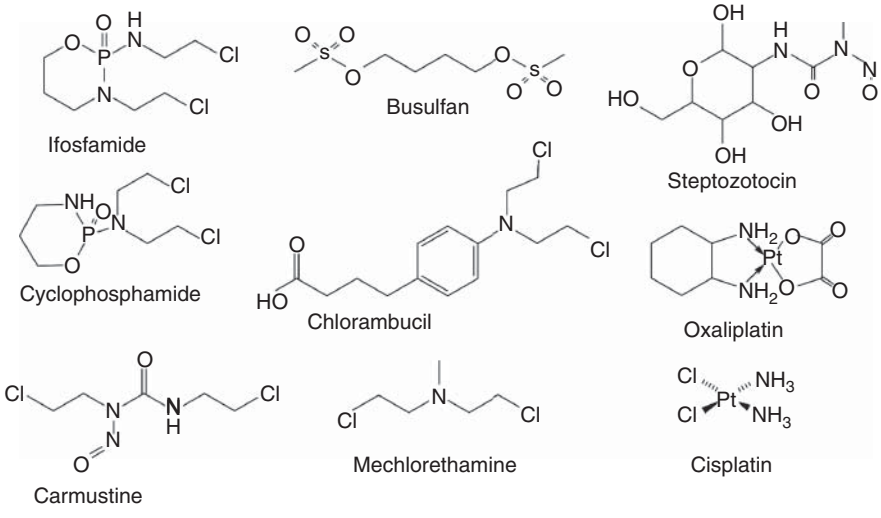


Figure 1.6 Examples for DNA alkylating anticancer drugs.

affect parts or molecules of the cell that are necessary not only for the active cell cycle but also for cell metabolism and cell survival (Figure 1.5, top). Most of these drugs induce toxic effects against both tumor and normal cells. Because most tumor cells have decreased ability to detect and to repair DNA damages caused by these drugs, more tumor cells than normal cells are killed. This decreased DNA repair ability is caused by inactivating mutations in genes that coordinate DNA repair and control DNA integrity. Examples are the tumor suppressor genes *TP53* and *MSH2*, which are mutated in more than 50% of all tumors. DNA in tumor cells with such defects is irreversibly damaged and destroyed by DNA alkylating agents such as mechlorethamine. DNA in normal cells is also damaged, though it is repaired faster and more efficiently than the DNA in tumor cells, because of the presence of intact repair systems.

1.2.4.1 DNA Alkylating Drugs

Today, many synthetic derivatives of mechlorethamine, such as the DNA-alkylating drugs cyclophosphamide, ifosfamide, and chlorambucil, are used in cancer therapy (Figure 1.6). Cyclophosphamide is an example for an inactive prodrug. It is metabolized in liver cells to the active molecule phosphoramide mustard, which forms covalent DNA interstrand bonds. Phosphoramide mustard is inactivated by oxidation by aldehyde dehydrogenases. Cells with high levels of aldehyde dehydrogenases are less sensitive against the toxic effects of phosphoramide mustard than cells with low levels of these enzymes. Because hematopoietic stem cells and stem cells in mucous membranes express high levels of aldehyde dehydrogenases, cyclophosphamide is less toxic against bone marrow and mucous membranes than other alkylating compounds.

Similar to phosphoramide mustard, the two metalorganic complexes cisplatin and oxaliplatin form DNA interstrand bonds. In addition, these two molecules are able to form covalent bonds between bases of the same strand. Such intrastrand bonds prohibit the complementary base pairing and thus block transcription and replication. Cisplatin is widely used in single drug therapies and combination therapies. More than 90% of all nonseminoma types of testicular tumors can be successfully treated by the combination therapy BEP (bleomycin, etoposide, cisplatin).

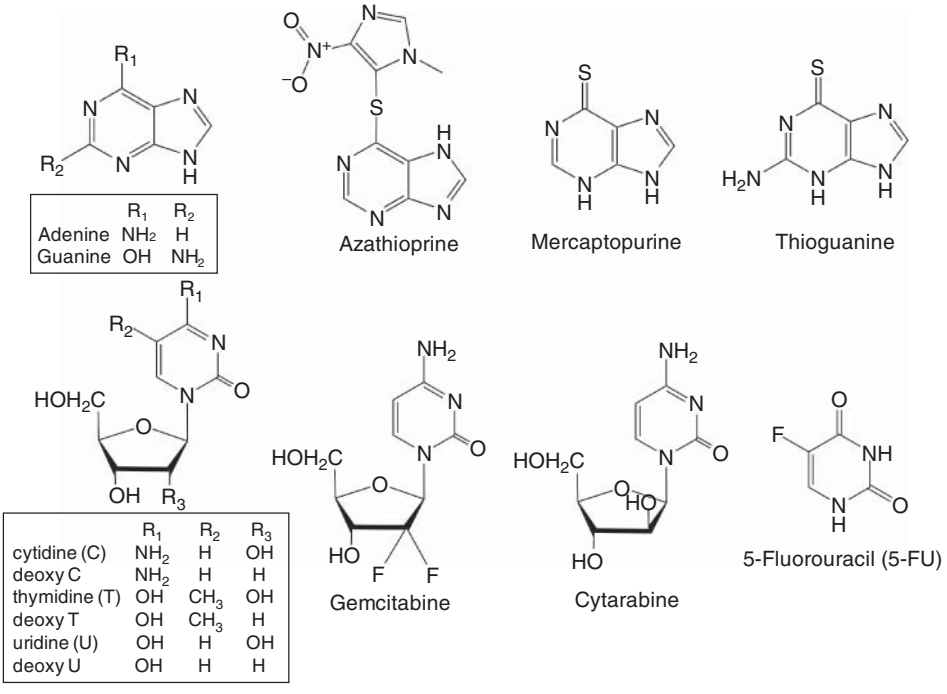
Other groups of alkylating drugs are the group of alkylsulfonates, with its main representative busulfan, and the large group of nitrosourea derivatives, including carmustine and streptozotocin. There are also some DNA-modifying antibiotics used in cancer therapy. Examples are the polypeptide dactinomycin (actinomycin D) and the large group of anthracyclines. Both are polyaromatic molecules that intercalate between neighbored base pairs in the DNA and thus inhibit the separation of the double strand into single strands. Thereby, intercalation blocks both replication and transcription. Because of its high toxicity, dactinomycin is used today against only a few cancers, such as rhabdomyosarcomas. An important representative of anthracyclines is doxorubicin (adriamycin), which is used in the therapy of Hodgkin's disease and several solid tumors.

1.2.5 Process-Blocking Drugs

With the increasing knowledge of the cellular processes that underlie biological functions, such as apoptosis and proliferation, novel potential target sites for anticancer drugs have emerged. Anticancer drugs of the second generation, which have been developed since the 1970s, interfere with RNA transcription, DNA replication, or chromosome segregation.

1.2.5.1 Drugs Blocking Synthesis of DNA and RNA

An antimetabolite is structurally related to a molecule that is a necessary substrate (metabolite) of a physiological biochemical reaction. The structural difference between the metabolite and the antimetabolite is small enough to allow the binding of the antimetabolite by the enzyme, but large enough to inhibit its metabolization in the reaction. As a consequence, the enzyme is blocked and the reaction is inhibited. The largest group of antimetabolites among the cytostatic drugs are the analogs of bases and nucleosides (Peters, Schornagel, and Milano, 1993). Typical examples are the analogs of purines (azathioprine, mercaptopurine), of pyrimidines (fluorouracil), or of pyrimidine nucleosides (cytarabine) (Figure 1.7).



	R_1	R_2
Adenine	NH_2	H
Guanine	OH	NH_2

	R_1	R_2	R_3
cytidine (C)	NH_2	H	OH
deoxy C	NH_2	H	H
thymidine (T)	OH	CH_3	OH
deoxy T	OH	CH_3	H
uridine (U)	OH	H	OH
deoxy U	OH	H	H

Figure 1.7 Examples for purine analogs (upper row), pyrimidine analogs, and pyrimidine nucleoside analogs (lower row). Residues of the physiological nucleobases and nucleosides are shown in the boxes. (Wagener and Müller, 2009), with permission.

Analogs of nucleosides are able to inhibit cell proliferation and tumor growth via several different mechanisms. First, some of these molecules compete with physiological precursor molecules and block biochemical pathways that lead to the synthesis of the DNA and RNA monomers. Some analogs are converted into the triphosphate form of the corresponding nucleotide analog. As such they can bind to DNA or RNA polymerases and inhibit the polymerization reaction by competing with the physiological substrates. Alternatively, they are incorporated by the polymerases into the growing DNA or RNA strands and block their further elongation.

1.2.5.2 Drugs Blocking the Synthesis of DNA and RNA Precursor Molecules

A common mechanism of base analogs is the inhibition of enzymes that catalyze the generation of nucleosides, the precursor molecules of the building blocks of DNA and RNA. Fluorouracil (5-FU) is an important anticancer drug used against tumors of the stomach and the colon. Fluorouracil is metabolized to 5-FdUMP, which blocks irreversibly the enzyme thymidylate synthase. This enzyme produces dTMP, which is a precursor molecule of dTTP, a substrate of the DNA polymerase (Figure 1.8).

Cytarabine was the first nucleoside of the analogs with an altered sugar component. This cytidine analog carries arabinose instead of ribose or deoxyribose (Figure 1.7). It is incorporated into the growing DNA strand instead of deoxycytidine. The following repair reactions lead to strand breaks and misincorporations. Cytarabine is used against several leukemias in adults.

Methotrexate (amethopterin), abbreviated MTX, is another standard drug working as an antimetabolite (Figure 1.9). As an antagonist of folic acid, MTX

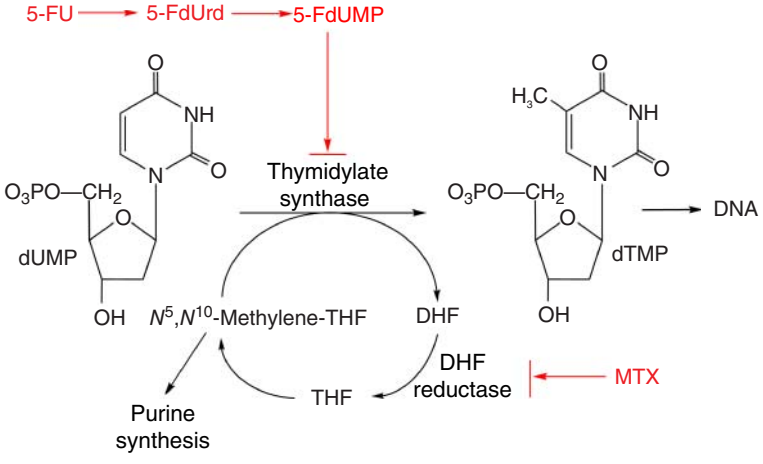


Figure 1.8 Effects of fluorouracil (5-FU) and methotrexate (MTX). 5-FU is metabolized to 5-FdUMP, which blocks irreversibly the enzyme thymidylate synthase. MTX blocks DHF reductase, which delivers the cofactor THF for purine and thymidylate synthesis. DHF, dihydrofolate and THF, tetrahydrofolate. (Wagener and Müller, 2009), with permission.

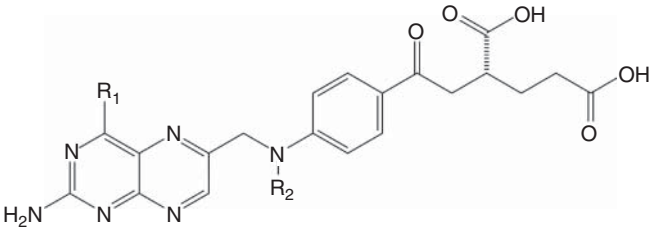


Figure 1.9 Basic structures of folic acid also called vitamin B9 ($R_1 = OH$, $R_2 = H$) and its two antagonists aminopterin ($R_1 = NH_2$, $R_2 = H$) and methotrexate ($R_1 = NH_2$, $R_2 = CH_3$).

blocks irreversibly the enzyme dihydrofolate reductase and thus prohibits the formation of tetrahydrofolate (THF) from dihydrofolate (DHF) (Figure 1.8). DHF in turn is produced from folic acid (vitamin B9). THF is the precursor molecule of N^5,N^{10}-methylene-THF, which is the coenzyme in purine and thymidylate synthesis and thus is essential for production of both DNA and RNA. In the presence of MTX and the resulting lack of thymine, the enzyme DNA polymerase incorporates uracil instead of thymine during DNA elongation. The removal of uracil by the enzyme uracil glycosylase and its replacement by thymine lead to strand breaks and misincorporations. MTX is used in single drug therapies and in combination therapies against acute lymphatic leukemia (ALL), carcinomas of the urothelium and the breast, and several other tumors.

1.2.5.3 Drugs Blocking Dynamics of Microtubules

Microtubules are intracellular protein fibers with several functions. Besides being a component of the cytoskeleton, microtubules are responsible for the equal segregation of chromosomes to the daughter cells during the mitotic anaphase. Microtubules are polymers that are built from heterodimers of one α- and one β-tubulin monomer. Dynamics of microtubules describes the regulated equilibrium between polymerization of α-/β-tubulin dimers to microtubules and depolymerization of the microtubules (Figure 1.10).

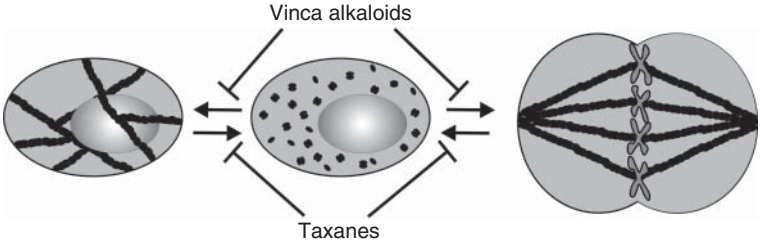


Figure 1.10 Dynamics of microtubules and the effects of vinca alkaloids and taxanes. Microtubules are components of the cytoskeleton (left) and serve as fibers for chromosome segregation during mitotic anaphase (right). Vinca alkaloids block polymerization of tubulin dimers, whereas taxanes block depolymerization of the fibers. (Wagener and Müller, 2009), with permission.

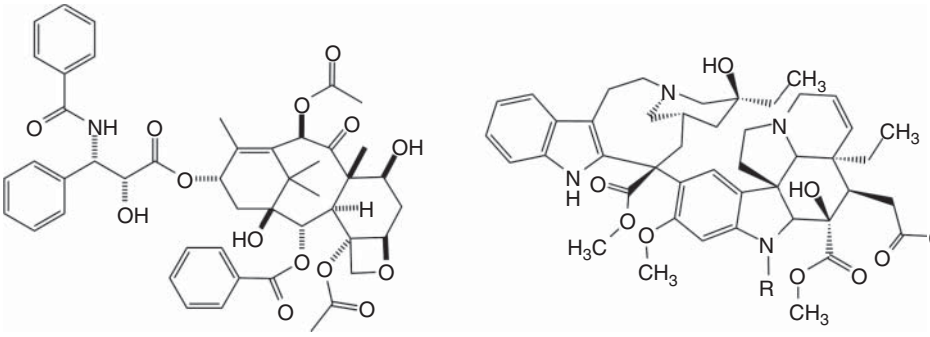


Figure 1.11 Paclitaxel and vinca alkaloids. (Wagener and Müller, 2009), with permission.

The dynamics of microtubules serves as therapeutic target of two groups of natural alkaloids. Vinca alkaloids from the Madagascar periwinkle have been used in traditional medicine against different diseases including diabetes (Figure 1.11). In systematic trials of their antidiabetic effects in the 1950s, treated laboratory animals displayed a reduced activity of the bone marrow (myelosuppression). Based on this finding, clinical trials with leukemia patients proved the antineoplastic effects of vincristine and vinblastine. Today, vincristine (Oncovin) is used in the combination therapy CHOP (cyclophosphamide, hydroxydaunorubicin, oncovin, prednisolone) for non-Hodgkin's lymphoma, as well as in the combination therapies of Hodgkin's lymphoma and ALL. Vinca alkaloids bind to the α-/β-dimer of tubulin and inhibit the polymerization of the dimers to microtubules (Figure 1.10). In addition, vincristine and vinblastine cause the detachment of the microtubules from the spindle pole. Consequently, chromosomes cannot segregate and the mitotic cell is frozen in the metaphase.

The second group of alkaloids with effects on microtubules dynamics is the taxane group from the bark of the Pacific yew tree (Taxus) with its best-known representatives docetaxel (Taxotere) and paclitaxel (Taxol) (Figure 1.11). These compounds were identified in the 1960s in a screen for new natural compounds with antineoplastic effects (Leistner, 2005). Today, the complex molecule paclitaxel is produced by semisynthesis starting from a natural precursor molecule from the European yew tree. Paclitaxel binds to the GDP-bound β-tubulin monomer in the polymerized microtubules and, in this way, leads to its stabilization. By this mechanism, the depolymerization of microtubules, which is necessary for proliferation and chromosome segregation, is blocked. Thus, taxanes shift the dynamic equilibrium of tubulin polymerization and microtubule depolymerization into the opposite direction as compared to vinca alkaloids (Figure 1.10). Paclitaxel is used in single drug and combination therapies of many solid tumors, including carcinomas of the ovary, lung (together with cisplatin), breast (together with Herceptin), and prostate.

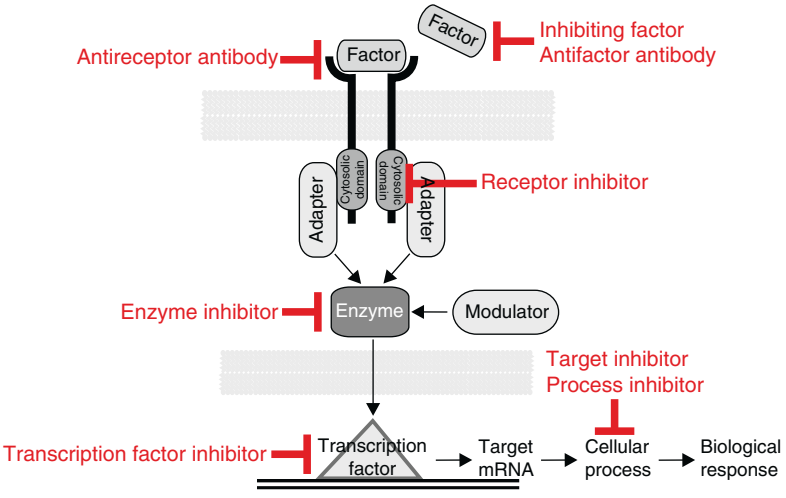


Figure 1.12 Potential drugs interfering at different levels of a general signal transduction pathway.

1.2.6
Innovative Molecule-Interfering Drugs

The antitumor effects of most cell-killing and process-blocking drugs were identified in undirected searches by chance. In contrast, anticancer drugs of the most recent generation were the results of directed searches for molecules that specifically interfere with tumor specific proteins. During the 1990s, our insights into intracellular signal transduction increased rapidly. With the knowledge that signal transduction pathways control all cellular processes and that deregulated pathways cause tumorigenesis, many new potential targets for drug interference were identified. These findings led to the development of the new group of molecule-interfering anticancer drugs. These third-generation drugs interfere specifically with molecules involved in intracellular signal transduction (Figure 1.5). Imatinib (Gleevec), which was FDA-approved in 2001, is a paradigm for such a drug. It was purposefully developed as a selective inhibitor of the enzyme BCR-ABL1, which phosphorylates many substrates in distinct leukemic cells and promotes their proliferation (Chapter 6). Other anticancer drugs of the third generation are directed against signaling factors, extracellular or intracellular domains of receptors, transcription factors, or target proteins of signaling pathways (Figure 1.12). Examples for molecule-interfering drugs are introduced in the chapters of the corresponding signal transduction pathways.

1.2.7 Fast-Dividing Normal Cells and Slowly Dividing Tumor Cells: Side Effects and Relapse

The typical differences between the cell cycle in tumor cells versus fast-dividing normal cells or somatic stem cells are very small. Therefore, drugs targeting the cell cycle of tumor cells also affect a large group of normal cells. Consequently, such drugs induce toxic effects on normal cells. Typical side effects include hair loss, alteration of mucous membranes, nausea, vomiting, and immune suppression.

Furthermore, tumor cells that proliferate slowly, or not at all, at the time point of therapy are not affected by drugs that selectively attack the active cell cycle. These cells survive and might contribute to tumor relapse after the therapy is stopped. For this reason, slowly growing tumors, such as prostate and renal cell carcinomas, are treated with low-drug doses over a long time period. Although this low-dose therapy does not lead to complete tumor eradication, tumor growth is suppressed.

1.2.8 Drug Resistance

Knowledge of the mechanism of a cytostatic drug already facilitates understanding how a tumor cell might defy the drug effects (Lippert, Ruoff, and Volm, 2008) (Table 1.4). A common mechanism for cellular drug resistance is the export of the drug. The gene *MDR1* (Multidrug resistance protein 1) encodes for P-glycoprotein 1 (P-gp), which is a glycosylated ATP-dependent efflux pump in the plasma membrane with a broad substrate specificity. P-gp is highly expressed in normal cells of the intestinal epithelium and the liver, where it exports potentially harmful xenobiotics. A tumor cell overexpressing *MDR1* is resistant and invulnerable against many anticancer drugs.

Secondary resistance might develop during long-term therapies. This type of resistance is caused by the therapy itself. An example is the induction of the gene encoding for thymidylate synthase under therapy with 5-FU. As a consequence, the level of thymidylate synthase rises to the point that it can no longer be blocked by intracellular 5-FU.

The more specific a therapy is, the easier it is for a cell to develop resistance. When a drug attacks only one or a few targets, a tumor cell may escape by a simple single-step mechanism. A single point mutation in the fusion gene *BCR-ABL1* may lead to resistance against imatinib (Gleevec) (Chapter 6).

1.2.8.1 Drugs Circumventing Resistance

Over the past few years, several compounds that prevent or inhibit drug resistance have been developed. Two general principles to prevent resistance can be distinguished. First, the therapy can be designed in a way that lowers the likelihood of resistance. The most common and most effective way is to combine two or more therapeutic strategies that attack the tumor from different directions. An example

Table 1.4 Exemplified causes and mechanisms of resistance against anticancer drugs.

Principle and gene responsible for resistance	Biochemical mechanism	Acquired resistance against
Gene amplification		
MDR1 (P-glycoprotein 1)	Export of hydrophobic compounds	Dactinomycin, doxorubicin
DHFR (dihydrofolate reductase)	Excess of free dihydrofolate	Methotrexate
Increased gene expression		
MT1 (Metallothionein) genes	Complexing of metals and export	Cisplatin
GSTP1 (glutathione S-transferase P)	Complexing by glutathione and export	Several different
BCL2	Inhibition of apoptosis induced by extrinsic stimuli	Apoptosis-inducing compounds
Genes for DNA repair enzymes	Repair of DNA damages	DNA alkylating agents
TYMS (thymidylate synthase)	Replacement of blocked enzymes	5-FU
PDGFRB (PDGF receptor)	Activation of BRaf independent pathway (pathway shift)	Vemurafenib
Gene mutation		
BCR-ABL1	Insensitivity of the enzyme	Imatinib

is the combination of a cytostatic therapy with an immune therapy. The simultaneous administration of 5-FU and interferon-α represents a combination therapy of patients with progressed solid tumors (Mitchell, 2003).

The second strategy is to directly inhibit the mechanism that is responsible for resistance. Examples of resistance-inhibiting compounds are the cyclic peptide cyclosporine and its derivatives, which block the cellular export of foreign substances by the MDR1 gene product P-gp. Another example is therapy with drugs or drug derivatives that block the mutated protein that escaped the regular drug. Novel derivatives of imatinib with broader substrate specificity, such as nilotinib, are able to inhibit mutated forms of the protein BCR-ABL1.

1.3 Outlook

As in the past, anticancer therapy in the future will also likely be based on the mechanisms represented by the drug groups: cell-killing, process-blocking, molecule-interfering, DNA-repairing, and resistance-inhibiting. The fine art of

anticancer therapy is to find the right balance between attacking the entire cell and inhibiting a single molecule, while preventing resistance. From today's point of view, four trends in research for novel drugs against cancer will be followed in the future.

Identifying and targeting new specific targets. The new techniques of molecular diagnosis, including the methods of whole-genome sequencing, are providing increased information with regard to the mutation spectra and molecular events of every tumor. Based on these data, each patient can be theoretically treated by one or a few highly selective drugs. In order to reach this goal, however, many more molecule-interfering drugs need to be developed to target the growing number of proteins that are deregulated in tumor cells. The major advantage of this approach is that these drugs destroy less normal cells and are thus accompanied by fewer side effects than classical drugs.

Combating tumor heterogeneity. Unfortunately, progressed tumors are generally heterogeneous at the cellular and molecular levels. Because of the diversity and the high number of deregulated targets, it is unrealistic to attack each potential target within the heterogeneous cell population of a tumor with a specific molecule-interfering drug. Thus, more and better cell-killing and process-blocking drugs are needed in order to fight tumors successfully. The broader target spectrum of these two types of drugs has the additional advantage that the likelihood of resistance is lower.

Circumventing and fighting drug resistance. In parallel to the groups of cytostatic and cytocidal drugs, new compounds have to be developed to fight resistant tumor cells. These include mainly drugs and drug derivatives that inhibit targets and mechanisms of resistance, such as overexpressed genes or mutated proteins.

Reversing mutations. The still visionary group of DNA-repairing drugs would induce the reversion of the tumorigenic DNA mutations. This approach, involving somatic gene therapy, would be the only way to fight a tumor at its roots, to convert tumor cells back into normal cells, and to cure a tumor ultimately (Figure 1.5). A specific drug for directed DNA repair has been a dream of cancer therapists for decades. With the new techniques of the CRISPR/Cas9 technology, this dream might become reality (Sander and Joung, 2014).

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2
Tumor Cell Heterogeneity and Resistance to Targeted Therapy

Summary

Tumor cells are heterogeneous. The heterogeneity of tumor cells may be explained by (i) clonal evolution driven by spontaneous mutations, (ii) various stages of cell differentiation within the tumorigenic tissue, and (iii) the tumor environment. In general, tumors evolve from a single cell and thus are clonal in nature. During clonal evolution, tumor cells acquire genetic and epigenetic lesions, which may differ between different tumor subclones within the same tumor. Tumors may also contain immature and more mature cells, indicating differentiation from tumor stem or progenitor cells. Within the microenvironment of solid tumors, the supply of oxygen and nutrients by newly formed blood vessels may differ in different areas of the tumor, providing selective pressure for different tumorigenic subclones. The microenvironment may also separate tumor cell clones from each other and may generate niches for tumor-stem-like cells. Tumor cell heterogeneity

Cancer Signaling: From Molecular Biology to Targeted Therapy, First Edition.
Christoph Wagener, Carol Stocking, and Oliver Müller.

Companion Website:www.wiley.com/go/wagener/cancersignaling

is the major reason for therapy resistance. Combinatorial therapies may overcome therapy resistance.

2.1 The Genetic Basis of Tumorigenesis

It is well established that human cancer is a genetic disease caused by mutations in DNA. Germline mutations are present in the zygote, whereas somatic mutations are acquired during development or after birth, accumulating throughout life. Only a limited set of normal genes, if mutated, affect tumor initiation or tumor progression. A mutant gene that is actively involved in tumor initiation or progression is called an *oncogene*. If an inactivating mutation in a cellular gene drives tumorigenesis, the respective gene is designated *tumor suppressor gene*.

In the human genome, base pairs are mutated at a defined rate. For normal colonic stem cells, a mutation rate of 5×10^{-9} mutations per base pair per cell generation has been estimated (Loeb, Loeb, and Anderson, 2003) and for cells of the pancreatic duct, a rate 5×10^{-10} per base pair per generation (Yachida *et al.*, 2010). Mutations occur during the replication of a cell. Considering a constant mutation rate per nucleotide, there is a close relation between the number of cell divisions and the number of cells hit by mutations. Mutation rates can also be accelerated by environmental factors, including exposure to chemical carcinogens and radiation.

If a mutation is causally involved in the initiation or in the progression of a tumor, it is called a *driver mutation*. Some driver mutations may be essential for the entire course of the disease; other driver mutations may confer a growth advantage only at certain stages of the disease. If a mutant protein is to serve as a therapeutic target, its role through disease course needs to be considered. The ideal therapy should hit all tumor cells at all stages of tumor progression (Stratton, Campbell, and Futreal, 2009).

The majority of mutations do not confer a growth or "fitness" advantage. If such a mutation occurs in the genome of a cell harboring a driver mutation, it will be present in the progenitors of the cell hit by the mutation. These mutations are designated passenger or hitchhiker mutations.

In human malignant tumors, many driver mutations interfere with cellular signaling pathways. General characteristics of signaling pathways are outlined in Chapter 1.

2.2 Clonal Heterogeneity

2.2.1 Clonal Origin of Tumors

Knowledge of whether a tumor cells arises from a single cell (clonal) or has a multicellular origin has important implications for understanding its origins

(etiology) and pathogenesis. It was long postulated that tumors arose as the consequence of a rare event in a single cell. However, to verify clonality in human neoplasms (cancers), it was necessary to find markers that could distinguish between cells with distinct genetic or epigenetic traits, the so-called mosaicism. In the 1960s and 1970s, several technologies were established, including analysis of genes suppressed by X-inactivation, immunoglobulin production by B-cells, or abnormal chromosomal karyotypes, which could be used to study mosaicism (Fialkow, 1976). Although such approaches were not applicable to all cancer types or patient samples, they provided compelling evidence of the clonal nature of cancers. A few key examples are presented as follows.

Immunoglobulin (Ig) production was the first mosaic system used to determine the clonal origin of a tumor. Multiple myeloma or plasma cell myeloma is a malignant tumor arising from plasma cells. Plasma cells are cells of the B-cell lineage, which reside in the bone marrow. Plasma cells produce antibodies, which circulate in the peripheral blood. When the proteins of human serum are separated by electrophoresis, most antibodies move as a broad fraction, the fraction of gamma globulins (IgG). The wide mobility range of the gamma globulin fraction reflects the fact that different clones of plasma cells produce antibodies of distinct primary structure. In most instances, the malignant plasma cells retain the capacity to produce antibodies. When the serum proteins of patients with multiple myeloma are separated by electrophoresis, a slender peak in the range of the gamma- or beta-globulin fractions is observed in the great majority of cases. The slender peak reflects the fact that each of the malignant plasma cells produces an antibody of identical primary structure. As the tumor cells were derived from a single transformed plasma cell, they form a tumor cell clone. The protein underlying the slender peak in the serum protein electrophoresis is designated monoclonal or "M" protein (Figure 2.1). Recognition that the variability in immunoglobulin proteins was due to somatic rearrangements of the Ig gene loci provided additional tools to extend clonal analysis to lymphoid tumors not expressing antibodies.

Gene expression mosaicism caused by X-inactivation is a system applicable to a wider range of tumor types. In somatic cells of women, either the paternal or the maternal X chromosomes is inactivated by methylation. The inactivation is random, which means, in a somatic cell, either the paternal or the maternal X-chromosome is inactivated. In a seminal publication, Bert Vogelstein, Eric R. Fearon, and Stanley R. Hamilton reported on the analysis of X chromosomal genes in normal colonic mucosa and colonic carcinomas of female patients (Fearon, Hamilton, and Vogelstein, 1987). In cells of the normal colonic mucosa, an X chromosomal gene in either the paternal or maternal chromosome was inactivated. However, in colonic adenomas or carcinomas, it was always the gene on the paternal or the maternal X chromosome, which was inactivated. These results indicate that the carcinoma cells were derived from a single cell (Figure 2.2).

Based on these and many other findings, it is now well established that human tumors originate from a single transformed cell, indicating that the initiating event is a rare event.

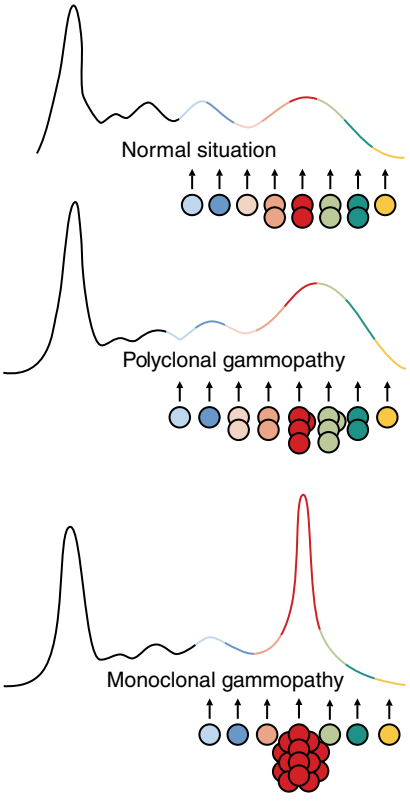

Figure 2.1 Monoclonal origin of multiple myeloma. Under normal conditions and in polyclonal gammopathy, multiple unique plasma cell clones secrete antibodies of different electrophoretic mobility. In multiple myeloma, the clonal plasma cell population secretes antibodies of identical primary structure. This results in a defined slender peak in the electropherogram of serum proteins. (Wagener and Müller, 2009), with permission.

2.2.2 Clonal Evolution

(*Video*: https://www.youtube.com/watch?v=XmZdPoZb804)

In 1976, Peter C. Nowell suggested the model of clonal evolution of tumor cell populations (Nowell, 1976). This was based on the observation that some tumor types had evidence of several genetic mutations (e.g., chromosomal deletions, translocations, etc.). The model implies that the cell initiating a tumor is hit by an event that provides the tumor cell with a growth advantage in comparison with the cells not hit by this event. One of the progenitors of this cell is hit by a second event, which again confers the cell with a growth advantage, and so forth. After sufficient events, the parental clone of a tumor is born. Although not explicitly postulated in the text, the figure in Nowell's seminal publication suggests that the more advanced clone will overgrow the less advanced clones (Figure 2.3). This view of tumor cell evolution is in accordance with Darwin's view of evolution: *Multiply, vary, let the strongest live and the weakest die* (Darwin, 1859).

At that time Nowell proposed the model of clonal evolution of tumor cell populations, the genes responsible for the initiation and progression of tumors had

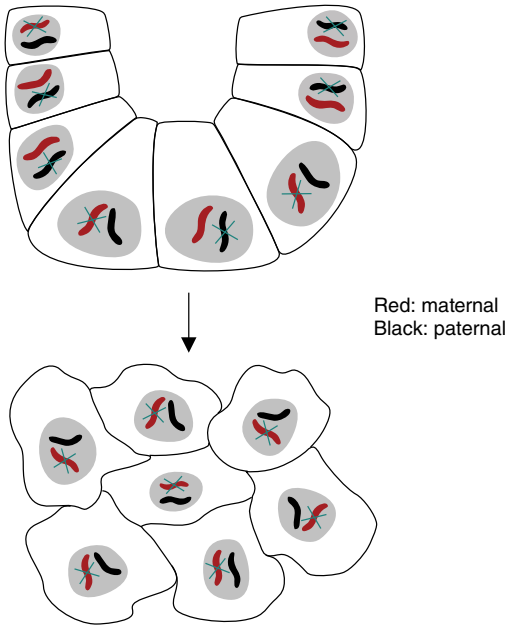

Figure 2.2 X-chromosome inactivation in normal colonic mucosa and in colonic tumors. In cells of the normal colonic mucosa of women, a polyclonal pattern of X-inactivation is observed. In colorectal carcinomas of women, either the maternal or the paternal X-chromosome is inactivated. This finding indicates a monoclonal origin of colorectal carcinomas (based on results by Fearon, Hamilton, and Vogelstein, 1987). (Wagener and Müller, 2009), with permission.

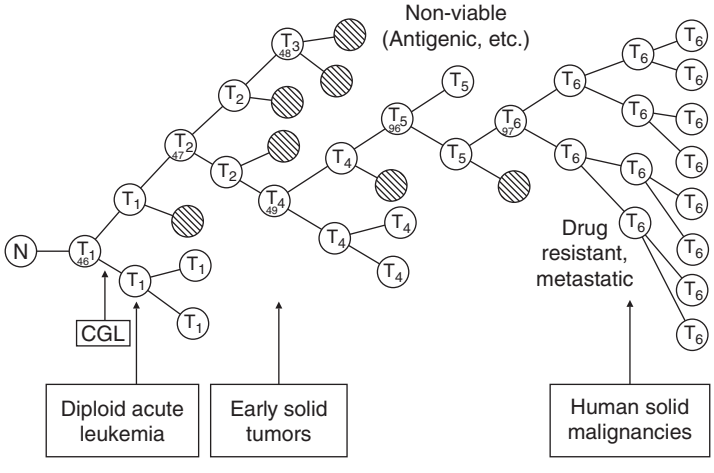

Figure 2.3 Nowell's model of clonal evolution in neoplasia. T_1, T_2, T_3, and so on indicate the evolving clones. The double-digit numbers indicate the number of chromosomes. Hatched circles indicate extinguished variants. CGL, chronic granulocytic leukemia (chronic myeloid leukemia). (Nowell, 1976), with permission.

not yet been identified. However, in 1960, Nowell and his colleague Hungerford described the first chromosomal abnormality that was regularly encountered in a human malignancy, the Philadelphia chromosome. The Philadelphia chromosome is observed in tumor cells of the great majority of patients with chronic myeloid leukemia (CML) (Nowell and Hungerford, 1960). Based on this and other findings, Nowell suggested that the "events" that drive the evolution of tumor cell populations are chromosomal and genetic lesions. Meanwhile, this assumption has been proven by advanced techniques of genomic analysis. In the following, the results of two exemplary investigations are described.

In childhood acute lymphoblastic leukemia (ALL), cell clones were distinguished with respect to their accumulation of genomic aberrations using fluorochrome-labeled probes. All of the leukemia cells investigated contained the *ETV6-RUNX1* fusion gene, the product of the translocation t(12;21). This genomic aberration is predominately a prenatal and presumed initiating event. In leukemic cells with this fusion gene, the allelic status of the nonfused *ETV6* and *RUNX1* genes as well as that of the *PAX5* gene were analyzed. The genes *ETV6*, *RUNX1*, and *PAX5* encode transcription factors and are subject to secondary deletions or amplifications in t(12;21) leukemia. In individual cells, copy number alterations were enumerated in reference to the *ETV6-RUNX1* fusion. In this way, the genetic signatures of individual tumor cell subclones could be distinguished, and the evolutionary relationship between the subclones was inferred. In Figure 2.4, the subclonal architecture of an ALL is shown. Two major conclusions

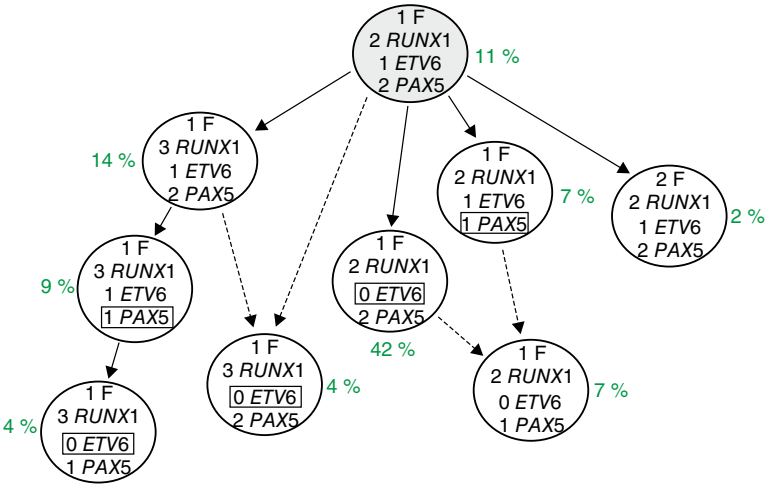

Figure 2.4 Subclonal architecture in a case of acute lymphoblastic leukemia. The different clones were distinguished by the detection of lesions of the genes *RUNX1*, *ETV6*, and *PAX5* using fluorescence in situ hybridization probes. "F" indicates the *ETV6-RUNX1* fusion gene, which is considered the initiating genetic lesion. The number before each gene indicates the number of gene copies (not including those in the gene fusion) and thus reflects amplifications or deletions events. (Anderson *et al.*, 2011), with permission.

can be drawn from this analysis: (i) the leukemia consists of subclones that differ with respect to their genomic aberrations and (ii) cell clones evolve from one another (Anderson *et al.*, 2011).

Gerlinger and coworkers asked the question if different sections of primary renal carcinomas and associated metastases exhibited different genomic aberrations. Indeed, they found that 63–69% of detected mutations are not found in every tumor region analyzed. These findings indicate that the tumor contained different subclones of tumor cells. Comparable to the evolution of species, the evolution of different clonal populations was analyzed based on ancestral relationships, and a phylogenetic tree of tumor regions was constructed by clonal ordering. The phylogenetic tree of one of the renal carcinomas is shown in Figure 2.5. The evolution of clonal populations within the primary tumor and in its metastatic sites is represented by a branching tree. Only one genetic abnormality is present in the stem and in each of the branches, which means that each cell of the primary carcinoma and the different metastases contain this mutation. From the stem of the evolutionary tree, one branch diversifies into primary tumor regions, whereas the other branch evolves into the clones present in metastatic sites. Interestingly, specific genes showed different mutations in distinct parts of the tumor, with each mutation conferring comparable gains of function to the encoded proteins, suggesting convergent phenotypic evolution. In three additional renal carcinomas and metastatic sites, comparable patterns of clonal evolution were reported. The

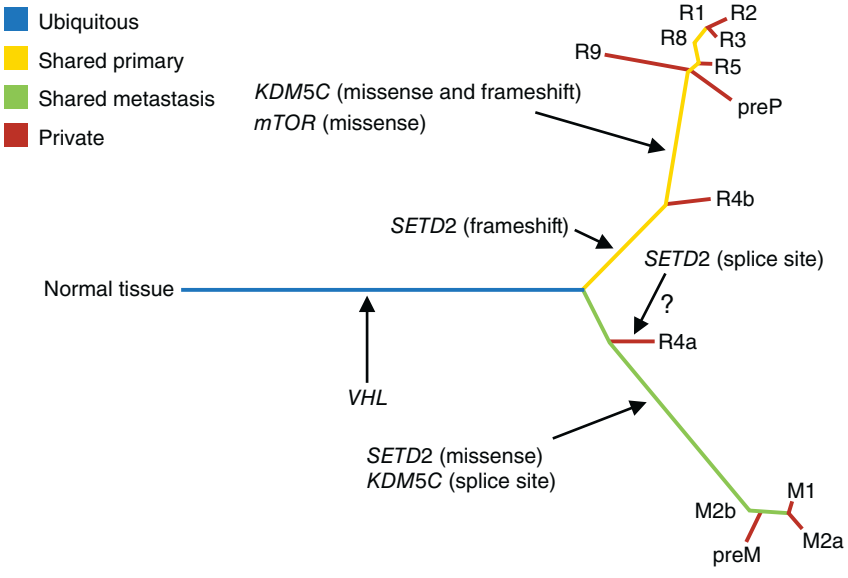

Figure 2.5 Phylogenetic relationship in regions of a primary renal carcinoma and its metastases. Branch lengths are proportional to the number of nonsynonymous mutations separating the branching point. R, regions of the primary tumor; M, metastasis; "Pre" indicates sampling before the start of therapy. (Gerlinger *et al.*, 2012), with permission.

genomic diversity of different tumor cell clones drives Darwinian selection and evolutionary adaptation (Gerlinger *et al.*, 2012).

These and other investigations provide evidence that a human tumor and its metastatic seedlings may contain clonal populations of tumor cells, which differ with respect to their genomic lesions. These findings differ from the message of the figure in Nowell's publications, which implies that the strongest clone outgrows the weaker clones. There are several explanations for the persistence of clonal heterogeneity of human tumors as observed in the two studies outlined earlier:

- The genomic lesions, which differ between different clones, are irrelevant or do not confer significant differences in fitness.
- The clones differ in their fitness over time. If the tumor had more time to grow, the fittest clone would have outgrown the other clones.
- Clones depend on each other, for example, by providing growth factors to each other.
- Clones adapt to different environments, for example, relative to the supply of oxygen and nutrients or at different metastatic sites.

From a Darwinian point of view, adapting to the environment is of utmost importance for survival: *In the struggle for survival, the fittest win out at the expense of their rivals because they succeed in adapting themselves best to their environment* (Darwin, 1859). The fitness of tumor cell clones may differ dramatically when the environmental conditions change, for example, by therapeutic interventions.

2.2.3 The Time Course of Clonal Evolution

It is well known that the evolution of cancer, particularly of carcinomas, takes years or even decades. Cutaneous malignant melanoma and hereditary nonpolyposis colorectal carcinomas serve as good examples. Often, the initiating carcinogenic event in cutaneous malignant melanoma is the exposure to UV radiation in early childhood. The malignant tumors arise decades later (Miller and Mihm, 2006). In hereditary nonpolyposis colorectal cancer, the predisposing mutation is already present in the germ line. Colonic carcinomas arise between the ages of 25 and 40 years (Rustgi, 2007).

Based on the advances in DNA sequencing technologies, it is possible to sequence all or at least the majority of genes in human cancer. If this approach is taken for primary tumors and their metastases, it is possible to calculate the time course of clonal evolution. Bert Vogelstein and coworkers first applied this approach to colonic and pancreatic cancer (Jones *et al.*, 2008; Yachida *et al.*, 2010). It is assumed that a normal pancreatic duct cell, the tumor-initiating cell, acquires mutations until the parental cell of the carcinoma is born. The parental cell forms the first tumor cell clone. This clone gives rise to further clones with additional mutations. The clones extend into different parts of the

primary carcinoma and into its metastases. When the DNA in different parts of the primary tumor and in metastases is sequenced, one is able to delineate a set of mutations that is common to all lesions. This set of mutations should have been present in the founder cell of the carcinoma. If one knows the mutation rate per base pair and per cell generation, as well as the time between subsequent cell divisions, the time it takes from the time point of tumor initiation until the point at which the parental clone is born can be extrapolated. It is similarly possible to calculate the time it takes from the birth of the parental clone until the birth of the clone that gives rise to a metastasis. In this case, the mutations in a metastatic site, the index lesion, are analyzed. The calculation is based on those mutations that are present in the index lesion, but not in the parental clone.

It should be noted that the mutations, on which the calculations are based, should be in those genes reflecting the stochastic error rate in DNA replication. For this reason, the calculations were restricted to passenger mutations. In self-renewing tissues such as colonic mucosa or the hematopoietic system, a significant fraction of passenger mutation accumulates before the onset of neoplasia. In the pancreas, however, normal pancreatic ductal cells, which are the precursors to pancreatic adenocarcinomas, do not self-renew (Tomasetti, Vogelstein, and Parmigiani, 2013). In Figure 2.6, the time course of the genetic evolution of pancreatic cancer is shown.

Most sporadic colorectal carcinomas arise from benign adenomas. In most resected carcinoma specimens, adenomatous tissue has been overgrown by the carcinomatous tissue. Sometimes, however, adenoma tissue is still present and

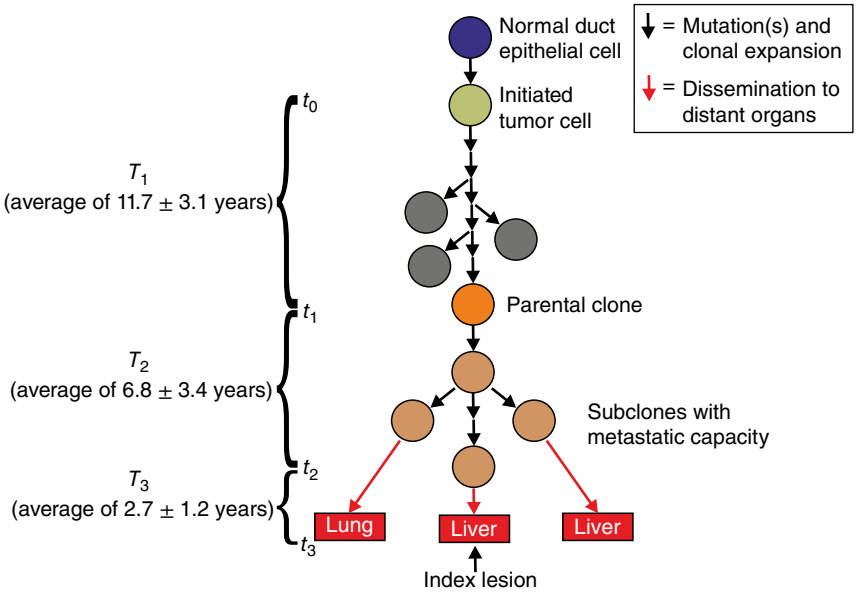

Figure 2.6 Scheme of the genetic evolution of pancreatic cancer. (Yachida *et al.*, 2010), with permission.

available for mutational analysis. It has been shown that approximately one-third of the mutations present in the carcinoma were not present in the adenoma. In contrast, few, if any, additional mutations were present in metastases compared to the primary carcinoma from which the metastases had arisen. Using a mathematical approach similar to the approach taken in pancreatic cancer, the average time interval between the birth of the founder cell of the adenoma and the birth of the founder cell of an advanced carcinoma was determined to be 17 years. The average interval between the founder cell of the advanced carcinoma and founder cell of the liver metastases is only 1.8 years. These findings indicate that it takes many more clonal expansions from the benign adenoma to the carcinoma than from the primary carcinoma to distant metastases (Jones *et al.*, 2008).

2.2.4 Clonal Evolution and Resistance to Therapy

Many oncogenic mutations alter signal transduction pathways in tumor cells. For example, mutations may increase the activity of a kinase protein, either by increasing absolute protein levels or by directly impacting on negative or positive regulators of its activity (see Chapter 5). Compounds have been developed that target the amplified or mutant kinases in order to reduce their activity. Therapies that specifically block the activity of mutant proteins or block the signaling pathways of mutant proteins are known as *targeted therapies*. The first great success of a targeted therapy was achieved in patients with CML. The drug imatinib blocks the activity of a fusion protein containing the ABL1 phosphotyrosine kinase (see Chapter 6). After an initial response to imatinib, which may last years, the disease may recur due to acquired drug resistance (Druker, 2002). Resistance to targeted therapies has turned out to be a recurring and sobering phenomenon. A particularly striking example is cutaneous melanoma treated with the drug vemurafenib. The drug inhibits the kinase activity of Braf, which is mutated in about half of the patients with metastatic cutaneous melanoma. Within 2 weeks after start of treatment, metastatic nodules disappear in patients harboring the *BRAF* mutation. Invariably, however, melanoma metastases recur after about half a year (Flaherty *et al.*, 2010).

Determining the genomic aberrations that drive clonal evolution of tumors will provide a conceptual framework to identify the mechanism(s) by which tumors become resistant to targeted therapeutic approaches. In theory, there are two possibilities for drug resistance that occurs within a short timeframe: the therapeutic drug may induce resistance itself or resistant clones may be present prior to therapy. These two possibilities were investigated by Luria and Delbrück more than 60 years ago, and the results are still relevant today.

In 1943 the Nobel prize laureates Max Delbrück and Salvador E. Luria published a seminal paper on the resistance of bacteria to viral infection (Luria and Delbruck, 1943). When a bacterial culture is exposed to bacteriophage, resistant bacterial variants appear readily in cultures grown from a single cell. The authors tested two possibilities that may explain the phenomenon: (i) resistance is induced

by infection or (ii) spontaneous mutations confer resistance prior to infection. The authors reasoned that each culture plate should contain roughly the same number of resistance clones if the first theory were correct. If the second theory were true, mutations conferring resistance would be induced constantly with a low probability. If the mutations occurred late in culture, when bacteria have multiplied extensively, resistant colonies would be small. Sometimes, however, a mutation hits a bacterium early after the start of culture. This would lead to a large colony of a resistant clone. The fluctuation in the size of colonies was what Delbrück and Luria observed. The authors published a probability distribution that fits their results (Luria–Delbrück distribution). Importantly, the distribution also applies to bacterial resistance against treatment with antibiotics and to resistance of tumor cells against therapeutic drugs (Diaz *et al.*, 2012).

In contrast to some of the cytotoxic drugs used in conventional chemotherapy, the drugs used in targeted therapeutic approaches are probably not mutagenic. For this reason, it is difficult to imagine how these drugs would actively induce resistance in a cell. Considering the long time interval between the initiation of a precancerous cell and the diagnosis of cancer, as observed in pancreatic cancer, the number of cell divisions prior to the start of therapy far exceeds the number of cell divisions after the start of therapy. Thus, the chance of generating mutant clones is exceedingly higher in the pretherapy than in the posttherapy time interval for most malignant diseases. The assumption that resistant cell clones are present in the tumor tissue prior to the start of a targeted therapy has been experimentally proven. Therapies involving inhibitors of the receptor for the epidermal growth factor (EGF) will serve as examples.

Tumor cells in patients with non-small-cell lung cancer (NSCLC) may harbor mutations in the gene encoding the epidermal growth factor receptor (*EGFR*). The EGFR belongs to the family of receptor phosphotyrosine kinases (see Chapter 5). Tumors with mutant EGFR may respond to drugs targeting the kinase domain of the receptor. However, in the course of therapy, drug resistance develops. Amplification of the MET gene encoding a different receptor tyrosine kinase, designated hepatocyte growth factor (HGF) receptor, is one of the mechanisms leading to therapy resistance. Amplifications of the *MET* gene have been detected in recurrent tumor specimens treated with an EGFR antagonist. Applying sensitive techniques, *MET* amplification could be detected in rare (<1%) tumor cells before drug treatment (Turke *et al.*, 2010).

As described in detail in Chapter 5, the EGF signaling pathway involves the KRas protein, which acts downstream of the EGFR. The KRAS gene is the oncogene most often mutated in human carcinomas. In some cases of NSCLC, the EGF signaling pathway is activated by mutant KRas, in which inhibitors of the EGFR tyrosine kinase would be ineffective (Karapetis *et al.*, 2008). In a cohort of patients with NSCLS, *KRAS* genes were analyzed in the tumor tissues. In patients with wild-type *KRAS* genes, therapy with an EGFR antibody was initiated. Most of the patients achieved either a partial response or stable disease with antibody therapy. Approximately 25 weeks after the start of therapy, the patients developed clinically

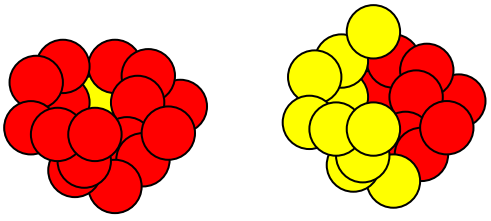

Figure 2.7 Two possibilities how a resistant tumor cell clone (yellow) escapes detection. (a) The cells of the resistant cell clone are present in the tumor tissue analyzed; however, the detection method is not sensitive enough to detect a mutant allele at high excess of wild-type alleles. (b) The cells of the resistant cell clone are not amenable to analysis, for example, in a region of the primary tumor used for histopathological inspection or in distant metastases.

evident progressive disease. Using highly sensitive detection methods, it was possible to detect mutant *KRAS* genes in the patient's peripheral blood. Approximately 22 weeks after the start of antibody therapy, mutant *KRAS* genes could be detected in the blood plasma in about onethird of the NSCLC patients. Under a generalized Luria–Delbrück distribution, it was estimated that the mutant cells were already present before the start of therapy (Diaz *et al.*, 2012).

There are two reasons why resistant tumor cell clones can be missed prior to the initiation of a targeted therapy: (i) the analytical method is not sensitive enough to detect the mutant alleles in high excess of wild-type alleles or (ii) the resistant clone is present in areas of the primary tumors or metastases that are not amenable to analysis. For example, the resistant clone may be in a region of the primary tumor not excised, or in distant metastases (Figure 2.7).

2.2.5
Targeting Essential Drivers (Driver Addiction)

Although it is true that resistant cell clones limit the long-term success of targeted drugs, there is a prominent exception to this rule. Treatment with imatinib, the first drug used in targeted therapies, has resulted in long-lasting response rates in patients with CML. After 5 years of follow-up, the progression-free survival was 93%, and the overall survival was 86% (Druker *et al.*, 2006). Imatinib-resistant clones do arise, but the incidence has been remarkably low. A decisive factor for this success may reflect the essential role of BCR-ABL1, the target of imatinib, in driving CML.

In CML, the gene encoding the ABL1 phosphotyrosine kinase is translocated from chromosome 9 to chromosome 22. The derivate chromosome 22 is known as *Philadelphia chromosome*, the first cytogenetic abnormality regularly found in a human malignancy (Nowell and Hungerford, 1960) (see Chapter 6). As a result of this translocation, the *ABL1* gene is fused with the so-called *breakpoint cluster region (BCR)* gene on chromosome 22. The Philadelphia chromosome or

the *BCR-ABL1* fusion gene is an essential criterion for the definition of CML in the WHO classification (Vardiman, Harris, and Brunning, 2002). The *BCR-ABL1* fusion gene encodes a fusion protein with a deregulated ABL1 tyrosine kinase. In most cases of resistance, the exchange of an amino acid blocks the binding of imatinib to the kinase domain in the ABL1 protein (Gambacorti-Passerini *et al.*, 2003).

The *BCR-ABL1* fusion gene and the deregulation of the ABL1 phosphotyrosine kinase are essential for the pathogenesis of CML; the tumor cells appear to be addicted to this particular mutation. A comparable mutation has been found in renal carcinomas (Gerlinger *et al.*, 2012). As discussed earlier, not all mutations found in renal carcinoma are shared by all tumor cells, but mutations of the von Hippel–Lindau (VHL) gene are present in each cell clone of the carcinoma. Similar to the *BCR-ABL1* mutation, the tumor cell clones appear to be addicted to mutations in this particular gene. However, in contrast to the *BCR-ABL1* mutation, which deregulates and activates the encoded kinase, the *VHL* mutation is a loss-of-function mutation. In this case, it is much more challenging to develop targeted therapeutic approaches. The *VHL* gene product plays a key function in negatively regulating factors that allow cell survival under low oxygen (hypoxia), thus its tumor suppressor activity likely relates to this function. Current therapeutic strategies aim to block downstream effectors that would be normally inhibited by VHL.

As shown in Figure 2.8, a targeted therapy can be successful if the mutation in a defined driver gene is essential for each of the tumor cell clones ("essential drivers") (Weinstein, 2002). If different clones are driven by different mutant genes, a targeted monotherapy will fail.

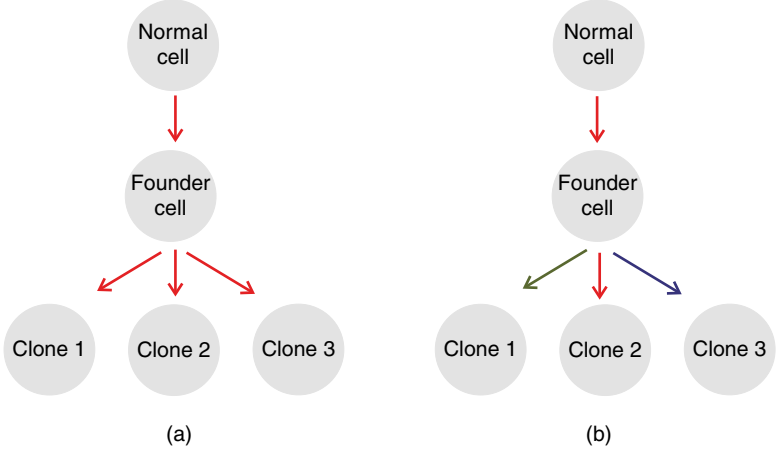

Figure 2.8 "Driver gene addiction" of tumor cell clones. (a) The growth of the founder cell and of each of the tumor cell clones depends on a single mutated driver gene. The tumor is said to be addicted to this activated gene. A targeted therapy will reach each of the clones and (b) the growth of different tumor cell clones may depend on different driver genes as indicated by the different colors of the arrows. To be effective, a combinatorial therapy must target each of the clones.

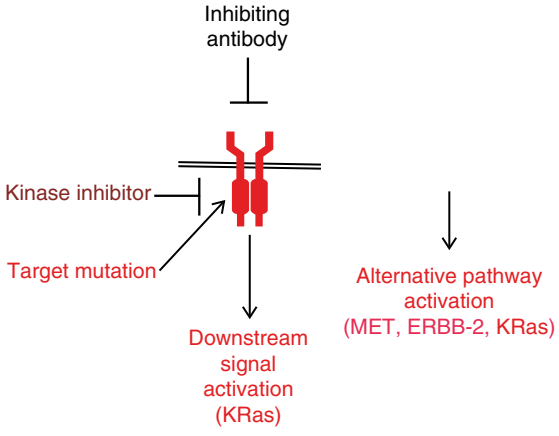

Figure 2.9 Resistance mechanisms in patients treated with EGFR inhibitors.

2.2.6
Resistance by Alternative Pathway Activation

As explicated in this book, targeted therapies interfere with signaling pathways activated by gene mutations. If a mutant signaling protein is essential for the activity of a particular pathway, and the pathway is essential for the growth of each of the tumor cell clones, blocking the activity of this particular protein will result in longer lasting remissions.

As outlined earlier, the *BCR-ABL1* oncogene encodes a fusion protein that is essential for the chronic phase of CML. Resistance against ABL1 tyrosine kinase inhibitors is primarily due to mutations within the kinase domain that are directly targeted by the inhibitors. Similarly and as mentioned before, NSCLC often harbors mutations in the EGFR that activate the EGFR pathway. Initially, tumors with these activating mutations respond to inhibitors. However, the gain in median progression-free survival is less than a year due to drug resistance. Similar to the BCR-ABL1 fusion protein, *de novo* mutations in the EGFR may block the interaction of the drug with the kinase domain. In addition, signaling pathways downstream of the EGFR may be activated by mutations in the *KRAS* gene. The activation of bypass signaling pathways such as the HGF/MET pathway are alternative mechanisms of resistance. Principal mechanisms of resistance against EGFR inhibitors are summarized in Figure 2.9. For further details on inhibitors of the EGFR signaling pathway, see Chapters 5, 7, and 8.

2.2.7
Overcoming Resistance by Combinatorial Therapies

Although the mortality of cancer patients remained fairly constant over the last 50 years, there are some exceptions. For example, the 5-year survival of children with ALL is in the range of 80–90%, and similar survival rates are obtained in men

suffering from testicular cancer. In these and other malignant diseases, the success was brought about by the combination of classical cytostatic and cytocidal drugs (DeVita and Chu, 2008).

So far, single drugs were applied in clinical trials of targeted therapies. With the exception of CML, resistance to therapy inevitably develops within few months. These results indicate that resistant cell clones most probably were present already at the start of therapy. In these resistant clones, critical pathways may have been activated downstream of the drug target or alternative pathways leading to essential targets may have been active. If longer lasting successes in the application of targeted therapies will be reached, resistant cell clones must be targeted already at the start of therapy. Mathematical models indicate that combination therapy with two drugs given simultaneously is far more effective than sequential therapy where the drugs are applied one after the other (Bozic *et al.*, 2013).

2.3 Tumor Stem Cells and Tumor Cell Hierarchies

In tissues such as the hematopoietic system, the gastrointestinal and genitourinary tracts, as well as the skin, self-renewal occurs constantly. Somatic stem cells replenish the functional mature cells of the tissue during the entire lifetime of the organism. Stem cells are defined as cells that have the ability to perpetuate themselves through self-renewal and to generate mature cells of a particular tissue through differentiation (Reya *et al.*, 2001). Somatic stem cells divide asymmetrically, that is, one of the daughter cells remains a stem cell, whereas the second daughter cell gives rise to progenitors, which differentiate into mature cell lineages. As explicated earlier, the initiation of tumors starts from a single cell. Since it takes years to decades for the transition from the first initiating genetic event to the growth of a tumor, the tumor-initiating cell must reside in the organism for an extended time period. In regenerating tissues, the somatic stem cell fulfills this precondition. The argument that somatic stem cells give rise to tumor-initiating cells is strengthened by the finding that the number of passenger mutations in tumors of self-renewing tissues is positively correlated with the age of the patient at diagnosis (Tomasetti, Vogelstein, and Parmigiani, 2013).

As a result of somatic stem cell differentiation, a tissue consists of different cells and tissue structures. In Figure 2.10, the differentiation of blood cells from hematopoietic stem cells is shown. Tumors may contain cells or differentiated tissue structures that resemble cells or tissue elements of the normal counterpart. In CML, for example, myeloid cells at various differentiation stages may be present in the peripheral blood. In contrast to the normal situation, however, immature cells such as promyelocytes and myeloblasts are also shed into the peripheral blood (Figure 2.11). Similarly, mature cells and tissue component may be present in solid tumors. In the carcinoma section shown in Figure 2.12, immature glandular structures document a process of differentiation.

The fact that tumors may contain a hierarchy of cells and tissue components indicates that the process of cellular differentiation is still at work, though not in

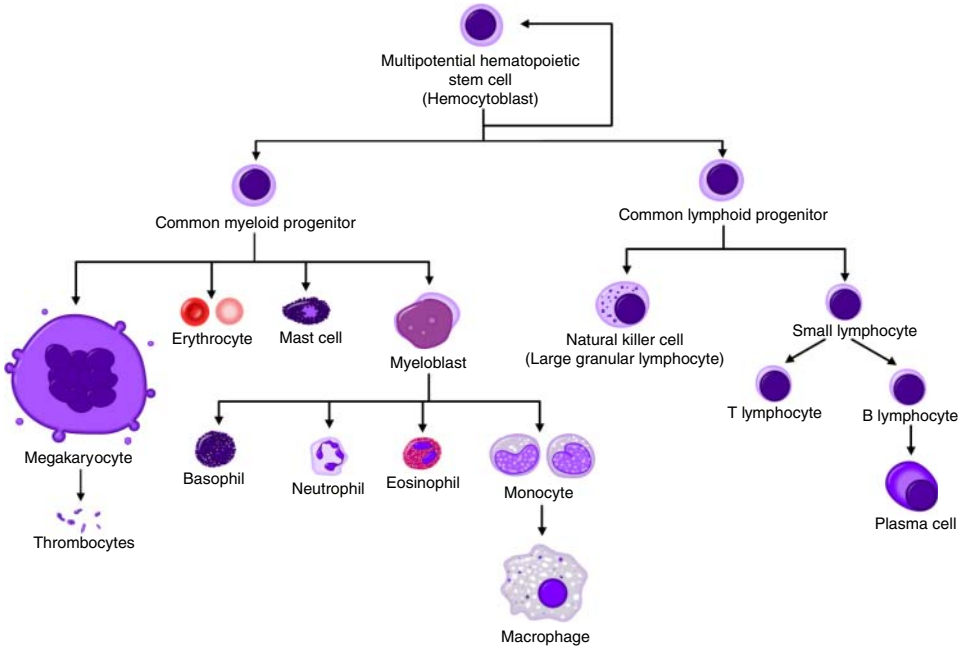

Figure 2.10 Normal hematopoiesis.

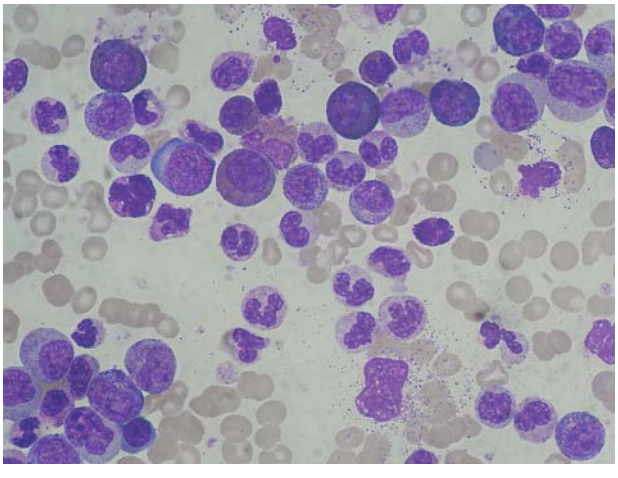

Figure 2.11 Peripheral blood smear from a patient with chronic myeloid leukemia (CML). Myeloid cells at different levels of differentiation are present, ranging from myeloblasts to segmented granulocytes.

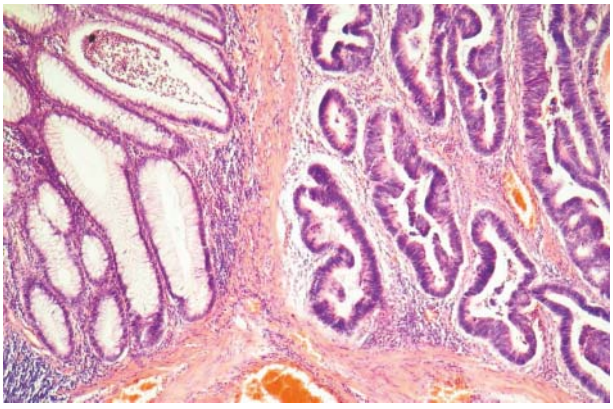

Figure 2.12 Section of a well-differentiated colonic adenocarcinoma with adjacent normal colonic mucosa. In the tumor section, glandular structures are present. (Wagener and Müller, 2009), with permission.

a normal and regulated fashion. If this is true, tumors must contain cells, from which the more mature cells and tissue structures originate. These cells have been coined tumor stem cells or cancer stem cells. Similarly to normal somatic stem cells, tumor stem cells divide asymmetrically. One daughter cell remains stem cell, whereas the second cell proliferates and gives rise to more mature cell lineages. When tumor cells are transplanted into a suitable host, for example, into an immune-deprived mouse, tumors can be propagated only when tumor stem cells are present. If tumor stem cells are absent, initial tumor growth may be observed, because progenitors of the stem cells proliferate. In the long term, however, the tumors collapse (Figure 2.13).

It should be stressed that the tumor stem cell concept does not imply that tumor stem cells are always derived from somatic tissue stem cells. Stem cells are characterized by two essential properties: self-renewal and asymmetric division. It has been shown that the property of asymmetric division can be induced in proliferating progenitor cells. In this way, progenitor cells are able to acquire properties of stem cells (Krivtsov *et al.*, 2006).

The tumor stem cell concept bears important implications for targeted therapies. Many therapies target signaling pathways that stimulate proliferation or prevent apoptosis. Such therapies act on the proliferating cell compartment only. Stem cells exhibit a low proliferation rate. In addition, they have powerful mechanisms for eliminating toxic components, for example, transporters of the activated B-cell (ABC) family, such as the multi-drug-resistant protein 1. In CML, specific inhibitors (e.g., imatinib) can block the tyrosine kinase activity of the BCR-ABL1 fusion protein. Although patients are under complete remission for years, imatinib never eliminates the CML stem cell compartment completely, and thus, treatment cannot be stopped (Chu *et al.*, 2011).

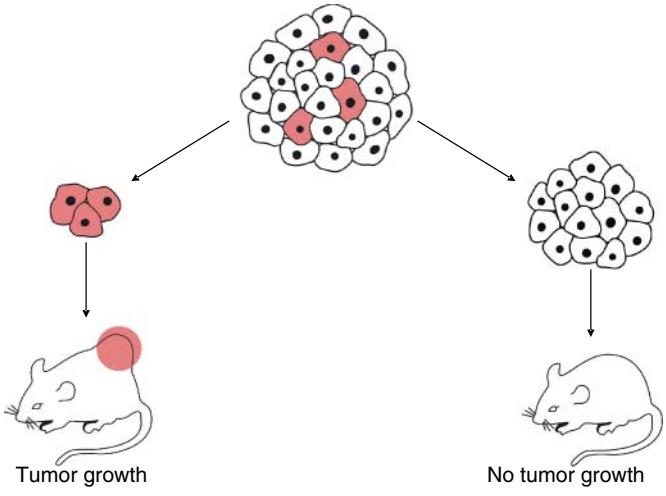

Figure 2.13 Xenotransplantation of tumor cells indicates the presence of tumor stem cells. A tumor contains few self-renewing stem cells (red), which produce progenitors differentiating into more mature cells. If stem cells are present in the transplanted tissue, the tumor grows in the recipient animal. In the absence of tumor stem cells, tumor growth is not sustained. (Wagener and Müller, 2009), with permission.

2.4 Epigenetics and Phenotypic Plasticity

One year after the Luria and Delbrück paper was published, Lieutenant Colonel Joseph W. Bigger reported on an observation on the exposure of staphylococci to penicillin. When large numbers of bacteria were exposed to the antibiotic, a small number of cocci survived. In contrast to the resistant viral populations described in the Luria–Delbrück experiment, the descendants of the bacteria that had survived high doses of penicillin lost their resistance to penicillin if they were cultured for 2 days in medium lacking penicillin. Bigger named the bacteria that survived the penicillin exposure "persisters." Interestingly, persisters themselves have no greater tendency than normal forms to produce persisters. Obviously, the property to be a persister is not genetically fixed (Bigger, 1944).

Decades later, the observation made on bacteria found its counterpart in reports on the behavior of tumor cells. As mentioned before, the tyrosine-kinase receptor for the growth factor EGF may be mutated in NSCLC. When NSCLC cells are exposed to tyrosine kinase inhibitors, a small percentage of cells survive. This population of cells has been named "drug-tolerant persisters" (Sharma *et al.*, 2010). Similar to the bacterial persisters, the drug-tolerant persisting tumor cells resumed growth and rapidly reacquired sensitivity toward kinase inhibitors when propagated in drug-free medium. The drug tolerant state was associated with reduced histone acetylation. Acetylation of certain histones leads to an open chromatin structure, which favors transcription. When the drug-tolerant

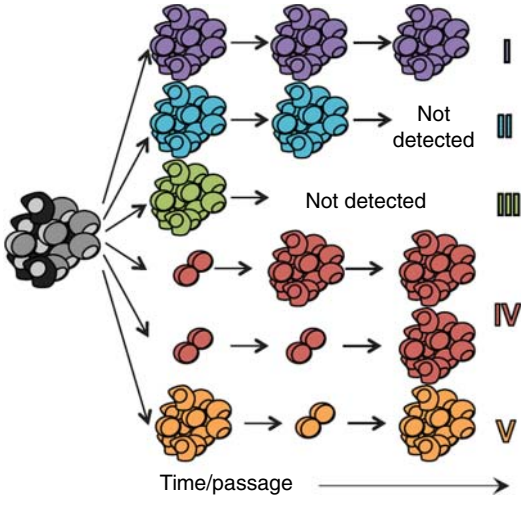

Figure 2.14 Clonal behavior of lentivirally marked cells from primary human colorectal cancer. Single-cell-derived clonal populations were serially transplanted into the renal capsule of immunodeficient mice. The following growth patterns were observed: type I clones were present in all serial transplants; type II clones exhausted before reaching the final passage; type III or transient clones were only detected in the first recipient; type IV clones were initially below detection limits in the first recipient but could be identified at later transplants; and type V clones fluctuated. Treatment with oxaliplatin favored the growth of type IV clones. (Kreso *et al.*, 2013), with permission.

persisters were exposed to an inhibitor of histone deacetylases, they rapidly died. These and other findings indicate that a drug-tolerant state can be mediated by epigenetic mechanisms.

The observations made on drug exposed tumor cell lines appear to apply also to the growth of solid tumors. John Dick and coworkers transduced patient-derived colorectal cancer cells with a lentiviral vector expressing green fluorescent protein (Kreso *et al.*, 2013). In this way, the repopulation kinetics of multiple single-cell derived clones could be traced after xenograft passage in immune-deprived mice. Genetic analyses distinguished individual tumor cell clones. In addition, these analyses indicated that the clones remained genetically stable through serial xenograft passage in mice. As shown in Figure 2.14, five distinct patterns of clonal growth behavior were observed: type I clones were present in all serial transplants; type II clones exhausted before reaching the final passage; type III or transient clones were only detected in the first recipient; type IV clones were initially below detection limits in the first recipient but could be identified at later transplants; and type V clones fluctuated, for example, they were sometimes detectable and sometimes not detectable in the serial transplants. Interestingly, clones remained undetected for 2–4 months and then recurred to dominate tumor growth. When the tumor-bearing mice were treated with the chemotherapeutic drug oxaliplatin, the growth properties of the regrown clones changed significantly. The proportion of type I persistent clones was reduced, whereas clones that were below the

detection limit in primary xenograft recipients appeared in secondary recipients. These data indicate that the variable response of individual cancer cells to the therapy with oxaliplatin was most probably due to epigenetic mechanisms.

2.5 Microenvironment

Tumor cells of solid tumors such as carcinomas are embedded in a matrix, which consist of connective tissue elements and a variety of cells such as fibroblasts, endothelial cells, macrophages, and lymphocytes. The matrix surrounding tumor cells has been named tumor stroma or tumor (micro) environment. The designations tumor stroma and microenvironment will be used mutually.

Carcinomas are the most frequent solid tumors. For this reason, carcinomas will serve as example for the interaction between tumor cells and their environment. Expanding carcinomas need oxygen and nutrients, which are delivered by blood vessels. The blood vessels are embedded in the tumor stroma. During the clonal expansion of epithelial tumor cells, the tumor stroma is constantly being remodeled and new blood vessels form. This process leads to the separation of clonal subpopulations that experience different environmental conditions, for example, differences in the supply of oxygen and nutrients. In this way, the microenvironment shapes the clonal expansion of tumor cell populations. Those clones will expand that adapt best to the microenvironment. The expansion of genetically diverse tumor cells clones is reminiscent of the evolution of species in separated environmental niches: *In the struggle for survival, the fittest win out at the expense of their rivals because they succeed in adapting themselves best to their environment.* (Darwin, 1859).

Asymmetric division is a fundamental property of stem cells, including tumor stem cells. In order to divide asymmetrically, polarity must be induced in the stem cell. Often, polarity and asymmetric cell division rely on the immediate microenvironment, which is known as *stem cell niche*. Considering the possibility that clonal tumor cell populations are hierarchically organized, the stem cell of a tumor cell clone similarly depends on the environment, which forms the tumor stem cell niche. In this way, the expansion of a particular clone depends on the immediate environment of the tumor stem cell, which replenishes the clone.

During the expansion of tumors, the growth of blood vessels must keep pace with the growth of tumor cells. If this is not accomplished, tumor cells experience lack of nutrients and particularly lack of oxygen. As a result, the cells may die. Indeed, necrotic tissue areas are often observed in fast-growing tumors. In order to avoid cell death, survival programs are activated in the tumor cells. These programs may stimulate the growth of blood vessels. Importantly, tumor cells may acquire increased motility and invasive growth properties in order to escape the regions of low oxygen pressure.

The microenvironment may influence the resistance of tumor cells to targeted therapeutic approaches in a number of ways (Figure 2.15). (i) The environment may support the expansion of resistant clonal tumor cell populations. (ii) The

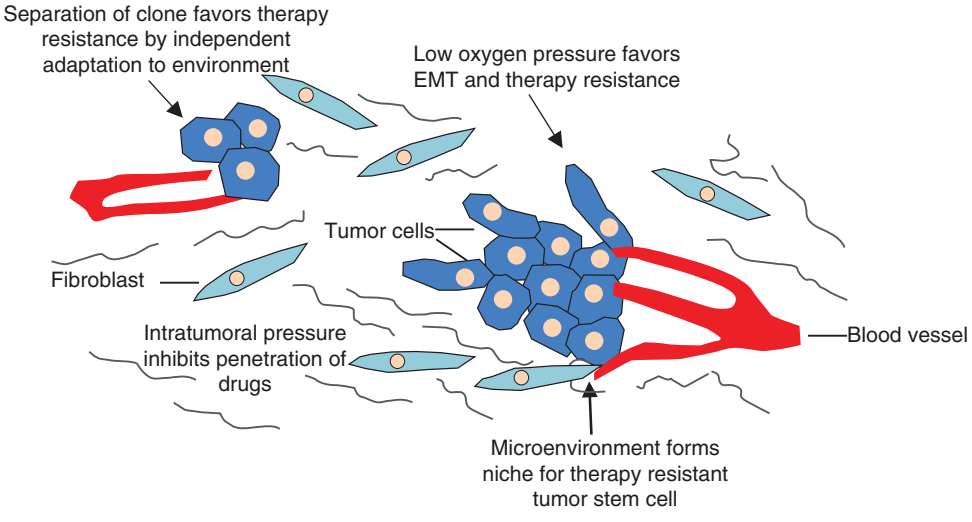

Figure 2.15 Impact of the tumor environment on tumor growth and therapy resistance. EMT, epithelial mesenchymal transition.

microenvironment may supply the niches to stem cells, which are resistant to targeted therapies. (iii) Because of inadequate or retarded growth of blood vessels, tumor cells may activate a stress response in order to survive. This response may affect the response of therapeutic targets. (iv) Because of the unregulated growth and irregular organization of blood vessels, the pressure in tumor tissues may be increased, and drugs may not be able to penetrate from the peripheral blood into the tumor tissue (Marusyk and Polyak, 2010).

2.6 Outlook

Tumors may contain different clones that differ with respect to genetic and epigenetic properties. Clonal heterogeneity poses significant problems to targeted therapeutic approaches. In order to target each and every clone, all clones must be identified. However, clones may escape detection if the sensitivity of detection methods is too low or if sampling is not possible. In order to detect minor clones, the sensitivity of sequencing techniques must be improved. Multiple sampling of primary tumors increases the probability of sampling all clones that are present in the tumor. In the case where distant metastases are present, multiple sampling is not feasible. Tumor DNA that circulates in peripheral blood may represent different clones in primary tumors and metastases. Sequencing of circulating tumor DNA (liquid biopsy) may assist to identify occult clones. Based on the identification of different tumor cell clones, combinatorial therapies may be designed, in this way preventing resistance against targeted therapies.

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3
Cell Cycle of Tumor Cells

Summary

Undoubtedly, there are many differences between tumor cells and normal cells. One of them is the high proliferation rate of tumor cells based on their fast passage through the interphase between two mitoses. In order to illuminate the details of the cell cycle and its different phases, we describe the general cell cycle with its major checkpoints. The roles of cyclins and cyclin-dependent kinases together with their inhibitors are illustrated. In the last part of this chapter, we introduce several examples of small molecules with therapeutic potential that exhibit inhibitory activity on the cell cycle and are currently under evaluation in clinical trials.

Cancer Signaling: From Molecular Biology to Targeted Therapy, First Edition.
Christoph Wagener, Carol Stocking, and Oliver Müller.

Companion Website:www.wiley.com/go/wagener/cancersignaling

3.1 Properties of Tumor Cells

There are several general features that demarcate tumor cells from normal cells (Hanahan and Weinberg, 2000) (Table 3.1). Tumor cells also show several similarities to stem cells, though there are also some striking differences (Table 3.2). Both cell types are undifferentiated and have no organ-specific function. Most tumor cells show proliferation rates, which are similar to those or even higher than those of normal stem and progenitor cells, based on the fact that they pass fast through the interphase between two mitoses. In contrast to a stem cell, a typical tumor cell is insensitive to signals that inhibit proliferation. Tumor cells are able to proliferate unlimitedly, even in the absence of external growth signals. Similar to stem cells, most tumor cells do not age and do not reach senescence that would be characterized by a permanent and irreversible block of proliferation. Furthermore, tumor cells do not react to internal or external apoptotic signals. Finally, cells of a progressed tumor are able to induce growth and branching of blood vessels, to invade into neighboring tissue and to metastasize.

Table 3.1 Disabilities and abilities that discriminate tumor cells from nonstem cells.

Disabilities	Differentiation
	Organ-specific function
	Growth arrest or slowing down of growth in response to growth-inhibiting signals
	Apoptosis in response to internal or external apoptotic signals
	Block of cell cycle by senescence
Abilities	Growth and proliferation without external growth signals
	Unlimited proliferation
	Induction of angiogenesis
	Invasion into neighboring tissue
	Detachment and colonization at another site (metastasis)

Table 3.2 Tumor cells in comparison to stem cells.

Property	Tumor cell	Stem cell
Organ-specific function	None	None
Proliferation rate	High	High
Proliferation inhibiting signals	Insensitive	Sensitive
Apoptotic signals	Insensitive	Sensitive
Replicative senescence	No	No
Induction of angiogenesis	Yes	No
Invasion into neighboring tissue	Yes	No
Metastasis	Yes	No

Table 3.3 Differences between normal cells and transformed cells *in vitro*.

Normal cells	Transformed cells
Monolayer	Multilayer
Contact inhibition	No contact inhibition
Dependent on substrate	Growth in the absence of substrate
Limited life span	Immortal
Limited number of proliferations	Unlimited number of proliferations
Dependent on growth factors	Independent of growth factors

3.1.1 Differences between Tumor Cells and Normal Cells In vitro

In cancer research, cultured tumor cells are useful *in vitro* models, as they share many of the typical characteristics distinct to normal cells (Table 3.3). The transition of a normal cell into a tumor cell is called *transformation*. A normal cell in cell culture can be transformed experimentally by transfection of cancer genes, by virus infection, or by exposition to carcinogenic compounds. Normal cells show limited viability outside of an organism. Most normal cells need a solid support ("substrate") to survive in culture. They need specific growth factors, are limited in the number of cell divisions, and die after a few weeks. As soon as the cells form a confluent monolayer, they stop dividing. This phenomenon is known as *contact inhibition*. In contrast to a normal cell, a tumor cell in culture is immortal. It shows no or only few signs of cellular aging. A tumor cell is able to proliferate unlimitedly, as long as there is sufficient supply of nutrition and energy. The proliferation of tumor cells is not inhibited by intercellular contacts. In fact, they are able to grow without substrate and to form cellular multilayers. Additionally, transformed cells need less growth factors than normal cells do and are able to grow into a solid tumor when injected into an immune-deficient animal.

3.1.2 Regulation of Cell Number

In a normal organ, a controlled balance between proliferation, differentiation, and regulated cell death by apoptosis or autophagy is necessary to keep the number of cells constant. In tumor tissue, the homeostasis of the cell number is shifted from differentiation and cell death toward proliferation and survival, resulting in an increasing cell number. Therefore, the cellular processes of mitosis and cell death have to be analyzed in detail in order to understand the growth of a tumor.

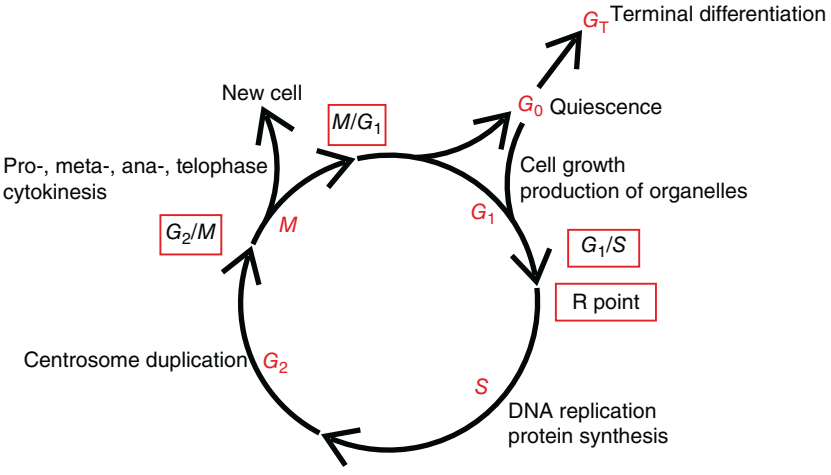

Figure 3.1 The cell cycle with phases (red) and checkpoints (red framed boxes). After mitosis (*M* phase), the cell enters the interphase consisting of the phases G_1, *S*, and G_2. From the G_1 phase, the cell can enter the quiescent or G_0 phase, which is a reversible cell cycle arrest. From the G_0 phase, the cell can either reenter into G_1 phase or terminally differentiate into the G_T phase. The cell cycle can be paused or stopped at the checkpoints G_1/S (also called *restriction point* or *R point*), G_2/M, and M/G_1.

3.2 The Cell Cycle

(Video: The cell cycle – Part 1: Phases and Checkpoints – enhanced ebook and closed website: Cell_Cycle_Video_01_ebook.mp4)

A proliferating cell passes through typical phases between cell divisions. These phases can be illustrated in a simplified cyclic model called the *cell cycle* (Figure 3.1). Once a cell has divided, it enters the so-called interphase. The interphase consists of the phases G_1 (gap), *S* (synthesis), and G_2. The interphase ends with the beginning of the next mitosis (*M* phase). The G_1 phase is the most important phase for the regulation of proliferation and differentiation by external factors. In this phase, the growth of the cell and its production of organelles depend on the presence of external growth factors. In the absence of growth factors, the cell can exit the cell cycle into a cell cycle arrest phase, called the G_0 phase. Either the cells in the G_0 phase can return into the G_1 phase and thus back into the active cell cycle, or they can differentiate to reach the terminally differentiated phase G_T. Cells in the G_T phase normally fulfill specific functions and do not proliferate any more. G_T cells of neuronal and other nonregenerating tissue might survive over decades, while the life spans of G_T cells in regenerating tissues such as blood or epithelia are limited. After the G_1 phase, the proliferating cell enters the *S* phase, during which the DNA is replicated and the levels of housekeeping proteins are doubled. As soon as the

Table 3.4 Cell cycle checkpoints and criteria for passage and block.

Checkpoint	Necessary criteria for passage	Criteria for block
G_1/S point or *R* point	Sufficient number of organelles Activation by growth factors	TGFβ ATP deficiency
G_2/M point or metaphase/anaphase checkpoint	Large cell volume Completely replicated DNA	DNA damage
M/G_1 point	Equal distribution of chromosomes to daughter cells	Aneuploidy

genome is completely replicated, the cell enters the G_2 phase. In the G_2 phase, the cell prepares for the upcoming mitosis by protein synthesis and doubling of the centrosome.

3.2.1 Checkpoints

Under physiological conditions, the cell cycle can only stop at specific checkpoints (Lapenna and Giordano, 2009). The most important checkpoint is the so-called restriction point, also called *R point*, at the transition from the G_1 to the *S* phase.

The cell can only pass this point when certain requirements are fulfilled, such as the formation of sufficient number of organelles, or when the cells are stimulated by external growth factors (Figure 3.1 and Table 3.4). Growth factors are necessary for both transition of the G_1 phase and passage through the *R* point. Once the cell progresses past the *R* point, growth factors are no longer required for cycle progression. In the absence of growth factors or in the presence of proliferation inhibitors such as TGFβ, the cell cycle stops at the *R* point.

Once the cell has passed the *R* point, it is obligated to undergo mitosis. The G_2/M checkpoint allows the cell cycle to pause only for a defined period of time before initiation of mitosis. This point is passed when the genome is completely replicated and the cell is large enough for proliferation.

Shortly before the *M* phase is finished, there is another checkpoint called M/G_1 or *metaphase/anaphase checkpoint*. This checkpoint is passed as soon as the chromosomes are equally distributed between the two new daughter cells. At this point, an ubiquitin ligase, as a component of the anaphase-promoting complex, marks the protein Securin, leading to its proteolytic degradation. Because intact Securin inhibits the disruption of the sister chromatids, the anaphase-promoting complex promotes the segregation of the chromatids to the new daughter cells in the anaphase.

3.2.2 Cyclins

(Video: The cell cycle – Part 2: Cyclins, cyclin dependent kinases (CDKs), CDK inhibitors – enhanced ebook and closed website: Cell_Cycle_Video_02_ebook.mp4)

The cell cycle is driven and regulated by proteins called *cyclins* (Malumbres and Barbacid, 2005). Cyclins are something similar to the fuel for driving the cell cycle. Within a very short time, the intracellular cyclin concentration can be increased or decreased over several orders of magnitudes by gene induction, gene repression, or ubiquitinylation and subsequent proteolytic degradation. Expression of cyclins is induced by mitogenic pathways. The cyclin D1 gene (*CCND1*) is an important proto-oncogene, which is activated by mutations in tumors such as breast cancer and lymphatic leukemias. Furthermore, the overexpression of cyclin genes, which can be caused by dysregulation of relevant pathways such as the mitogen-activated protein kinase (MAPK) and the Wnt pathways, is causally involved in the development of many other tumors.

Cyclins themselves have no enzymatic activity on their own. They are rather able to activate serine/threonine kinases of the family of cyclin-dependent kinases (CDKs). The catalytic domain of a CDK is only active in association with a cyclin (Figure 3.2). A human cell expresses many different cyclins, which form defined complexes with CDKs during the cell cycle.

Cyclins are named with capital letters and numbers. Well-characterized cyclins are the cyclins A, B1, B2, D1–D3, and E. All cyclins are only expressed during distinct phases of the cell cycle and are degraded by ubiquitin-mediated proteolysis.

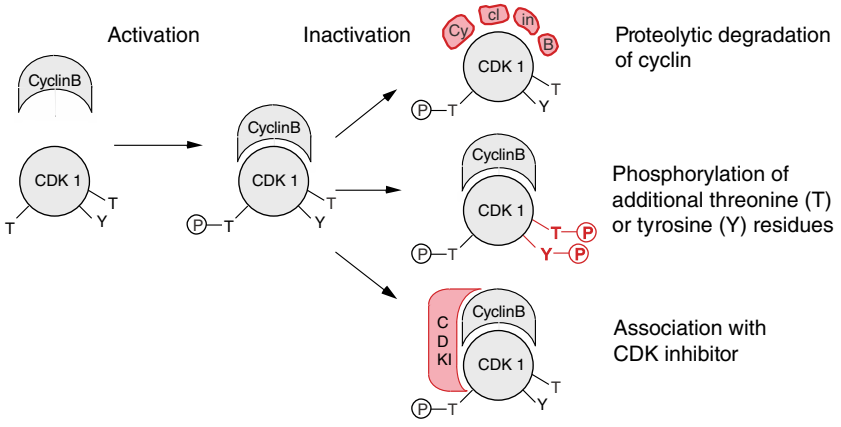

Figure 3.2 Mechanisms of regulation of the cyclin-dependent kinase 1 (CDK1). The regulatory mechanisms of other CDKs are comparable. (Wagener and Müller, 2009), with permission.

3.2.3
Cyclin-Dependent Kinases (CDKs)

There are different mechanisms for regulating CDK activity in addition to association with cyclins (Figure 3.2). Most CDKs must be phosphorylated at a conserved threonine residue (position 160 in CDK1), in order to unfold full activity. CDK activity can be decreased by phosphorylation of a conserved tyrosine and a threonine residue (T14 in CDK1). Another mechanism for blocking the activity of CDK–cyclin complexes is through association with inhibiting proteins, the cyclin-dependent kinase inhibiting proteins or CDKIs (see below).

CDKs induce the passage through the cell cycle phases by phosphorylating key proteins that regulate gene expression. Depending on the cell cycle phase during which they are active, CDKs of the G_1, M, or S phase can be distinguished. The genes for CDK4 and CDK6, which are CDKs of the G_1 phase, are amplified and overexpressed in many tumors. The tumor suppressor protein Rb (retinoblastoma-associated protein) is one of the most important substrates of the CDKs (Figure 3.3).

During the G_2 phase, the level of the complex between cyclin A and CDK2 decreases, while the levels of the complex between cyclin A and CDK1 and of the complex between cyclin B and CDK1 increase. After dephosphorylation of the cyclin B/CDK1 complex by the phosphatase Cdc25A, the cyclin B/CDK1 is

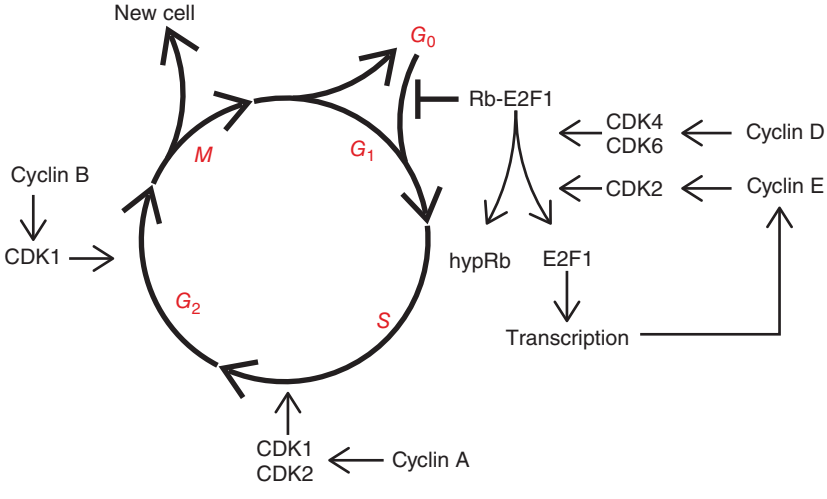

Figure 3.3 Regulation of the cell cycle by cyclins and cyclin-dependent kinases (CDKs). By complexing the transcription factor E2F1, Rb blocks the re-entry of the cell from the G_0 phase into the active cell cycle and the passage of the restriction point between G_1 and S phase. The hyperphosphorylation of Rb by CDK4, CDK6, or CDK2 leads to the release of E2F1 and its activation. CDK1 and CDK2 activate the transition through other cell cycle phases.

able to phosphorylate the protein Cdh1. In its nonphosphorylated state, Cdh1 inhibits the anaphase-promoting complex. Phosphorylation of Cdh1 leads to the activation of the anaphase-promoting complex, which controls the transition of the metaphase to the anaphase of mitosis and the passage of the *M* into the G_1 phase.

3.2.4 The Retinoblastoma-Associated Protein Rb as Regulator of the Cell Cycle

The retinoblastoma gene was isolated in 1986. It was the first tumor suppressor gene that was isolated based on knowledge of its chromosomal location: chromosome 13 band q14. Germline mutations in the retinoblastoma gene *RB1* predispose to a pediatric malignancy of the eye: retinoblastoma. In addition, loss of the *RB1* gene predisposes to a variety of other tumors later in life, with osteosarcoma being the most prominent secondary tumor. Loss of Rb function is also seen in a variety of spontaneous human tumors, including lung cancer, lymphoma, and breast cancer. The retinoblastoma gene *RB1* encodes the 110 kDa retinoblastoma-associated protein Rb that is found in almost every cell of the human body and contributes to growth regulation in these cells. Reintroduction of a functional *RB1* gene in retinoblastoma tumor cells results in growth arrest, indicating that the function of the gene is to restrict proliferation.

Between the *R* checkpoint and the beginning of the G_1 phase, the Rb protein binds to the transcription factor E2F1 and thereby blocks its activity (Figure 3.3). During the G_1 phase, Rb is partially phosphorylated by CDK4 and CDK6. CDK4 and CDK6 are themselves activated by cyclins of the cyclin D family as a response to external growth factors. Partially phosphorylated Rb releases E2F1. Free E2F1 induces the expression of several important genes, including the genes encoding cyclin E (*CCNE1* and *CCNE2*). Cyclin E activates CDK2, which phosphorylates Rb until it is hyperphosphorylated. Hyperphosphorylated Rb cannot block the cell cycle at the *R* checkpoint any more, and the cell passes further to the *S* phase.

3.2.5 Inhibitors of CDKs

The CDKIs are protein inhibitors of CDKs. As functional antagonists, CDKIs can be regarded as brakes of the rotating cell cycle. CDKIs associate with the cyclin/CDK complexes and inhibit their activity (Figure 3.4). CDKIs can be grouped according to their specificity. First, the inhibitors of kinase CDK4 (INK4) group includes the CDKIs p16-INK4, p15-INK4, p18-INK4, and p19-INK4. CDKIs of the INK4 group inhibit the kinases CDK4 and CDK6 and are thus able to block the cell cycle at the *R* point.

The second group called the Cip/Kip (CDK-interacting protein/cyclin-dependent kinase inhibitor protein) group includes the CDKIs p21, p27Kip1, and p57Kip2 (Schwartz and Shah, 2005). CDKIs of the Cip/Kip group are able to block the cell cycle at all three checkpoints.

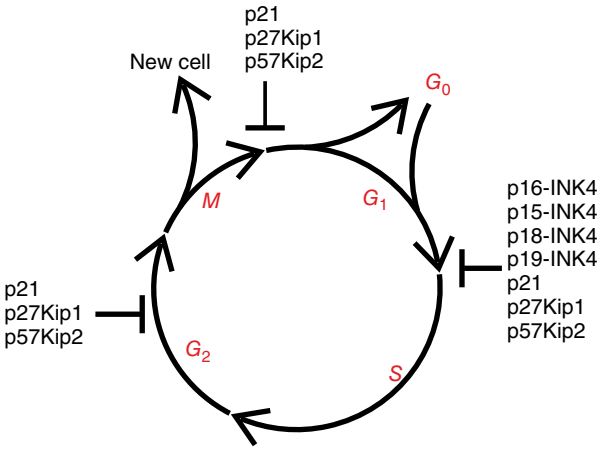

Figure 3.4 Inhibition of the cell cycle by cyclin-dependent kinase inhibitors (CDKIs). Inhibitors of the INK4 family block the cycle at the restriction point between G_1 and S phase, and inhibitors of the Cip/Kip family block the cycle at all three checkpoints.

3.2.6 Checkpoints and DNA Integrity

DNA is permanently subject to mutagens from both endogenous (e.g., reactive oxygen species, ROS) and exogenous sources (e.g., radiation). To avoid transmission of the resulting DNA lesions to the next cell generation, the DNA has to be repaired before the next cell division (Branzei and Foiani, 2008). The DNA lesion triggers a block at a cell cycle checkpoint and the upcoming mitosis is delayed. During cycle arrest, the cell can repair the mutation before it continues through the cycle. After successful repair, the block is released by the checkpoint machinery, and the cell cycle can continue.

DNA lesions occurring during the active cell cycle lead to the activation of two serine/threonine kinases, ATR (ataxia telangiectasia and Rad3 related) and ATM (ataxia telangiectasia mutated homolog) (Figure 3.5) (Lapenna and Giordano, 2009).

Whereas ATR is activated mainly in the presence of single-strand breaks during the *S* phase, ATM is activated by double-strand breaks. Both ATR and ATM phosphorylate and thereby activate several proteins, including the two checkpoint kinases CHK1 and CHK2. CHK1 phosphorylates the phosphatase CDC25, leading to its ubiquitinylation and subsequent proteolytic degradation. CDC25 removes two inhibitory phosphates from CDK1. After CDC25 degradation, CDK1 is no longer dephosphorylated. As a consequence, CDK1 is inactivated and the cell cycle stops in the *S* phase or at the G_2/M point. The activation of the checkpoint kinase CHK2 leads to the activation of the tumor suppressor protein p53 (Figure 3.5). P53 activates the expression of the CDKI p21, which inhibits CDK4.

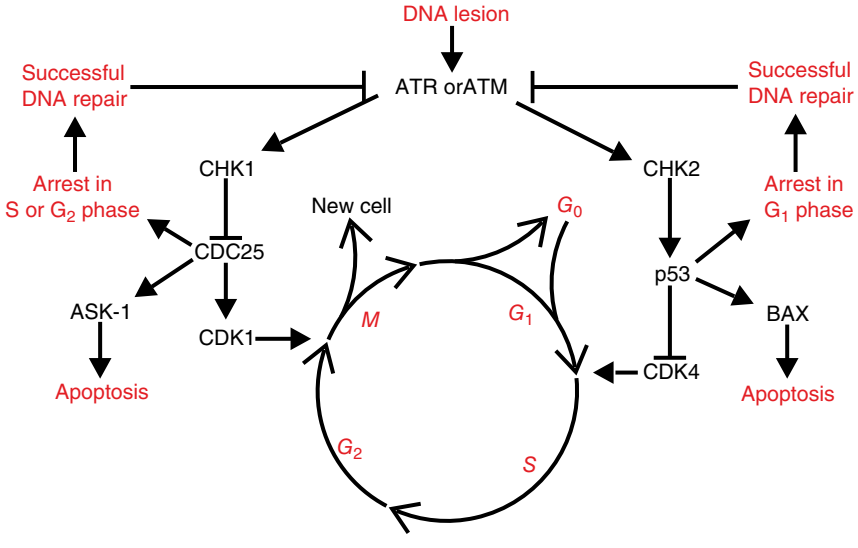

Figure 3.5 Cell cycle arrest and apoptosis as a result of DNA damage. The detection of a DNA lesion leads via activation of CHK1 to the degradation of the phosphatase CDC25. The lack of CDC25 leads to the block of the cell cycle and also – in the case of unsuccessful DNA repair – to apoptosis (far left). The detection of a DNA lesion can also lead to the activation of the tumor suppressor protein p53 via activation of CHK2. p53 blocks the cell cycle by inducing the expression of the CDKI p21. If DNA repair is not successful, p53 can activate BAX protein and induce apoptosis (far right).

The resulting block at the *S* phase, or at the *R*, or at the G_2/M checkpoints gives the cell time to repair its DNA.

As soon as the DNA is successfully repaired, ATM and ATR are no longer active. The checkpoint kinases CHK1 and CHK2 are thus inactivated. CDK1 and CDK4 are not inhibited, and the cell cycle proceeds. If DNA repair fails or the DNA lesion is irreparable, apoptosis is activated via two parallel mechanisms. First, the constant absence of CDC25 leads to dimerization and activation of MEK kinase 5, also known as ASK-1 (apoptosis signal-regulating kinase 1). Active ASK-1 activates a kinase cascade resulting in apoptosis. Secondly, apoptosis can also be activated by the increased levels of p53. High levels of cytosolic p53 activate directly the pro-apoptotic protein BAX and mediate mitochondrial outer membrane permeabilization (MOMP), which results in the activation of a cascade leading to apoptosis (Chipuk and Green, 2004).

Germline mutations in the *ATM* gene are responsible for the neurodegenerative hereditary disease ataxia telangiectasia (AT), also called *Louis-Bar syndrome* or *Border–Sedgwick syndrome*. Because of its deficient DNA repair, the DNA of AT patients is unstable and very sensible to mutagenic influences. This fact explains the high risk of AT patients for breast cancer, leukemia, and Hodgkin's lymphoma.

Table 3.5 Repair mechanism and cell cycle phases.

Repair mechanism	G_1 phase	*S* phase	G_2 phase
Homologous recombination (HR)		DSB, SSB	DSB, SSB
Nonhomologous end joining (NHEJ)	DSB, SSB		
Base excision repair (BER)	Oxoguanine		
Mutation mismatch repair (MMR)		Base mismatches	

DSB, double-strand break and SSB, single-strand break.

3.2.7
The Repair Mechanism Depends on the Cell Cycle Phase

Both the cell cycle phase and the type of the DNA lesion influence the activation of the different repair mechanism (Table 3.5) (Bernstein *et al.*, 2002).

Double- and single-strand breaks during *S* phase and early G_2 phase are repaired by homologous recombination (HR) of intact sister chromatids. During G_1 phase, the chromatin is condensed and there is no second chromatid, thus strand breaks are closed directly by nonhomologous end joining (NHEJ). Because NHEJ is independent of homology at the strand ends, NEHJ is more error-prone than HR. Oxoguanine, which originates directly from oxidation of guanine by ROS, is repaired by base excision repair (BER) during G_1 phase. Single mismatches are repaired during S phase when the DNA is replicated by the MMR (Mutation-Mismatch-Repair) system. Inactivating mutations in the genes coding the MMR proteins lead to DNA instability and are found in both inherited and sporadic colon tumors.

3.2.8
Tumor-Relevant Proteins in the Cell Cycle

In normal cells, the expression of genes encoding CDKIs is activated by proliferation inhibitors, such as TGFβ (transforming growth factor β), and stress signals, such as DNA lesions or ROS. Mitogenic signaling pathways such as the Wnt or the PI3K/Akt pathway inhibit the expression of CDKIs (see chapters 8 and 11). Regulatory mechanisms of the cell cycle show defects in many tumors (Table 3.6).

Genes coding for CDKIs belong to the most frequently mutated tumor suppressor genes. Lack of mutation and a high intracellular concentration of the CDKI p27Kip1 correlate with good prognosis of patients with tumors of the breast, esophagus, lung, and colon, whereas a high concentration of the CDK activator cyclin E correlates with bad prognosis (Tsihlias, Kapusta, and Slingerland, 1999). Many tumors show activating mutations in the genes coding for CDK4 or CDK6. The corresponding mutated proteins are resistant to inhibition by CDKIs.

Table 3.6 Tumor-relevant proteins regulating the cell cycle and gene mutations.

Protein	Biochemical activity	Cellular function	Gene alterations in tumors
Cyclin B1	CDK1 activation	G_2-M passage	Overexpressed in colorectal tumors
Cyclin D1	CDK4 and CDK6 activation	G_1-S passage	Mutated in mamma carcinomas and leukemias Overexpressed in tumors with activated Wnt pathway
Cyclin E	CDK2 activation	G_1-S passage	Rearrangements and overexpression in breast cancer
CDK4	Phosphorylation and inactivation of Rb and others	G_1-S passage	Amplification in gliomas Missense mutation in inherited form of melanoma
CDK6	Phosphorylation and inactivation of Rb and others	G_1-S passage	Amplification in lymphoma, squamous cell carcinoma, and glioma
p21	Inhibition of CDK2, CDK4, CDK6	Block at *R* point between G_1 and *S* phase	Mutated and epigenetically inactivated in many tumors
p16-INK4	Inhibition of CDK4 and CDK6	Block at *R* point between G_1 and *S* phase	Inactivated and mutated in melanoma and other tumors
p27Kip1	Inhibition of different CDKs	Block at all three checkpoints	Inactivated and mutated in breast, lung, colon tumors, and others

3.3 The Cell Cycle as Therapeutic Target

Functional differences between tumor cells and normal cells can be exploited as therapeutic targets. Because the increased proliferation rate is a major difference, the different phases of a tumor cell cycle with its fast rates of DNA, RNA, and protein synthesis represent important targets for anticancer drugs (Eckhardt, 2002).

According to the classification of anticancer drugs described in Chapter 1, compounds with effects on the cell cycle belong to the group of drugs with effects on intracellular processes. Drugs affecting the cell cycle can be grouped according

Table 3.7 Anticancer drugs affecting different phases of the cell cycle.

Affected phase	Effect	Examples
S phase	Block of DNA replication	Base and nucleotide analogs such as 5-FU
M phase	Block of chromosome segregation	Drugs interfering with depolymerization of microtubules or polymerization of tubulin such as paclitaxel or vincristine
Checkpoints	Inhibition of cyclin-dependent kinases	Small CDK inhibitors such as flavopiridol

to the phase, in which they unfold their activity (Table 3.7). Analogs of bases and nucleotides block DNA replication and RNA transcription in the *S* phase. Drugs blocking polymerization or depolymerization of microtubules lock the cell in the metaphase. Both groups are described in Chapter 1.

3.3.1 Small Compounds Inhibiting Cell-Cycle-Dependent Kinases as Anticancer Drugs

CDKs are overactive in many cancers because of genetic or epigenetic lesions in their coding regions or in their promoter/enhancer regions that regulate their activities or expression levels. Small molecules that inhibit the enzymatic activity of CDKs might be able to simulate the effects of the physiological CDKIs and to decelerate the cell cycle and thereby decrease the proliferation rate. Over the past two decades, many small compounds with inhibitory effects on CDKs have been discovered, either in targeted screens or accidentally while searching for inhibitors for other kinases (Blagden and de Bono, 2005). More than a dozen small CDK inhibitors have entered clinical trials, though none of them has been approved for commercial use until now (Lapenna and Giordano, 2009). Four promising examples are introduced here (Figure 3.6).

The flavonoid alkaloid flavopiridol, also known as *alvocidib*, shows inhibitory activity against different kinases, including CDK1, CDK2, CDK4, CDK7, and CDK9. CDK9 is part of the transcription elongation factor P-TEFb, which phosphorylates RNA polymerase II and thereby stabilizes the nascent RNA transcript. Due to its inhibitory effect on P-TEFb, flavopiridol inhibits synthesis of mRNA, stops the cell cycle, and induces apoptosis in tumor cells. Clinical trials show that flavopiridol prolongs the life span of patients with chronic lymphocytic leukemia (CLL).

The synthetic drug candidate roscovitine, also known as *seliciclib*, inhibits kinases CDK1, CDK2, CDK5, CDK7, and CDK9. First clinical trials showed that patients with hepatocellular carcinoma or with small-cell lung carcinoma may benefit from treatment with roscovitine.

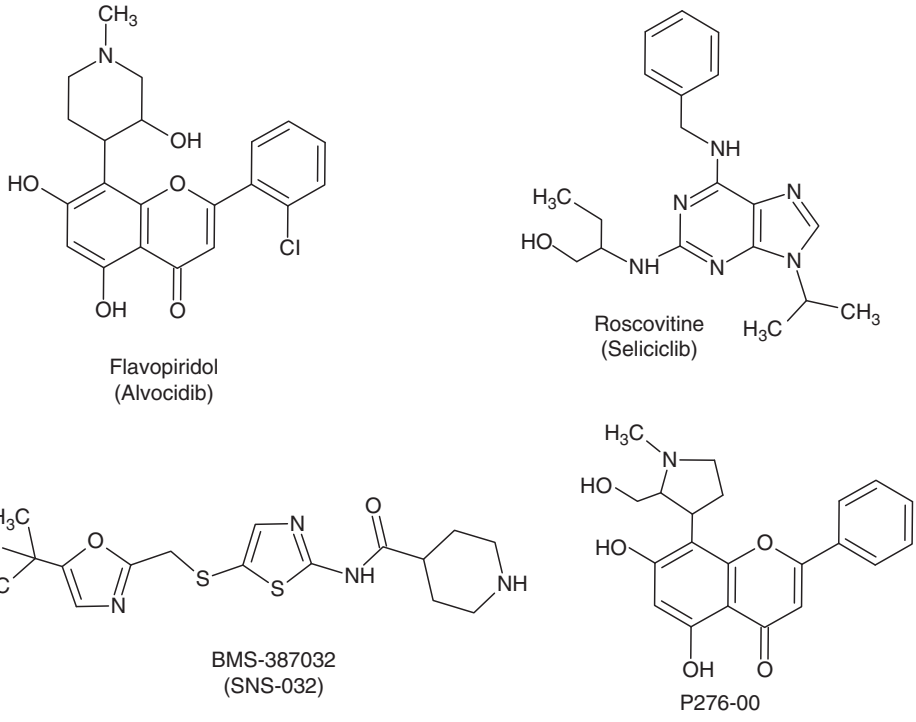

Figure 3.6 Examples for small inhibitors of cyclin-dependent kinases that have entered clinical trials.

Similarly to roscovitine, BMS-387032, also known as SNS-*032*, inhibits CDK2, CDK7, and CDK9. BMS-387032 inhibits the growth of different tumor cell lines and shows positive effects in combination therapies of patients with metastasizing tumors (Heath *et al.*, 2008).

P276-00, which is a synthetic derivative of flavopiridol, inhibits CDK1, CDK2, and CDK9. Remarkably, this compound induces cell cycle arrest and also caspase-dependent apoptosis in many tumor cell lines including cisplatin-resistant cell lines. In phase I and II studies, P276-00 was effective against multiple myeloma, head and neck cancer, and melanoma.

3.4 Outlook

Slowing down the pace of the cell cycle is an attractive concept for lowering the proliferation rate of tumor cells. This is why the search for novel cell cycle inhibitors and their evaluation will continue in the next years. Cell cycle inhibitors with therapeutic potential include not only inhibitors of CDKs but also inhibitors of checkpoint kinases, aurora kinases, and polo-like kinases, which have not been

further explained in this chapter. Actually, some of these show promising results in preclinical and clinical trials. Some of the new compounds not only effectively slow down proliferation rate, but also induce apoptosis. Nevertheless, it has to be noted that cell cycle inhibitors are neither able to turn tumor cells into normal cells nor eradicate the tumor itself. In other words, cancer therapy with cell cycle inhibitors can only be successful when it is combined with therapeutics that block additional aspects of malignancy, including the invasion of tumor cells into neighboring tissue, and the formation of metastases. Clinical data show that the combined therapy with CDK inhibitors and differentiation inducers might be an alternative to the therapy with conventional cytotoxic drugs.

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4
Cell Aging and Cell Death

Cancer Signaling: From Molecular Biology to Targeted Therapy, First Edition.
Christoph Wagener, Carol Stocking, and Oliver Müller.

Companion Website:www.wiley.com/go/wagener/cancersignaling

Summary

Normal cells go through only a limited number of cell divisions, whereas tumor cells keep their replicative potential over many rounds of replication. This is because tumor cells do not enter replicative senescence, a phase in which the cell cycle is irreversibly blocked. Controlled cell death is an important process for sorting out unnecessary cells and for keeping the number of cells constant within a healthy organ. The loss of susceptibility to external and internal signals leading to controlled cell death is one of the major and specific properties of tumor cells. In the past few years, many molecules and mechanisms underlying the processes of cell aging and death have been used as starting points for the development of new anticancer drugs.

4.1 A Cell's Journey through Life

A normal cell has many options: it can proliferate, differentiate, and perform its specific function; alternatively, it can enter quiescence, a temporary arrest of cell cycle (G_0 phase) or, finally, it can die (Figure 4.1). After multiple rounds of cell division, a normal cell enters replicative senescence, which is the irreversible block of the cell cycle. A typical tumor cell is not able to differentiate in a physiological way, to enter G_0 phase, or to die in a regulated manner. This is because most tumor cells have acquired resistance against signals that induce differentiation, inhibit growth and proliferation, or induce processes leading to cell death. These insensitivities are based on defects in the signaling pathways that are responsible for regulating cell cycle and cell death.

4.2 Cellular Aging and Senescence

In the 1960s, the American gerontologist Leonard Hayflick observed that the number of divisions of human fibroblasts is limited (Hayflick, 1965). He showed

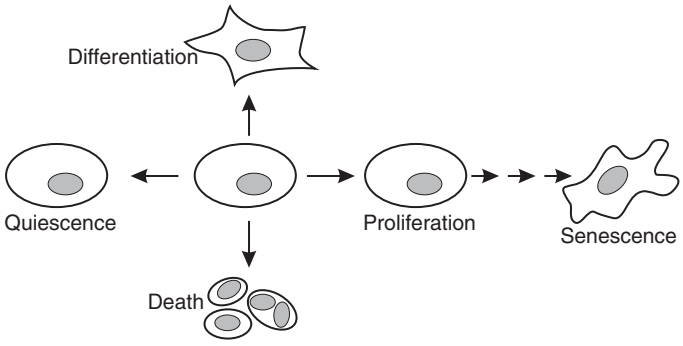

Figure 4.1 Potential fates of a normal cell. A normal cell can enter a reversible phase of cell cycle arrest (quiescence), differentiate, die, or proliferate. After multiple divisions, the cell enters into replicative senescence. A cell can also enter nonreplicative senescence (not shown).

that normal cells are not able to replicate more than 40–60 times. Hayflick called the *state* cellular senescence, in which the cells are still alive but unable to proliferate any further. Cellular senescence is often used as a synonym for age-related apoptosis. Nevertheless, senescence is not a form of cell death, but rather an irreversible stop of the active cell cycle. Cells in senescence are still metabolically active. As opposed to the reversible cell cycle stop during cellular quiescence in G_0 phase, the cell cycle stop in senescence cannot be overcome by mitogens.

Senescence is verified by the detection of several different markers (Table 4.1). In addition to high levels of the enzyme senescence-associated β-galactosidase (SA-β-Gal), senescent cells show a typical gene expression pattern and a specific chromatin structure with the so-called senescence-associated heterochromatin foci (SAHF). Most senescent cells in culture show flat and outspread morphology.

4.2.1 Replicative Senescence

Replicative senescence can be distinguished from the nonreplicative forms, which have a similar phenotype but have a distinct origin. By definition, replicative senescence is caused by shortened chromosomes.

The process of replicative cell aging can be divided into two phases (Figure 4.2) (Shay and Wright, 2005). Progression through the first phase (M1) of mortality into the second phase (M2) is determined by the extent of chromosome shortening and the activity of the p53/Rb network. Cells with telomeres shortened by around 5000 bp enter M1 stage (Cheung and Deng, 2008). The further reduction of telomere lengths activates the so-called DNA damage response pathways. As a consequence, the cell cycle is irreversibly stopped by the proteins p53 and Rb. In the case that the cell cycle cannot be stopped, for example,

Table 4.1 Markers of senescence (Ewald *et al.*, 2010).

Marker	Method
Cell	
Flat outspread morphology	Microscopy
Mitochondrial fusion	Microscopy
Senescence-associated β-galactosidase	
Enzymatic activity	ELISA, staining
Expression	Real-time PCR, RNA-FISH
Intracellular level	Immune staining
Cell cycle arrest	
BrdU incorporation	Immune staining, flow cytometry
Decreased Ki67	Immune staining
Apoptosis exclusion	
Annexin V	Immune staining, flow cytometry
Level/activity of caspases	ELISA, immune staining
TUNEL	Staining
Protein or RNA levels	
p27Kip1, p21, p16-INK4a	Immune staining
IGF2, IGFBP3, IGFBP5, IGFBP7	Immune staining, real-time PCR, RNA-FISH
IL-6, IL-8	Immune staining, real-time PCR, RNA-FISH

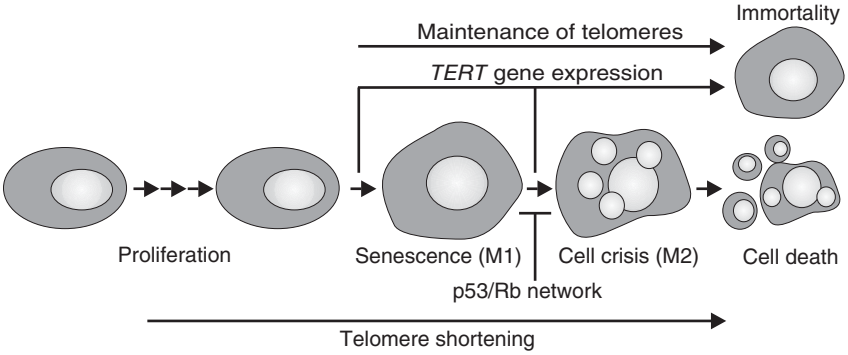

Figure 4.2 Replicative cell aging. After multiple divisions, a normal cell enters into senescence (M1). At this point, the cell cycle is blocked permanently by the p53/Rb network. Defects in this network lead to cell crisis (M2), which results in cell death. Senescence and cell crisis can be overcome by expression of *TERT* in stem cells or in tumor cells. TERT: telomerase reverse transcriptase. (Wagener and Müller, 2009), with permission.

because of a defective p53/Rb network, the cell proliferates further. The telomeres are shortened further and the cell enters the second phase of mortality (M2), the so-called cell crisis. This phase leads to irreversible chromosomal lesions and to cell death. Thus, two mechanisms, chromosome shortening and the p53/Rb network, control the total number of cell divisions. Both mechanisms are overcome in tumor precursor cells, a prerequisite for cell transformation and tumor development (Hanahan and Weinberg, 2000, 2011).

4.2.2 Shortening of Chromosomal Telomeres during Replication

During replication, both strands of the parental DNA serve as templates for the synthesis of the new strands. All known DNA polymerases form new bonds only at the free hydroxyl group at the 3′ end of an existing polynucleotide. Thus, a new DNA strand can only be extended in the 5′–3′ direction. Because the two DNA strands are running antiparallel, one daughter strand runs from 5′ to 3′ and the other from 3′ to 5′. As a consequence, only one daughter strand, the so-called leading strand, can be synthesized continuously at the replication fork. The synthesis of the lagging strand is performed by parallel elongation of single RNA primers of 8–12 bp. These primers are extended to discrete RNA-DNA Okazaki fragments that are named after the Japanese couple Okazaki who suggested the mechanism of replication in 1968. As soon as a growing Okazaki fragment hits the 5′ end of a RNA primer, the primer is removed and degraded. Gaps between Okazaki fragments are filled and the fragments are ligated to form the new continuous strand.

At the chromosomal end, there is no polynucleotide primer with a free 3′ end. A gap remains that cannot be extended by the DNA polymerase. Thus, each replication leads to a DNA double strand with an overhanging 3′ end of the parental DNA strand (Figure 4.3). The single-stranded overhang is degraded and thereby the telomere shortens. Strictly speaking, when chromosomal length is used as the only criterion for the age of a cell, a daughter cell will always be older than its mother cell.

Theoretically, each chromosomal end shortens by around 20 bp with every cell division. Actually, a shortening of around 50–100 bp is observed after each division. The difference is explained by the influence of free radicals, which damage DNA preferentially at the chromosomal ends.

4.2.3 Chromosomal Telomeres

Chromosomal DNA ends in a telomere, which consists of DNA with a specific telomeric sequence and a complex of several proteins. The intact telomere is essential for chromosomal integrity. A missing or mutated telomere might lead to fusion with another chromosome or to instability and to the loss of the

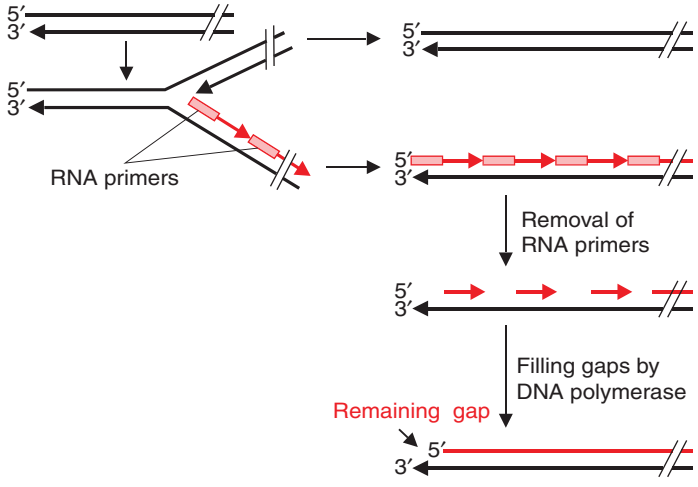

Figure 4.3 DNA replication at chromosomal ends. The upper leading strand (black) is synthesized continuously and runs into the replication fork. The lagging strand (red) is synthesized discontinuously by extension of RNA primers at their 3′ ends. After removal and degradation of the primers, the gaps are filled in by a DNA polymerase. At the 5′ end of the lagging strand, a gap remains, because there is no primer that can be extended. (Wagener and Müller, 2009), with permission.

chromosome. Human telomeres consist of repetitive TTAGGG motifs, which end in a guanine-rich single strand. The mechanisms described earlier lead to the shortening of the telomere DNA with every cell division. The magnitude of chromosome shortening correlates inversely with the time period between two mitoses. With each mitosis, the interphase becomes longer and longer. After 40–60 cell divisions, a normal cell has reached cellular senescence and does not proliferate any further. The limit in the number of divisions is called *Hayflick limit* (Hayflick and Moorhead, 1961). The single-stranded 3′ end of the telomeres can invade the double-stranded DNA of the telomere and replace the homologous DNA strand. Thereby, the so-called t-loop forms at the chromosomal end. This loop inhibits the formation of a free DNA end.

Telomeres in mammalian cells associate with a protein complex called *shelterin*, also called *telosome*. This complex consists of six proteins: TRF (telomeric repeat-binding factor) 1 and TRF2, TIN2 (TRF-interacting nuclear factor 2), POT1 (protection of telomeres protein 1), TPP1 (telomeric POT1-binding protein 1), and TRF2IP (telomeric repeat-binding factor 2 interacting protein) (Figure 4.4). The shelterin complex stabilizes the chromosomal end by forming the so-called t-loop. Telomeres can lose their protecting function on chromosomal ends by progressive shortening or by the loss of the shelterin complex. A free chromosomal end lacking the t-loop or an inactive shelterin complex or a chromosome shortened by replicative senescence might be recognized as double-strand break by the DNA repair system. The following "repair," either by nonhomologous end-to-end

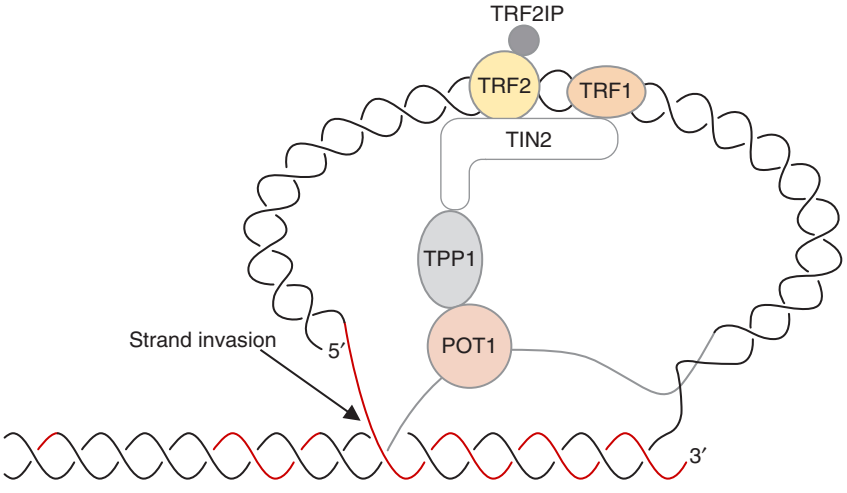

Figure 4.4 The chromosomal telomere with the shelterin complex. A telomere ends in a single strand 3′ overhang, which invades the double strand and protects the chromosomal end by forming a t-loop. Six proteins form the "shelterin" complex that is part of the telomere. The TPP1-POT1 heterodimer regulates the association of the telomerase with its substrate. TRF1, 2: telomeric repeat-binding factor 1, 2; TRF2IP: telomeric repeat-binding factor 2 interacting protein; TIN2: TRF-interacting nuclear factor 2; TPP1: telomeric POT1-binding protein 1; and POT1: protection of telomeres protein 1. (Wagener and Müller, 2009), with permission.

joining or by homologous recombination, may lead to chromosomal fusions (Figure 4.6).

Embryonal growth and tissue regeneration do not conform to the Hayflick limit. Indeed, embryonal and also somatic stem cells of regenerating tissues are able to proliferate beyond the Hayflick limit. This finding can be explained by the fact that embryonal and adult stem cells harbor mechanisms that antagonize telomere shortening. The most important mechanism is based on the activity of an enzyme complex called telomerase, which can prolong the telomeres after each cell division and restore the original chromosomal length.

4.2.4 Telomerase

The catalytic subunit of the telomerase is a reverse transcriptase, which is able to synthesize a DNA strand by using an RNA strand as template. The telomerase complex has its own RNA template, which is complementary to the repetitive DNA sequence of the telomere. The complementary RNA hybridizes with the 3′ end of the DNA. The reverse transcriptase subunit of the telomerase extends the 3′ end of the telomere DNA by using the nonpaired sequences of the RNA as template. This process is repeated several times. The complementary DNA strand can be filled by a DNA polymerase (Figure 4.5). This mechanism implies that two genes are responsible for the formation of the enzyme telomerase. The gene *TERC*

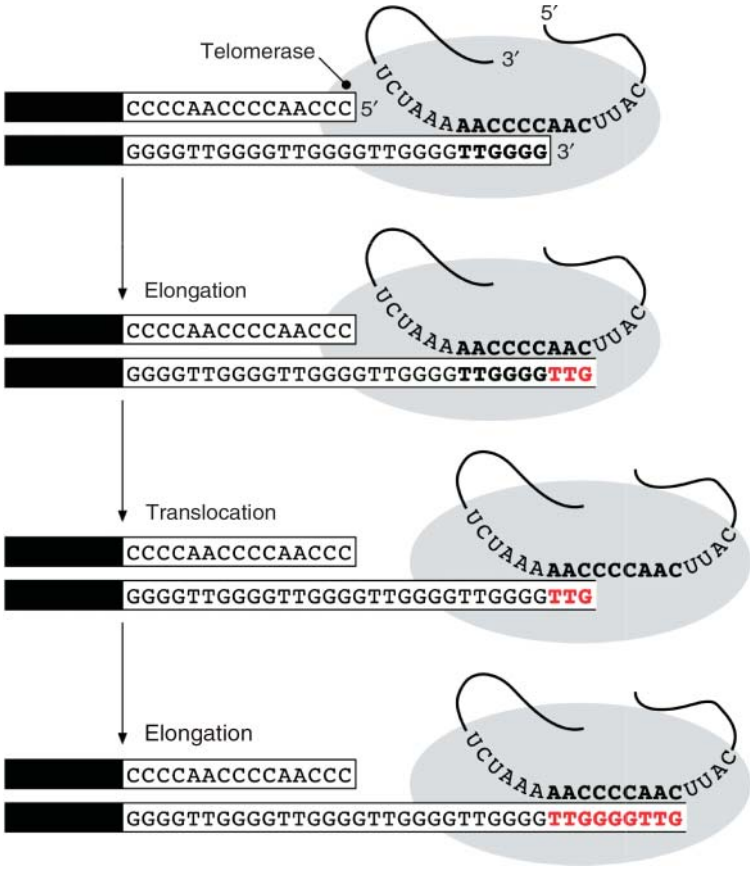

Figure 4.5 Extension of the 3′ end of telomeric DNA by the telomerase enzyme complex. A partial sequence of the telomerase RNA binds to a complementary sequential motif at the 3′ end of the DNA. The nonpaired RNA sequence serves as template for extension of the 3′ end and DNA synthesis. (Wagener and Müller, 2009), with permission.

expresses the RNA template, and the gene *TERT* (telomerase reverse transcriptase) codes for the catalytic subunit of the reverse transcriptase.

As soon as the Hayflick limit is reached, signaling pathways are activated that inhibit mitosis. In the case that these pathways are inactive, the cell continues to proliferate until it reaches the stage of the so-called cell crisis. In the absence of telomerase, chromosomal ends erode and chromosomes can fuse by mechanisms of DNA repair. Because of the fusion, sister chromosomes cannot be separated during mitotic anaphase. New double-strand breaks are generated and dicentric chromosomes evolve. This cycle is called the *bridge–fusion–breakage cycle* (Figure 4.6) (Maser and DePinho, 2002).

In the absence of an active telomerase, the cell dies in cell crisis. The activation of telomerase in a single cell with an aberrant genome may lead to its survival

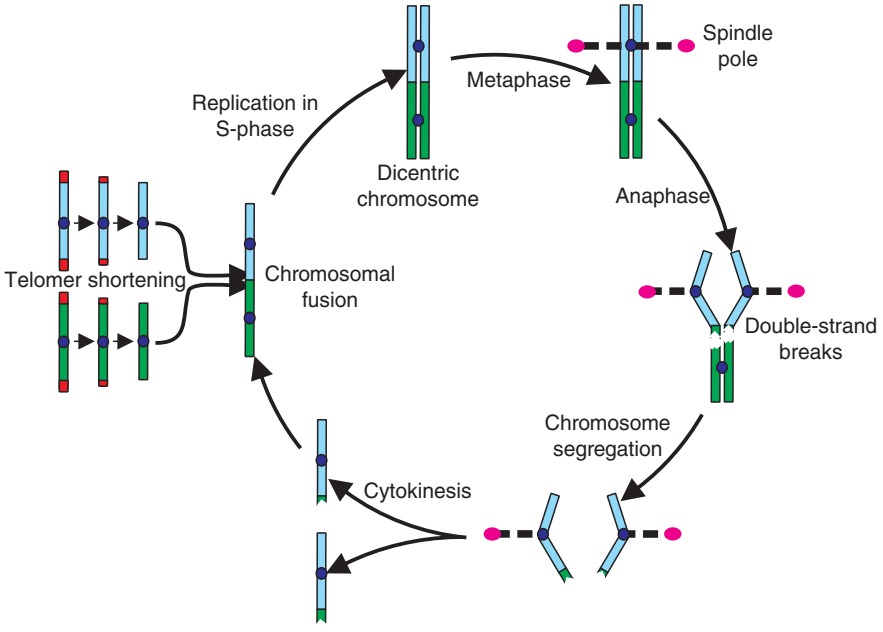

Figure 4.6 Cycle of chromosomal fusion and chromosomal breakage. Chromosomal ends shorten in normal proliferating cells due to the absence of telomerase (left). Shortened ends fuse by the DNA repair mechanism of end-to-end joining. The dicentric chromosome breaks in the mitotic anaphase, when it is drawn to opposite cell poles. This may lead to chromosomal rearrangements and double-strand breaks. DNA repair mechanisms may lead to additional fusions. The cycle of chromosomal breakage and chromosomal fusion is perpetuated. (Wagener and Müller, 2009), with permission.

Table 4.2 Telomerase activity, telomere length, genome instability, and number of DNA aberrations in different stages of mammary carcinomas (Chin *et al.*, 2004).

Stage of mammary carcinoma	Telomerase	Telomere length	Genome	Number of DNA aberrations
Normal	Inactive	Long	Stable	Low
Hyperplasia	Inactive	Medium	Stable	Low
Carcinoma *in situ*	Active	Short	Instable	High
Invasive carcinoma	Active	Short	Instable	High

and the initiation of tumor development. These processes play a crucial role in human carcinogenesis, as has been proven in early stages of mammary carcinomas (Chin *et al.*, 2004). Erosion of telomeres and anaphase bridges were observed in carcinomas *in situ*, but not in hyperplastic tissue regions (Table 4.2).

4.2.5 Animal Models

In order to analyze the causality of telomere shortening in tumor development, transgenic knockout mice without the *Terc* gene were generated. Because murine chromosomes harbor relatively long telomeres, no phenotype was observed until the sixth generation. In these animals, the apoptosis rate was increased in tissues with high proliferation rates, such as skin and blood. In addition, the function of these tissues was disturbed. Surprisingly, telomere shortening did not result in increased tumor risk. With an established model of chemical carcinogenesis, the number of skin tumors in $Terc^{-/-}$ mice of late generations was found to be significantly lower than in wild-type mice (Gonzalez-Suarez *et al.*, 2000). Similar results were observed in a second mouse tumor model. Inactivation of the tumor suppressor gene adenomatous polyposis coli protein (APC) in APC^{min} mice results in both microadenomas and macroadenomas in the small intestine. However, in double-mutant $Terc^{-/-}$ Apc^{min} mice, microadenomas only developed after six generations of breeding. These results show that eroding telomeres have a tumor suppressing effect, at least as long as the tumor suppressor protein p53 is active.

The erosion of telomeres and the loss of the shelterin complex activate the DNA double-strand repair mechanism. The tumor suppressor p53 is an important effector of this mechanism. If p53 is inactive, the effect of eroding telomeres reverses to a tumor-promoting effect. $Terc^{-/-}$ mice that carry a heterozygous deletion of the *Trp53* gene ($Trp53^{+/-}$) develop tumors that are common in human cancer patients, including tumors of the breast, the colon, and the skin. In contrast, $Trp53^{-/-}$ mice develop lymphomas and sarcomas, but no carcinomas (Artandi and DePinho, 2000).

4.2.6 Overcoming Replicative Senescence in Tumor Cells

Arithmetically, 40 cell divisions result in 10^{12} cells and a tumor of around 10 cm in diameter, which is the average size and average cell number of a potentially fatal, malignant tumor. However, in solid tumors, proliferation is balanced by necrosis and apoptosis (Figure 4.7). Thus, more than 80 cell divisions are likely necessary to reach the mentioned cell number. Consequently, tumor cells have mechanisms to overcome replicative senescence. More than 85% of all analyzed tumors showed defects in the p53/Rb network, which allows tumor cells to overcome the cell cycle block in the M1 phase. In a similar proportion of tumors, the *TERT* gene is overexpressed, which facilitates the circumvention of senescence and cell crisis and thereby inhibits apoptosis (Rhyu, 1995). In normal cells of the adult organism, expression of the *TERT* gene, encoding the enzymatic subunit of telomerase, is inhibited by epigenetic mechanisms. In contrast, the *TERC* gene, which expresses the RNA template, is expressed in all cells (Aubert and Lansdorp, 2008). Telomeres in cells overexpressing the *TERT* gene keep their original lengths. Nearly 85% of stomach tumors are telomerase-positive. Most of the telomerase-positive

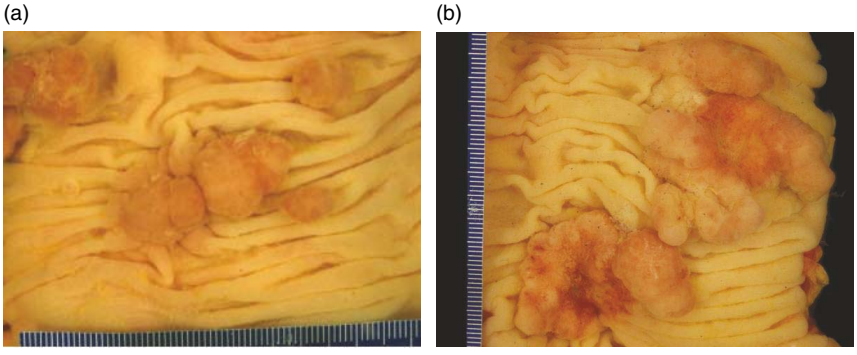

Figure 4.7 Colon tumors of different stages. (a) Early stage of tumorigenesis. Several small and regional adenomas (polyps). (b) Progressed stage. Large tumors with macroscopically visible signs of inflammation (reddish) and indications of necrosis (sunken tissue regions). Figure kindly provided by Professor Dr. Cornelius Kuhnen.

tumors were in progressive stages and had already metastasized. The survival rates of patients with proven telomerase activity in tumors were significantly lower than that of patients with telomerase negative tumors. There is no connection between telomerase activity in kidney carcinoma cells and clinical pathological parameters such as differentiation grade, tumor stadium, and clinical course (Mehle *et al.*, 1996). In contrast, the telomerase activity in mammary carcinomas correlates significantly with tumor progression (Chin *et al.*, 2004) (Table 4.2).

4.2.7
Nonreplicative Senescence

There are three major causes for nonreplicative senescence. First, accumulation of DNA alterations, caused by, for example, reactive oxygen species (ROS), may lead to activation of the p53/Rb network and to a permanent cell cycle stop (Figure 4.8). Secondly, nonreplicative senescence might be caused by the induction of the genes encoding cyclin-dependent kinase-inhibiting proteins (CDKIs) p21 and p16-INK4a. The degree of induction depends on cell age (Herbig and Sedivy, 2006). The age-related expression of CDKIs is independent from the telomere length. The expression of CDKIs limits the number of replications in embryonal stem cells and also tumor cells, even if they express high levels of telomerase. This means that a cell with intact DNA and full-length telomeres might also enter into senescence. Thirdly, cytotoxic compounds including DNA-damaging compounds, as well as kinase-inhibiting anticancer drugs, may induce nonreplicative senescence. Because this form of senescence is often observed in tumor cells during anticancer therapy, it is called therapy-induced senescence (TIS) (see below).

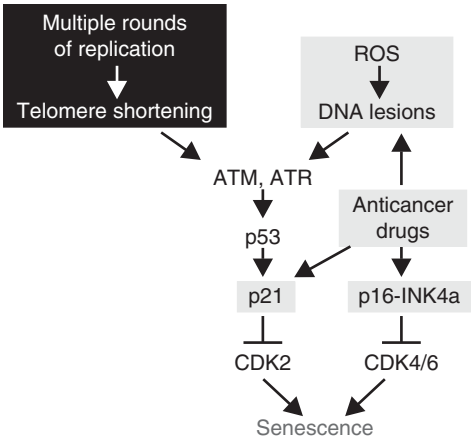

Figure 4.8 Induction of cellular senescence. Replicative (black box) and nonreplicative (gray boxes) senescence run via partially shared molecular mechanisms. Nonreplicative senescence can be induced by DNA lesions (e.g., induced by ROS) or anticancer drugs that either damage DNA or induce expression and activation of p21 and p16-INK4a, or by age-related expression of p21 or p16-INK4a. ROS: reactive oxygen species; ATM: Ataxia telangiectasia mutated; ATR: Ataxia telangiectasia and Rad3-related protein; and CDK: cyclin-dependent kinase.

4.3 Cell Death

Five main ways of cellular dying can be distinguished (Table 4.3): necrosis (ancient Greek: killing), apoptosis (ancient Greek: downfall), their hybrid form called necroptosis, autophagy (ancient Greek: self-devouring), and cornification (conversion into horn) (Zhivotovsky and Orrenius, 2010; Nikoletopoulou *et al.*, 2013). Cornification denotes the conversion of squamous epithelial cells into a keratinized horny material, such as hair, nails, or feathers. Because cornification is limited to epidermal cells, it is not further described here.

Table 4.3 Principle pathways of cell death.

Principle of cell dying	Trigger example	Target cell example
Necrosis	Accidental injury, e.g., burn	Various cells
Necroptosis	TNF-α together with low ATP level	Various cells
Apoptosis induced by		
External signals	Granzyme B on cytotoxic T-cells	Tumor cell
Absence of vitality factors	Lack of erythropoietin	Precursor erythrocyte
Internal signals	Irreparable DNA mutations	Aging cell
Autophagy	Low level of essential amino acids	Cells in a newborn

4.4
Morphologies of Dying Cells

4.4.1
Morphology of Necrotic Cells

Necrotic regions can be seen macroscopically in many solid tumors (Figure 4.7). On the cellular level, necrosis begins with cell swelling and proceeds with perforation and lysis of the membranes (Figure 4.9). The complete cellular and nuclear contents are released into the surroundings in an uncontrolled manner. Antigens, which are normally located inside the cell, reach the intercellular space, where they attract immune cells and cause inflammation. Such molecules are called DAMPs (damage-associated molecular pattern molecules). Necrosis is uncontrolled and independent from energy and metabolism.

4.4.2
Morphologies of Apoptotic and Necroptotic Cells

In the 1970s, Andrew Wyllie and coworkers observed that cells of the adrenal cortex die when deprived of the adrenocorticotropic hormone (ACTH). They

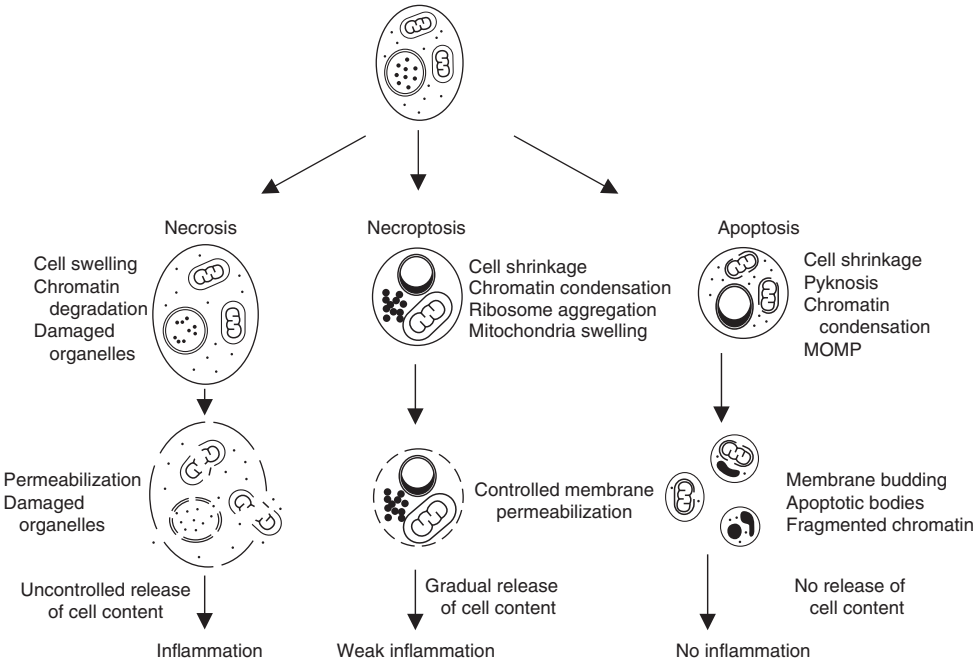

Figure 4.9 Cell morphological differences between necrosis, necroptosis, and apoptosis. MOMP: mitochondrial outer membrane permeabilization. (Wagener and Müller, 2009), with permission.

also observed that these dying cells show morphological features that are distinct from the features shown by necrotic cells, which die by accidential injury (Wyllie, Kerr, and Currie, 1972). Wyllie called this form of dying apoptosis. Apoptotic cells can be clearly differentiated from necrotic cells by light microscopical analysis (Figure 4.9, Table 4.4) (Hacker, 2000). The first visible sign of beginning apoptosis is the cell shrinkage. Regions of the cell membrane cave in and form intracellular blebs, called *apoptotic blebs*. Simultaneously, the nucleus shrinks, a phenomenon known as *pyknosis*. The genomic DNA, which is found in the periphery of the nucleus, is degraded into sections of approximately 180 bp length or multiples thereof. This length corresponds to the length of the DNA strand as part of a nucleosome, which is a complex consisting of a histone multimer and the DNA. Next, the membrane buds and forms vesicles called *apoptotic bodies*. Apoptotic bodies are surrounded by intact membranes and contain intact organelles, for example, mitochondria. Budding continues until the entire cell is fragmented in apoptotic bodies, which stay intact and metabolically active until they are incorporated by macrophages. During the entire apoptotic process, transcription, ATP production, and protein synthesis are still active. These processes are necessary to keep the process controlled. In contrast to necrosis, intracellular antigens are not released into the intercellular space and the surrounding tissue. Therefore, there is no inflammatory reaction.

Necroptotic cells show fewer striking morphological changes in comparison to apoptotic cells. Many necroptotic cells harbor swollen mitochondria and aggregated ribosomes. During the entire process, the cell remains attached to the intercellular matrix.

4.4.3 Morphology of Autophagy

Autophagy is mainly induced by low levels of essential nutrients during periods of starvation. Major morphological characteristics of an autophagous cell are the autophagosomes, which are organelles surrounded by a double membrane. In a later stage, autophagosomes fuse with lysosomes and the content is degraded. In the scenario that this self-digestion is not stopped by increased levels of nutrients, it continues until the entire cytoplasm and all organelles are degraded into autophagosomes and the cell dies.

4.5 Necroptosis

For many years, it was thought that only apoptosis and autophagy are gradual and regulated ways of cell dying and that only these forms of cell death require

Table 4.4 Differences between necrotic, necroptotic, apoptotic, and autophagous cells.

	Necrosis	Necroptosis	Apoptosis	Autophagy
Nucleus	Perforation Chromatin degradation	Shrinkage (pyknosis) Chromatin condensation	Shrinkage (pyknosis) Nuclear fragmentation DNA laddering Chromatin condensation	
Mitochondria	Lysis and degradation	Swelling Loss of membrane potential	Mitochondrial outer membrane permeabilization (MOMP) Loss of membrane potential	Surrounded by membrane
Cytoplasm	Released into surrounding tissue	Ribosome aggregation	Formation of apoptotic blebs Cleavage of caspase targets Degradation of cytoskeleton	Autophagosome formation
Plasma membrane	Permeabilization Annexin V negative	Controlled permeabilization Annexin V negative	Annexin V positive Membrane blebbing	Annexin V negative
Cell	Swelling	Remains attached to matrix Removal through unknown mechanism	Detachment from matrix Formation of apoptotic bodies Phagocytosis	Autophagocytosis of organelles
Energy Inflammation	Independent Strong	Partly dependent Weak	Dependent No	Dependent No

energy and gene expression. Necrosis was regarded as the result of an accidental and uncontrolled process. Today, we know that certain forms of necrotic cell death can be mediated by signaling pathways and controlled mechanisms, which are similar to those signals inducing apoptosis. In 2005, Junying Yuan and coworkers showed that T-cells, which have been treated with a specific inhibitor for caspase 8 (cysteinyl aspartate cleaving protease 8), still die by a gradual mechanism that is similar to apoptosis, but independent from caspases (Degterev *et al.*, 2005). This process was called *necroptosis* to describe the regulated and programmed form of dying, which is yet distinct from apoptosis.

In addition to the maintenance of T-cell homeostasis, necroptosis is seen during bacterial and viral infections, in inflammatory and neurodegenerative diseases, and also in maintenance of intestinal homeostasis. Stimulation of death receptors, including CD95 (Fas), tumor necrosis factor receptor (TNFR), and TRAILR leads to activation of caspases and thereby to apoptosis, whereas low ATP levels or inhibition of caspases may lead to necroptosis (Figures 4.10 and 4.11). The TNFR-associated death protein (TRADD) activates receptor-interacting protein 1 (RIP-1) (receptor-activated serine/threonine kinase 1), which recruits RIP-3 to

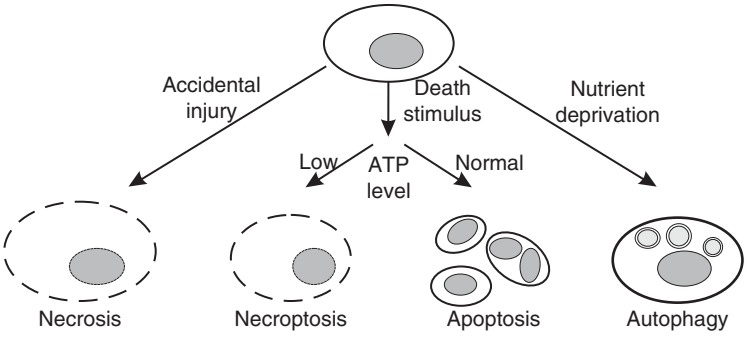

Figure 4.10 Stimuli and causes leading to the different forms of cell death.

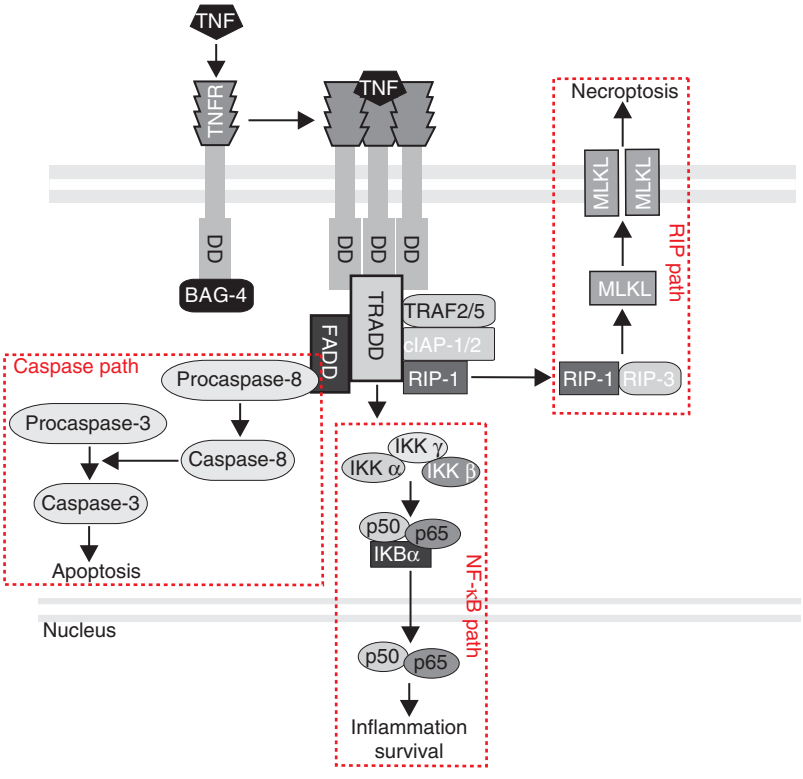

Figure 4.11 Pathways activated by TNFR, the receptor for tumor necrosis factor (TNF). Depending on the interaction partner of TRADD (TNFR type 1-associated death domain protein), apoptosis, inflammation, or necroptosis can be activated. DD: death domain; BAG-4: Bcl-2-associated athanogene 4; FADD: FAS-associated death domain protein; TRAF: TNF receptor-associated factor; cIAP: cellular inhibitor of apoptosis protein; RIP: receptor-interacting protein; MLKL: mixed lineage kinase domain-like protein; IKK: inhibitor of nuclear factor κ-B kinase subunit beta; and IKB: NF-κB inhibitor. The NF-κB pathway is described in detail in Chapter 10. (Wagener and Müller, 2009), with permission.

form the necrosome. Phosphorylation of MLKL (mixed lineage kinase domain-like protein) by the necrosome drives oligomerization of MLKL, allowing MLKL to insert into and to permeabilize the plasma membrane.

4.6 Apoptosis in the Healthy Organism

Apoptosis plays an important role in organ development, organ homeostasis, and maintenance of organ health (Penaloza *et al.*, 2008) and is often designated "programmed cell death." Because neither the process leading to death nor the death as such is programmed, a more precise term would be "controlled and gradual dying process." Every normal cell holds intrinsic "dying programs," which – when activated – may start as a gradual and regulated process leading to cell death. "Dying programs" are intracellular signaling pathways, which can be activated in distinct situations by the presence or the absence of external or internal factors.

An example of the importance of apoptosis during cell development is the formation of red blood cells. More than 95% of all precursor erythrocytes die by apoptosis in the bone marrow. When the blood level of oxygen is low and the number of erythrocytes needs to be elevated, the kidney produces erythropoietin. This growth factor has strong inhibitory activity on the apoptosis of precursor erythrocytes. Another example of apoptosis in the absence of external factors is the regression (involution) of the mammary gland during weaning. Here, the decreasing prolactin levels, in connection with other stimuli, cause cessation of milk production and apoptosis of gland cells. The consequence is the involution of the mammary gland, while the level of involution is highly individual.

An example of apoptosis that is stimulated by external factors with the aim of organ health is that induced by cytotoxic T-cells. These cells recognize and associate with cells that present nonphysiological peptides on their surfaces. Such peptides may result from internal DNA mutations or from viral infection. After binding to the target cell, T-cells may activate membrane-bound receptors that activate pathways leading to apoptosis of the target cell. Apoptosis can also be induced by internal factors. An example is the activation of apoptosis in cells with modified proteins that cannot be degraded or in cells with DNA mutations that can no longer be repaired.

In the adult organism, apoptosis can be regarded as the antagonist of cell proliferation. Both proliferation and apoptosis are responsible for homeostasis of cell numbers. It is self-evident that, in an adult organism, the number of dying cells must be approximately equal to the number of newly formed cells, which is estimated to be around 7×10^4 per minute. An example for apoptosis in homeostasis is that of cells in the regenerating colon epithelium. Here, the cells die when they reach the top of the crypt. Apoptosis is stimulated by the increasing levels of modified proteins, the breaking of cell-to-cell contacts, and the decreasing levels of growth factors. Nonapoptotic conditions are only present at the crypt base, where the somatic stem cells proliferate and thereby replace the dead cells.

4.6.1
The Four Phases of Apoptosis

The apoptotic process runs through four phases: initiation, execution, phagocytosis of the apoptotic blebs, and their degradation (Jin and El-Deiry, 2005) (Figure 4.12). Initiation is induced by the presence or absence of extracellular or intracellular factors (Tables 4.5 and 4.6).

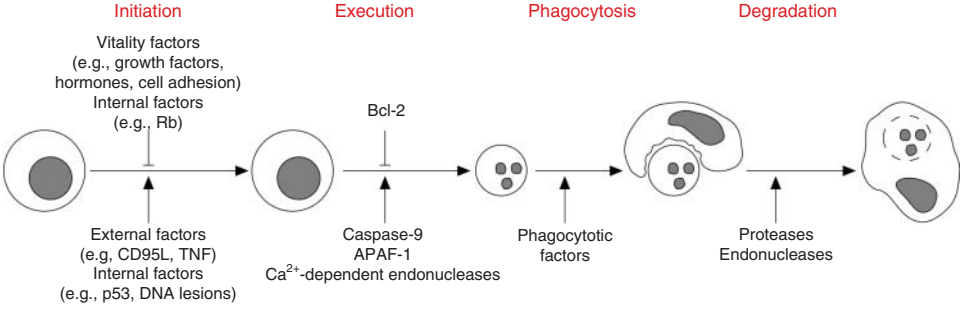

Figure 4.12 The four phases of apoptosis. TNF: tumor necrosis factor; APAF-1: apoptotic protease-activating factor 1. (Wagener and Müller, 2009), with permission.

Table 4.5 Triggers of apoptotic initiation.

Trigger	Source	Target cells
TNF-α	Macrophages	Various
CD95L (Fas ligand)	Cytotoxic T-cells	Lymphocytes, colon epithelial cells, others
Glucocorticoids	Adrenal cortex	Lymphocytes
p53 activation	DNA lesion	Various
Granzyme B	Cytotoxic T-cells	Virus infected cells

Table 4.6 Factors causing apoptosis when deprived.

Factor	Target cell
Growth factor	
IL-3, Kit ligand	Hematopoietic precursor cell
IL-2	T-cell
NGF	Neuronal cell
Hormone	
Prolactin	Mammary gland cell
Testosterone	Prostate cell
Erythropoietin	Erythrocyte precursor
Extracellular matrix	
Laminin	Epithelial cell
Fibronectin	Mesenchymal cell

In accord with the source of the trigger, intrinsic and extrinsic mechanisms of initiation, which are not completely independent of each other, can be distinguished. The tumor suppressor protein p53, which is a major initiator of the intrinsic initiation, also activates expression of CD95 (Fas) and thus increases the sensitivity of the cell for CD95L (Fas ligand), a main inducer of the extrinsic pathway of initiation. Initiation leads to the activation of the execution caspases. During the execution phase, intracellular proteins and DNA are degraded. Execution ends with the decomposition of the cell into apoptotic bodies, which are incorporated by surrounding macrophages and other cells.

4.6.2 Extrinsic Initiation

There are two major pathways of extrinsic initiation. The direct extrinsic pathway starts with the association of the cell targeted for apoptosis with a cytotoxic T-cell or a natural killer cell. Both cell types are able to attach the protease granzyme B to the outer side of the cell membrane. After endocytosis into the target cell, granzyme B directly cleaves the execution protease caspase 3. The second extrinsic pathway starts with the activation of the so-called death receptors by extracellular ligands. Such ligands include tumor necrosis factor (TNF)-α, which is released from macrophages, and CD95L (Fas ligand), which is present on the outer surface of cytotoxic T-cells.

4.6.2.1 TNF Pathway

The TNF-α and TNF-β (lymphotoxin-α) were among the first identified cytokines. Members of the TNF family mediate and regulate inflammatory reactions (Bertazza and Mocellin, 2008). TNF pathways stimulate cell proliferation, differentiation, and angiogenesis. Some TNF cytokines, such as TNF-α, TNF-β, and CD95L (Fas ligand), activate apoptotic initiation.

The family of TNF receptors comprises at least 12 members. The extracellular carboxyterminal domains of TNF receptors show high sequence homology. The extracellular domain is connected to a short amino-terminal intracellular domain via a single transmembrane domain (Figure 4.11). As opposed to other cytokine receptors, TNF receptors do not have their own catalytic activity. Each TNF receptor carries a so-called death domain, which consists of an intracellular stretch of 65 amino acids, which is necessary for its apoptosis-activating activity. Cytoplasmic proteins with their own death domains can bind to the death domain of TNF receptors. In addition, some death domain proteins carry a 15 amino acids long, negative regulatory domain, which is able to inactivate the apoptosis-inducing activity of the death domain. This so-called survival domain associates with a protein tyrosine phosphatase indicating that signals from TNF receptors are transferred via phosphorylation.

As a result of ligand binding, TNF receptor monomers associate to trimers. Thereby, the cytoplasmic domains of the monomers converge, which leads to the dissociation of the inhibitor protein BAG-4 (Bcl-2-associated athanogene 4, also called SODD for silencer of death domains). Next, the adapter protein TRADD binds to the death domain.

4.6.2.2 TNF Receptor Downstream Signaling

Starting at TRADD, different signaling pathways and the corresponding cell biological functions can be activated: the caspase cascade, which activates apoptosis; the NF-κB pathway, which activates inflammation and inhibits apoptosis; and the RIP way, which leads to permeabilization of the plasma membrane and to necroptosis (Wajant, Pfizenmaier, and Scheurich, 2003). The specific interaction partner of TRADD determines which of the three pathways is activated.

For activation of the NF-κB pathway, TRADD recruits the proteins TRAF2 (TNF receptor-associated factor 2) and RIP-1. The NF-κB path is described in detail in Chapter 10. The caspase pathway starts with the binding of the protein FADD (Fas-Associated Death Domain) to TRADD. FADD recruits the initiation protease caspase-8. Caspase-8 cleaves and activates execution caspases, such as caspase-3, leading to the beginning of the execution phase of apoptosis.

4.6.2.3 Caspases

Caspases are cysteine proteases that cleave the peptide bond directly behind aspartate. The 14 known caspases can be grouped into three categories according to their functions: initiation caspases (e.g., caspase-9), execution caspases (e.g., caspase-3), and inflammation caspases (e.g., caspase-1 also called ICE for interleukin converting enzyme). Caspases have homologous structures and similar substrate specificities. Caspases are synthesized as inactive proenzymes, which consist of an aminoterminal prodomain and a large and a small subunit. Procaspases are processed by the degradation of the prodomain and the cleavage between the large and the small subunit. Small and large subunits form a heterodimer. The active enzyme is a tetramer of two heterodimers (Figure 4.13). Caspases show high substrate specificity, which is based on the four amino acids located at the carboxyterminal side of the aspartate of the substrate. Table 4.7 shows examples for substrates of caspases.

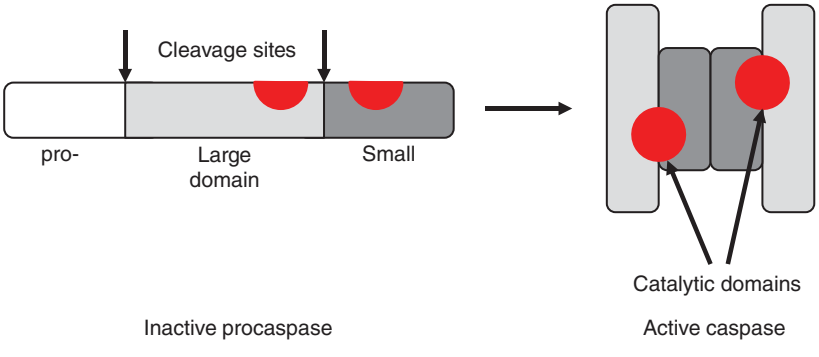

Figure 4.13 Caspase domains in the inactive proform and the tetramerized active form. (Wagener and Müller, 2009), with permission.

Table 4.7 Substrates of caspases (examples).

Protein	Function
Structural proteins	
Actin	Cytoskeleton
Keratin-18	Intermediate filament
Lamin	Nuclear membrane
Signaling proteins	
PLA_2	Phospholipase
MEKK1	MAP kinase
PKCδ	Protein kinase
Bcl-2	Apoptosis inhibitor
p120GAP	RAS inhibitor
Cell cycle proteins	
Rb	Cell cycle inhibitor
Mdm2	p53 inhibitor
Cyclin A	Cyclin-dependent kinase activator
p21	Cyclin-dependent kinase inhibitor
DNA/RNA metabolism	
Topoisomerase	DNA structure
DNA-PK	DNA repair
hnRNP-C	mRNA splicing
RFC	DNA replication
ICAD	DNA degradation inhibitor

4.6.3 Intrinsic Initiation

(Video: Apoptosis – open website: https://www.youtube.com/watch?v=wREkXDiTkPs*)*

Intrinsic initiation is induced by internal signals such as DNA lesions. Double-strand breaks and other DNA damages activate p53. Active p53 inhibits the expression of the BCL2 gene and acivates the expression of the *PMAIP1* (*NOXA*) and BAX genes. The apoptosis-inhibiting protein Bcl-2 inhibits the association of homodimers of the proteins BAX and BAK (Figure 4.14). In the absence of Bcl-2, BAX/BAX and BAK/BAK dimers form pore complexes in the outer mitochondrial membrane, which leads to MOMP (mitochondrial outer membrane permeabilization) (Green and Kroemer, 2004). MOMP is the key event of intrinsic initiation. MOMP positive cells inevitably execute apoptosis. Execution is initiated by the activation of execution caspases by molecules released from

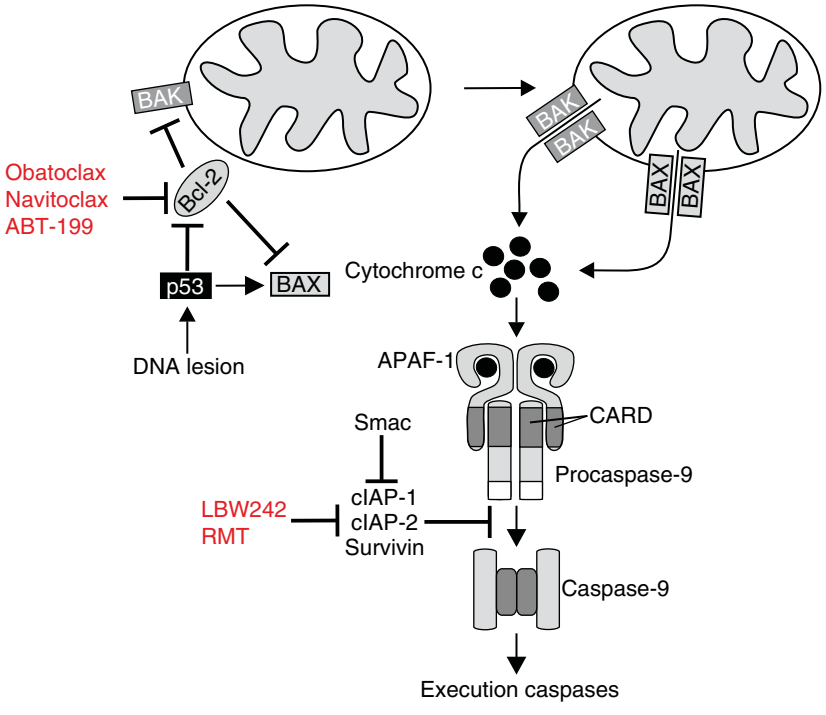

Figure 4.14 Intrinsic pathways that initiate apoptosis. APAF-1: apoptotic protease-activating factor 1; CARD: caspase recruiting domain; cIAP-1/-2: cellular inhibitor of apoptosis protein 1/2; and Smac: second mitochondria-derived activator of caspase. Red: compounds with therapeutical potential. (Wagener and Müller, 2009), with permission.

the intermembrane space between outer and inner mitochondrial membranes. These include cytochrome c, protons, and Ca^{2+} ions. As a consequence, the proton gradient over the inner membrane collapses, leading to inactivation of the respiratory chain and the accompanying ATP synthesis. In the cytosol, cytochrome c forms complexes with the protein APAF-1 (apoptosis protease-activating factor-1). Interaction with cytochrome c leads to the activation of the caspase recruiting domain (CARD), which is also present in several caspases. The activated CARD binds to procaspase-9. This association leads to the autocatalytic cleavage and activation of procaspase-9. Caspase-9 cleaves and activates procaspase-3 and other execution caspases. Activation of caspase-9 is inhibited by cIAP-1 (cytosolic inhibitor of apoptosis-1), cIAP-2, and survivin, which leads to the block of apoptosis.

4.6.4 Execution Phase

The execution phase begins with the activation of two execution caspases, caspase-3 and caspase-6. This activation is enhanced by the mitochondrial protein

Smac (second mitochondria-derived activator of caspase), which is released from the mitochondria and inhibits the inhibitory effect of cIAPs on caspases (Figure 4.14). The execution caspases cleave more than 300 different proteins with different functions, for example, regulators of cell cycle (Rb), proteins of the cytoskeleton (actin), and DNA repair proteins (DNA-PK) (Table 4.5). The cleavage of ICAD (inhibitor of caspase activated DNase) in the nucleus activates DNases. In consequence, the DNA strand is cleaved between the nucleosomes, leading to sections of 180 bp length or multiples thereof. The degradation of the cytoskeletal proteins vimentin and actin leads to the loss of cellular architecture and to the formation of intracellular apoptotic blebs. The degradation of the proteins FAK (focal adhesion kinase), PAK2 (p21-activated kinase 2), and Gelsolin leads to the loss of cell adhesion and to the blebbing of vesicles, the apoptotic bodies. In addition, membrane proteins are reallocated and the phospholipid phosphatidylserine (PS), which is exclusively located on the inner side of the membrane of healthy cells, is translocated to the outer side. This is why apoptotic blebs are recognized by macrophages and other immune cells. Since Annexin V specifically associates with PS, this interaction can be used to detect apoptotic cells, for example, in microscopical or flow cytometrical analysis.

4.6.5 Phagocytosis and Degradation

At the end of the execution phase, the entire cell is decomposed in apoptotic bodies, which are phagocytosed by neighboring cells and macrophages. Because no cellular antigens are released, there is no inflammatory reaction. Experiments with the model organism *Caenorhabditis elegans* showed that many genes and gene products regulate phagocytosis. After phagocytosis, the apoptotic bodies are degraded by proteases and endonucleases.

4.7 Apoptosis of Tumor Cells

Development, growth, and progression of a tumor are only possible if processes leading to apoptosis are inactive or dysregulated. In some tumors, such as in B-cell lymphoma and early prostate carcinoma, decreased rates of apoptosis are the actual cause for the apparent fast growth of tumors. Dysregulation of signaling pathways regulating apoptosis is mostly caused by mutations in corresponding genes that lead to decreased sensitivity against internal and external apoptotic signals (Tables 4.8 and 4.9). An example is the Wnt pathway, which, in its activated form, is able to block apoptosis induced by internal factors. Because the intrinsic pathway of apoptosis normally leads to the death of cells with DNA lesions, tumor cells with dysregulated apoptosis are able to acquire mutations without triggering apoptosis. As a consequence, the tumor development proceeds.

Table 4.8 Causes for decreased sensitivity against external apoptotic signals.

Cause	Consequence
TNF receptor inactivation	No transfer of apoptotic signal
FAS gene (CD95) mutation	No transfer of apoptotic signal
NF-κB signaling pathway activation	Induction of antiapoptotic genes
FADD deficit	No activation of caspase-8
CASP8 gene (caspase-8) mutation	No activation of execution caspases

Table 4.9 Causes for decreased sensitivity against internal apoptotic signals like DNA lesions.

Cause	Direct consequence
MDM2 gene promoter mutation	Inhibition of p53
TP53 gene mutation	No inhibition of *BCL-2* expression
Bcl-2 excess	Block of mitochondrial pore formation
BAX deficit	No formation of mitochondrial pores
NF-κB signaling pathway activation	Induction of antiapoptotic genes
MAPK signaling pathway activation	Inhibition of *CASP8* gene expression

The so-called *molecular camouflage* serves as an example for a mechanism circumventing externally induced apoptosis. In this scenario, expression of tumor specific antigens is blocked by one of several mechanisms, including deregulation of MHC I antigen presentation. Cytotoxic T-cells are no longer able to recognize the tumor cells, and thus, tumor cells may be able to escape from externally induced apoptosis.

Although a decreased rate of apoptosis is a universal feature of nearly all tumors, there might also be regions of increased apoptosis rates. Especially, many large solid tumors contain regions where low supplies of oxygen and of nutrients induce apoptosis.

4.8 Autophagy

Autophagy has been identified in all multicellular organisms and was originally described as cellular answer to low levels of nutrients. Autophagy denotes the controlled intracellular degradation of proteins and cellular organelles (Gozuacik and Kimchi, 2007). The degradation may be limited to a single or a few organelles, but it may also precede complete self-digestion and cell death.

Autophagy in a normal cell fulfills three functions. First, autophagy can help to overcome starvation periods. For example, in the neonatal organism, autophagy is induced by low concentrations of essential amino acids in different tissues, allowing survival of the organism until the first enteral food intake. Secondly,

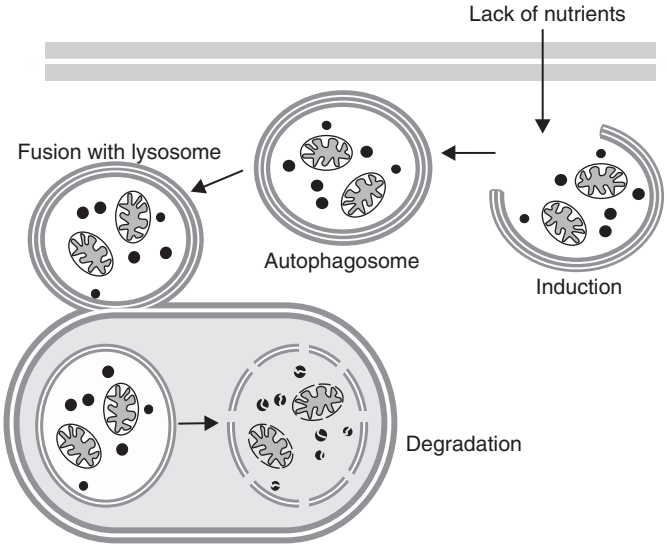

Figure 4.15 Steps of the autophagous process. Cytosolic proteins and organelles are surrounded by a double membrane. The resulting autophagosome fuses with a lysosome, where it is degraded. (Wagener and Müller, 2009), with permission.

autophagy serves to recycle inactive mitochondria and other organelles in normal cells. Third, autophagy may serve specific functions in distinct tissues. Erythrocytes, for example, eject their nuclei and degrade their residual organelles by autophagy.

At the beginning of autophagy, cell organelles are surrounded by double membranes that are either newly synthesized or blebbed from the endoplasmatic reticulum or from the Golgi (Figure 4.15). Next, the new autophagosome fuses with an endosome or a lysosome. Lysosomal hydrolases degrade the inner leaf of the two membranes and the included macromolecules. The resulting small monomers are either recycled or completely degraded. The beginning of autophagy is inhibited by the kinase mTOR (mammalian target of rapamycin) (Petiot *et al.*, 2002). mTOR inhibits the association of protein multimers that regulates the formation of the double membrane (Figure 4.16). Proteins included in this complex in yeast cells are the so-called ATGs (autophagy related proteins), of which only a few have been identified in mammalian cells. One of these proteins is ATG6 or Beclin-1, which binds to and inhibits phosphatidylinositol-4,5-bisphosphate 3-kinase (PI3K). Since Beclin-1 blocks the activating effect of the PI3K/AKT pathway on mTOR, its activity can be regarded as negative feedback loop.

4.8.1 Autophagy in Tumor Development

Several studies have proven that there is a connection between dysregulation in the autophagy process and tumorigenesis (Fulda and Kogel, 2015).

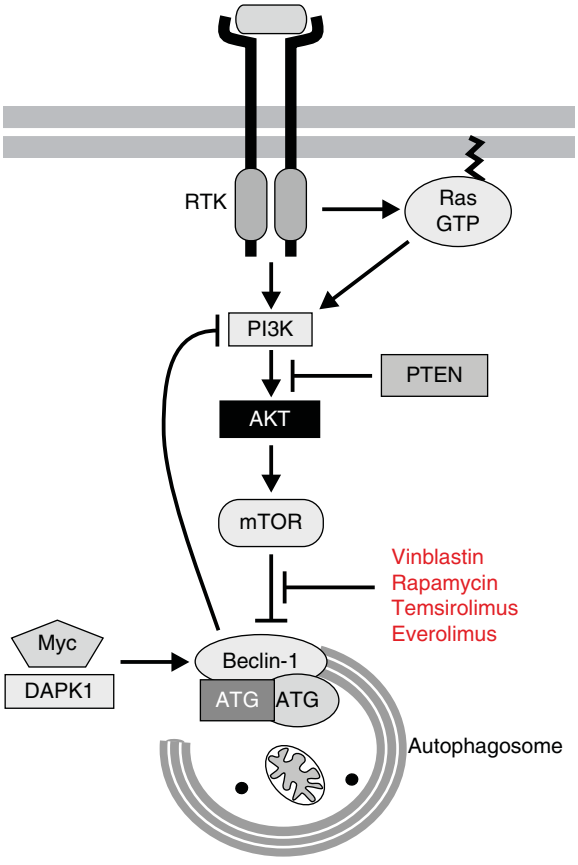

Figure 4.16 Signaling pathways and factors, which regulate autophagy. Activation of receptor tyrosine kinases may lead to inhibition of autophagosome formation. Red: compounds with therapeutic potential to induce autophagy. RTK: receptor tyrosine kinase: PI3K: phosphatidylinositol-4,5-bisphosphate 3-kinase; mTOR: mammalian target of rapamycin; ATG: autophagy related protein; and DAPK1: death associated protein kinase 1. (Wagener and Müller, 2009), with permission.

Overexpression of the Beclin-1 coding tumor suppressor gene *BECN1* leads to an increased number of autophagocytic vesicles in mammary tumor cells (Liang *et al.*, 1999). *BECN1* is mutationally inactivated in nearly half of all analyzed mammary carcinomas. Proliferation rate and tumorigenicity of mammary cancer cells can be decreased by reactivation of *BECN1* expression. Mice with monoallelic mutation in *BECN1* develop lymphomas, lung, and liver tumors. As opposed to normal cells, cultured tumor cells do not activate autophagy when deprived of essential amino acids. Additionally, the extent of autophagy is lower in carcinogen-induced murine tumors of the liver as compared to normal liver tissue.

4.8.2
Regulation of Autophagy

Autophagy is regulated by several tumor relevant proteins and signaling pathways (Figure 4.16). Proto-oncogenes of the RAS family and the oncogenic mitogen-activated protein kinase (MAPK) signaling pathway inhibit autophagy in gliomas and in gastric and colorectal carcinomas. Induction of the proto-oncogene MYC activates autophagy in cultured cells with blocked caspases. This effect is independent of the proliferation activating and transforming activity of *MYC.*

Additional factors regulating autophagy are the tumor suppressor protein phosphatase and tensin homolog (PTEN) as autophagy-activating protein and the tumorigenic PI3K/AKT signaling pathway as autophagy-inhibiting pathway. PTEN is a direct antagonist of the PI3K and the PI3K/AKT pathway (Chapter 8) and also prohibits its effects on mTOR.

The tumor suppressing effect of the tumor suppressor gene DAPK1 (death associated protein kinase 1) is mainly found in its capacity to activate autophagy (Bialik and Kimchi, 2008). The serine/threonine kinase gene *DAPK1* is inactivated in many tumors of the bladder, the kidney, and the breast by inactivating nonsense and missense mutations. Additionally, progression of these tumors correlates with the hypermethylation of the *DAPK1* gene promoter. Metastatic potential and growth rate of lung tumors can be decreased by induction of *DAPK1* gene expression.

4.9
Cell Death and Cell Aging as Therapeutic Targets in Cancer Treatment

4.9.1
Induction of Apoptosis by Radiation

(Video: Molecular action of p53 in carcinogenesis – open website: https://www.youtube.com/watch?v=RxwYoevYZYs*)*

Ionizing radiation in low or moderate doses induces apoptosis rather than necrosis in normal cells. Especially, fast proliferating cells and somatic stem cells are sensitive to radiation-induced apoptosis. Radiation also forces tumor cells into apoptosis, although the extent of apoptosis induced by radiation varies enormously between different tumors. One explanation for the different susceptibilities is that the radiation effects depend on the expression of the wild-type tumor suppressor gene *TP53.* Radiation at low doses causes DNA mutations without killing the cell. As a consequence, the level of the p53 protein rises, what leads to a cell cycle arrest, giving the cell extra time for DNA repair. If the mutations cannot be repaired, p53 may trigger apoptosis by inducing MOMP via blocking Bcl-2 and inducing BAX (Figure 4.17). Radiation is used in single or adjunctive therapy of Hodgkin lymphoma, seminoma, breast, rectal and prostate

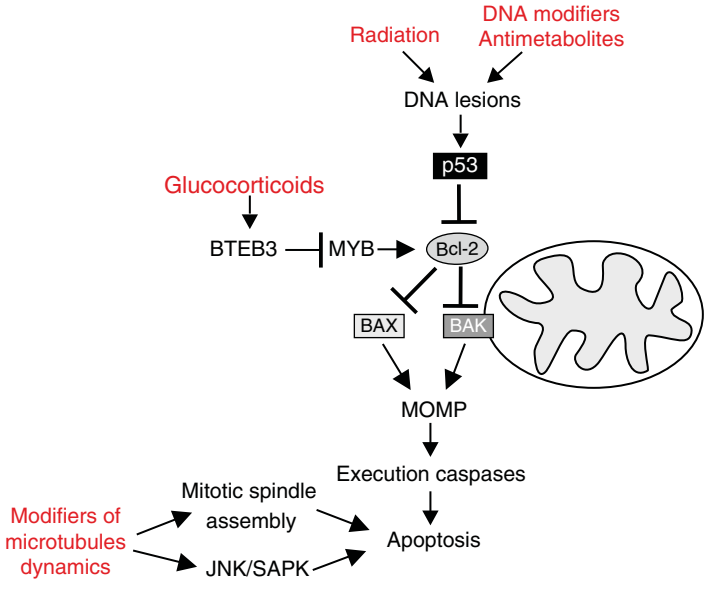

Figure 4.17 Mechanisms leading to apoptosis that are induced by radiation, glucocorticoids and conventional anticancer drugs. MOMP: mitochondrial outer membrane permeabilization and JNK/SAPK: Jun N-terminal kinase/stress-activated protein kinase.

cancer, and other cancer types. Because radiation-induced apoptosis depends on intact p53, radiation shows only low effectiveness against tumor cells with inactivating mutations in the *TP53* gene.

4.9.2 Induction of Apoptosis by Conventional Anticancer Drugs

Conventional anticancer drugs are compounds that attack the tumor cell in its entirety or block complex biological processes, for example, transcription or replication (Chapter 1). A wide range of conventional drugs including DNA-alkylating agents, antimetabolites, and hormones induce apoptosis in tumor cells (Table 4.10). These drugs unfold various primary effects and induce diverse mechanisms, which lead to apoptosis.

DNA-modifying agents induce DNA alterations, which, when not repairable, may lead to apoptosis via activation of p53, repression of *BCL2,* and induction of *BAX,* which leads to MOMP (Figure 4.17). The apoptotic mechanisms induced by antimetabolites are similar. Such drugs block intracellular processes such as DNA replication, and the resulting unrepairable DNA aberrations induce apoptosis via p53 activation and MOMP.

Paclitaxel and vincristine interfere with the dynamics of microtubules. Whereas paclitaxel blocks the depolymerization of microtubules, vincristine blocks their formation from tubulin monomers (Chapter 1). Both drugs lead to an arrest

Table 4.10 Examples for conventional anticancer drugs inducing apoptosis.

Drug	Primary effect	Apoptotic mechanism
DNA modifiers		
Cyclosphosphamide, chlorambucil	DNA alkylation	p53, Bcl-2, BAX, MOMP
Cisplatin	DNA strand cross-linking	
Adriamycin	DNA intercalation	
Antimetabolites		
Cytarabine, gemcitabine, 5-fluorouracil	Block of transcription and replication	p53, Bcl-2, BAX, MOMP
Methotrexate	Block of nucleobase synthesis	
Modifiers of microtubules dynamics		
Vincristine	Block of tubulin polymerization	Mitotic spindle assembly, cyclin-dependent kinases, JNK/SAPK
Paclitaxel	Block of microtubules depolymerization	
Hormones and hormone antagonists		
Glucocorticoids, tamoxifen, finasteride	Induction/repression of gene expression	BTEB3, MYB, Bcl-2, BAX, MOMP

of the cell cycle at mitotic phase, which is the cause for the cytotoxic effects. However, the biochemical mechanisms induced by vincristine and paclitaxel that lead to apoptosis are only poorly understood. Paclitaxel-induced apoptosis involves the checkpoint of mitotic spindle assembly and the JNK/SAPK (c-Jun N-terminal kinase/stress-activated protein kinase) pathway (Wang, Wang, and Soong, 2000).

Glucocorticoids regulate development and selection of many cell types including T- and B-cells, osteocytic and osteoblastic cells, pancreatic β-cells, and many others through induction of apoptosis. Recently, the biochemical mechanisms, by which glucocorticoids induce apoptosis in leukemic cells in patients with acute lymphoblastic leukemia (ALL), have been described (Jing *et al.*, 2015). Glucocorticoids activate expression of the transcription factor BTEB3 (basic transcription element-binding protein 3). BTEB3 represses expression of the gene MYB, which is a transcriptional activator of *BCL2*. The repression of *MYB* results in a reduced level of Bcl-2 allowing the apoptosis regulator BAX to activate apoptosis via MOMP. This mechanism is consistent with the finding that the glucocorticoid-responsive promoter of the *BTEB3* gene is absent in patient-derived leukemia cells, which are resistant to glucocorticoids.

4.9.3 Innovative Drugs Targeting Aging and Death Pathways

Innovative drugs are compounds that interfere specifically with a specific molecule or a single pathway. During recent years, several compounds have been identified that target directly and specifically proteins and pathways regulating apoptosis, autophagy, or senescence.

4.9.3.1 Targeting TRAIL (TNF-Related Apoptosis-Inducing Ligand)

TNF-related apoptosis-inducing ligand TRAIL is able to induce cell death in cancer cells (Koff, Ramachandiran, and Bernal-Mizrachi, 2015). Thus, mechanisms regulating the TRAIL receptor, TRAIL ligands, or signaling effectors might be potential targets in cancer treatment. Unfortunately, promising results from animal studies with TRAIL targeting drugs, recombinant TRAIL ligand, or antibodies against the TRAIL receptor as single agents could not be confirmed in clinical trials. Therefore, TRAIL targeting was tested in combination therapies. These studies showed that conventional chemotherapy together with inhibitors of TRAIL signaling might be an interesting strategy in cancer treatment. This combined effect might be caused by the fact that DNA-damaging agents or radiotherapy induces the expression of genes coding for TRAIL receptors.

It has to be kept in mind that there are both agonistic receptors (TRAIL-R1 and TRAIL-R2) and antagonistic decoy receptors (TRAIL-R3 and TRAIL-R4) in human cells. Thus, stimulation of TRAIL receptors with recombinant TRAIL ligands may activate or inhibit apoptosis. Actually, in some cancer cells, TRAIL induces NF-kB and thereby promotes survival of cancer cells rather than their apoptotic death. Thus, the effects on different TRAIL receptors explain the unexpected lack of efficacy, which was seen in clinical studies of TRAIL-targeted anticancer therapy.

4.9.3.2 Targeting Bcl-2

Members of the Bcl-2 protein family play a crucial role in regulating apoptosis. Individual members may either promote or inhibit apoptosis. Several attempts have been made to mimic the apoptosis-promoting effects of the Bcl-2 proteins. Obatoclax (GX15-070) and Navitoclax (ABT-263) block the apoptosis-inhibiting effects of Bcl-2 proteins (Figure 4.14) and show positive effects in clinical studies of therapies of leukemia, lymphoma, and other cancers. Other Bcl-2 modulators, such as the recent drug ABT-199, show even more promising results. Such modulators are able to enhance the effects of cytostatic drugs (e.g., cytarabine) and kinase inhibitors (e.g., imatinib). Additionally, ABT-199 alone or in combination with the anti-CD20 antibody (rituximab) could be successfully used to treat patients with chronic lymphocytic leukemia.

4.9.3.3 Simulating the Effects of cIAP Inhibitors

The experimental peptidomimetic LBW242 targets cIAPs by competing with high affinity with Smac (Figure 4.14) (Sharma, Straub, and Zawel, 2006). LBW242

induces apoptosis in cells of many tumor types, such as multiple myeloma, acute myeloid leukemia, glioblastoma, and melanoma. Thus, LBW242 enhances the apoptotic effects of conventional anticancer drugs. Furthermore, it improves the sensitivity of ovarian cancer to cell death induced by either TRAIL or anticancer drugs such as Topotecan through an effect related to a potentiation of caspase-8 activation.

The novel Smac mimetic RMT (RMT5265.2HCL) binds to cIAPs with similar affinity as endogenous Smac. RMT induces apoptosis in virus-associated lymphoma models that are otherwise resistant to receptor-mediated apoptosis. Besides inhibiting cIAP, RMT induces the release of endogenous Smac and cytochrome c from mitochondria, which can be regarded as a positive feedback loop for activation of apoptosis.

4.9.3.4 Targeting Autophagy Pathways

The potential of several autophagy-activating compounds as anticancer drugs has been proven in several studies (Amaravadi and Thompson, 2007). The cytostatic compound vinblastine and the antifungal agent rapamycin (sirolimus) inhibit mTOR and thus activate autophagy (Figure 4.16). Rapamycin was used to successfully treat Kaposi sarcomas in immune-suppressed patients recovering from kidney transplantations. Rapamycin might also be used in the treatment of patients with tumors that are resistant against conventional cytostatic drugs. Lymphomas with an activated PI3K/AKT signaling pathway are not sensitive to doxorubicin or cyclophosphamide. Because rapamycin inhibits the PI3K/AKT signaling pathway, it might restore sensitivity to these drugs. Indeed, mice with AKT-positive lymphomas could be successfully treated with a combined therapy of doxorubicin and rapamycin. Because rapamycin also activates autophagy of normal immune cells, it unfolds a strong immune-suppressive effect, which might qualify the drug for immune-suppressive therapies. Temsirolimus, a derivative of the mTOR-inhibiting compound rapamycin, increased the survival rate of patients with progressed kidney carcinomas and is also used in the treatment of patients with mantle cell lymphoma.

4.10 Senescence in Anticancer Therapy

Cellular senescence is the result of telomere shortening, DNA alterations, or age-related expression of cell cycle inhibitors. Recent data showed that cancer cells lacking functional p53 and Rb show phenotypic criteria of the senescence phenotype when treated with selected anticancer compounds or radiation (Ewald *et al.*, 2010) (Table 4.11 and Figure 4.18). This so-called therapy-induced senescence (TIS) might be a therapeutic aim, as it produces reduced toxicity-related side effects and increased tumor-specific immune activity, thereby TIS improves long-term outcomes. Nevertheless, senescent tumor cells have to be eliminated, because of their potentially harmful properties. Recently, it was shown that TIS is

Table 4.11 Compounds leading to therapy-induced senescence (TIS).

Drug	Molecular effect	Tumor
Carboplatin + docetaxel	DNA lesion	Lung
Cyclophosphamide + doxorubicin + 5-FU	DNA lesion	Breast
K858	Kinase inhibition	Various xenografts
MLN8054	Kinase inhibition	Colon xenografts
VO-OHpic	PTEN inhibition	Prostate xenografts
Diaziquone	DNA lesion	Prostate xenografts

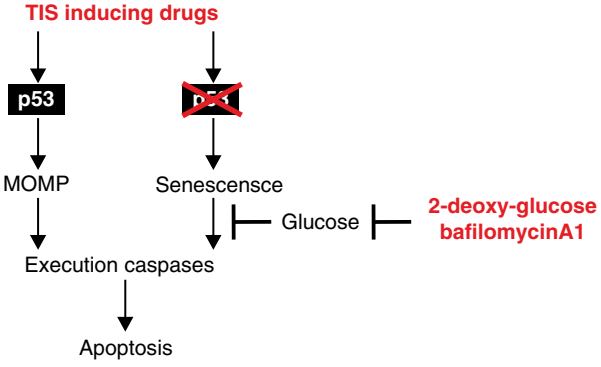

Figure 4.18 Mechanisms leading to apoptosis that are induced by drugs causing therapy-induced senescence (TIS). MOMP: mitochondrial outer membrane permeabilization.

a highly glucose-consuming and energy-producing condition and that cells in TIS are sensitive to a block of glucose utilization (Dorr *et al.*, 2013). Inhibiting glucose metabolization by 2-deoxy-glucose or by bafilomycin A1 leads to apoptotic death of cells in TIS. These data indicate that the induction of TIS combined with a block of glucose utilization might represent a novel strategy in anticancer therapy.

4.11 Outlook

Studies over the last few years have revealed many new insights into the processes of cellular aging and dying. Today, we know that dysregulated pathways that initiate or regulate these processes are involved in tumor development and progression in a significant fraction of malignancies. Targeted manipulations of these pathways are opening new possibilities in cancer therapy. Particular hopes rest on new Smac simulators inducing apoptosis, new rapamycin derivatives activating autophagy, and additional compounds that induce TIS. The major advantage of these therapeutic approaches is that they are potentially applicable even in tumors that are resistant to conventional anticancer drugs.

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5
Growth Factors and Receptor Tyrosine Kinases

Summary

Growth factors and their membrane-bound receptors play an essential role in the activation of cell growth and proliferation. Most growth factor receptors are membrane-bound protein tyrosine kinases. Dysregulation of signaling originating from these receptors may cause tumor development and progression. Such dysregulation may be caused by ectopic expression or abnormally high expression levels of the receptor and/or ligand or by constitutive activation of its enzymatic activity caused by mutations in the corresponding gene. This chapter introduces

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Christoph Wagener, Carol Stocking, and Oliver Müller.

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the most important receptor protein tyrosine kinases (RTKs) that are causally involved in tumorigenesis, their activities on intracellular signaling, and the biological effects upon their activation. Additionally, therapeutic strategies to treat tumors with dysregulated RTKs and some examples for drugs targeting RTKs are presented.

5.1 Growth Factors

Growth factors, also called *mitogens* because of their proliferation activating function, are extracellular signaling proteins. In contrast to hormones, which affect mainly cells in distant tissues, most factors are effective on the same cell (autocrine) or on adjacent cells (paracrine) (Table 5.1).

Many growth factors such as epidermal growth factor (EGF) or stem cell factor (SCF) are synthesized at the rough endoplasmic reticulum and transferred to the cell membrane, where they are anchored as inactive profactors facing the outer side of the membrane. Some factors are probably able to bind in this membrane-bound form to their cognate receptors located on the same cell or on adjacent cells. After a proteolytic enzyme has cleaved the membrane anchor, the growth factor is released and can thus reach distant receptors on the same cell or on other cells. Most growth factor receptors stimulate intracellular signal transduction pathways activating proliferation and cell survival. Several factors stimulate cell differentiation and cell motility. The biological effects caused by growth factors implicate that many of these factors, as well as the proteins that transmit or intensify their effects, receptors, kinases, and transcription factors, are encoded by proto-oncogenes.

5.2 Protein Kinases

Most growth factors transmit their signal into the cell via membrane-bound receptor protein kinases. Protein kinases form the largest functionally related group of proto-oncoproteins. Around 10% of all tumor-relevant proteins are kinases. Protein kinases catalyze the transfer of the γ-phosphate from ATP to an amino acid residue of a protein (Figure 5.1). Phosphorylation is the most common covalent protein modification. Serine and threonine make more than 90% of all phosphorylated amino acids. About 10% of phosphorylated amino acids are phosphocysteines and the aminophosphorylated phosphoarginine, phospholysine, and phosphohistidine. Phosphotyrosine residues make up only 0.1% of all phosphorylated amino acids. Tumor-relevant protein kinases are grouped according to the amino acid residue that they phosphorylate, for example, serine/threonine protein kinases, tyrosine protein kinases, and dual-specificity protein kinases. Within these groups, there are receptor protein

Table 5.1 Examples for tumor-relevant growth factors and their receptors.

Factor	Principle structure	Effect on	Receptor
EGF (epidermal growth factor)	Soluble monomer formed by cleavage from transmembrane profactor	Proliferation, differentiation	EGFR
TGFα (transforming growth factor)	Soluble monomer formed by cleavage from transmembrane profactor	Proliferation, differentiation	TGFR-1
PDGF-A, PDGF-B (platelet-derived growth factor)	Homo- or heterodimer, formed by cleavage from profactor	Proliferation, chemotaxis	PDGFR-α, PDGFR-β
SCF (stem cell factor)	Soluble homodimer or monomer formed by cleavage from transmembrane profactor	Proliferation, differentiation	KIT
IGF-1 (insulin-like growth factor 1)	Monomer	Proliferation, differentiation	IGF-1R
NGF (nerve growth factor)	Homodimer	Proliferation, differentiation, survival of neuronal cells	Trk-A
GDNF (glial cell line–derived growth factor)	Homodimer, glycoprotein	Proliferation, differentiation, survival of neuronal cells	Ret
VEGFs (vascular endothelial growth factors)	Homodimers, glycoproteins	Proliferation of endothelial cells, chemotaxis	VEGFR-1, VEGFR-2, VEGFR-3
HGF (hepatocyte growth factor)	Heterodimer	Motility and proliferation of epithelial and endothelial cells, chemotaxis	Met
TNF-α (tumor necrosis factor)	Transmembrane trimer	Proliferation, angiogenesis, apoptosis, cell adhesion	TNF-R

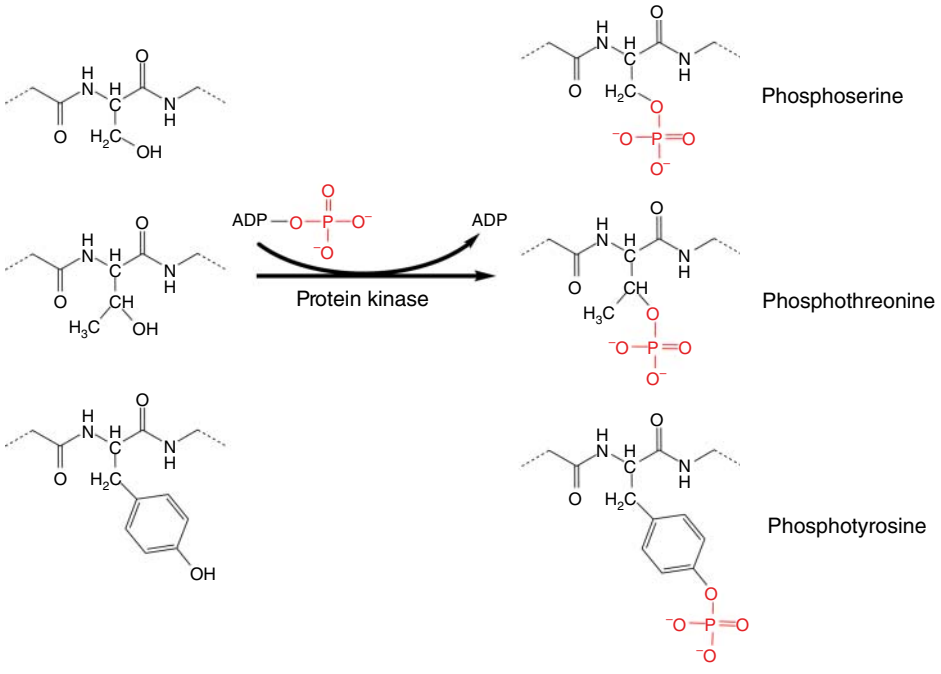

Figure 5.1 The phosphorylation reaction. The terminal phosphate of ATP (red) is transferred to a serine, threonine, or a tyrosine residue within the peptide chain of a protein. (Wagener and Müller, 2009), with permission.

kinases and nonreceptor protein kinases (Table 5.2). Receptor protein tyrosine kinases (RTKs) for growth factors play a significant role in tumor development (Table 5.3).

5.2.1 Receptor Protein Tyrosine Kinases

The lipid bilayer of the cell membrane is impermeable for most proteins, including growth factors. Membrane-anchored receptors bind growth factors at the outer side of the cell and transfer the signal into the cell by activating an intracellular signaling pathway. Most growth factor receptors belong to the class of receptor protein tyrosine kinases (RTKs).

All RTKs show similar structural principles (De Bacco, Fassetta, and Rasola, 2004) (Figure 5.2): a glycosylated extracellular domain for ligand binding, a membrane spanning hydrophobic domain and an intracellular part with a juxtamembrane domain, and one or two catalytic tyrosine kinase domains. Some RTKs also have an extracellular dimerization domain. The catalytic domain consists of an amino-terminal ATP-binding region and a carboxy-terminal phosphotransferase domain. There are about 60 different RTKs, which can be grouped into subfamilies according to the principle composition of their extracellular domains

Table 5.2 Examples for tumor relevant protein kinases with substrate, signaling pathway that is activated within the cell, and tumors with mutation in the corresponding gene.

Kinase	Kinase type with substrate	Signaling pathway	Tumors
BRaf	Nonreceptor serine/threonine	MAPK	Melanoma, colon, and others
PI3K	Nonreceptor phosphatidylinositol(4,5)bisphosphate	PI3K/AKT	Breast, ovary, and others
Src	Nonreceptor tyrosine	PI3K/AKT and others	Breast
ABL	Nonreceptor tyrosine	PI3K/AKT and others	Leukemia, breast
MEK	Nonreceptor dual specificity	MAPK	Melanoma, colon, and others
TGF receptor 2	Receptor serine/threonine	TGF-β	Colon
EGF receptor	Receptor tyrosine	MAPK, PI3K/AKT	Mamma, lung, colon
PDGF receptor	Receptor tyrosine	MAPK, PI3K/AKT	Gastrointestinal
Eph receptor	Receptor dual specificity	PI3K/AKT and others	Glioma

Table 5.3 Receptor protein tyrosine kinases involved in tumor development.

Receptor	Intracellular signaling	Tumors	Genetic alterations in tumors
EGF receptor	MAPK and others	Lung	Amplification, point mutation
ERBB2	MAPK and others	Breast	Amplification
PDGF receptor	PLC/PKC, PI3K/AKT, and others		Recombination
Ret (GDNF receptor)	MAPK and others	Neuronal	Recombination, point mutation
Met (HGF receptor)	PI3K/AKT and others	Gastrointestinal	Amplification, point mutation
NGF receptor	MAPK, PLC/PKC, and others		Recombination
KIT (SCF receptor)	PI3K/AKT and others	Gastrointestinal stroma tumor, mastocytoma, leukemia	Point mutations
FMS (CSF-1 receptor)	PLC/PKC, PI3K/AKT, and others	Leukemia	point mutations
FLT3 (Fms-like tyrosine kinase receptor)	MAPK, PI3K/AKT, and others	Leukemia	Internal tandem duplications
Orphan receptor ALK	MAPK and others		Recombination

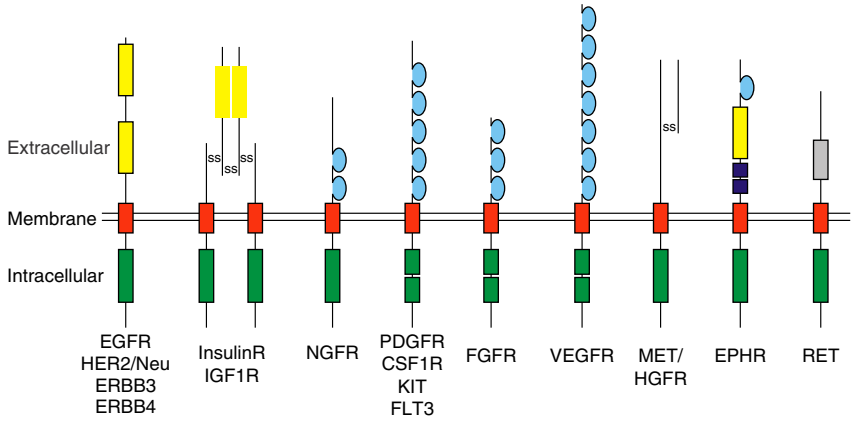

Figure 5.2 Principle domain structures of exemplified receptor protein tyrosine kinases (RTKs). Yellow, cysteine-rich domain; light blue, immunoglobulin-like domain; dark blue, fibronectin type III-like domain; gray, cadherin-like domain; red, transmembrane domain; and green, enzymatic domain. SS, disulfide bridge and R, receptor. (Wagener and Müller, 2009), with permission.

(Figure 5.2). As examples, members of the platelet-derived growth factor receptor (PDGFR), the fibroblast growth factor receptor (FGFR), or the vascular endothelial growth factor receptor (VEGFR) families have five, three, or seven extracellular immunoglobulin-like domains, respectively.

5.2.2 Receptor Protein Tyrosine Kinase Activation

(Video: Oncogenic activation of receptor tyrosine kinases – open website: https://www.youtube.com/watch?v=3nODx3cT1RU*)*

Based on the activation mechanism of several different receptors, a general model was designed for RTK activation (Figure 5.3). In the absence of a ligand, most RTKs are monomeric polypeptide chains. Exceptions are the receptors of the insulin receptor family, which consist of four peptide chains that are connected via disulfide bridges. According to the activation model for monomeric receptors, there is an equilibrium between the inactive monomers and active dimers of the receptor. Ligand binding leads to dimerization and shifts the equilibrium toward the active form of the receptor. Dimerization can also be induced directly by a ligand, which interacts with two monomers at the same time. An example for such a ligand is the EGF inducing the dimerization of its receptor EGFR (epidermal growth factor receptor). Alternatively, ligand binding may induce a conformational change of the extracellular domain, leading to the exposition of sites for interaction between monomers. An example is the SCF inducing the dimerization of its receptor Kit.

As a consequence of dimerization, the intracellular parts of the monomers are juxtaposed. This juxtaposition leads to a transient (temporary) activation of the

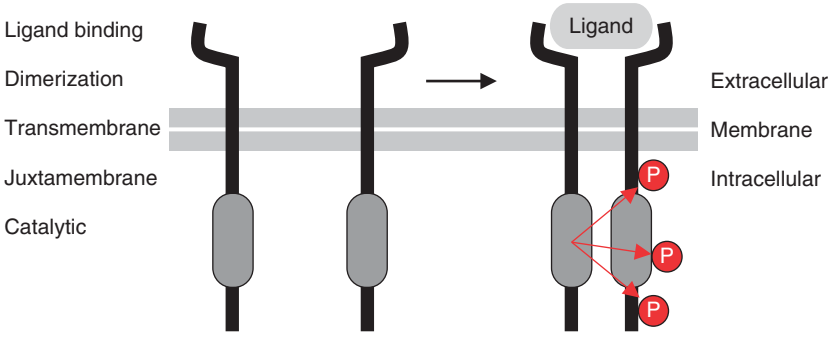

Figure 5.3 Principle structure, functional domains (left) and activation of a receptor tyrosine kinase (right). Ligand binding induces dimerization, which in turn activates autophosphorylation and transphosphorylation of tyrosine in the intracellular sections of the monomers. For simplification, only transphosphorylation of one protomer is shown. (Wagener and Müller, 2009), with permission.

intrinsic tyrosine kinase activity of the catalytic domain. The consequence is the reciprocal transphosphorylation of tyrosine residues in the activation loop of the catalytic region and in regulatory domains of the receptor protomers. Thus, receptor phosphorylation leads to full enzymatic activity, but also provides docking sites for other proteins. Phosphorylated tyrosines are recognized by proteins containing SH2 (Src homology 2) and PTB (phosphotyrosine-binding) domains. SH2 and PTB domains and their principle functions are described in Chapter 1. Association of SH2/PTB proteins with the phosphorylated receptors may activate the enzymatic activity or change the binding properties of the corresponding proteins.

5.2.3 The Family of EGF Receptors

(Video: The EGF receptor tyrosine protein kinases – enhanced ebook and closed website: egfr_Video_01_ebook.mp4)

The ERBB family of receptors includes four receptor tyrosine kinases, which are structurally related (Figures 5.2 and 5.4). The designation for the *ERBB* gene goes back to the avian erythroblastosis oncogene B, which is a retroviral oncogene with sequential homology to all four receptor genes. In human cells, the ERBB family includes EGFR (ERBB1, HER1), HER2/Neu (ERBB2, CD340), ERBB3 (HER3), and ERBB4 (HER4). EGFR was the first discovered receptor of the ERBB family and is the major receptor for EGF in normal cells as well as in tumor cells. ERBB1 through ERBB4 are also called HER1 through HER4 (human epidermal growth factor receptor). The gene *ERBB2* is also called NEU because of its homology to the transforming gene of a neuroglioblastoma (neu) rat cell line.

All ERBB proteins comprise two cysteine-rich regions within their extracellular part, a transmembrane domain, and a continuous tyrosine kinase domain in

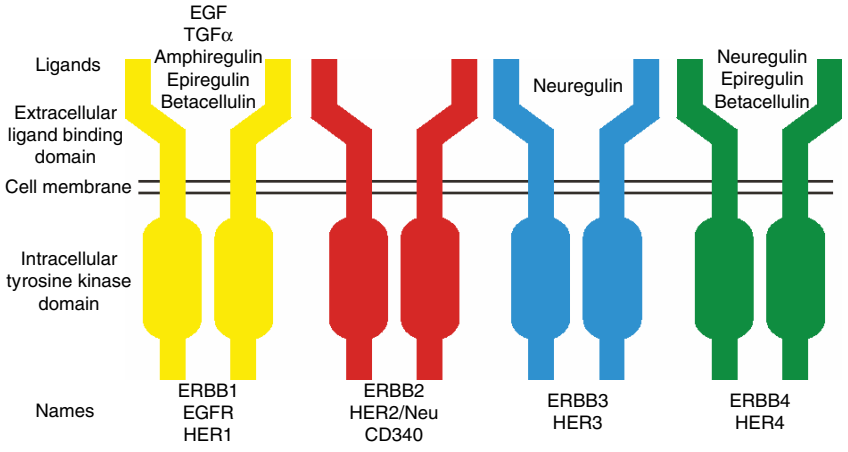

Figure 5.4 Members of the family of EGF receptors. EGF, epidermal growth factor; TGF, transforming growth factor; ERBB, avian erythroblastosis oncogene B; EGFR, EGF receptor; HER, human EGF receptor; and Neu, neural tumor.

the cytoplasmic part (Figure 5.2). Known ligands of the ERBB family of receptors include EGF, transforming growth factor alpha (TGFα), amphiregulin, epiregulin, betacellulin, and neuregulin (Figure 5.4).

5.2.4 The Family of PDGF Receptors

The platelet-derived growth factor (PDGF) receptor family is composed of four receptors (Figure 5.2). In addition to the receptor for PDGF, it includes FMS and KIT proto-oncoproteins, which are receptors for the (macrophage) colony-stimulating factor-1 (CSF1), and for the growth factor SCF (also called MCF for mast cell factor), respectively. A more recently identified member is FLT3 (fms-like tyrosine kinase receptor). All receptors of the PDGF receptor family contain five domains with immunoglobulin-like domains and two kinase domains, which are interrupted by a noncatalytic section.

PDGF activates proliferation of smooth muscle cells, fibroblasts, nondifferentiated oligodendrocytes, and distinct epithelial cells. The two PDGF monomers A and B may associate to form a homodimer (AA or BB) or a heterodimer (AB) (Figure 5.5). The different combinations of the two PDGF receptor monomers (α and β) result in three different receptor dimers (αα, αβ, and ββ). The different receptor dimers differ in ligand specificity and expression pattern. The PDGF-AA dimer binds only to the receptor αα-dimer, the heterodimer AB binds to the αα- and to the αβ-dimer, and the BB homodimer binds to all receptor dimers. The different homo- and heterodimers of PDGF and PDGF receptors allow complex mechanisms of regulation.

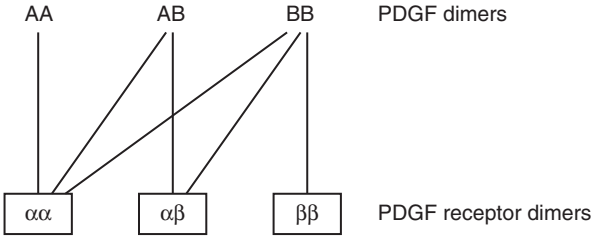

Figure 5.5 Interactions between PDGF and PDGF receptor dimers. (Wagener and Müller, 2009), with permission.

The relation of PDGF to a retroviral oncogene had striking influence on our concepts of the ways oncogenes may act. In 1983, Waterfield and coworkers proved that the amino acid sequence of the PDGF-B monomer is nearly identical to the sequence of the transforming protein p28-sis of the Wooley monkey sarcoma virus (Simian sarcoma-associated virus) (Waterfield *et al.*, 1983). The protein p28-sis, which is encoded by the gene v-*sis*, binds to the PDGF receptor and stimulates the growth of the secreting cell by an autocrine mechanism (Figure 5.6).

The gene coding for the CSF1 receptor helped to identify the effects of proto-oncogenes. Sherr and coworkers found that an antiserum against the protein encoded by the retroviral oncogene v-fms also recognizes a receptor located on the cell membrane of macrophages. Based on the specific binding of CSF1 and the tyrosine kinase activity of the binding protein, the receptor could be identified (Sherr *et al.*, 1985). From these studies, it was concluded that oncogene-encoded growth factors as well as their corresponding receptors are able to stimulate cell growth in an autocrine manner.

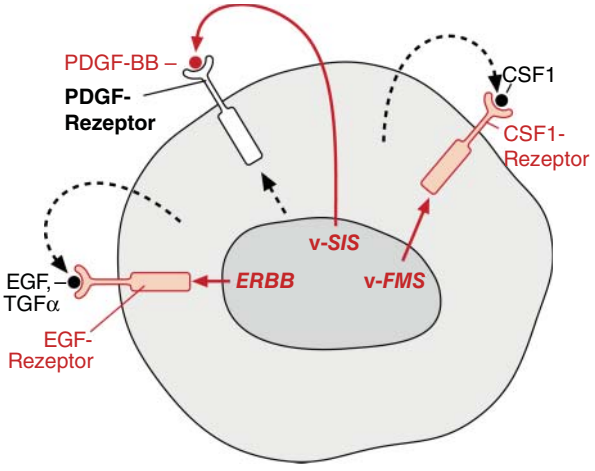

Figure 5.6 Autocrine growth stimulation via receptors of the ERBB family and the PDGF receptor family by retroviral oncogenes. Dashed lines: physiological synthesis by the cell; red arrows: synthesis by oncogenes. (Wagener and Müller, 2009), with permission.

The oncogene v-kit was first identified in the sarcoma inducing feline virus FSV (feline sarcoma virus). The homologous human proto-oncogene *KIT,* which codes for the membrane-bound receptor for the growth factor SCF, controls development and maturation of several different cell types including germ cells, blood cells, mast cells, and melanocytes. The knockout of either one of the genes coding for SCF or for its receptor in mice is lethal. The protein Kit consists of an extracellular domain, a transmembrane segment, a juxtamembrane segment, and a protein tyrosine kinase domain, which is split into two parts (Figure 5.2). Binding of SCF to the extracellular domain leads to receptor dimerization and activation of the intracellular protein tyrosine kinase. The activated receptor autophosphorylates several tyrosine residues, which serve as binding sites for proteins with SH2 domains. The phosphorylated intracellular domains serve as binding sites for adaptor proteins (APS, SHC, GRB2 (growth factor receptor bound)), kinases (Src, PI3K (phosphatidylinositol-4,5-bisphosphate 3-kinase)), substrates for tyrosine phosphatases (SHPS-1 and SHPS-2), and a phospholipase-Cγ1 (PLCγ1) (Figure 5.7).

The high number and wide range of Kit-binding partners intimate that many differential effects may be induced by Kit activation in the cell. In many human tumors, high *KIT* gene expression correlates with bad prognosis. A retrospective study of patients with small-cell lung cancer showed that patient samples that expressed *KIT* and its ligand had a mean survival time of less than 3 months, whereas patients without *KIT* expression survived 10 months after diagnosis (Micke *et al.*, 2003). Somatic activating mutations in *KIT* are involved in the

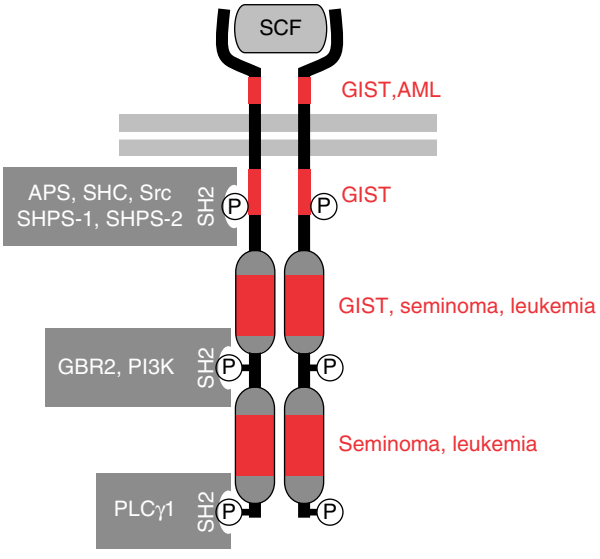

Figure 5.7 The receptor protein tyrosine kinase Kit with interaction partners (left) and conserved domains, in which most mutations are found in the indicated tumors (red). GIST, gastrointestinal stroma tumor and AML, acute myeloid leukemia. (Wagener and Müller, 2009), with permission.

development of acute myeloid leukemia (AML), gastrointestinal stroma tumors (GISTs), and malignant germ cell tumors of testis (seminomas) (Figure 5.7). Activating point mutations in *KIT* are found in nearly all GISTs. Mutations in the kinase domain lead to increased enzymatic activity, mutations in the dimerization domain to constitutive dimerization.

The fourth PDGF receptor family member, FLT3, is one of the most frequently mutated proteins in leukemia, with about 30% incidence in AML and 5% in acute lymphoblastic leukemia (ALL). FLT3 mutations are associated with poor prognosis. Two types of mutations are found. Internal tandem duplications (ITD) are the most frequent and are in-frame duplications of 5–400 bp either within the juxtamembrane region (60% of the cases) or within the first tyrosine kinase domain (30% of the cases). Approximately 5–10% of patients have a single-point mutation within the second tyrosine kinase domain (TKD mutations). Both ITD and TKD mutations lead to constitutive FLT3 activation, leading to aberrant activation of multiple downstream signaling pathways.

5.2.5 The Insulin Receptor Family and its Ligands

Prominent members of the insulin receptor family are the insulin receptor and the receptor for the insulin-like growth factor I (IGF-I). Receptors of the insulin receptor family are heterotetramers, which consist of two α- and two β-subunits (Figure 5.2). The extracellular ligand-binding domain consists of two α-subunits, which are connected via a disulfide bridge. Each of the α-chains is connected via a disulfide bridge with a β-subunit. The β-subunit crosses the membrane with its hydrophobic section. Similar to the EGF receptor family, β-subunits of the insulin receptor family have an uninterrupted catalytic domain. Ligand binding to the α-subunit leads to autophosphorylation of β-subunits on the intracellular side.

Upon binding of the growth factor IGF-I or IGF-II, the activated receptor stimulates proliferation. Cells of the tumor stroma, which has properties of connective tissue, may secrete IGF-I and/or IGF-II. Cells of several carcinomas express the IGF-I receptor. Thus, IGF-I and IGF-II secreted by the tumor stimulate tumor growth via a paracrine mechanism (Werner and LeRoith, 1996). IGFs are transported in the blood in a complex with transport proteins called insulin-like growth factor binding proteins (IGFBPs) are comparable to their affinities to IGF receptors. This means that IGF has to be released from its IGFBP before it can bind to the IGF receptor. This release is executed by proteolytic degradation of the IGFBP (Figure 5.8).

5.2.5.1 Prostate-Specific Antigen

A prominent example for such an IGFBP degrading protease is PSA (prostate-specific antigen). The kallikrein-like protease PSA, also called kallikrein-3, is secreted by the epithelial cells of the prostate. PSA degrades IGFBP and thus releases IGFs, which are either secreted by stroma cells of the carcinoma or

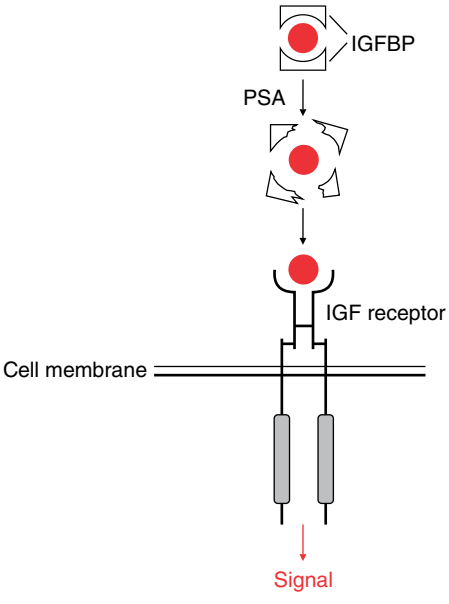

Figure 5.8 Release of IGF (red) from the IGF binding protein (IGFBP) by the protease PSA and interaction with the IGF receptor. (Wagener and Müller, 2009), with permission.

originate from circulating blood. Released IGFs are then available for interaction with the IGF-I receptor present on epithelial cells of the carcinoma (Kolibaba and Druker, 1997). The potential importance of IGFs in the development of prostate cancer was confirmed by a prospective study that showed a significant correlation between the IGF-I concentration in the plasma and the risk to develop prostate carcinoma (Chan *et al.*, 1998). Today, PSA is the most important marker for the diagnosis of tumors of the prostate. Unfortunately, the PSA serum level may be significantly increased by different unspecific conditions, which are independent of tumor development, for example, mechanical stress or benign hyperplasia of the prostate. Nevertheless, the alteration of the PSA serum level is a very sensitive and specific marker to detect relapse or metastasis in the follow-up examination of prostate cancer patients after prostatectomy.

5.2.6
Signaling from Receptor Protein Tyrosine Kinases

Many tumor-relevant signaling pathways start with binding of an extracellular growth factor to a membrane anchored RTK. In response to RTK stimulation, formation of molecular bridges facilitates activation of specific effectors, induces amplification of single transduction pathways, and promotes connections between different cascades, thus eliciting a network of receptor-dependent effects. It is still not completely clear how the stimulation of relatively common repertoires of signal transducers can activate one specific response out of

many different biological responses. Pathway specificity is probably determined through different strategies. These include the following: the combinatorial recruitment of specific molecular subsets at each step of the pathway; insulation through scaffolding and subcellular compartmentalization of molecular assemblies; differences in signal threshold caused by variations in input duration and amplitude; differences in the molecular complement of specific signal transducers expressed in a given cell in a certain moment; and signal redundancy due to cross talks with other metabolic pathways (Schlessinger, 2000).

5.2.7
Association of PDGF and EGF Receptors with Cytoplasmic Proteins

The downstream signaling from the PDGF- and EGF-receptor families has been characterized in detail. The physiological activation of an EGF receptor and the transmission of the signal into the cell are processes of several steps (Figure 5.3). After ligand binding, the receptor monomers dimerize, the intrinsic tyrosine kinase is activated, and several tyrosine residues of the intracellular domain are phosphorylated. Phosphorylated and thereby activated RTKs are able to phosphorylate other cytosolic proteins and are also able to recruit cytosolic proteins.

Many phosphorylated tyrosine residues in cytoplasmic domains of the RTK may serve as binding sites for cytoplasmic proteins. This interaction is mediated by the SH2 and the PTB domains of the cytoplasmic proteins. Many proteins binding to phosphotyrosine containing sequence motifs of receptors have also other characteristic domains (Figure 5.9). Figure 5.10 shows schematically the phosphotyrosine residues and the associating proteins of PDGF and EGF receptors (Yaffe, 2002).

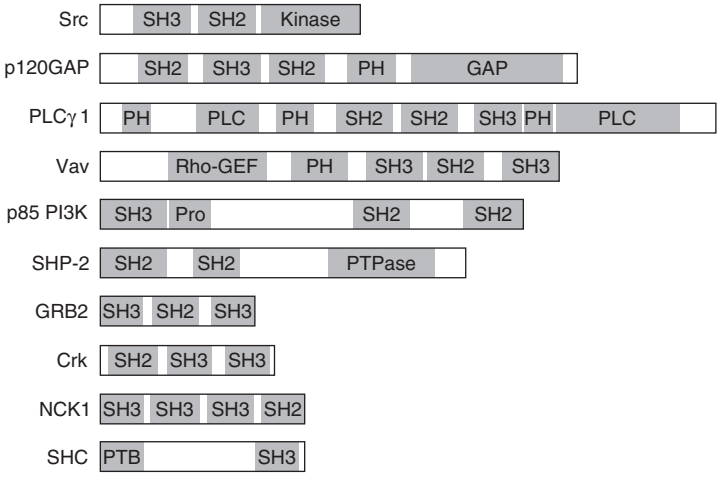

Figure 5.9 Domain structures of binding partners of receptor tyrosine kinases. PH, pleckstrin homologous domain; Pro, proline-rich sequence; PTB, phosphotyrosine-binding domain; PLC, phospholipase C; PTPase, protein tyrosine phosphatase and SH, Src homology domain (from Yaffe (2002). (Wagener and Müller, 2009), with permission.

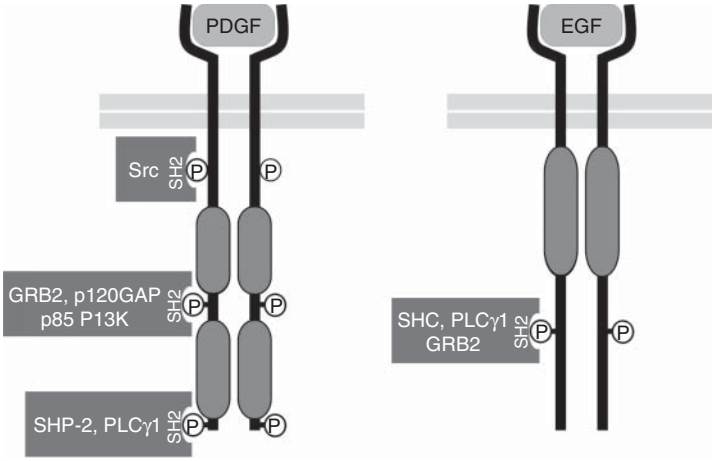

Figure 5.10 Binding of cytoplasmic proteins with SH2 domains to phosphotyrosines of receptors of the PDGF and EGF receptor families. The indicated proteins bind to different phosphotyrosine-containing motifs. For simplification, the collective interactions of all proteins binding to the corresponding phosphotyrosine are shown. P, phosphotyrosine. (Wagener and Müller, 2009), with permission.

In PDGF receptors, the two phosphotyrosines in the juxtamembrane region recruit SH2 domains of tyrosine kinases belonging to the Src family. The adaptor protein GRB2 binds to the phosphotyrosine located between the catalytic partial domains. Other more carboxyl-terminally located phosphotyrosines are able to recruit the 85 kD subunit of the PI3K. Alternatively, these phosphotyrosines may recruit the adaptor protein NCK via its SH2 domain. Another phosphotyrosine within the insertion section binds to the GTPase-activating protein p120GAP. Binding sites for the phosphotyrosine phosphatase SHP-2 and the PLCγ1 are found close to the carboxyl terminus.

In EGF receptors, the cytoplasmic sequences comprise peptide motifs with tyrosines, which when phosphorylated interact with PLCγ1, SHC, and GRB2.

Specificity of proteins binding to phosphotyrosine containing motifs is generally determined by both the structure of the SH2 domain of the ligand and the three amino acids located carboxyl-terminally to the phosphotyrosine. In contrast, the specificity of interaction of the PTB domain is primarily determined by amino acids located amino-terminally to the phosphotyrosine (Figure 5.11). However, PTB domains may also tightly interact with nonphosphorylated tyrosine containing binding motifs.

Different mechanisms transfer the signal from the activated RTK to the associating protein (Figure 5.12). Firstly, an interacting partner can be phosphorylated by the tyrosine kinase of the receptor and thereby can be activated. An example is PLCγ1. Secondly, binding can lead to a conformational change, which may result in either direct activation or the dissociation of regulatory (inhibiting) subunits from a catalytic subunit (PI3K). Thirdly, the membrane translocation of an effector protein without SH2 domain, such as the guanine nucleotide exchange factor

Figure 5.11 Binding specificity of the SH2 and the PTB domains. SH2 domains bind to the phosphotyrosine (Y) and the three amino acid residues (R_{+1}, R_{+2}, R_{+3}) that are located in the carboxy-terminal direction. PTB domains bind to the phosphotyrosine and the three residues (R_{-1}, R_{-2}, R_{-3}) that are located in the direction of the amino terminus. (Wagener and Müller, 2009), with permission.

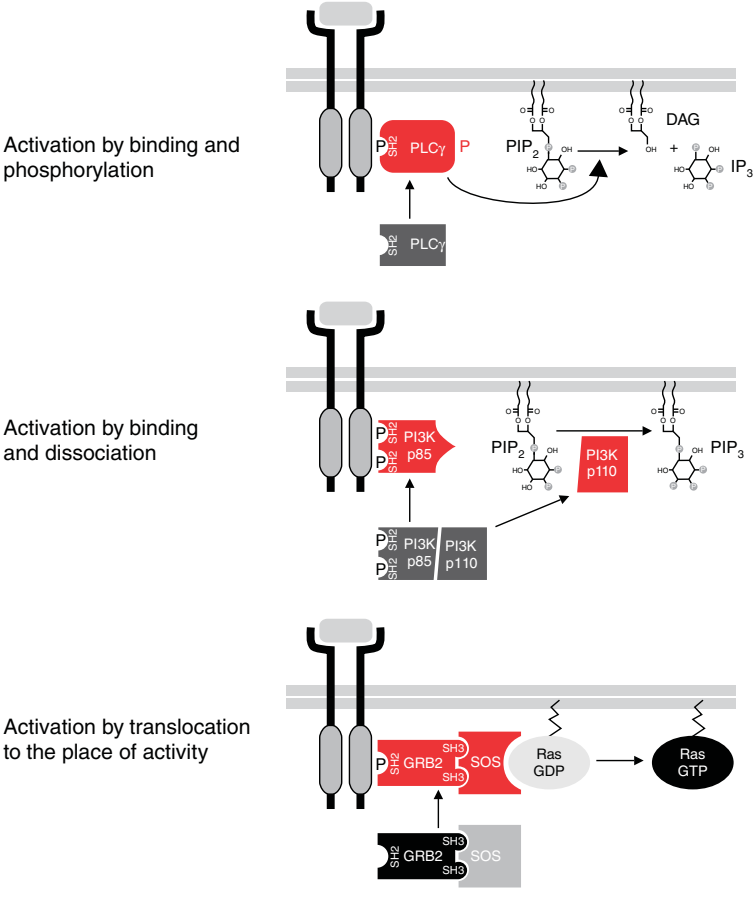

Figure 5.12 Mechanisms of signal transduction right after activation of a receptor tyrosine kinase. Binding and phosphorylation of the binding partner (a), binding and dissociation of a heterodimeric protein consisting of a regulatory and a catalytic monomer (b), or translocation to the location, where it unfolds its effects (c), may lead to activation of a downstream protein. (Wagener and Müller, 2009), with permission.

son of sevenless (SOS), might be mediated by an SH2 containing adaptor protein, for example, GRB2. As a consequence, the effector protein reaches the place of activity.

5.2.7.1 Signaling from PDGF and EGF Receptors

(Video: The JAK-STAT pathway – enhanced ebook and closed website: JAK_STAT_ebook.mp4)

Within a few minutes after activation of the RTK, several metabolic alterations occur in the cytoplasm, including the increase in concentrations of inositol trisphosphate (IP3) and Ca^{2+}. About 10 min after receptor activation, the mRNAs of the proto-oncogenes FOS, MYC, and other "early-response" genes are detectable. Block of the enzymatic activity of the receptors by site-directed mutagenesis or deletion of the kinase domain prohibits these effects and also DNA synthesis and mitosis. These experiments show that tyrosine kinase activities are necessary for the effects induced by PDGF and EGF and their receptors. The activation of the EGF receptor system leads to several biological effects, including activation of proliferation, growth, differentiation, migration, and cell survival. These effects are performed via several downstream signaling pathways, which are activated upon receptor activation by ligand binding.

Four major signaling pathways are activated by proteins, which are recruited by the EGF receptor and its homologs: the MAPK pathway (Chapter 7), the PI3K/AKT pathway (Chapter 8), the PLCγ-PKC pathway, and the Janus kinase and signal transducer and activator of transcription (JAK-STAT) pathway (Figure 5.13). In addition, several pathways are activated by proteins that are

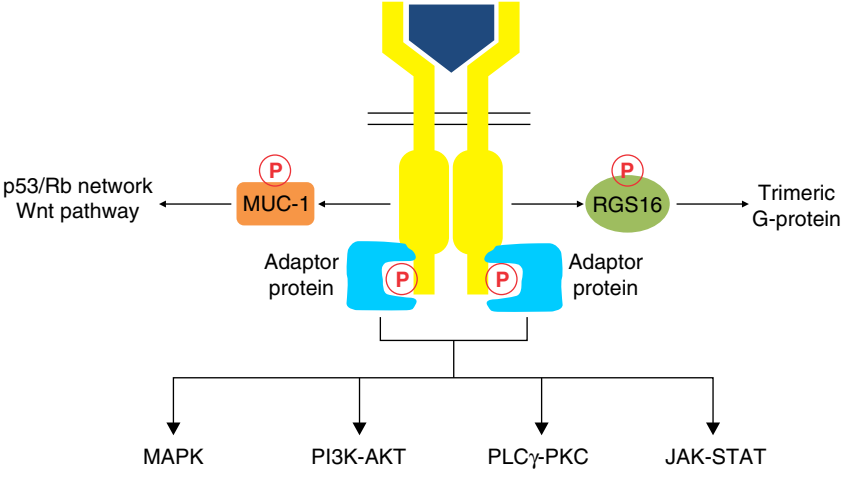

Figure 5.13 The two principles of signaling from active receptors of the ERBB family. First, the active RTKs are able to phosphorylate cytosolic proteins, such as the β-subunit of Mucin-1 or RGS16, which themselves regulate other pathways. Secondly, the phosphate groups of the receptor are able to recruit adaptor proteins. The bound adaptor proteins may signal in the cytosol and activate different downstream signaling pathways.

phosphorylated by activated receptors of the ERBB family. These include the regulator of G-protein signaling RGS16, which leads to activation of its GTPase activity. Thereby, EGF receptor signaling is coupled to the G-protein-coupled receptor signaling. Finally, EGF receptors also phosphorylate the β-subunit of the internalized form of the cell surface glycoprotein Mucin-1 (MUC-1). Phosphorylated MUC-1 β-subunit is able to modulate the activities of different pathways including the p53/Rb network and the Wnt signaling pathway.

5.2.8
Constitutive Activation of RTKs in Tumor Cells

Excessive signaling from RTKs may result in tumor development and progression. Many tumor cells synthesize growth factors or their receptors that are absent or only present at low levels in a normal cell. Thereby, tumor cells may stimulate proliferation by an autocrine mechanism. But several other mechanisms leading to constitutive activation of RTKs also exist, as outlined below. (Figure 5.14).

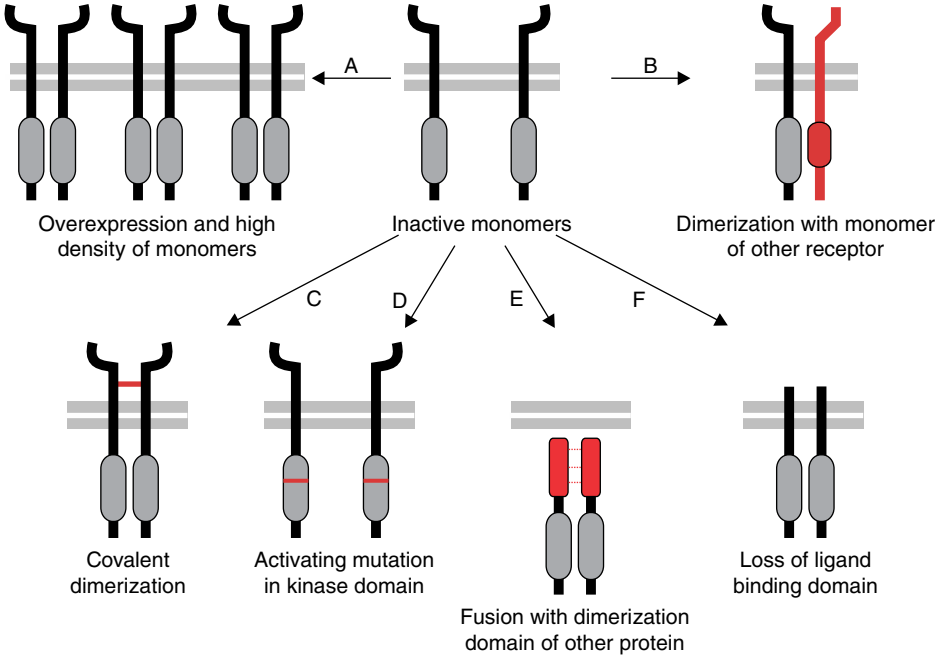

Figure 5.14 Mechanisms leading to constitutive activation of receptor tyrosine kinases (RTK) in tumor cells. Overexpression and high receptor density (e.g., EGFR) (A), heterodimerization with other receptor monomers (e.g., HER2/Neu) (B), covalent dimerization (e.g., Ret) (C), activating mutation in the kinase domain (e.g., Kit) (D), fusion with dimerization domain of other proteins (e.g., NGFR, PDGFR, Ret, Trk with Tel/Etv6 or with TPR) (E), and loss of ligand-binding domain (e.g., v-erbB) (F) may lead to constitutive activation. (Wagener and Müller, 2009), with permission.

The EGF receptor-encoding gene *EGFR* is expressed in many types of normal cells at a low level. In more than 50% of all glioblastomas, the *EGFR* gene is amplified and the number of EGF receptors on the surfaces of glioblastoma cells is increased (Collins and James, 1993). Because the corresponding growth factors EGF and TGFβ are also expressed in glioblastoma cells, these tumor cells are able to stimulate their own growth via an autocrine mechanism.

In contrast to the other members of the EGF receptor family, the HER2/Neu receptor has no cognate ligand; however, HER2/Neu monomers are able to form heterodimers with monomers of other receptors of the EGF receptor family. Thereby, HER2/Neu is able to transmit signals into the cell. In around 25% of all mammary carcinomas, the HER2/Neu coding gene is amplified and overexpressed (Lohrisch and Piccart, 2001). Because of their increased density on the cell membrane, HER2/Neu monomers form heterodimers with EGFR monomers at low extracellular concentrations of EGF. These heterodimers stimulate signaling pathways in the tumor cell. Overexpression of the *HER2/NEU* gene in mammary carcinomas correlates with an increased rate of relapses and bad prognosis. The transforming oncogene *NEU*, which was found in the cell line of a rat neuroglioblastoma, shows a single-point mutation in the transmembrane domain that leads to its constitutive homodimerization (Bargmann, Hung, and Weinberg, 1986).

Nucleotide point mutations within the extracellular domain of a receptor gene may lead to dimerization and receptor activation independently of ligand binding. Examples are mutations in the *RET* gene, which play a role in the development of thyroid tumors.

Point mutations within the intracellular kinase domain may activate enzymatic activity independently of dimerization and ligand binding. Examples are mutations in the gene *KIT*, which are found in GISTs, mutations in the FLT3 gene, associated with a high incidence of acute myeloid leukemias (AMLs), and mutations in the cytoplasmic kinase domain of the *EGFR* gene, which are found in human lung tumors.

Chromosomal aberration may lead to fusion of an RTK coding gene with another gene, whose expression product forms a dimer. Example is the fusion of the hepatocyte growth factor (HGF) receptor, also called Met, to TPR (translocated promoter region). The name Met relates to the cytotoxic compound *N*-methyl-*N*′-nitro-*N*-nitrosoguanidine (MNNG), which was used to treat the osteosarcoma, in which the gene was originally identified. The normal TPR protein forms a homodimer in the healthy cell. Fusion TPR–MET proteins also form dimers independently of the HGF ligand. The permanent dimerization leads to the constitutive activation of the intracellular kinase domain of Met. Another example for dimerization by chromosomal aberration is the fusion of the PDGF receptor with the transcription factor Tel/Etv6. The translocation t(5;12)(q33;p13) was found in cells of a patient with chronic myelomonocytic leukemia. Consequence of this translocation is the fusion of the sequence for the α-subunit of the PDGF receptor with the sequence coding for the transcription factor Tel1/Etv6. While the DNA-binding domain of Tel1 is deleted, the fusion

protein still harbors the pointed motif, which mediates dimerization of the transcription factor. Dimerization of the TEL-PDGFR fusion protein leads to constitutive activation of the PDGF receptor independently of PDGF ligand binding (Golub *et al.*, 1994).

The loss of the ligand-binding domain may also lead to dimerization of monomers. Example is the protein v-erbB of the avian erythroblastosis virus, which forms dimers, but has no ligand-binding domain. This observation implies that the ligand-binding domain of an RTK, which promotes dimerization in the presence of a ligand, may also have domains that inhibit dimerization (Hayman and Enrietto, 1991).

5.3
Therapy of Tumors with Dysregulated Growth Factors and their Receptors

Because of their dramatic activities and effects in tumor cells, activated RTKs and growth factors represent important targets for cancer therapy. Over the last few years, many drugs have been developed against overexpressed or hyperactivated receptors and their ligands. Preferred strategies to target the effect of growth factors are to interfere with their production or with their posttranslational maturing or to catch the soluble factor with a specific antibody to circumvent its interaction with its receptor.

Regarding the inhibition of RTKs, therapeutic strategies follow two general principles, which can be distinguished according to the exact location of the therapeutic attack (Chapter 1). First, the extracellular part may serve as a target for specific antibodies, which inhibit receptor activation by blocking ligand binding, by preventing signal transmission from the extracellular to the intracellular domain of the receptor, or by blocking dimerization of two monomers. Secondly, the intracellular enzymatic domain may serve as a target for small molecules that inhibit the kinase activity of the active receptors. Several antibodies and kinase inhibitors against dysregulated forms of the EGF receptor found their ways into clinical applications.

5.3.1
Targeting Growth Factors

Bevacizumab (trade name Avastin), which was the first antibody against a growth factor involved in tumor growth, is used for therapy of certain metastatic cancers including colon, lung, and kidney cancers. Bevacizumab is a recombinant humanized monoclonal antibody against the human VEGF that stimulates blood vessel growth (Shih and Lindley, 2006). By binding to VEGF, bevacizumab blocks its angiogenesis activating activity and inhibits the growth of new blood vessels. Blood vessels are necessary for the supply of oxygen and nutrients to solid tumors, because tumors larger than a few millimeters in diameter cannot be nourished by diffusion. The effect of bevacizumab proves Judah Folkman's hypothesis, proposed in 1971, that angiogenesis inhibition might be useful to stop tumor growth.

5.3.2 Targeting EGF Receptors by Antibodies

(Video: The EGF receptor tyrosine kinases and the therapy of tumors with activated EGF receptors – Part 1: Inhibition of receptor activation via antibodies – only closed website: egfr_Video_02_ebook.mp4)

The antibody cetuximab, with the registered trade name Erbitux, is a prominent example for an antibody against EGF receptors. Cetuximab binds to an antigenic site within the extracellular domain of the EGFR and inhibits the binding of its physiological ligand EGF. Thus, in the presence of cetuximab, neither the EGFR homodimer nor the heterodimer with HER2/Neu can be formed. Cetuximab is used in the treatment of colon cancer as well as head and neck cancers.

The antibody panitumumab, registered trade name Vectibix, binds to an antigenic site within the extracellular domain of EGFR and inhibits the activation of the receptor after ligand binding and dimerization. Thus, in the presence of panitumumab, the receptor can bind to its ligand and dimerize, but is unable to phosphorylate the intracellular tyrosine residues.

The sensitivity against cetuximab and panitumumab correlates negatively with the number of EGFR proteins in the cell membrane. In other words, tumor cells that overexpress the *EGFR* gene are resistant to these drugs. Furthermore, mutations in genes located downstream of EGFR lead to resistance against panitumumab. This is why panitumumab is used only in the treatment of colon tumors without mutations in the proto-oncogene *RAS.*

The antibody pertuzumab, registered trade name Omnitarg, binds to an antigenic site within the extracellular domain of the receptor HER2/Neu and inhibits its heterodimerization with all other members of the EGF receptor family. No matter whether a ligand (e.g., EGF) is present or not, two monomers cannot dimerize anymore. Pertuzumab is active in tumor cells independently of the expression level of the *ERBB2* gene. It is used in the therapy of several tumor types such as lung and breast tumors.

Trastuzumab with the registered trade name Herceptin was the first antibody that was developed against tumors with activated EGF receptors. It is the best-characterized antibody used in cancer therapy. Trastuzumab binds to an antigenic site within the extracellular domain of the receptor HER2/Neu and inhibits the activation of the receptor independently of ligand binding or heterodimerization. Thus, in the presence of trastuzumab, the receptor does not phosphorylate its intracellular tyrosine residues. Trastuzumab is indicated against breast and stomach tumors with amplification or increased expression of the *ERBB2* gene or with positive HER2/Neu immunohistochemical staining, respectively. The antibody is also effective in patients whose tumors show *ERBB2* gene amplification but do not show HER2/Neu protein, as assayed by immunohistochemistry. This might be explained by the shedding of the HER2/Neu protein by tumor cells resulting in a lower immunohistochemical signal. Under treatment with Herceptin, the relapse rate in patients with breast cancer could be lowered by more than 50%.

5.3.3
Targeting EGF Receptors by Kinase Inhibitors

(Video: The EGF receptor tyrosine kinases and the therapy of tumors with activated EGF receptors – Part 2: Blocking of EGF receptor signaling via tyrosine inhibitors – only closed website: egfr_Video_03_ebook.mp4)

The second principle to block dysregulated signaling by EGF receptors is represented by small molecules that inhibit the enzymatic activity of the intracellular protein tyrosine kinase domain. Several RTK inhibitors have been developed for cancer therapy. Among them, the two drugs erlotinib, with the registered trade name Tarceva, and gefitinib, with the registered trade name Iressa, are mainly used against tumors with activated EGF receptors. Most small kinase inhibitors block enzymatic activity via a competitive mechanism. As an example, erlotinib binds to the intracellular ATP binding site of the protein tyrosine kinase domain and thus prevents the binding of the substrate ATP.

Both gefitinib and erlotinib act independently of EGF receptor gene expression and its protein level. Since the EGF receptor of normal cells may be also inhibited by RTK inhibitors, significant side effects may occur under the treatment. These include mainly diarrhea and skin rash.

EGF receptor inhibition may be overcome by somatic mutations in the EGF receptor gene, which lower the affinity of the drugs to the ATP binding site. Additionally, activating mutations in the *RAS* gene or inactivating mutations in *PTEN,* which stimulate signaling downstream of EGF receptors, might lead to resistance against kinase inhibitors such as gefitinib and erlotinib.

5.4
Outlook

Because of their high impact on tumorigenesis, hyperactivated and mutated growth factor receptors will stay within the focus of cancer research and drug development in the next years. In addition to the search for new antireceptor antibodies and new kinase inhibitors, the search will continue for compounds that block the interaction of the activated receptors with adaptor and effector proteins. Such drugs are supposed to inhibit receptor signaling independent of mutations that affect the ATP binding site, as discussed for inhibitors of the kinase BCR-ABL1 in the next chapter.

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6
The Philadelphia Chromosome and BCR-ABL1

Summary

The Philadelphia chromosome was the first specific genetic aberration that could be causally connected to tumorigenesis. On this aberrant chromosome, which is the defining marker of chronic myeloid leukemia (CML), two genes from physiologically different chromosomes are fused to form the fusion gene *BCR-ABL1*. *BCR-ABL1* codes for a cytosolic tyrosine kinase, which phosphorylates many intracellular substrates leading to increased cell survival and activation of proliferation. Research on the Philadelphia chromosome and the fusion gene *BCR-ABL1* advanced not only the diagnosis but also the therapy of leukemia. The small-molecule imatinib, which is a competitive inhibitor of BCR-ABL1, was the first kinase inhibitor in clinical applications and paved the way toward a whole new generation of anticancer drugs.

Cancer Signaling: From Molecular Biology to Targeted Therapy, First Edition.
Christoph Wagener, Carol Stocking, and Oliver Müller.

Companion Website:www.wiley.com/go/wagener/cancersignaling

6.1 Analysis of Chromosomes

The human genome comprises 23 pairs of chromosomes. During the mitotic metaphase, chromosomes are condensed and compacted. After fixation and staining with a suitable dye such as Giemsa stain, metaphase chromosomes can be visualized by light microscopy. For analysis, chromosomes are sorted and numbered according to their lengths. The resulting arrangement is called a *karyogram* (Figure 6.1). Because of the staining properties of Giemsa stain, each chromosome has a characteristic banding pattern. Homologous chromosomes in a pair have the same banding pattern. Cells of many tumors show chromosomes of abnormal size or banding pattern. Frequent abnormalities include alterations in number, lengths, and structures of the chromosomes. Figure 6.2 shows an exemplified karyogram of a leukemic cell from a female patient with several chromosomal abnormalities. In addition to the classic method of Giemsa stain karyotyping and analysis by light microscopy, chromosomes can be analyzed by fluorescence microscopy after labeling with chromosome-specific fluorescent probes.

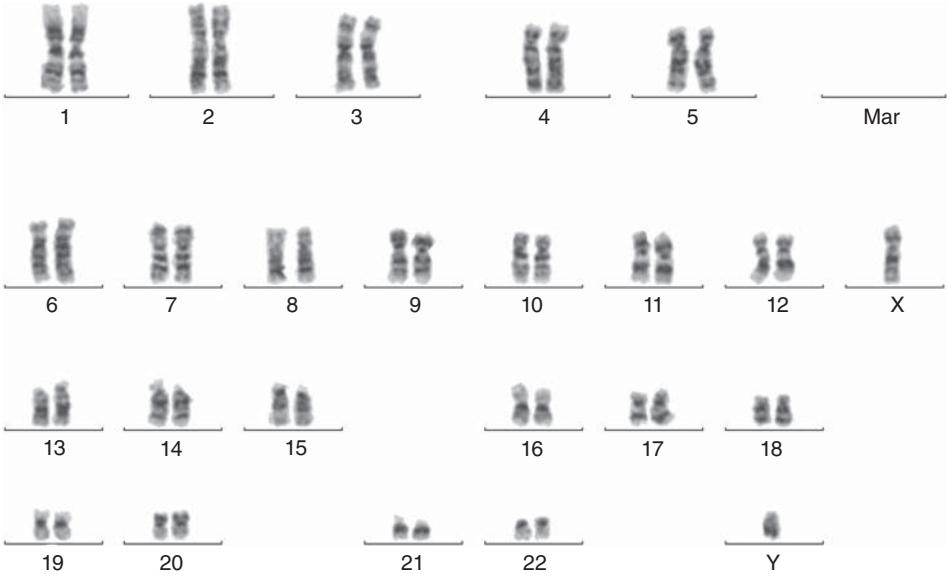

Figure 6.1 Normal human male karyogram. Metaphase chromosomes are stained with Giemsa stain, microscopically photographed, and ordered by size. The male genome comprises 22 pairs of autosomes and 1 pair of allosomes (X and Y). Figure kindly provided by Professor Dr. Dr. Judith Dierlamm.

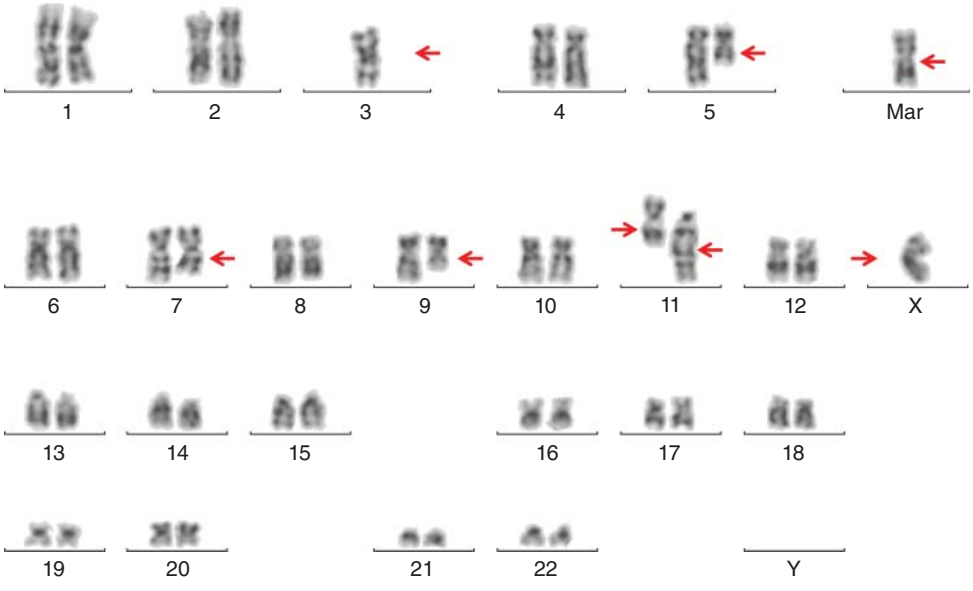

Figure 6.2 Karyogram of a leukemic cell of a female patient. Loss of chromosomes, translocations, deletions, and the additional chromosome called mar (marker chromosome) are indicated by red arrows. Figure kindly provided by Professor Dr. Dr. Judith Dierlamm.

6.2 Aberrant Chromosomes in Tumor Cells

(*Video*: *BCR-ABL1 and the Philadelphia chromosome – Part 1: Normal and aberrant karyograms – only closed website: BCR-ABL_01_Video_01_ebook.mp4*)

At the beginning of the twentieth century, the German biologist Theodor Boveri discovered that chromosomal numbers are equal and constant in all individuals of the same biological species and also in all cells of an organism. Analyzing tumor cells, Boveri found that chromosomes may differ in numbers, lengths, and structures compared to those in healthy cells (Harris, 2008). Boveri already proposed a causal connection between chromosomal aberrations and tumorigenesis. Nevertheless, it took several decades until this connection could be finally proven.

In 1960, two cancer researchers Peter Nowell and David Hungerford analyzed karyograms of patients with leukemia in a hospital in Philadelphia. They detected an abnormally small chromosome in leukemic blood cells from nearly all patients with chronic myeloid leukemia (CML) (Figure 6.3). The so-called Philadelphia chromosome was the first defined genetic mutation described in a human malignancy. These findings were extended by a study in 1973 by Janet Rowley, in which she could demonstrate that the aberrant chromosome was not due to a loss of

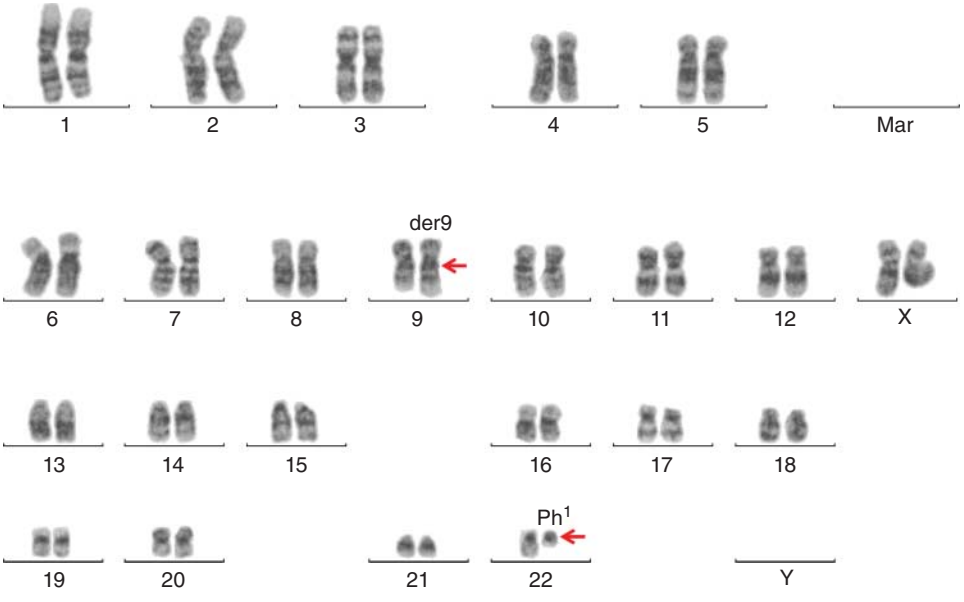

Figure 6.3 Karyogram of a leukemic cell of a female patient with chronic myeloid leukemia (CML). The aberrant chromosome 9q+, also called derivative chromosome 9 or der9, and the tiny Philadelphia chromosome 22q, also called Ph^1, are indicated by red arrows. Figure kindly provided by Professor Dr. Dr. Judith Dierlamm.

genetic material, but rather because genetic material from one chromosome had been translocated to another and vice versa.

The translocation (9;22) is found in the leukemic cells of more than 95% of all CML patients, making it the hallmark of this disease. If left untreated, CML can progress to fatal acute myeloid leukemia (AML) or B-cell precursor acute lymphoblastic leukemia (ALL). In both scenarios, the Philadelphia chromosome is still present, demonstrating that the chromosome occurs in a multipotent stem cell. The Philadelphia chromosome also occurs at a low incidence in sporadic B-cell precursor ALL.

The discovery of the Philadelphia chromosome established the field of cancer genetics as a novel chapter of cancer research. Even more importantly, the Philadelphia chromosome was the first diagnostic marker molecule for a distinct type of leukemia and its expression product was among the first tumorigenic molecules that could be successfully attacked by a specific drug.

6.3 The Philadelphia Chromosome

(*Video*: *BCR-ABL1 and the Philadelphia chromosome – Part 2: The Philadelphia chromosome – enhanced ebook and closed website: BCR-ABL_01_Video_02_ebook.mp4*)

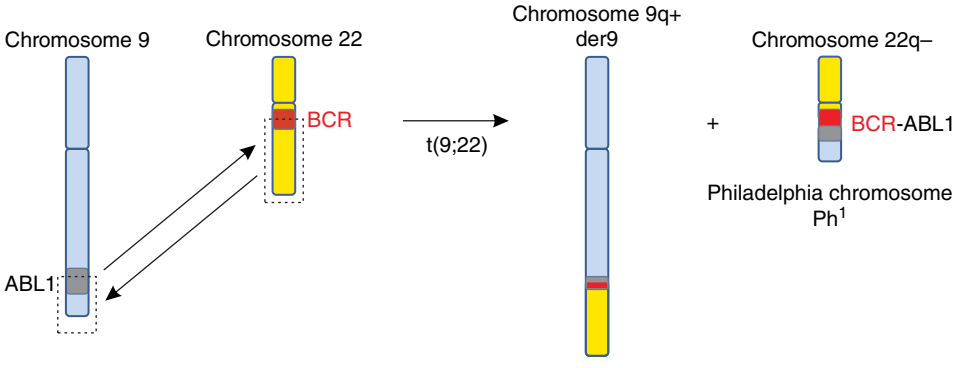

Figure 6.4 Translocation t(9;22) between chromosome 9 and 22 leading to the aberrant chromosome 9q+ (der9) and the Philadelphia chromosome 22q– (Ph^1). The translocation leads to fusion of the two genes *BCR* and *ABL1*, resulting in the *BCR-ABL1* fusion gene on the Philadelphia chromosome.

The Philadelphia chromosome is the result of a translocation between the chromosomes 9 and 22 (Figure 6.4). A major part of the gene ABL1 on chromosome 9, which codes for the tyrosine kinase ABL1, is translocated to chromosome 22. The fusion occurs in nearly all cases within a short region on the long arm of chromosome 22, which was named "Breakpoint Cluster Region." Realization that the region was situated between exons with open reading, frames prompted naming the gene *Breakpoint Cluster Region*, shortly BCR. Concurrently, a part of the *BCR* gene together with downstream sequences on the long arm of chromosome 22 is translocated to the long arm of chromosome 9. Consequence of the t(9;22) translocation is the formation of two new chromosomes: the aberrant chromosome 9q+, also called der9 for derivative chromosome 9, and the tiny Philadelphia chromosome 22q−, also called Ph^1. The plus and the minus indicate the extension and the shortening of the corresponding q arm of the two chromosomes caused by the translocation.

The reciprocal translocation results in the fusion gene *BCR-ABL1* on the Philadelphia chromosome. The *BCR-ABL1* gene consists of the 5′-sequences of the *BCR* gene fused at the breakpoint region to the 3′-sequences of the *ABL1* gene (Figure 6.5). The exact breakpoint, where the two genes are fused to each other, varies at the DNA level. In leukemic cells of most CML patients, exon 13 or 14 of the *BCR* gene is fused to exon 2 of the *ABL1* gene. Circa 20% of CML patients, and in 30% of B-cell precursor ALL, the breakpoint occurs further upstream in the *BCR* gene, resulting in the expression of exon 1 of the *BCR* gene fusing to exon 2 of the *ABL1* gene. A rare translocation in CML occurs upstream of exon 19. Nevertheless, after transcription and splicing, only a few different mRNA fusion transcripts are found.

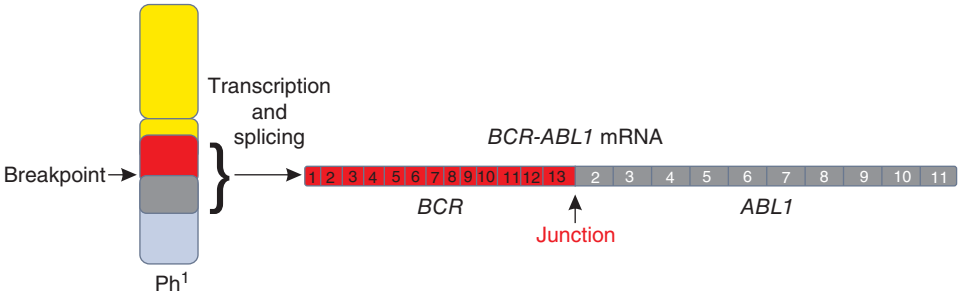

Figure 6.5 The Philadelphia chromosome Ph^1 codes for the fusion gene BCR-ABL1, which consists of the 5′-part of the *BCR* gene fused at the breakpoint to the 3′-part of the *ABL1* gene. In the *BCR-ABL1* fusion mRNA that is most often found in leukemic cells of CML patients, exon 13 of the *BCR* gene is spliced to exon 2 of the *ABL1* gene.

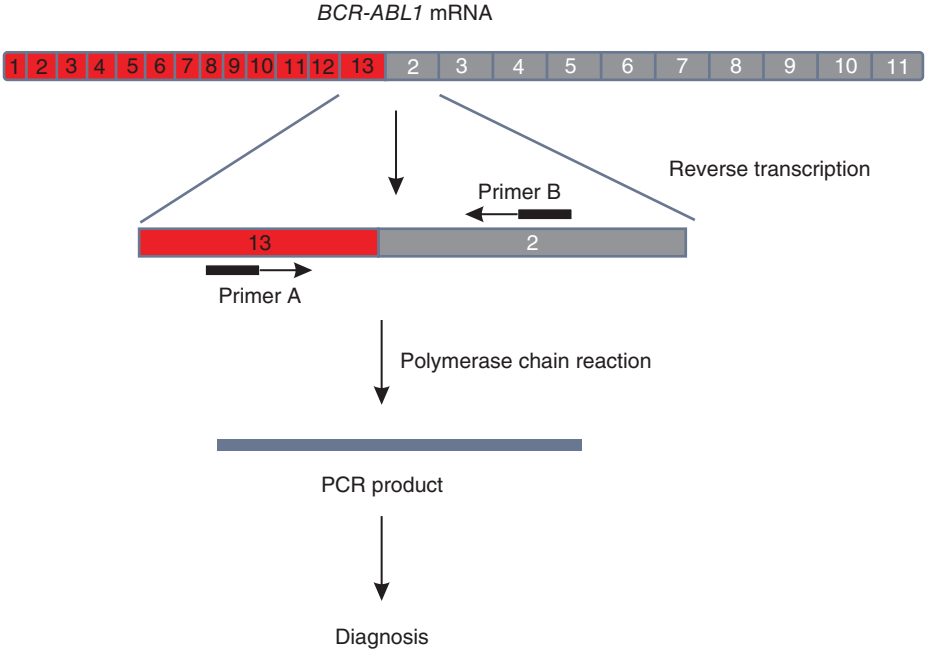

Figure 6.6 Molecular detection of the mRNA transcript of the fusion gene *BCR-ABL1*. RNA isolated from leukemic cells is reverse transcribed and then a fragment spanning the breakpoint of the *BCR-ABL1* mRNA is amplified by polymerase chain reaction (PCR). The resulting PCR product indicates the presence of the *BCR-ABL1* mRNA and of the *BCR-ABL1* fusion gene.

6.3.1 Molecular Diagnosis of the *BCR-ABL1* Fusion Gene

(*Video*: *BCR-ABL1 and the Philadelphia chromosome – Part 3: The BCR-ABL1 fusion protein – enhanced ebook and closed website: BCR-ABL_01_Video_03_ebook.mp4*)

The typical *BCR-ABL1* transcript allows the sensitive and specific detection of leukemic cells in CML patients on the molecular level (Figure 6.6). The first step of this analysis is the reverse transcription of mRNA. The resulting cDNA is amplified by PCR between exon 13 or exon 14 of *BCR* and exon 2 of *ABL1*. Because this reaction gives no product in normal cells without the *BCR-ABL1* translocation, a PCR product proves the presence of leukemic cells. This test detects even very low numbers of leukemic cells, which are not detectable by optical or cytogenetic methods. A positive molecular test indicates incomplete remission, due to the presence of tumor cells. Due to the sensitivity of the molecular test in comparison to conventional methods, it is indispensable for therapeutic decisions.

6.4 The BCR-ABL1 Kinase Protein

The *BCR-ABL1* mRNA encodes the tyrosine kinase BCR-ABL1 (Figure 6.7). The size of the protein depends on the exons fused. Either of two main proteins can

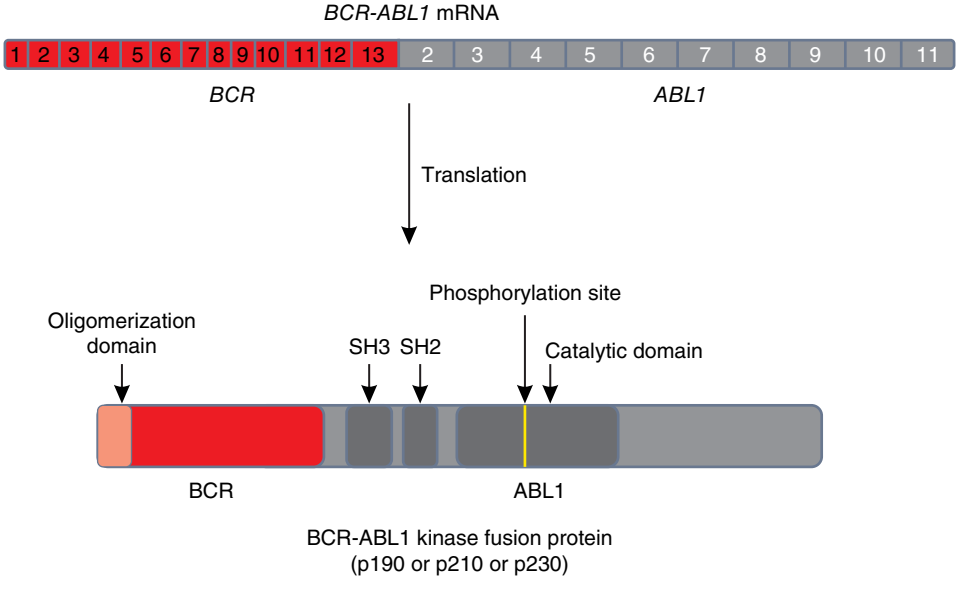

Figure 6.7 The tyrosine kinase BCR-ABL1 as translation product of the *BCR-ABL1* mRNA. Depending on the exact position of the breakpoint, proteins of different sizes may evolve. The BCR-ABL1 protein includes an oligomerization domain, an SH3 domain, an SH2 domain, and a catalytic domain with a regulatory phosphorylation site.

be detected, which are named according to their molecular weights p185 or p210. p185 is the major translation product in B-cell precursor ALL, whereas p210 is the major one in CML. Most rare is the p230 isoform generated by fusions to exon 20 of the *BCR* gene. All fusion enzymes share key structural elements including the oligomerization domain of BCR and the SH3 (SRC homology), SH2, and catalytic domain of ABL1. Within its catalytic domain, there is a phosphorylation site that is important for the regulation of its enzymatic activity.

6.4.1 Structural Aspects of BCR-ABL1 Kinase

ABL1 kinase consists of several structural domains. As shown in Figure 6.8, the domains are packed in a compact tertiary structure (Nagar *et al.*, 2003). In this structure, the catalytic domain is locked in its closed state by the so-called cap domain. This stabilization is due to hydrogen bonds and ionic interactions between the cap domain and the SH3 and catalytic domains, respectively. The catalytic domain of the ABL1 kinase can switch between two conformations, the closed and the open conformation (Figure 6.9). Only the open conformation of the catalytic domain is able to bind ATP, thus allowing enzymatic activity. The native ABL1 kinase is locked in the closed conformation, resulting in autoinhibition of its enzymatic activity.

In the BCR-ABL1 protein, the oligomerization domain of BCR replaces the cap domain of the ABL1 kinase (Figure 6.10). As a consequence, the closed conformation is destabilized, while the open conformation is stabilized by two different

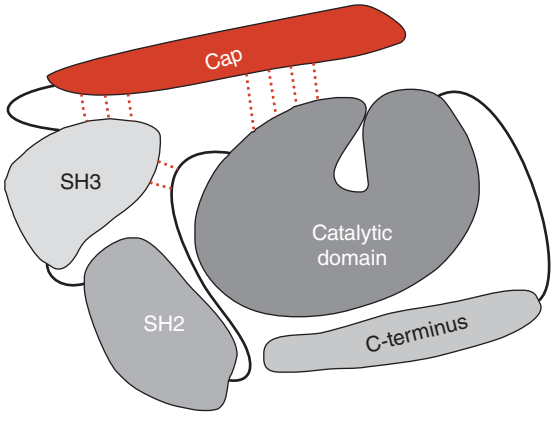

Figure 6.8 Simplified representation of the tertiary structure of the native ABL1 kinase. Domains are packed in a compact structure. The catalytic domain is stabilized in its closed state by the cap domain forming hydrogen bonds and ionic interactions with the SH3 domain and the catalytic domain, respectively (red dotted lines). This closed conformation has only low enzymatic activity. (Wagener and Müller, 2009), with permission.

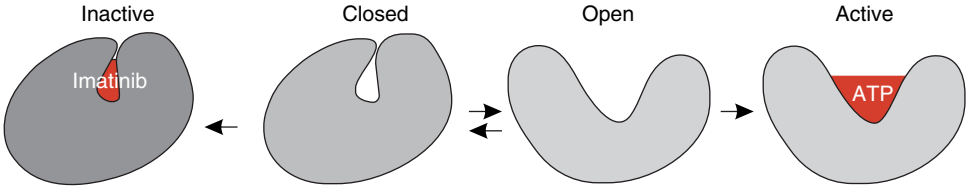

Figure 6.9 Simplified representation of the different conformations of the catalytic domain of BCR-ABL1. There is an equilibrium between the closed and the open conformation. ATP can only bind to the open conformation turning it into the active state. Imatinib binds to the closed conformation and fixes the inactive state, thereby lowering the enzymatic activity of the kinase. (Wagener and Müller, 2009), with permission.

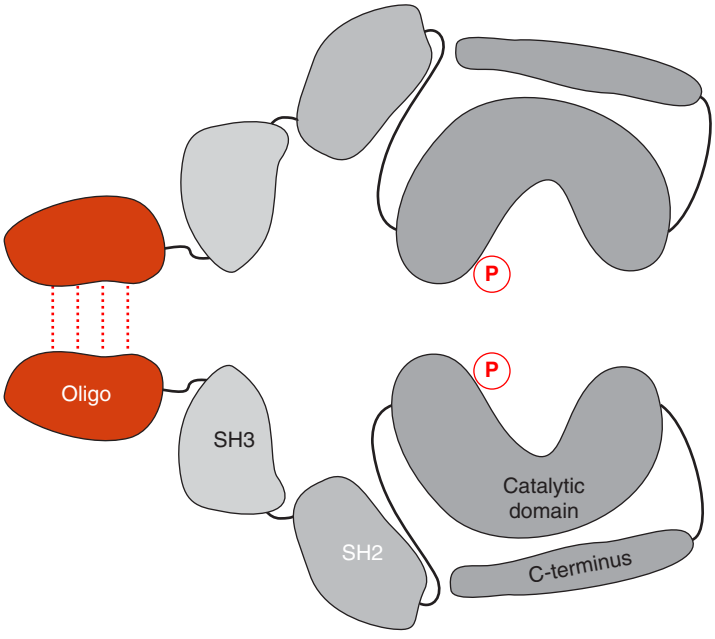

Figure 6.10 Schematic structures of two dimerized BCR-ABL1 kinase proteins. The open conformation of the catalytic domain is stabilized by dimerization of the oligomerization domains (oligo) and by a covalently bound phosphate in each catalytic domain. The open conformation has an increased enzymatic activity. (Wagener and Müller, 2009), with permission.

mechanisms. First, two monomers of the BCR-ABL1 kinase dimerize via hydrogen bonds. Secondly, the open conformation is stabilized by transphosphorylation of each monomer by the other monomer. Thus, the final result of the fusion of the *BCR* and the *ABL1* genes is a dimerized and phosphorylated BCR-ABL1 kinase protein with a catalytic domain in an open conformation with high enzymatic activity.

6.4.2 Substrates and Effects of BCR-ABL1 Kinase

When the tyrosine 177 residue of the BCR-ABL1 fusion protein is autophosphorylated, it associates with the adaptor protein GRB2 (growth factor receptor-bound protein 2) via its proximal SH2-binding site (Figure 6.11). When bound to BCR-ABL1, GRB2 interacts with the son of sevenless (SOS) protein. The resulting BCR-ABL1/GRB2-SOS complex activates the protein Ras and thereby the MAPK pathway and growth factor–independent proliferation. BCR-ABL1 also associates and activates the phosphatidylinositol (4,5)-bisphosphate 3-kinase (PI3K) pathway, suppressing programmed cell death and increasing cell survival. BCR-ABL1 associates and activates the adaptor protein CRKL (CRK-like) thereby modulating the activities of proteins regulating cell adhesion including actin, paxillin, and FAK (focal adhesion kinase). Cell adhesion is inhibited, while cell motility is promoted. BCR-ABL1 also associates with the JAK-STAT (Janus

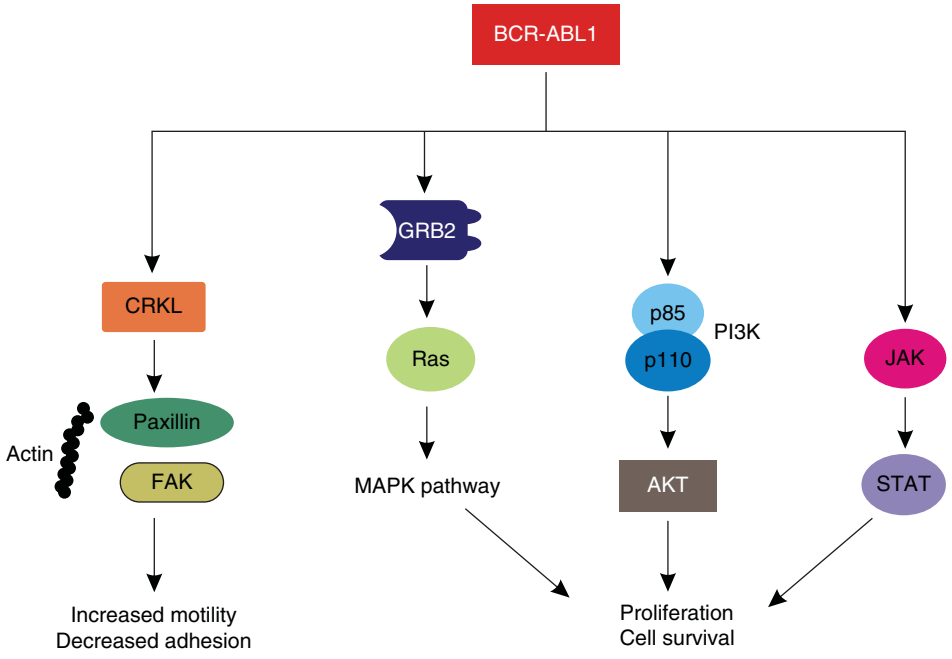

Figure 6.11 Substrates and effects of the tyrosine kinase BCR-ABL1. BCR-ABL1 modulates the functions of components of focal adhesion (actin, paxillin, FAK (focal adhesion kinase)) via the adaptor protein CRKL. BCR-ABL1 interacts with GRB2, which interacts with the son of sevenless (SOS) protein and thus activates Ras and the MAPK pathway. BCR-ABL1 also activates PI3K and JAK (Janus activated kinase) and thereby the JAK–STAT pathway. Through these effectors, BCR-ABL1 promotes proliferation and cellular motility, but also inhibits cell adhesion and programmed cell death.

kinase and signal transducer and activator of transcription) pathway, leading to activation of proliferation and cell survival.

6.4.3 The BCR-ABL1 Kinase Inhibitor Imatinib

(*Video*: *BCR-ABL1 and the Philadelphia chromosome – Part 4: Therapy of tumors with BCR-ABL1 fusion proteins – only closed website: BCR-ABL_01_Video_04_ebook.mp4*)

In most if not all cases of CML, tumorigenesis is due to the t(9;22) translocation and the fusion of the genes *BCR* and *ABL1.* Therefore, the BCR-ABL1 kinase protein represents an attractive target for therapy of these tumors. In the 1990s, several high-throughput screens for inhibitors of the BCR-ABL1 kinase were performed. Scientists at the pharmaceutical company Novartis identified the small-molecule 2-phenylaminopyrimidine as potential inhibitor. The further development of this molecule by rational drug design led to the molecule imatinib, also called *Gleevec* or *Glivec* by registered trade name (Figure 6.12). After a few years of use in second-line indication, imatinib was licensed for use in first-line indication in 2003 (Gambacorti-Passerini and Piazza, 2015). Imatinib was the first anticancer drug with selective inhibitory activity against a tumor-specific enzymatic activity. Because of its high specificity, negative side effects against normal cells are limited. Thanks to imatinib and its derivatives, the percentage of CML patients surviving more than 5 years after diagnosis was more than doubled.

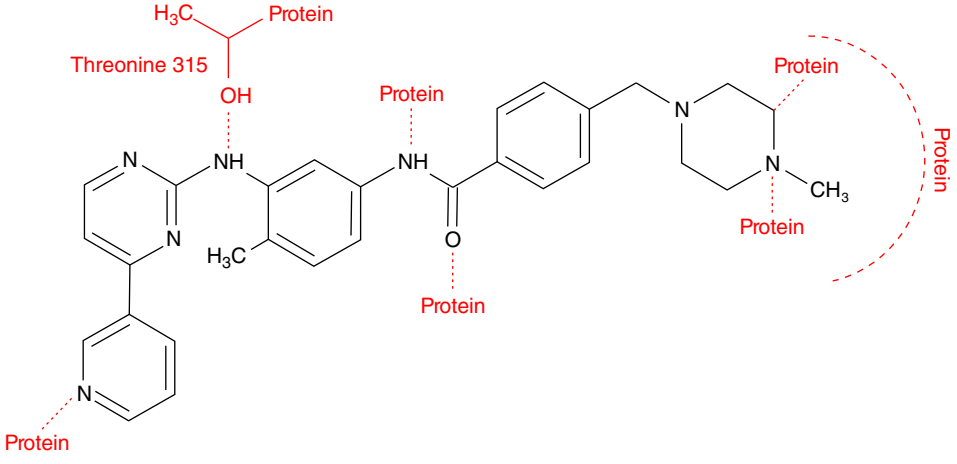

Figure 6.12 Imatinib (Glivec, Gleevec). Interaction sites of the molecule with the catalytic domain of the protein BCR-ABL1 are shown in red. The hydrogen bond between the amino acid threonine at position 315 and imatinib is important for the inhibitory effect.

6.4.4 Imatinib in Treatment of Tumors Other than CML

Not only CML patients may benefit from treatment with imatinib. Patients with Philadelphia chromosome–positive B-cell precursor ALL, which represent more than 30% of adult cases, have a poor prognosis. Most of these patients do not respond to classical chemotherapy. Studies have demonstrated long-term survival of a significant proportion of BCR-ABL1-positive ALL patients treated with combinations of chemotherapy and imatinib.

Imatinib inhibits the enzymatic activity not only of the kinase BCR-ABL1 but also of other tyrosine kinases. A prominent example for a kinase inhibited by imatinib is Kit, a homolog of the PDGF receptor tyrosine kinase. The increased activity of mutant Kit is a major cause for development and progression of gastrointestinal stroma tumors (GISTs). Life span of patients with these tumors can be significantly increased by treatment with imatinib.

6.4.5 Mechanism of Imatinib Action

The mechanism of imatinib action can be explained in a simplified manner by an equilibrium between the two conformational states of the catalytic domain of the kinase BCR-ABL1 (Schindler *et al.*, 2000). Imatinib is able to bind selectively to the active center of the closed conformation and to fix the enzyme in its inactive state (Figure 6.9). Because the closed conformation is selectively fixed, molecules in this conformation are removed out of the equilibrium. Thereby, the equilibrium is further shifted toward the closed and inactive conformation, which is not able to bind ATP. As a consequence, the number of enzymatically active kinase molecules in the open conformation decrease.

6.4.6 Resistance against Imatinib

Despite its high potency, imatinib is ineffective in some CML patients. Resistance against imatinib results either from amplification and elevated expression or from mutation of the *BCR-ABL1* fusion gene. More than 50 different *BCR-ABL1* mutations have been identified in patients with imatinib-resistant CML and *in vitro* through random mutagenesis assays. Remarkably, different imatinib-resistant *BCR-ABL1* point mutants may have different transforming potentials in cells and different prognostic outcomes. Point mutations leading to imatinib-resistant BCR-ABL1 have been found to pre-exist in newly diagnosed patients with CML, as well as to be acquired owing to selective pressure of imatinib. Most mutations responsible for imatinib resistance impair drug binding. The three-dimensional structure of the catalytic domain of the BCR-ABL1 kinase in complex with imatinib shows that the drug binds to the catalytic domain via hydrogen bonds (Figure 6.12). Additional interactions are van der Waals interactions and hydrophobic interactions between a hydrophobic pocket of the kinase around

one of the rings of the imatinib molecule. A very potent and frequent mutation in the imatinib-resistant BCR-ABL1 kinase is a missense mutation leading to the substitution of the amino acid threonine 315 with isoleucine (T315I). This substitution not only eliminates one of the major hydrogen bonds between imatinib and the BCR-ABL1 kinase but also leads to steric hindrance of imatinib binding to the protein BCR-ABL1.

6.4.7 BCR-ABL1 Kinase Inhibitors of the Second and the Third Generation

Over the last few years, the so-called second-generation inhibitors of BCR-ABL1 have been developed (Weisberg *et al.*, 2007). Examples are the imatinib derivatives nilotinib (registered trade name Tasigna) (Figure 6.13) and dasatinib (registered

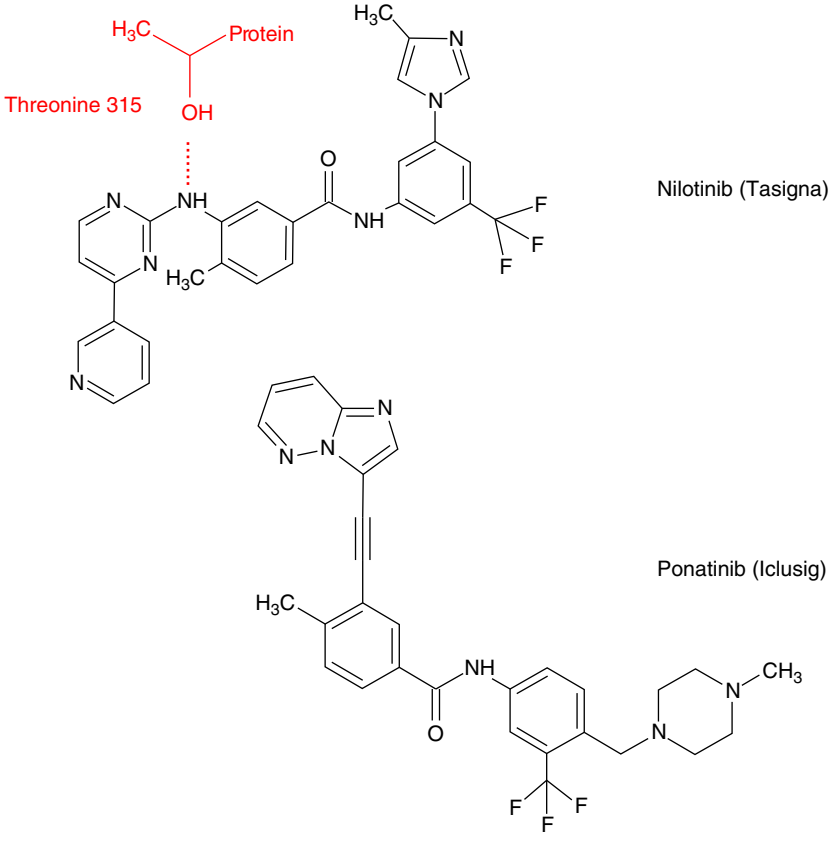

Figure 6.13 The second-generation BCR-ABL1 inhibitor nilotinib and the third-generation inhibitor ponatinib. Whereas nilotinib needs the intact threonine at position 315 of the protein for its inhibitory effects, ponatinib is also effective against BCR-ABL1 mutants with missense mutations at this position.

Table 6.1 Tyrosine kinase inhibitors in use or in clinical trials for therapy of BCR-ABL1-positive leukemias (Weisberg *et al.*, 2007).

Drug	Targets
Imatinib	ABL1, Kit, PDGFR
Nilotinib	ABL1, Kit, PDGFR, EphB4
Dasatinib	ABL1, Kit, PDGFR, EphB4, FGR, FYN, HCK, LCK, LYN, Src, YES
Bosutinib	ABL1, FGR, LYN, SRC
Ponatinib	ABL1, FGFR, VEGFR, SRC
INNO-406	ABL1, Kit, PDGFR, LYN
MK-0457	ABL1, Aurora kinases, FLT3, JAK2
PHA-739358	Aurora A, B, and C
AZD0530	SRC family kinases
GNF-2, GNF-5	BCR-ABL1

trade name Sprycel) and several others (Table 6.1). The latter has a broad target spectrum including kinases of the Src family. Both drugs inhibit several different BCR-ABL1 mutants. Nevertheless, nilotinib and dasatinib need the intact threonine at position 315 to interact with the BCR-ABL1 protein and are therefore ineffective against leukemic cells with an amino acid exchange at this position. Dasatinib has recently been approved for the treatment of patients with CML or Philadelphia chromosome–positive ALL resistant or intolerant to imatinib. Future studies will show whether treatment with dasatinib leads to unacceptable side effects, which might be caused by the broad target spectrum.

Drug resistance based on the T315I mutation can be overcome by the third-generation molecules of BCR-ABL1 inhibitors. One example is the compound ponatinib (registered trade name Iclusig). This drug binds independently of threonine 315 to the BCR-ABL1 protein and unfolds high inhibitory activity against the T315I mutant form of BCR-ABL1 kinase. In addition, ponatinib blocks the activity of tyrosine kinases of the Src, VEGF receptor, and FGF receptor families. Ponatinib was approved for the treatment of patients with CML or Philadelphia chromosome–positive ALL resistant or intolerant to other BCR-ABL1 inhibitors.

6.4.8 Allosteric Inhibitors of BCR-ABL1

Several allosteric inhibitors of BCR-ABL1 have been developed (Zhang *et al.*, 2010). These drugs are able to inhibit resistant BCR-ABL1 mutants that harbor mutations in the active site. GNF-2 binds to the myristoyl binding pocket, which is distant from the active site and close to the carboxyl terminus of BCR-ABL1, and stabilizes the inactive form of the kinase. GNF-2 is highly specific for BCR-ABL1. It has no inhibitory activity against most other kinases, including native ABL1, and shows complete lack of toxicity toward cells not expressing BCR-ABL1. The novel GNF-2 analog GNF-5 showed better pharmacokinetic properties and also promising effects in preclinical testing, when utilized in combination with the ATP-competitive inhibitors imatinib or nilotinib. GNF-5 inhibited the emergence

of resistance mutations and displayed additive inhibitory activity against the T315I mutant of BCR-ABL1 *in vitro* and *in vivo*. Results from X-ray structural studies delivered a surprising explanation for this effect. Despite its allosteric mechanism, GNF-5 binding leads to a conformational change of the active site. This change locks the BCR-ABL1 protein in its closed and inactive state. Thus, the binding of GNF-5 to BCR-ABL1 at a position that is far away from the active site has a similar effect on the BCR-ABL1 protein as the binding of imatinib and its derivatives, which bind directly to the active site.

6.5 Outlook

Certainly, imatinib is one of the most important achievements in anticancer research. During the few years since its introduction, imatinib and its derivatives have significantly increased the life span of more than 100 000 patients. Nevertheless, these drugs do not represent the end point of therapeutic research on BCR-ABL1-positive tumors. In the future, efforts will be put into the search for novel compounds for the therapy of patients whose tumors are resistant to imatinib or who do not tolerate these tyrosine kinase inhibitors. Novel compounds will include novel imatinib derivatives, as well as molecules with divergent structures and mechanisms, for example, molecules that inhibit BCR-ABL1 by allosteric mechanisms (such as GNF-5) or that block activities and effects of proteins downstream of BCR-ABL1 (such as PHA-739358 and AZD0530). A growing concern is the resistance of rare quiescent leukemia stem cells to BCR-ABL1 inhibitors, which likely accounts for half of relapse cases in patients eligible for therapy cessation. Development of strategies to identify, activate, and successfully target these cells is another important research endeavor.

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MAPK Signaling

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Christoph Wagener, Carol Stocking, and Oliver Müller.

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Summary

In this chapter, we introduce the biochemical function of the proto-oncoprotein Ras. Its role as a molecular switch of signal transmission and the mechanisms by which it is activated or inactivated are described. Next, we present the mitogen-activated protein kinase (MAPK) signaling pathway and outline the function of the most relevant proteins in this important pathway. In the third section, drugs that interfere with the active MAPK signaling pathway are described, focusing on those that target the mutated forms of the proteins Ras and BRaf found in many types of cancer.

7.1 The *RAS* Gene

HRAS was the first oncogene identified in human tumors (summarized in (Stephen *et al.*, 2014)). In 1982, several groups independently recognized that the mutated gene was responsible for transformation in bladder carcinoma cells (Der, Krontiris, and Cooper, 1982; Parada *et al.*, 1982; Santos *et al.*, 1982). The gene name originated from the observation that rat-derived sequences were responsible for sarcoma induction in mice infected with the Harvey sarcoma virus, which arose through recombination events between murine retrovirus and rat genomic sequences. HRAS and its paralogs KRAS and NRAS code for small monomeric GTP hydrolyzing proteins, also called *small GTPases* or *small G-proteins*. Because all three Ras proteins have similar biochemical and cellular functions, we will use the terms Ras protein and *RAS* gene as generic terms for all three proteins and genes. *RAS* genes are the most frequently mutated oncogenes in human tumors. Additionally, genes encoding proteins that regulate Ras activity or transfer signals to and from Ras show significantly high mutation rates in many tumors. Despite more than 30 years of intense research on *RAS* genes and their products, many open questions remain. Among them is the question why the mutation rates of the three *RAS* genes differ between different tumors (Table 7.1), even though all three genes have transforming capacity *in vitro* and are expressed in most adult tissues and tumors.

7.2 The Ras Protein

Several functional domains have been identified within the 21 kD Ras protein. These include the GTPase catalytic domain and several interaction domains for activating proteins or exchange factors. The Ras protein is anchored in the inner leaflet of the plasma membrane by a farnesyl/geranylgeranyl anchor or by both a farnesyl/geranylgeranyl and a palmitoyl anchor, which are covalently

Table 7.1 Genes coding for Ras proteins, Ras regulating proteins, or Ras signal transmitting proteins that are mutated in human tumors.

Gene	Protein	Function of protein	Percentage of exemplified tumors with mutation, amplification, or overexpression
EGFR	EGFR	Growth factor receptor tyrosine kinase	20% of lung adenocarcinomas
ERBB2	ErbB2	Growth factor receptor tyrosine kinase	30% of breast tumors
BCR-ABL1	BCR-ABL1	Tyrosine kinase	80% of chronic myeloic leukemia 20% of acute lymphatic leukemia
KRAS	KRas	GTPase switch	71% of pancreas tumors 35% of colon tumors 35% of small intestine tumors
NRAS	NRas	GTPase switch	18% of melanomas 6% of colon tumors 5% of endometrial tumors
HRAS	HRas	GTPase switch	10% of urinary tract tumors 9% of cervical tumors
NF1	Neurofibromin	GTPase activator	10% of glioblastomas 13% of melanomas 10% of lung adenocarcinomas
BRAF	BRaf	Ras signal transmitting serine/threonine kinase	60% of melanomas 100% of hairy cell leukemias

bound to amino acids close to the carboxyl terminus of the protein (Figure 7.1). The protein is active only in this membrane anchored form. Prerequisite for membrane anchoring is the covalent prenylation of the protein by the enzyme farnesyltransferase (FT). FT transfers a farnesyl/geranylgeranyl group from farnesyl/geranylgeranyl pyrophosphate on the cytosine of the so-called CaaX box of the Ras protein. The CaaX box is located at the carboxyl terminus and consists of four amino acids: cysteine (C), two aliphatic amino acids (aa), and one variable amino acid (X). Upon prenylation, the three terminal amino acids are cleaved and the terminal cytosine is capped by methylation. NRas, HRas, and the two alternatively spliced Kras4A and Kras4B isoforms are subject to different posttranslational modifications. KRas4B is anchored to the inner side of the cell membrane directly after prenylation, proteolysis, and methylation. KRas4A, NRas, and HRas are additionally modified by transfer of one or, in the case of HRas, two palmitoyl acids on cysteine residues close to the carboxyl terminus. After modification in endosomal membranes, the Ras proteins are bound by phosphodiesterase δ and transported to the plasma membrane.

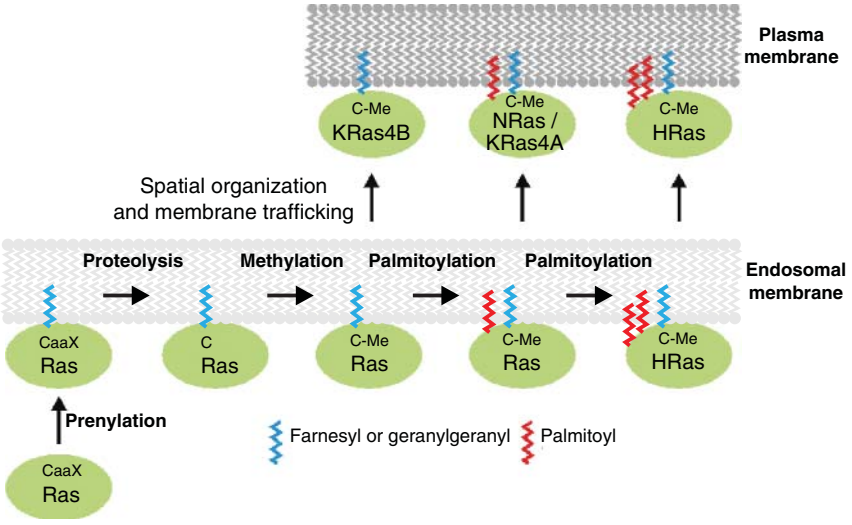

Figure 7.1 Posttranslational modifications of Ras. Ras isoforms show different modifications. All Ras isoforms are prenylated by either a farnesyl or a geranylgeranyl moiety. In addition, KRas4A and NRas are monopalmitoylated, HRas is dipalmitoylated. After modification in the endosomal membrane, the proteins are transferred through the cytoplasm and anchored in the plasma membrane.

7.2.1 The Ras Protein as a Molecular Switch

The Ras protein can be regarded as a molecular switch of signal transmission. In its active and GTP-bound state, Ras transfers the incoming signal from the membrane into the cell by interaction with downstream effectors and their consequent activation (Wittinghofer and Pai, 1991) (Figure 7.2). In its GDP-bound state, the Ras protein and its signaling activity are switched off. The exchange of the bound GDP to GTP and the switch from the inactive into the active state is catalyzed by guanine nucleotide exchange factors (GEFs). GEFs promote the release of the bound nucleotide GDP, thereby allowing activating GTP to take its place. There are several different signals and pathways, which result in GDP-GTP exchange and Ras activation (Figure 7.3) (Stephen *et al.*, 2014). The most prominent exchange factor is SOS-1, the human homolog of the Drosophila protein "son of sevenless." SOS-1 is comprised of a proline-rich domain, which interacts with two SH3 (Src homology 3) domains of the adaptor protein GRB2 (Growth Factor Receptor Bound 2). GRB2 itself can be activated by interaction of its SH2 (Src homology 2) domain with phosphorylated tyrosines of other proteins (Figure 7.4). Examples for GRB2-activating phosphotyrosine proteins are receptor tyrosine kinases (RTKs) such as the epidermal receptor growth factor receptor (EGFR), its homolog ERBB2 (HER2/neu), the autophosphorylated gene product BCR-ABL1 of the chromosome translocation 9;22 (Chapter 6), and the phosphoproteins SHC and SHP-2.

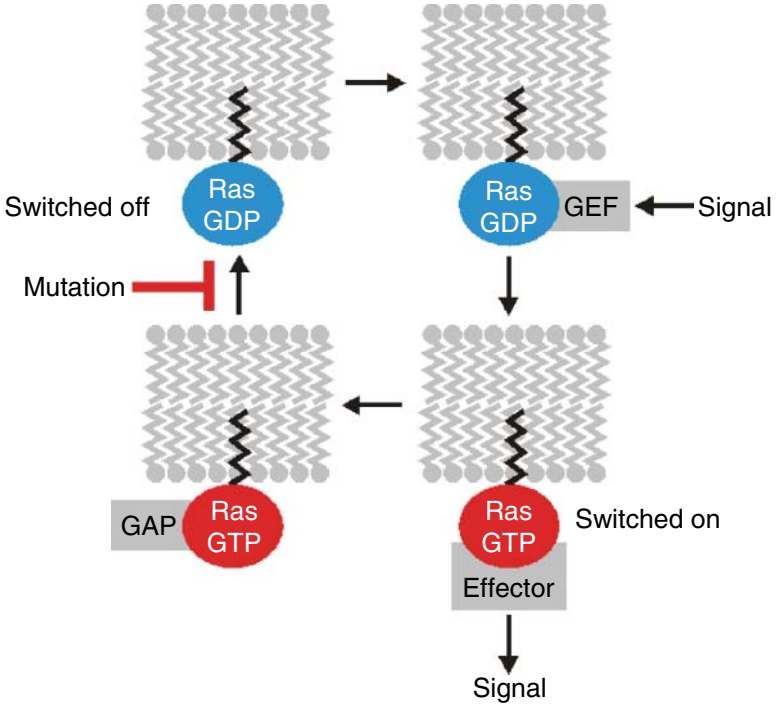

Figure 7.2 Ras as a molecular switch of signal transduction. The membrane-anchored Ras protein is switched off in its GDP-bound state (blue). A guanine nucleotide exchange factor (GEF), which is activated by an incoming signal, promotes the exchange of the bound GDP against GTP. GTP-bound Ras is active (red) and binds to an effector. The bound GTP is hydrolyzed by the intrinsic GTPase activity of the Ras protein to GDP leading to its inactivation. A GTPase-activating protein (GAP) can activate the intrinsic GTPase activity. Oncogenic mutations in the RAS gene block the GTPase reaction leading to a constitutively active Ras protein. (Wagener and Müller, 2009), with permission.

Other exchange factors associated with Ras activation are Ras-GRF1, which is activated by direct interaction with the NR2B subunit of the *N*-methyl-D-aspartate (NMDA) receptor (Krapivinsky *et al.*, 2003), and RasGRP1, which is activated by the lipid diacylglycerol (DAG) (Stone, 2011). DAG and inositol 1,4,5-trisphosphate (IP3) are products of the hydrolysis of the phospholipid phosphatidylinositol 4,5-bisphosphate (PIP2) by phospholipase C (PLC).

7.2.2
The GTPase Reaction in Wild-Type and Mutant Ras Proteins

As a small GTPase, the Ras protein is able to hydrolyze its bound GTP and to terminate its own signaling activity (Figure 7.3). The rate of the intrinsic GTPase activity of Ras is very slow, but it is accelerated by several orders of magnitude by GTPase-activating proteins (GAPs). Two RasGAPs are known: p120GAP

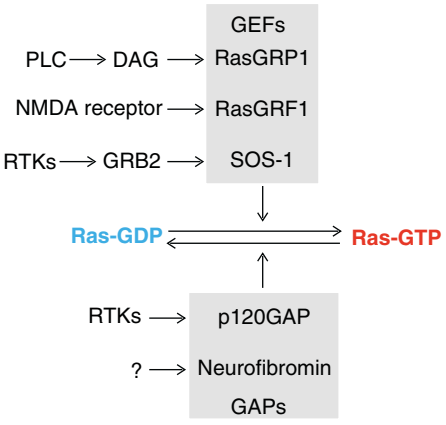

Figure 7.3 Activation and inactivation of the Ras protein. Inactive GDP-bound Ras (blue) is converted by GEFs (guanine nucleotide exchange factors) into active GTP-bound Ras (red). The intrinsic GTPase activity of Ras is activated by GAPs (GTPase activating proteins). GRF, guanine nucleotide releasing factor; GRP, guanine nucleotide releasing protein; PLC, phospholipase C; DAG, diacylglycerol; and RTKs, receptor tyrosine kinases.

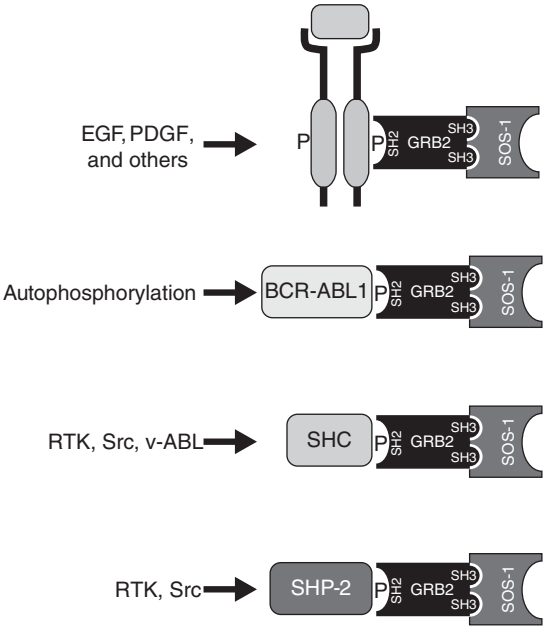

Figure 7.4 Mechanisms of GRB2 activation. GRB2 is activated by receptor tyrosine kinases (RTKs), BCR-ABL1, Src, and v-ABL. GRB2 is an adaptor between phosphotyrosine proteins and the Ras nucleotide exchange factor SOS-1. (Wagener and Müller, 2009), with permission.

(p120 GTPase–activating protein) and Neurofibromin (neurofibromatosis type 1 GTPase–activating protein).

Nearly all oncogenic mutations in RAS genes are found in codons 12, 13, and 61 (Figure 7.5). Why do these mutations have such a dramatic effect? On the biochemical level, this question can be answered by data from the GTPase reaction and from a detailed analysis of the structures of the Ras protein alone and in complex with p120GAP, as well as the structure of the Gα subunit of the trimeric guanine nucleotide-binding (G) protein (Vetter and Wittinghofer, 2001). Although there are several similarities between the GTPase reaction of the Gα protein and Ras, the reaction rate of Ras is much slower than that of the former. The explanation for this difference came from comparison of the three-dimensional structures of the two proteins. The GTP hydrolysis runs via an intermediate or transition state, where the water molecule attacks the phosphorus of the terminal γ-phosphate (Figure 7.6). The Ras protein is unable to promote this transitional state by its own, but in a complex with p120GAP, the reaction is favored by spatial stabilization of the glutamine residue at position 61 of the Ras protein, which coordinates the water molecule in the transition state (Figure 7.7). Additionally,

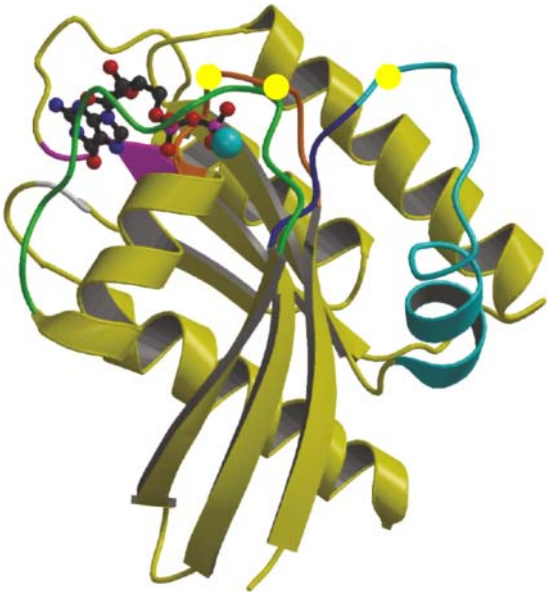

Figure 7.5 Three-dimensional structure of the active Ras protein in complex with a nonhydrolyzable form of GTP, which is depicted as a ball and stick model (Vetter and Wittinghofer, 2001). The most frequently mutated positions ("mutational hotspots") in the protein chain are positions 12, 13, and 61 (yellow balls). The structures of the so-called "switch I" and "switch II" regions are flexible and change with activation of the protein (green and blue-green). The Mg^{2+} ion, which increases the affinity to the nucleotide, is depicted as a blue-green ball (designed and kindly provided by Dr. Ingrid Vetter and Professor Dr. Alfred Wittinghofer).

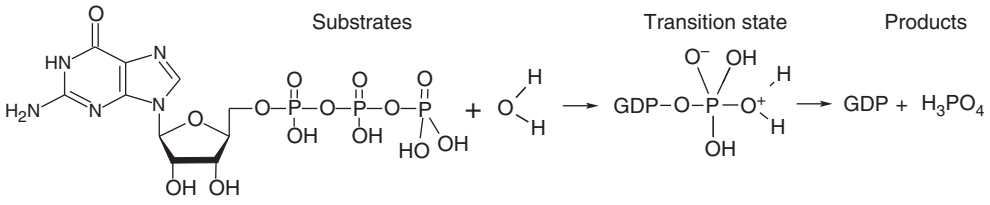

Figure 7.6 Schematic presentation of the GTP hydrolysis. The water molecule attacks the phosphorus atom of the terminal phosphate. A transition state with a fivefold coordinated phosphor is formed, which decomposes into GDP and phosphoric acid. (Wagener and Müller, 2009), with permission.

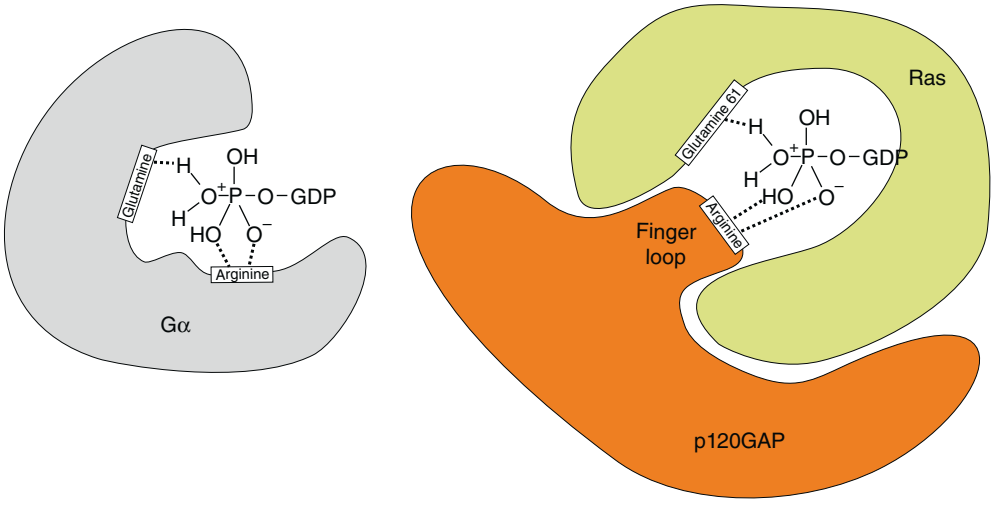

Figure 7.7 Stabilization of the transition state of the GTP hydrolysis in the Gα-subunit of a trimeric G protein and in the complex of Ras and p120GAP. The transition state is stabilized by the side chains of the amino acids arginine and glutamine. Glutamine at position 61 of the Ras protein is a mutation hotspot in many tumors. Arginine is an amino acid of the Gα-protein itself or provided as part of the "Finger Loop" of the associated p120GAP protein.

an arginine residue of p120GAP, the so-called arginine finger or finger loop, is shifted into the phosphate-binding pocket of Ras. The positively charged arginine side chain neutralizes the negative charge of the γ-phosphate and stabilizes the transition state (Scheffzek *et al.*, 1997) (Figure 7.8). In contrast to Ras, the Gα protein has its own arginine and is thus able to stabilize the transition state without an additional GTPase-activating protein. As a consequence, the rate of the GTPase reaction catalyzed by Gα is faster than the rate by Ras in the absence of p120GAP.

When glutamine 61 is mutated, the Ras protein is no longer able to stabilize the transition state of the hydrolysis reaction. Similarly, the exchange of one of the two glycine residues at position 12 or 13 against a larger amino acid residue prevents

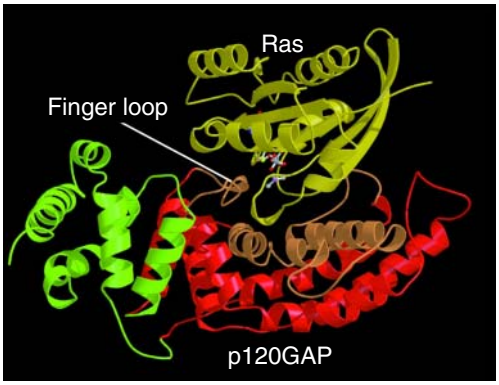

Figure 7.8 Three-dimensional structure of the Ras protein (olive green) in complex with p120GAP (light green, brown, and red). The p120GAP protein catalyzes the GTP hydrolysis using the "Finger Loop," which stabilizes the transition state of the reaction using its arginine residue (Scheffzek *et al.*, 1997) (designed and kindly provided by Dr. Ingrid Vetter and Professor Dr. Alfred Wittinghofer).

the exact spatial orientations required by glutamine 61 and the arginine provided by p120GAP to promote the transition state. Each of the three mutations leads to the block of the GTPase activation by p120GAP and to fixation of the Ras protein in its GTP-bound form. As a consequence, the Ras protein is constitutively active in its function as a signal transmitter.

7.3 Neurofibromin: The Second RasGAP

Neurofibromatosis type 1 (NF1), also known as *Morbus Recklinghausen*, was the first inherited tumor predisposition that could be genetically explained. The German physician Daniel Friedrich von Recklinghausen described the clinical and pathological characteristics of the disease for the first time in 1882. The main symptoms of NF1 patients are brown discolorations of the skin with diameters of a few centimeters, the so-called café-au-lait spots, and painless tumors of the optical nerve and neurofibromas. Neurofibromas are benign tumors, which stem from Schwann cells of neuronal axons passing the subcutis. However, malignant tumors may develop, such as fibrosarcomas, stemming from peripheral fibroblasts in the connective tissue, or glioblastomas, stemming from astrocytes in the brain. In most cases, clinical symptoms lead to diagnosis of the NF1 disease during childhood. In Western countries, NF1 belongs to the most frequent inherited cancer predispositions with a rate between 30 and 40 per 100,000 newborns. Most NF1 patients carry germline mutations in the *NF1* gene. Half of the NF1 cases are caused by *de novo* germline mutations. The high number of mutations in the *NF1* gene might be partially explained by the unusual size of the gene. The

12.394 bp long mRNA is assembled from 57 exons, which are distributed over more than 400,000 bp on chromosome 17. In most tumors of NF1 patients with a heterozygous germline mutation, the second *NF1* allele is inactivated or lost by various mechanisms (mitotic recombination or deletion) as evidenced by loss of heterozygosity (LOH). In addition, tumors with monoallelic mutations have been found. Tumorigenesis in these cases might be explained by haploinsufficiency (HIS). This would mean that the amount of the Neurofibromin protein expressed from a single allele is not sufficient to suppress tumor formation. Indeed, the concentration of Neurofibromin in tumors with monoallelic mutations was found to be very low. In addition to tumors caused by *NF1* germline mutations, many sporadic tumors show *NF1* mutations. Somatic loss of *NF1* gene expression by mutation, chromosomal deletion, or other mechanisms occurs in significant portions of glioblastomas, melanomas, and many other cancers. Thus, *NF1* is a classical and important tumor suppressor gene.

The 7.800 bp open reading frame of the *NF1* gene codes for the 300 kD protein Neurofibromin. Database comparison of the Neurofibromin sequence revealed high similarity of a domain of the protein with the GTPase-activating domain of the human p120GAP (Cichowski and Jacks, 2001). Biochemical experiments proved that this Neurofibromin domain stimulates the GTPase reaction of Ras. Many *NF1* gene mutations result in the loss of the GTPase stimulating activity of Neurofibromin. Accordingly, the amount of active GTP-bound Ras is higher in tumors with *NF1* mutation than in normal cells. Paradoxically, however, proliferation of tumor cells with *NF1* mutation cannot be blocked by Ras-inhibiting compounds, contradicting the proposed model of the tumor suppressing function of Neurofibromin being linked to its GTPase stimulating activity. This result indicates that the tumor-causing potential of mutated Neurofibromin is based on yet unknown functions in addition to its GTPase-stimulating activity.

7.4 Downstream Signaling of Ras

(Video: The MAP-Kinase (MAPK) signaling pathway – open website: https://www.youtube.com/watch?v=r7GoZ9vFCY8*)*

In its GTP-bound form, Ras is able to bind and to activate effector proteins. Important effectors are phosphatidylinositol-3 kinase (PI3K), the Ral exchange factor RalGDS, the phospholipase C (PLC) ε-1 isoform, and the T-lymphoma invasion and metastasis-inducing protein 1 (TIAM1). Depending on the interaction partner, binding leads to the activation of specific downstream effectors (Figure 7.9) (Karnoub and Weinberg, 2008). By modulating these various pathways, Ras exerts influence on many different biological functions, including cell cycle, proliferation, cell migration, differentiation, and apoptosis. The best-characterized Ras effector is the serine/threonine kinase BRaf, which activates the intracellular part of the MAPK signaling pathway.

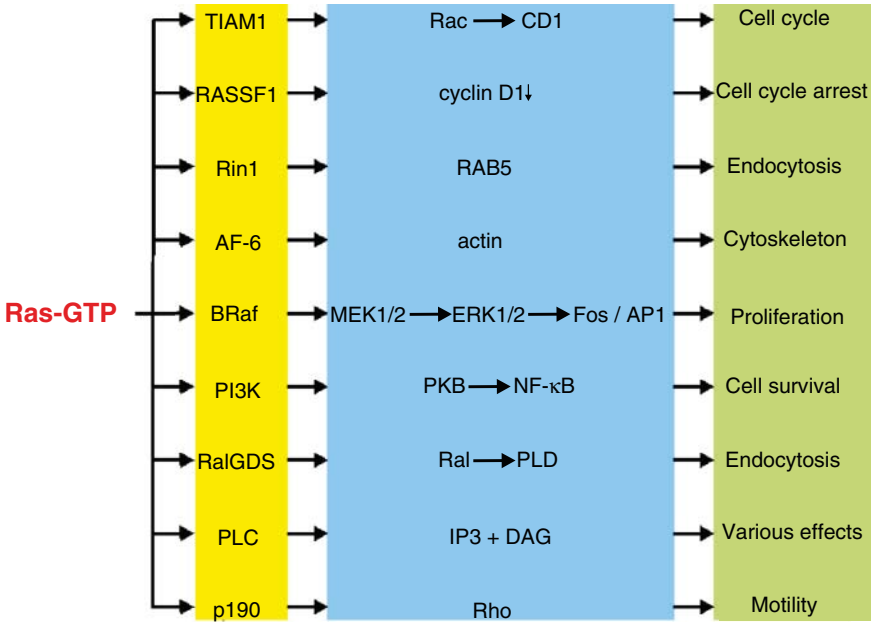

Figure 7.9 Downstream signaling of active Ras-GTP. Ras interaction partners (yellow), major downstream effectors (blue), and biological effects (green) are shown. AF-6, acute lymphoblastic leukemia-1 fused gene on chromosome 6; CD1, cadherin domain-1; DAG, diacylglycerol; ERK1/2, extracellular signal-regulated kinase1/2; IP3, inositol-1,4,5-trisphosphate; MEK 1/2, MAP/ERK kinase 1/2; NF-κB, nuclear factor-κB; PI3K, phosphatidyl inositol (4,5)-bisphosphate 3-kinase; PKB, protein kinase B; PLC/D, phospholipase C/D; RASSF, Ras association domain-containing family; Rin1, Ras interaction/interference protein-1; and TIAM1, T-cell lymphoma invasion and metastasis-1.

7.4.1 The BRaf Protein

Interestingly, the crucial event for BRaf activation is not its interaction with Ras-GTP *per se*, but rather its translocation to the inner side of the plasma membrane and its dimerization. When BRaf is experimentally anchored into the membrane, it is constitutively active, even in the absence of active Ras (Leevers, Paterson, and Marshall, 1994). This finding might be partially explained by the interaction of BRaf with ceramides at the membrane. Ceramides activate the ceramide-dependent kinase CeDK, which phosphorylates and activates membrane-bound BRaf. The BRaf protein possesses three conserved domains (CR1 to CR3) (Figure 7.10). CR1 is cysteine-rich, CR2 serine- and threonine-rich, and CR3 correlates with the kinase domain. Experimental data show that BRaf is locked in a closed conformation by the protein 14-3-3, which binds to two phosphoserines S259 and S621 (Figure 7.11). Dissociation of 14-3-3 and activation

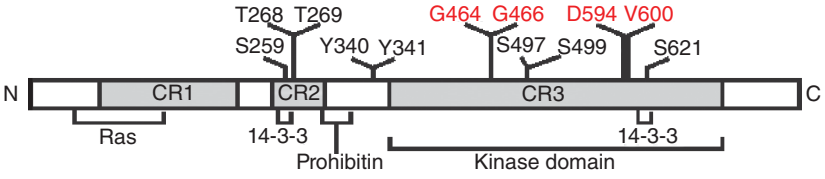

Figure 7.10 The BRaf protein with domains and interaction partners. Upper part: Phosphorylation sites (black) and positions of most frequent mutations (red). Lower part: Interacting proteins and kinase domain. CR1–CR3: conserved regions. (Wagener and Müller, 2009), with permission.

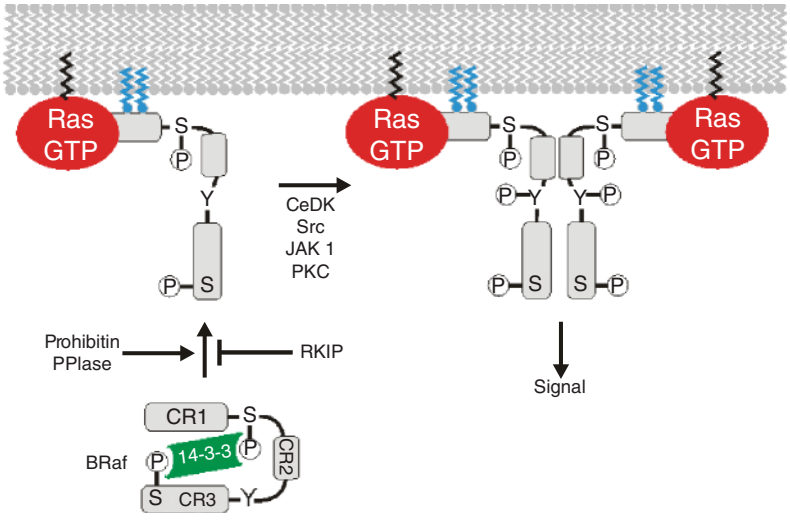

Figure 7.11 Activation of BRaf. The protein 14-3-3 (green) locks BRaf in a closed and inactive conformation. Dissociation of 14-3-3 and BRaf are promoted by Prohibitin and PPIase (peptidyl-prolyl *cis–trans* isomerase). The Raf kinase inhibitor protein (RKIP) inhibits dissociation and activation of BRaf. Upon binding to Ras-GTP, the protein BRaf unfolds to adopt an open conformation. BRaf is thus accessible for activating kinases. Membrane-localized BRaf interacts with ceramides (blue), which activate ceramide-dependent kinase (CeDK). BRaf is phosphorylated by CeDK, Src, JAK1, and PKC. Phosphorylated BRaf is able to dimerize, and dimerized BRaf is able to phosphorylate other proteins and to transmit the signal.

of BRaf are promoted by the proteins Prohibitin and PPIase (Peptidyl-prolyl *cis–trans* isomerase). The Raf kinase inhibitor protein (RKIP) inhibits activation of BRaf. Upon binding to Ras-GTP, the protein BRaf unfolds to adopt an open conformation. The protein is thus accessible for activating kinases. Positions T268 and T269 are phosphorylated by BRaf itself and by CeDK. Y340 and Y341 are phosphorylated by the kinases Src and JAK1. S497 and S499 are phosphorylated by protein kinase C (PKC) (Morrison and Cutler, 1997). As a consequence, BRaf dimerizes. When dimerization is blocked by mutations in the dimerization domain, signal transduction is blocked.

7.4.2 The *BRAF* Gene

The gene encoding BRaf is an important proto-oncogene. *BRAF* is the only gene of the three paralogous genes RAF1, ARAF, and *BRAF* that is mutated in human tumors. Originally, mutations within the *BRAF* gene were found in a genome-wide search for mutations in malignant melanomas (Davies *et al.*, 2002). *BRAF* is mutated in 50–60% of malignant melanomas. Besides melanomas, papillary carcinomas of the thyroid gland and hairy cell leukemia show high *BRAF* mutation rates (Dhomen and Marais, 2007). *BRAF* mutations can already be found in naevi, which are early benign stages of melanomas. Possibly, the mutation of the *BRAF* gene is the first and crucial event in UV-light-induced development of malign melanomas. Overall, nearly 7% of all human tumors carry *BRAF* gene mutations, and the BRaf kinase is the most frequently mutated protein kinase in human tumors. More than 40% of all sporadic colon carcinomas with microsatellite instability (MSI) carry *BRAF* mutations, whereas the mutation rate in MSI negative tumors is below 5%. More than 90% of all mutations are missense mutations within the kinase domain. The most frequent mutation is the mutation V600E, which is the replacement of a valine residue (V) at position 600 by a glutamic acid (E) in the activation segment of the kinase domain. A smaller fraction of melanomas harbor the V600K mutant. In the following, the replacement of valine in position 600 of BRaf by any other amino acids will be denoted as $BRaf^{V600}$. The V600 mutations lead to a monomeric and open conformation, which is no longer locked in an inactive state. The mutated kinase stimulates the MAPK signaling pathway independently of phosphorylation, membrane localization, and dimerization. The mutant is 500 times more active than the wild-type protein, and the mutated gene is able to transform NIH3T3 fibroblasts (Wan *et al.*, 2004). Thus, the $BRaf^{V600}$ mutant fulfills all criteria of a classical oncoprotein.

7.4.3 The MAPK Signaling Pathway

Ras and BRaf are key proteins of the MAPK (mitogen-activated protein kinase) pathway (Figure 7.12). The level of Ras and BRaf divides the pathway into two parts: an upstream section, including the receptor, adapter proteins, and exchange factors, and a downstream section consisting mainly of kinases and transcription factors. Activation of BRaf by Ras initiates the downstream MAPK pathway. Upon activation, the serine/threonine kinase BRaf phosphorylates and activates the kinases MEK1 (MAPK/ERK kinase 1) and MEK2. MEK1 and MEK2 activate the kinases ERK1 (extracellular signal-regulated kinase 1) and ERK2. ERK1 and 2 phosphorylate and activate several transcription factors, including Myc and AP-1 (composed of Fos and Jun proteins). These transcription factors bind to specific DNA sequence motifs and activate transcription of important target genes, such as CCND1 (coding for cyclin D1). The products of the target genes mediate the

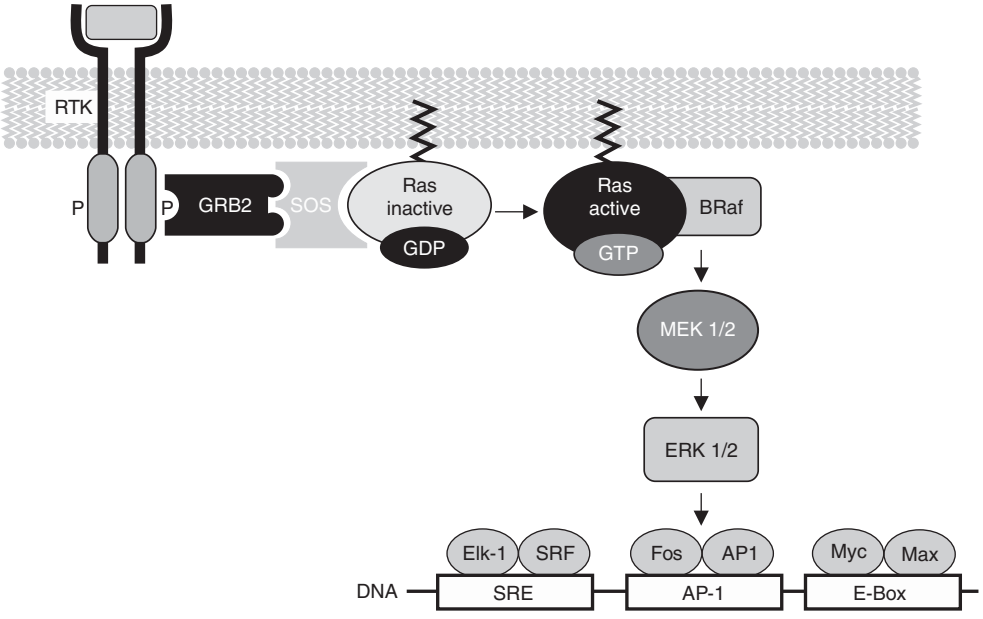

Figure 7.12 The canonical MAPK signaling pathway. The pathway begins with the activation of a receptor tyrosine kinase (RTK) by an extracellular factor. The activation signal is transferred via GRB2 to SOS. SOS catalyzes nucleotide exchange on Ras. Active Ras-GTP activates BRaf, which activates a kinase cascade involving MEK 1/2 and ERK1/2. The cascade leads to activation of different transcription factors (gray ovals), which bind to specific DNA motifs (white rectangles). (Wagener and Müller, 2009), with permission.

cellular effects; among these, the activation of the cell cycle and proliferation is most prominent.

Under normal conditions, the MAPK pathway is tightly regulated. In order to prevent excessive signaling, a number of negative feedback loops exist. ERK kinases phosphorylate many proteins of the RTK-RAS-MAPK pathway, thus attenuating the activity of the pathway. In addition, ERK induces transcription of phosphatases, which inactivate the pathway by dephosphorylating critical signaling proteins.

7.4.4 Mutations in Genes of the MAPK Pathway

Genes of the MAPK pathway that are mutated in human tumors are genes encoding receptor tyrosine kinases, all three Ras proteins, BRaf, and Neurofibromin (Table 7.1). Interestingly, activating mutations need only to occur at one level of the MAPK pathway to drive tumorigenesis. Papillary carcinoma of the thyroid gland serves as a good example. Two thirds of these carcinomas carry a single activating mutation in the receptor tyrosine kinase gene RET, in the *KRAS*

gene, or in the *BRAF* gene, but only very few carcinomas carry more than one mutation in the MAPK pathway. Why do different tumors harbor mutations in different genes of the MAPK pathway? The hierarchy and the branching of the MAPK pathway might answer this question. A mutation at a higher level of the pathway potentially activates the branched activities. Thus, a mutation in a *RAS* gene exhibits stronger and more expansive effects than a mutation in *BRAF*. For some tumor types, depending on inherent signaling pathways or collaborating mutations, a downstream mutation may be sufficient to induce abnormal growth. On the other hand, upstream mutations in *RAS* would be more advantageous for potential tumor cells of all cell types and mutational status, likely explaining its high frequency of mutation compared to genes at functionally lower levels.

7.5
Therapy of Tumors with Constitutively Active MAPK Pathway

Four general levels for therapeutic intervention of tumors with mutations in the MAPK pathway can be distinguished (Figure 7.13). At the first level, drugs that inhibit the activation or the activity of receptor tyrosine kinases can be employed. These drugs include therapeutic antibodies and tyrosine kinase inhibitors, which are described in Chapter 5. The second and third levels both target the Ras protein, one by the development of drugs that prohibit its modification and membrane anchoring and the other by directly inhibiting its signaling activity. The fourth level of interference is downstream of Ras. Inhibitors of the kinases BRaf or MEK1/2 show promising effects in experimental studies, as well as in clinical applications.

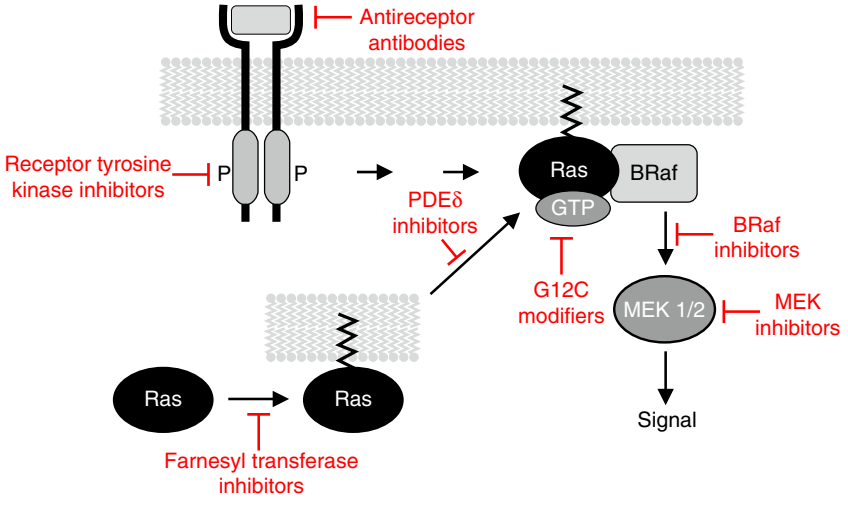

Figure 7.13 Sites of interferences of potential drugs inhibiting the MAPK signaling.

7.5.1 Ras as a Therapeutic Target

Activated *RAS* genes initiate tumorigenesis in cell models, in whole animal organisms, and probably also in humans, underlining its key role in this process. In addition, a number of experimental studies have demonstrated that activated Ras represents a promising candidate for therapeutic targeting of tumors. Cell transformation by mutated RAS could be reversed by microinjection of anti-Ras antibodies (Feramisco *et al.*, 1985; Kung *et al.*, 1986) and the incidence of tumors decrease significantly upon knockout of an activated *RAS* gene in animal models (Chin *et al.*, 1999; Fisher *et al.*, 2001). Despite these clear outcomes, the questions of whether mutated *RAS* is necessary for tumor maintenance in humans or whether *RAS*-mutated tumors can be treated by Ras protein inhibition or by *RAS* gene ablation have not been decisively answered. Indeed, knocking down *KRAS* in *KRAS* mutated tumor cells led to the demonstration that some cells were insensitive to *KRAS* inactivation, that is, they were not "addicted" to KRas signaling (Singh *et al.*, 2009).

Several features of the Ras protein complicate the approach toward an anti-Ras drug. Most importantly, all known signaling activities of the Ras protein are based on its direct interaction with other proteins. Because Ras does not transfer its signal by an enzymatic activity that leads to covalent modification of an interacting protein, there is neither a straightforward assay for measuring Ras' own activity nor can this activity be inhibited by an enzymatic substrate analog. Additionally, it is accepted that oncogenic Ras cannot be blocked by guanine nucleotide analogs, mainly because Ras possesses a high affinity toward guanine nucleotides and because the high intracellular GTP concentration prevents an effective competition by GTP analogs. Finally, the small and three-dimensionally compact Ras protein has no obvious binding site for allosteric regulators and inhibitors. Based on its properties, Ras as a tumor protein has been called "undruggable" for many years. Consequently, research on anti-Ras drugs has primarily focused on the enzymatic reactions that are necessary for an active Ras, including posttranslational modifications that are necessary for membrane anchoring. Surprisingly, it was recently shown that the signaling activity of oncogenic Ras could be successfully blocked by targeting its interaction with BRaf and by covalently modifying the guanine nucleotide-binding pocket.

7.5.1.1 Inhibiting Posttranslational Modification and Membrane Anchoring of Ras

Only membrane-anchored Ras can be activated by GEFs. Prerequisite for its membrane anchoring is its posttranslational farnesylation (Figure 7.1). The reactions leading to this modification can be effectively blocked by competitive analogs of both substrates of the farnesyl transferase (FTase) reaction (Konstantinopoulos, Karamouzis, and Papavassiliou, 2007). The first substrate is the CaaX box of the Ras protein. Based on the known sequence and the proposed three-dimensional structure of the CaaX box, efficient FTase inhibitors (FTIs) have been developed (Figure 7.14). The tricyclic and orally applicable molecule lonafarnib (Sarasar)

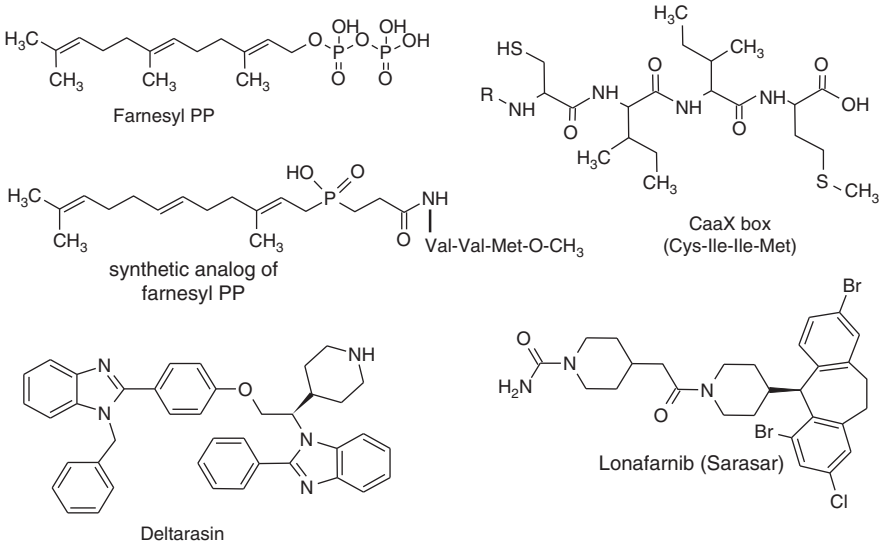

Figure 7.14 The physiological substrates of the farnesyltransferase reaction farnesyl pyrophosphate (PP) and CaaX box, and drugs, which inhibit farnesylation and membrane anchoring of Ras. Farnesyltransferase can be competitively inhibited by a synthetic analog of farnesyl PP as well as by the Caax-box analog Lonafarnib. Deltarasin blocks the prenyl binding site of PDE and thereby its interaction with farnesylated Ras. R: protein chain of Ras.

was the first anti-Ras drug that entered clinical tests. Besides a significant antitumor activity, the drug showed strong gastrointestinal toxicity. The combined low-dose therapy of lonafarnib with two cytostatic drugs, gemcitabine and paclitaxel, showed reduced toxicity and strong effects on pancreas and lung tumors of the NSCLC (non-small-cell lung cancer). The FTase reaction can also be inhibited with analogs of the second substrate, that is, farnesyl pyrophosphate. Because farnesyl pyrophosphate is a substrate not only of FTase but also of other enzymes including squalene synthase, its competitive analog inhibits essential biosynthetic reactions. This is why farnesyl pyrophosphate analogs show strong side effects.

Surprisingly, signaling activities of KRas and NRas still remain active in FTI-treated cells. Immunostaining of these cells proved that Ras is still localized at the membrane. Closer analysis revealed that in FTI-treated cells, Ras is modified by a geranylgeranyl rather than by a farnesyl residue. Based on these findings, a search for more general inhibitors that affect membrane anchoring of both farnesylated and geranylgeranylated Ras proteins was initiated. The phosphodiesterase δ (PDEδ) represents a potential target for this strategy. PDEδ is a prenyl-binding Ras chaperone and sustains the spatial organization of KRas by facilitating its diffusion in the cytoplasm. Proper PDEδ function is necessary for membrane trafficking and for the transport of modified KRas from endosomal membranes toward the plasma membrane. A high-throughput screen revealed a master structure that blocks the interaction between PDEδ and KRas with nanomolar

affinity. Combinatorial synthesis delivered deltarasin and other small compounds that selectively bind to the prenyl-binding pocket of PDEδ and thereby inhibit KRas/PDEδ interaction and block anchoring of KRas in the plasma membrane (Zimmermann *et al.*, 2013). Both downstream KRas signaling and proliferation are inhibited in cells with oncogenic *KRAS* mutation after treatment with these compounds.

7.5.1.2 Direct Targeting Mutant Ras

Although the Ras protein was long thought to be an "undruggable" oncoprotein due to its structure and its functional mechanism, the direct inhibition of mutated Ras recently succeeded (Ostrem *et al.*, 2013). Electrophilic molecules that selectively and irreversibly bind to the cysteine residue of the oncogenic G12C mutant of KRas were developed. The covalent modification impairs interaction of KRas to BRaf and thereby its signaling activity. While the new compounds showed promising effects in cultured tumor cells with *RAS* mutations, two caveats have to be considered. First, the antitumor effect of such compounds is limited to tumors with the G12C mutation in the *KRAS* gene. Secondly, it can be expected that the electrophilic G12C modifiers bind covalently to many other molecules besides KRas, which might lead to strong side effects.

7.5.1.3 Preventing Ras/Raf Interaction

The Ras/Raf interaction surface is too large to be selectively blocked by a single small molecule. Nevertheless, some derivatives of the nonsteroidal, anti-inflammatory drug sulindac were found to prevent binding of active Ras to BRaf (Waldmann *et al.*, 2004). Because this effect depends on high concentrations of these molecules, it is probably not specific. Unspecific binding of the aromatic compounds with the hydrophobic surface area of Ras, locking it in a conformation with lower BRaf binding affinity, may be a possible mechanism of action.

7.5.2 BRaf Inhibitors

Over the last 20 years, many companies have performed high-throughput screens to identify kinase inhibitors with therapeutic potential. In these screens, several unspecific kinase inhibitors have been found that affect BRaf and MEK kinases, among other kinases. Such unspecific kinase inhibitors, which found their way into clinical cancer therapy, include imatinib (Glivec) and sorafenib (Nexavar). The first selective BRaf inhibitor vemurafenib (registered trade name Zelboraf) (Figure 7.15) was identified in a high-throughput BRaf kinase screen by the company Plexxikon. Vemurafenib ("V to E mutated Raf inhibitor") was approved in 2011 for treatment of melanomas and its metastases with the BRaf V600E mutation (Jang and Atkins, 2014) (Figure 7.15). Vemurafenib inhibits selectively the V600E mutant of BRaf, which is the most frequent of all BRaf mutants, present in more than 60% of melanomas. In contrast to sorafenib, which inhibits and blocks the inactive form of BRaf, vemurafenib binds and blocks the ATP-binding site of

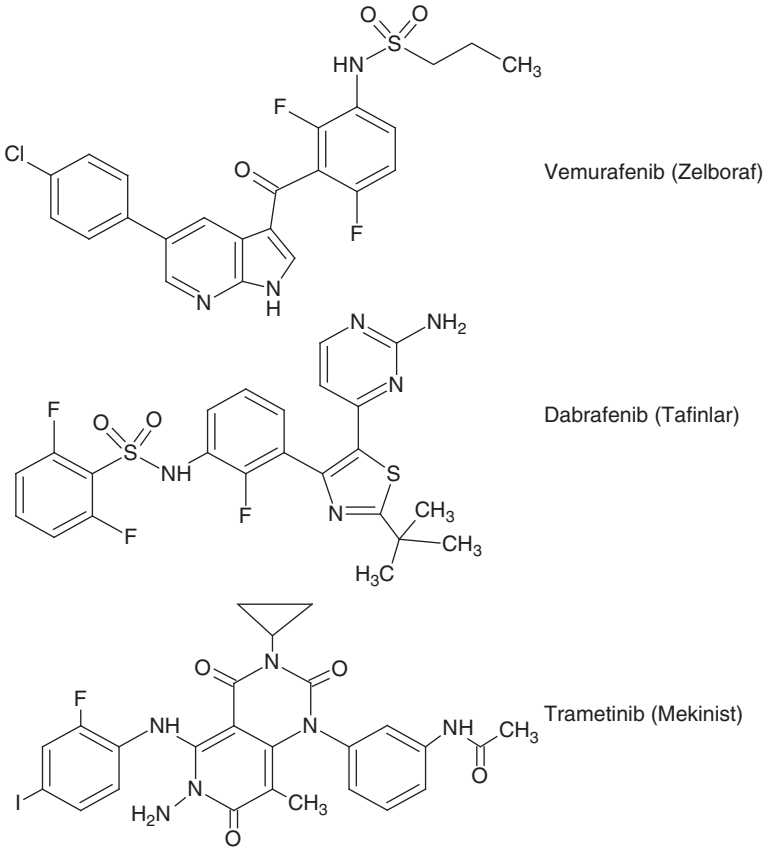

Figure 7.15 The BRaf kinase inhibitors vemurafenib and dabrafenib and the MEK 1/2 inhibitor trametinib.

the active monomeric form of the mutant. The monomeric form of BRaf is essential for the inhibitory action of vemurafenib and other ATP analogs. When BRaf dimers are exposed to vemurafenib, the activity of the drug-binding protomer is blocked. However, vemurafenib induces a conformational change, which transactivates the binding partner. Only when the inhibitor concentration is sufficiently high to bind to both protomers of the dimer, BRaf activity is inhibited. Because of its mechanism of action, vemurafenib activates MAPK signaling in *BRAF* wild-type tumors and in normal cells. Thereby, it inhibits the proliferation of cells with deregulated BRaf only.

The second selective BRaf inhibitor dabrafenib (Tafinlar) (Figure 7.15) was approved in 2013. Dabrafenib is similar to vemurafenib with regard to its pharmacological and biochemical properties, but with one important difference: in contrast to vemurafenib, which affects only V600E mutants of BRaf, dabrafenib is also effective against BRaf mutants with other mutations at position 600. Both vemurafenib and dabrafenib significantly prolong the regression-free

survival of melanoma patients. Unfortunately, most patients treated with one of these drugs develop therapy resistance already during the first year of therapy (Sosman *et al.*, 2012).

7.5.2.1 Consequences of BRaf Inhibition by Vemurafenib

The paradoxical effect of vemurafenib on BRaf has important consequences. Because active Ras induces dimerization of BRaf monomers, dimerization of BRaf monomers similarly increases when the activity of Ras increases. Thus, high activity of Ras decreases the therapeutic potency of vemurafenib. Since vemurafenib activates BRaf signaling activity in the dimerization state of nonmutated monomers, the drug increases the activity of MAPK signaling in the presence of mutant Ras. This may lead to the activation of cell clones with mutant KRas, for example, in keratoacanthomas and squamous cell carcinomas of the skin.

7.5.2.2 Resistance against BRaf Inhibitors Based on BRaf Dependent Mechanisms

Unfortunately, nearly all tumors develop resistance against vemurafenib and dabrafenib. Several underlying mechanisms have been identified (Figure 7.16) (Lito, Rosen, and Solit, 2013; Rizos *et al.*, 2014). Most resistant tumor cells show increased amounts of the BRaf protein, which are too high to be inhibited by the intracellular drug level. In around 10% of all $BRaf^{V600}$ tumors, the high BRaf

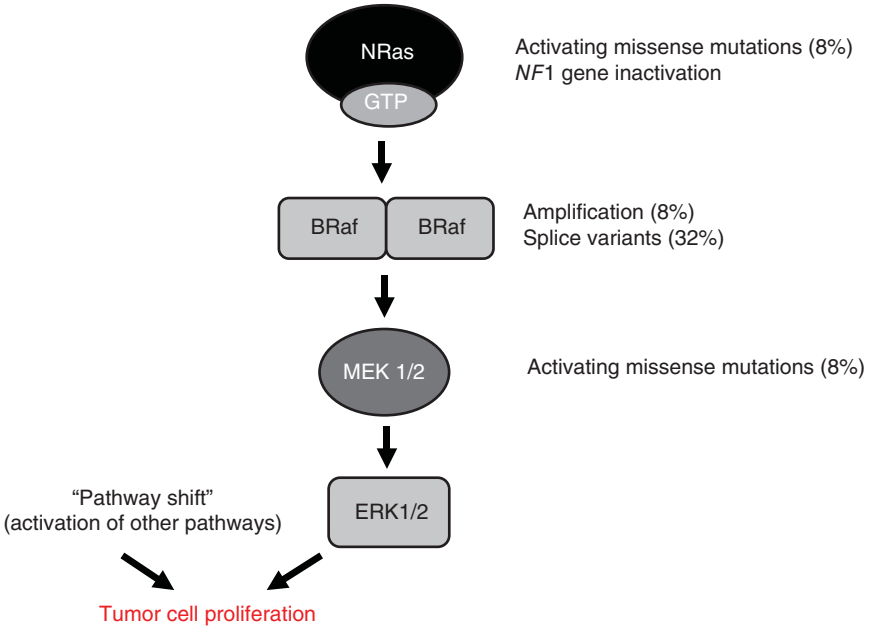

Figure 7.16 Development of resistance in $BRaf^{V600}$ mutant malignant melanomas in response to treatment with vemurafenib/dabrafenib. Frequencies of mutations or splice variants refer to tumors with $BRaf^{V600}$ mutations (Rizos *et al.*, 2014). *NF1* gene mutations have been described in other patient populations.

level is caused by amplification of the *BRAF* gene. Mutations in the binding pocket of inhibitors are other common mechanisms of resistance to kinase inhibitors. So far, such mutations have not been described for vemurafenib or dabrafenib. However, in about one third of $BRaf^{V600}$ melanomas, splice variants of BRaf lacking exons 4–8 have been identified. The protein isoforms, which lack the Ras-binding domain, dimerize independently of the Ras protein, in this way conferring resistance. The splice variants may be caused by mutations in splice sites or by epigenetic changes.

7.5.2.3 Resistance against BRaf Inhibitors Based on BRaf Independent Mechanisms

There are also mechanisms of resistance that are independent of BRaf. First, a so-called *pathway shift* leads to activation of survival pathways independently of the BRaf level. This shift might be explained by the relief of negative feedback loops upon downregulation of ERK in vemurafenib-treated cells. Cells of some vemurafenib resistant tumors show increased expression of the receptor genes *PDGFRB*, *EGFR*, *MET*, and *IGFI-R* leading to the activation of BRaf-independent pathways. In therapy-resistant melanomas, activating mutations of PI3 kinase isoenzymes (*PIK3CA*, *PIK3R1*) and inactivating mutations in the *PTEN* gene have been identified. However, in comparison to the MAPK pathway, the contribution of PI3K/AKT pathway activation to therapy resistance appears not to be well defined (Van Allen *et al.*, 2014).

Secondly, some vemurafenib-treated tumors show activating mutations in other genes of the MAPK pathway. In about 10–20% of primary malignant melanomas, *NRAS* is mutationally activated leading to activation of MAPK signaling via nonmutated BRaf, which is not inhibited by vemurafenib. In the case that mutations are not found at the beginning of therapy, minor mutant clones may become predominant during therapy. Occasionally, loss-of-function mutations have been found in the *NF1* gene. Since the *NF1* gene product Neurofibromin is a GTPase-activating protein (GAP), its inactivation increases the active GTP-binding state of NRas. Active NRas induces the dimerization of BRaf monomers, resulting in therapy resistance. Mutations in proteins downstream of BRaf may activate ERK. For example, activating point mutations have been identified in the *MAP2K1* or *MAP2K2* genes, which encode MEK1 or MEK2, respectively.

Third, *BRAF*-mutated cells may escape drug sensitivity by modulating their microenvironment. Such cells are able to produce immunosuppressive factors, which contribute to tumor evasion, and the vascular endothelial growth factor (VEGF), which increases vascularity.

7.5.2.4 Treatment of Vemurafenib-Resistant Tumors

Since MAPK pathway activation is a common mechanism of therapy resistance in $BRaf^{V600}$ melanomas treated with vemurafenib or dabrafenib, these drugs may be combined with MEK inhibitors. Indeed, the combination of dabrafenib with the MEK 1/2 inhibitor trametinib (Figure 7.15) led to a significant increase of median progression-free survival (Flaherty *et al.*, 2012).

7.6 Outlook

Due to its importance in many human tumors, the MAPK signaling pathway is currently and will continue to be one of the major topics of anticancer drug development. Based on the achievements of recent research, a drug that blocks the biochemical events leading to the activation of Ras will likely be available for cancer therapy soon. Furthermore, additional compounds that are more effective against tumorigenic kinases are necessary. Finally, the ability of the tumor to evolve, leading to more aggressive growth and drug resistance, will require staying abreast of the molecular mechanisms at play and the development of new potent inhibitors against drug-resistant effectors. Promising strategies to treat tumors that are resistant against Ras and BRaf inhibiting drugs include blocking downstream kinases, inhibiting parallel pathways, and reversing changes of the tumor microenvironment.

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8
PI3K-AKT-mTOR Signaling

Summary

The PI3K (phosphatidylinositol-4,5-bisphosphate 3-kinase)-AKT-mTOR (mechanistic target of rapamycin) pathway regulates growth, survival, and division of cells. Class I PI3-kinases are activated by extracellular signals, primarily through receptor tyrosine kinases, and mediate the conversion of the membrane lipid phosphatidylinositol-4,5-bisphosphate (PIP2) to phosphatidylinositol-3,4,5-trisphosphate (PIP3). The phosphatase PTEN (phosphatase and tensin homolog)

Cancer Signaling: From Molecular Biology to Targeted Therapy, First Edition.
Christoph Wagener, Carol Stocking, and Oliver Müller.

Companion Website:www.wiley.com/go/wagener/cancersignaling

can counteract class I PI3Ks by dephosphorylating PIP3. PIP3 binds the PH (pleckstrin homology) domain of the AKT protein kinase, as well as of other signaling proteins. AKT inhibits apoptosis and stimulates cell cycle progression through phosphorylation of diverse substrates. AKT also phosphorylates and inactivates TSC2 (tuberous sclerosis 2) in complex with TSC1. The TSC1/2 complex acts as a GTPase-activating protein (GAP) for the small GTPase designated "RAS homolog enriched in brain (Rheb)". Rheb in its GTP-bound form stimulates the kinase activity of mechanistic target of rapamycin complex 1 (mTORC1). AKT and mTORC1 coordinately regulate cell metabolism to produce new biomass needed for the generation of a new cell. In addition, mTORC1 inhibits autophagy. Genes encoding components of the pathway are mutated frequently in major human malignancies. In patients with B-cell tumors, such as chronic lymphocytic leukemia (CLL) and non-Hodgkin's lymphoma, inhibition of the catalytic PI3K p110δ subunit resulted in significant response rates. In major solid carcinomas, however, successes of monotherapies are rather limited. Considering the many inputs, extensive cross talks, feedback circuits, and redundancies of the pathway, rational combinations of therapeutic compounds are being tested in clinical trials. As a proof of principle, the rapamycin homolog everolimus combined with an aromatase inhibitor significantly increases progression-free survival of patients with breast cancer.

8.1 Discovery of the PI3K-AKT-mTOR Pathway

(Video: The PI3K/AKT pathway – open website: https://www.youtube.com/watch?v=ewgLd9N3s-4*)*

Cells destined to divide produce proteins, nucleic acids, and lipids, collectively called *biomass*. Production of biomass, passage through the cell cycle, apoptosis, and autophagy need to be precisely coordinated. The PI (phosphatidylinositol)-3-kinase-AKT-mTOR (mechanistic target of rapamycin) pathway executes this coordination. During the elucidation of this, several lines of research converged that historically were quite apart.

In the local language, Easter Island is known as *Rapa Nui*. In the 1970s, the bacterial strain *Streptomyces hygroscopicus*, which was isolated from a soil sample from Easter Island, was found to produce a potent antifungal metabolite. The compound was named rapamycin. The target of rapamycin (TOR) was identified in yeast mutants, by its ability to confer resistance to the growth inhibitory properties of rapamycin. The mammalian homolog of the yeast proteins was named mammalian TOR and later renamed mechanistic TOR (mTOR). Rapamycin and rapamycin homologs (rapalogs) were first applied as immunosuppressants. After mTOR was identified as central node in the regulation of cell growth, rapamycin and rapalogs were applied in cancer therapy (Wullschleger, Loewith, and Hall, 2006).

In 1977, Stephen P. Staal isolated a retrovirus from a T-cell lymphoma of the susceptible mouse strain AKR. The virus was named AKT8 (AKR thymoma #8)

(Staal, Hartley, and Rowe, 1977). The directly transforming properties of AKT8 are based on the cell-derived viral oncogene *akt.* In 1988, Staal identified the corresponding cellular oncogene *AKT* in mammalian cells. Now we know that the AKT protein acts as the major regulator of cell growth, division, and survival (Bellacosa *et al.*, 2005).

In the mid-1980s, researchers observed that several viral oncoproteins, such as Src, were associated with a lipid kinase. In 1988, a group led by Lewis Cantley made the surprising discovery that this oncoprotein-associated kinase is actually a "phosphatidylinositol-3-kinase" (PI3K), in that it phosphorylates the 3-OH group of the inositol ring of the membrane lipid phosphatidylinositol (Whitman *et al.*, 1988).

Since these early years, the PI3K-AKT-mTOR pathway has been analyzed much further. The discovery of both PI3-kinase and AKT occurred during research on oncoproteins. This led to the assumption that the pathway may be involved in human malignancies. Meanwhile, this assumption has been substantiated.

8.2 Phosphatidylinositol-3-Kinase (PI3K)

PI3-kinases phosphorylate the inositol ring of phosphatidylinositol at position 3. The two hydrophobic fatty acids anchor phosphatidylinositol in cellular membranes. In mammals, there are four classes of PI3 kinases: class IA, class IB, class II, and class III. The preferred substrate of class I PI3 kinases is phosphatidylinositol-4,5-bisphosphate (PIP2), which is converted into phosphatidylinositol-3,4,5-trisphosphate (PIP3) (Figure 8.1a). Class III and presumably also class II PI3-kinases phosphorylate the inositol ring of phosphatidylinositol in 3 position, generating PI-3-phosphate (Figure 8.1b).

PIP3 recruits proteins containing a pleckstrin homology (PH) domain to the plasma membrane. Class IA PI3-kinases are activated by receptor tyrosine kinases or their substrates, whereas class IB PI3-kinases are activated by G-protein-coupled receptors. With respect to tumorigenesis, class IA PI3-kinases are most important (Engelman, Luo, and Cantley, 2006). In the following, class IA PI3-kinases will be collectively called phosphatidylinositol-3-kinases.

Class I PI3Ks are heterodimers consisting of a regulatory and a catalytic subunit (Figure 8.2). The regulatory subunits of class IA PI3-kinases possess two SH2 domains, which bind to the corresponding recognition motif of the receptor or the receptor substrate IRS1 (insulin receptor substrate 1). There are three genes (PIK3R1, PIK3R2, PIK3R3), which encode the regulatory subunits p85α (with its splicing variants p55α and p50α), p85β, and p85γ, respectively. The genes PIK3CA, PIK3CB, and PIK3CD encode the highly homologous catalytic isoforms of 110 kD, which associate with any of the regulatory isoforms. After binding to a pYxxM motif in the cytoplasmic domain of receptor tyrosine kinases, the regulatory subunit induces an activating conformational change in the catalytic subunit. The activated catalytic subunit converts PIP2 into PIP3 (Fruman and Rommel, 2014; Thorpe, Yuzugullu, and Zhao, 2015).

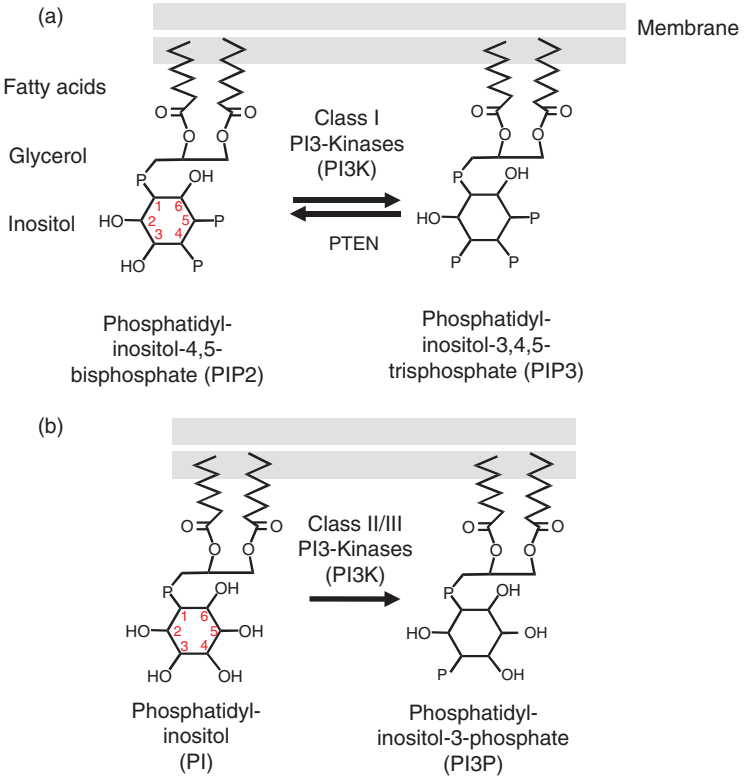

Figure 8.1 (a) Phosphatidylinositol (PI) consists of an inositol and phosphatidylglycerol moiety. Two fatty acid residues (jagged lines) anchor PI in the cell membrane (gray horizontal bars). Class I phosphatidylinositol-3-kinases (PI3Ks) generate phosphatidylinositol-3,4,5-trisphosphate (PIP3) by phosphorylating phosphatdylinositol-4,5-bisphosphate (PIP2) at position 3. The phosphatase PTEN (phosphatase and tensin homolog) reverses the phosphorylation at position 3. (b) Class III and presumably also class II PI3-kinases phosphorylate the inositol ring of phosphatidylinositol (PI) at position 3, generating PI-3-phosphate. (Wagener and Müller, 2009), with permission.

The p110 catalytic subunits harbor a GTPase-binding domain (GBD). Knock-in studies in mice indicate that the p110α isoform binds Ras and is an effector of Ras (Gupta *et al.*, 2007). The GBD of p110β interacts with RAC and CDC42, which are small GTPases involved in cytoskeletal reorganization. The GBD of p110δ interacts with the small GTPase TC21 (Fruman and Rommel, 2014). Additionally, the p110 catalytic subunits contain a calcium-binding C2 domain, which is responsible for targeting the enzyme to the membrane.

An enzyme named PTEN (phosphatase and tensin homolog) dephosphorylates PIP3 in 3 position of the inositol ring (Figure 8.1). As a consequence, binding of proteins with PH domains is reversed. PTEN has additional properties, which will be described later in this chapter.

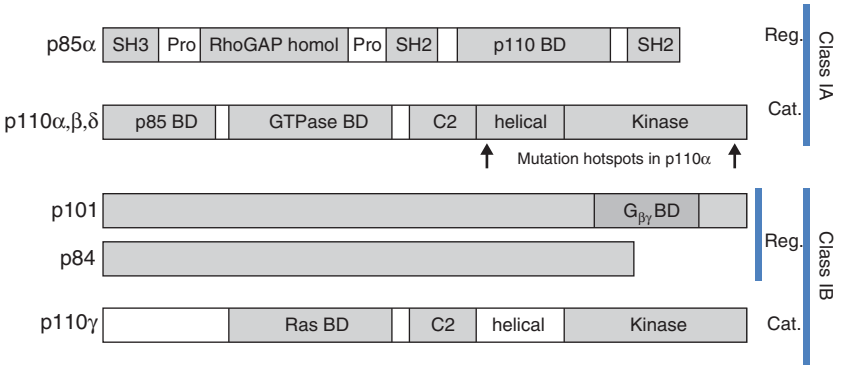

Figure 8.2 Domain structure of class I PI3-kinases. Class IA PI3-kinases comprise five regulatory subunits (p85α, p85β, p55α, p55γ, and p50α). The p85α subunit is shown as an example. The GTPase-binding domain of p110α binds Ras, the GTPase-binding domain of p100β interacts with RAC and CDC42. The GTPase-binding domain of p110δ binds the small G-protein TC21. Arrows indicate mutational hotspots in the p110α subunit. Class IB PI3-kinases comprise two regulatory subunits (p101, p84). The C2 domains target the proteins to the membrane. BD, binding domain.

8.3
Inositol Trisphosphate, Diacylglycerol, and Protein Kinase C (PKC)

Besides being a substrate for conversion into PIP3, PIP2 can be the source for two other cofactors. Phospholipase C (PLC) is able to cleave PIP2 into the second messengers DAG (diacylglycerol) and IP3 (inositol-1,4,5-trisphosphate) (Figures 8.3 and 8.5). While DAG stays within the membrane, IP3 is water soluble and diffuses from the membrane into the cytosol. DAG is an activating cofactor of the protein kinase C (PKC). IP3 leads to the opening of calcium channels in the membranes of the endoplasmic reticulum and storage vesicles. As a consequence, the cytosolic calcium concentration rises.

8.3.1
Protein Kinase C (PKC)

(Video: Protein kinase C – open website: https://www.youtube.com/watch?v=Lp-2mrijwwU*)*

The term *protein kinase C* (PKC) refers to a family of structurally related serine/threonine kinases that are involved in multiple cellular processes, including cellular proliferation, differentiation, survival, and motility (Garg *et al.*, 2014). In the 1980s, PKC was identified as the substrate of phorbol ester, a potent tumor promoter (Blumberg, 1988). Phorbol esters activate PKC by mimicking DAG, which is the physiological activator of PKC.

The PKC family can be divided into three subfamilies based on their second messenger requirements (Figure 8.4): Classical PKCs (isoforms α, βI, βII, and γ)

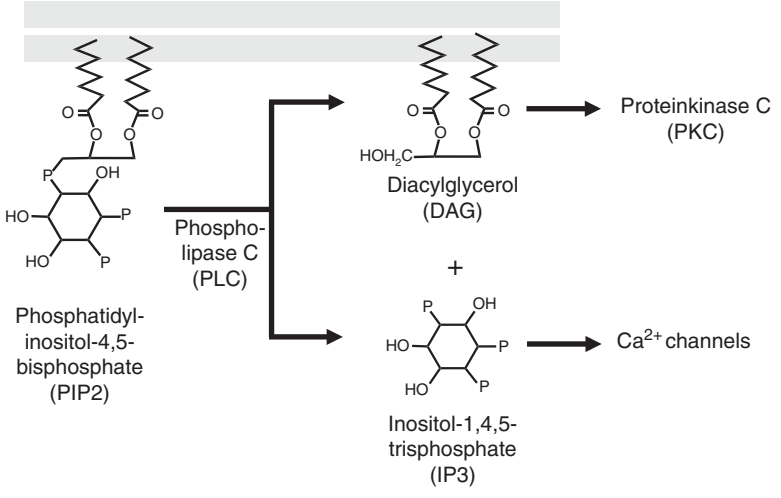

Figure 8.3 Hydrolytic cleavage of phosphatidylinositol-4,5-bisphosphate (PIP2) by phospholipase C into diacylglycerol (DAG) and inositol-1,4,5-trisphosphate (IP3). DAG activates protein kinase C, IP3 releases Ca^{2+} from the endoplasmic reticulum and storage vesicles. (Wagener and Müller, 2009), with permission.

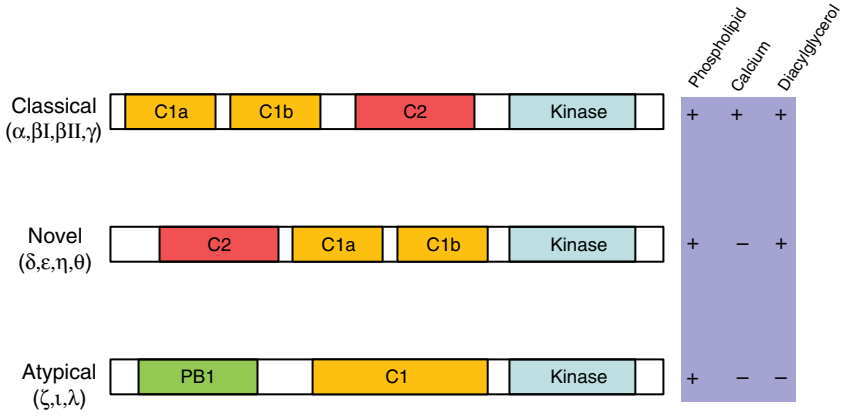

Figure 8.4 Domain structures of PKC isoforms. C1a and C1b domains interact with lipids such as phorbol esters and diacyl glycerol and with proteins; the C1 domain interacts only with lipids; the C2 domain interacts with calcium and phospholipids; the PB1 domain interacts with the proteins PAR6 and CDC42. All isoforms are activated by phospholipids, whereas classical isoforms are activated also by Ca^{2+} and DAG, and the novel isoforms are activated also by DAG. (Figure adapted from (Garg *et al.*, 2014), with permission.)

require Ca^{2+}, DAG, and a phospholipid such as phosphatidylserine for activation. The novel PKCs (δ, ε, η, and θ) require DAG, but not calcium for activation. Atypical PKCs (ζ, ι, λ) require neither calcium nor DAG for activation. PKC contains a large regulatory region with several domains. DAG and phorbol esters bind to the C1 domains. Only classical PKCs are activated by calcium,

which binds to the C2 domain. In contrast, the C2 domain in novel PKCs is calcium-insensitive. Atypical PKCs are atypical regarding their regulatory properties; these enzymes do not bind DAG or calcium and rather depend on protein–protein interactions and phosphorylation for their activation.

8.3.2 Activation and Functions of PKC

The activation process of PKC is rather complex and runs through three levels. First, the catalytic domain is phosphorylated by the tyrosine kinase PDPK1 (3-phosphoinositide-dependent protein kinase 1). Although PDPK1 is activated by the phosphatidylinositols PIP2 and PIP3, PDPK1 is believed to be constitutively active and does not always require phosphatidylinositols for its activities. Secondly, Ca^{2+} and DAG bind to C2 and C1 domains of phosphorylated PKC, which leads to its recruitment to the membrane where it is anchored by RACK proteins (membrane-bound receptor for activated PKC proteins). The third level of PKC activation is the maintenance of its activated state. PKC remains activated even after the original activation signal has ended or the calcium level has decreased. This is probably achieved by DAG alone, which is produced by phospholipase C.

Active PKC phosphorylates many different substrates that play key roles in multiple intracellular processes (Table 8.1). Thereby, PKC enzymes are effectors and regulators of many different signaling pathways, including the MAPK pathway, PI3K/AKT/mTOR pathway, JAK/STAT pathway, and the NF-kB signaling network. Active PKC may induce proliferation, apoptosis, differentiation, or migration.

8.4 AKT (Protein Kinase B)

AKT, also named protein kinase B (PKB), is a serine/threonine kinase with a PH domain. Three genes AKT1, AKT2, and AKT3 encode three members of the AKT protein family. In the following, the AKT family members are collectively referred

Table 8.1 Exemplified substrates of PKC.

Substrate	Function
IκB, vitamin D3 receptor (VDR)	Transcription factor
CDC42	Small GTPase
β-actin, α-tubulin, plectin	Cytoskeleton
MAP kinase, PAK2 (p21-activated kinase 2), Rho-activated kinase (ROCK), BRaf	Signaling kinase
EF-Tu	Translation elongation factor
Vasodilator-stimulated phosphoprotein (VASP)	Cytoskeleton remodeling protein
Calpain	Cysteine protease
EGF receptor	Growth factor receptor

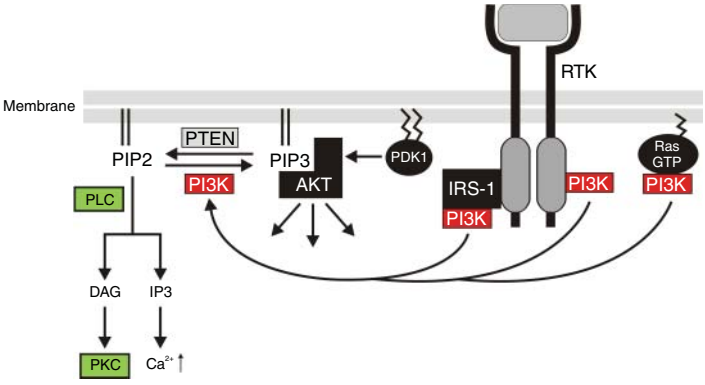

Figure 8.5 Signaling mediated by PIP2 and PIP3. Phospholipase C cleaves PIP2 into diacylglycerol (DAG) and inositol-1,4,5-trisphosphate (IP3). DAG activates protein kinase C, and IP3 mobilizes Ca^{2+} from cytosolic Ca^{2+} stores. The regulatory subunit of PI3K binds to phosphotyrosine peptide motifs in receptor protein tyrosine kinases (RTKs) or the insulin receptor substrate 1 (IRS1). Upon binding of the regulatory subunit, the inhibition of the catalytic p110 subunit is relieved. The catalytic subunit converts PIP2 to PIP3. PIP3 binds the pleckstrin homology (PH) domains of AKT and PDK1. Upon phosphorylation by PDK1, AKT is fully activated. PTEN counteracts PI3 kinases by dephosphorylating PIP3 at 3 position. (Wagener and Müller, 2009), with permission.

to as AKT. For properties of individual family members, digits will be used. The PH domain of AKT binds to PIP3 at the inner leaflet of the plasma membrane. In addition to AKT, PIP3 recruits the kinases PDK1 (phosphoinositide-dependent kinase 1). In this location, PDK1 phosphorylates and activates AKT (Figure 8.5). In addition to PDK1, mTOR in complex 2 phosphorylates and activates AKT (see the following text) (Bellacosa *et al.*, 2005; Fruman and Rommel, 2014).

AKT constitutes a central knot in signal transduction. The established substrates of AKT reflect the cellular processes that are regulated by the kinase. AKT interacts with several key proteins of the intracellular signaling network and affects major signaling pathways (Figure 8.6).

Cell survival. AKT inhibits apoptosis by phosphorylating regulatory proteins that bind and inactivate the antiapoptotic protein Bcl-2. Based on their domain structure, these proteins are named BH3 (Bcl-2 homology domain 3)-only proteins. For example, AKT phosphorylates and inhibits the BH3-only protein BAD. The transcription of BH3-only proteins is stimulated by FOXO (forkhead box O) transcription factors. AKT phosphorylates FOXO transcription factors. Upon phosphorylation, 14-3-3 proteins bind FOXO and displace the transcription factors from their promoters. In this way, the synthesis of the proapoptotic BH3-only proteins is blocked. Additionally, AKT phosphorylates and inactivates the ubiquitin ligase Mdm2. Upon phosphorylation, Mdm2 translocates into the nucleus and initiates the degradation of tumor suppressor protein p53. p53

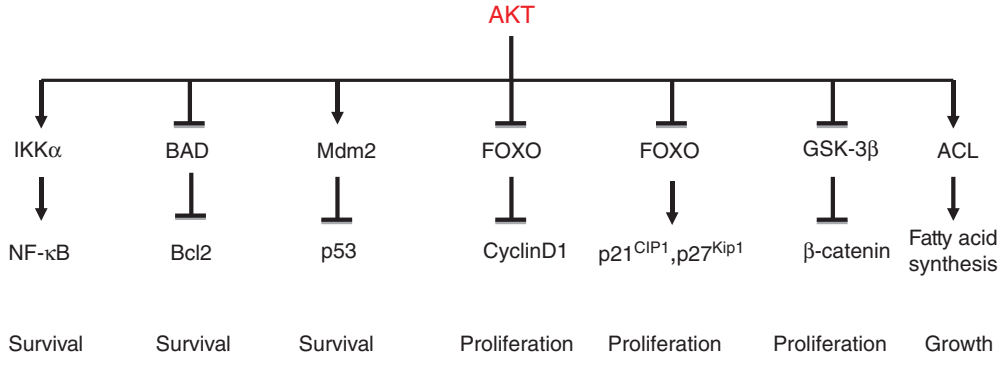

Figure 8.6 Substrates and effects of AKT kinases. For AKT-mTOR interactions see Figure 8.8. (Wagener and Müller, 2009), with permission.

stimulates the synthesis of proapoptotic BH3-only proteins. By degrading p53, activated Mdm2 inhibits their synthesis.

Inhibition of apoptosis is fostered further by cross talk to the NF-kB pathway. Under certain conditions, AKT assists the activation of the IkB kinase, which phosphorylates IkB. Phosphorylation of IkB leads to its proteasomal degradation, in this way liberating the NF-kB transcription factors (see Chapter 10).

Cell cycle. AKT controls the cell cycle at different levels. AKT phosphorylates the cyclin-dependent kinase inhibitor $p27^{Kip1}$. The cytosolic 14-3-3 protein binds phosphorylated $p27^{Kip1}$, thus attenuating its nuclear import and its inhibitory effect on the cell cycle. As mentioned before, AKT inhibits the activity of FOXO transcription factors. FOXO proteins activate the transcription of the CDN1A and *CDN1B* genes, which encode $p21^{CIP1}$, and $p27^{Kip1}$, respectively. Both proteins inhibit the cell cycle (see Chapter 3). On the other hand, FOXO represses the synthesis of cyclin D1. AKT is activated by the mechanistic target of rapamycin complex 2 (mTORC2), which responds to growth factors. Taken together, AKT signaling contributes to cell cycle progression and proliferation.

Cell growth. A cell destined to divide needs amino acids, nucleotides, and fatty acids (collectively, biomass) for building a new cell. AKT directly stimulates the production of lipids, such as fatty acids and cholesterol. The synthesis of these lipids starts from acetyl-CoA (ac-CoA) in the cytosol. After export of citrate from the mitochondrion into the cytosol, ATP-citrate lyase (ACL) catalyzes the conversion of citrate to ac-CoA. AKT phosphorylates and activates ACL, in this way stimulating the synthesis of essential lipids needed for the generation of biological membranes. In AKT-driven tumors, knockdown of the *ACLY* gene, which encodes ACL, slows tumor growth *in vivo*, supporting the importance of the activation of ACL by AKT (Ward and Thompson, 2012). AKT acutely stimulates the uptake of glucose in response to insulin. AKT2, the main AKT protein in insulin-responsive tissues, associates with vesicles containing Glut4

(glucose transporter 4) upon stimulation by insulin. Thus, activated AKT2 supports the translocation of Glut4 to the plasma membrane and thereby the extent of glucose uptake.

8.5 mTOR

The mTOR is a key indirect downstream target of AKT, by which the production of new biomass is stimulated. mTOR is an atypical serine/threonine kinase that comprises several conserved domains (Figure 8.7). The N-terminus possesses 20 tandem HEAT (for Huntington, EF3, A subunit of PP2A, TOR1) repeats, which mediate protein-protein interactions. The C-terminal half contains the kinase domain. In addition, two so-called FAT (for FRAP, ATM, TRAP) domains are present. mTOR forms two distinct complexes, mTORC1 and mTORC2. Both complexes share the mTOR kinase and the so-called LST8 protein, which is thought to facilitate mTOR signaling. The protein RAPTOR (regulatory-associated protein of mechanistic target of rapamycin) is specific to mTORC1, and the protein RICTOR (rapamycin-insensitive companion of mTOR) is specific to mTORC2.

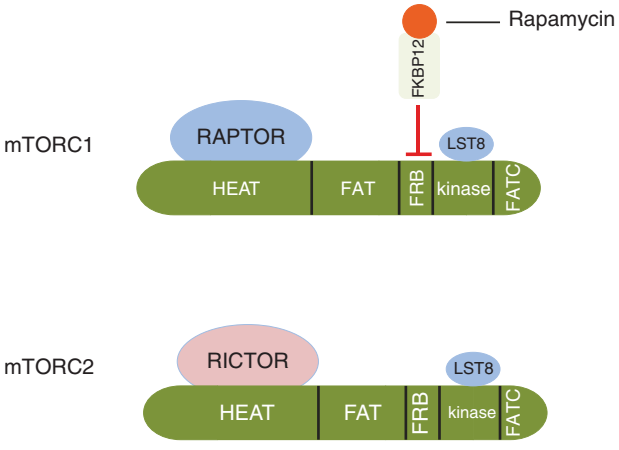

Figure 8.7 mTOR complexes 1 and 2. mTOR is an atypical serine/threonine kinase that comprises several conserved domains. The N-terminus possesses 20 tandem HEAT (for Huntington, EF3, A subunit of PP2A, TOR1) repeats, which mediate protein-protein interactions. The C-terminal half contains the kinase domain. In addition, two so-called FAT (for FRAP, ATM, TRAP) domains are present. Note that mTOR contains a binding domain for the complex of the 12 kD FK506-binding protein (FKBP12) and rapamycin (FKBP12–rapamycin-binding domain (FRB)) both in mTORC1 and mTORC2. However, the complex of FKBP12 and rapamycin inhibits mTOR in mTORC1 only. The LST8 protein binds to the kinase domain and is thought to facilitate mTOR signaling. FATC, C-terminal FAT domain; RAPTOR, regulatory-associated protein of mTOR; and RICTOR, rapamycin-insensitive companion of mTOR.

Rapamycin binds to a protein of 12 kD, which was first identified as binding protein of the immunosuppressive drug FK-506. Accordingly, the protein has been named 12 kD FK506-binding protein (FKBP12). The complex of rapamycin and FKBP12 binds to the FKBP12–rapamycin-binding (FRB) domain of mTOR and directly interacts with and inhibits mTOR when it is part of mTORC1, but not mTORC2. It is thought that the rapamycin–FKBP12 complex disrupts the interaction between RAPTOR and mTOR in complex 1.

mTORC1 simultaneously stimulates the biosynthesis of macromolecules and cell cycle progression; therefore, it is of major importance for the generation of new cells. mTORC2 is stimulated by growth factors and affects cell survival and cytoskeletal reorganization.

The key natural regulator of mTORC1 is a heterodimer consisting of the proteins tuberous sclerosis 1 (TSC1) and TSC2 (Figure 8.8). The TSC1/2 complex acts as a GTPase-activating protein (GAP) for the small GTPase-designated "RAS homolog enriched in brain (Rheb)." In its GTP-binding form, Rheb stimulates the kinase activity of mTORC1. Since the TSC1/2 complex acts as a GAP, it negatively regulates the activity of mTORC1 (Laplante and Sabatini, 2012) (Thomson, Turnquist, and Raimondi, 2009).

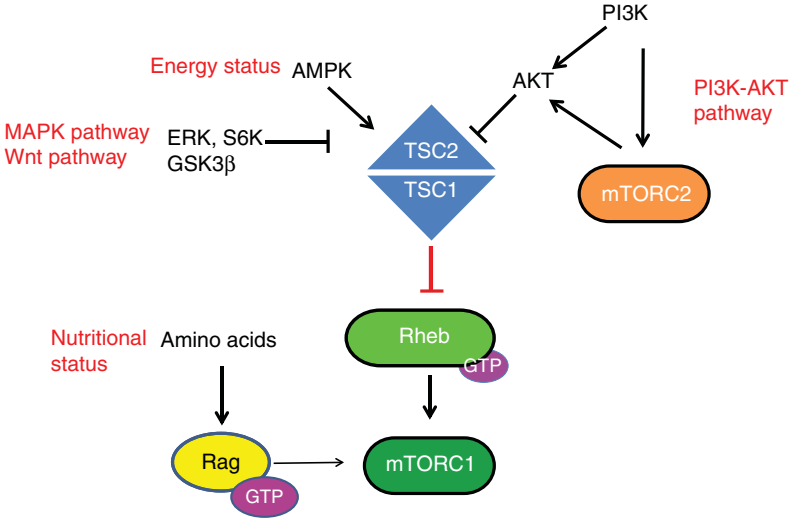

Figure 8.8 Stimulators and inhibitors of the mechanistic target of rapamycin complex 1 (mTORC1) and mTORC2. mTORC1 is stimulated by the small GTPase Rheb (Ras homolog enriched in brain) in its GTP-bound form. Rheb is inhibited by the TSC (tuberous sclerosis complex) 1/2. AKT activates mTORC1 by inhibiting TSC2. In addition, kinases of the MAPK pathway (ERK, S6K) and of the WNT pathway (GSK3β) inhibit TSC2 and activate mTORC1. Adenosine-monophosphate-activated kinase (AMPK) stimulates the GTPase activity of the TSC1/2 complex, thereby inhibiting mTORC1. Lysosomal amino acids induce activation of the small GTPase Rag, which directs mTORC1 to the lysosomal surface. PI3K stimulates mTORC2, which phosphorylates and activates AKT.

8.5.1 mTORC1: Inputs

The TSC1/2 regulation of mTORC1 can be stimulated or inhibited by several pathways, as illustrated in Figure 8.8 and described as follows.

AKT. AKT inhibits the GTPase activity of the TSC1/2 complex by phosphorylating TSC2. As net result, mTORC1 is activated. There exists a second route to mTORC1 activation that is independent of TSC1/2. AKT phosphorylates the RAPTOR subunit of mTORC1, which acts as an mTOR inhibitor. Phosphorylated RAPTOR dissociates from the complex, in this way relieving the inhibition of mTOR (Laplante and Sabatini, 2012).

MAPK and Wnt pathways. The MAPK and WNT pathways feed into the AKT/mTOR pathway. ERK, a kinase in the MAPK pathway (see Chapter 7), phosphorylates and activates the ribosomal S6 protein kinase (S6K). Both kinases phosphorylate TSC2 on residues distinct from AKT phosphorylation sites. Similarly, GSK3β (glycogen synthase kinase 3β), a component of the WNT pathway, can phosphorylate TSC2. By inactivating the GTPase activating activity of the TSC1/2 complex, mTORC1 is activated (Fruman and Rommel, 2014).

Energy status. The synthesis of biomolecules requires energy. mTORC1 senses energy via the adenosine-monophosphate-activated kinase (AMPK). A parameter for the intracellular energy status is the AMP/ATP ratio. A low ratio stimulates the AMP-activated protein kinase. AMPK directly phosphorylates TSC2 and stimulates the GTPase-activating activity of the TSC1/2 complex, thereby inhibiting mTORC1. On the one hand, AMPK stops energy-demanding processes such as protein synthesis, and on the other hand, AMPK stimulates the production of ATP by fatty acid oxidation. AMPK itself is activated by liver kinase B1, abbreviated as LKB1.

Amino acids. TORC1 senses the availability of amino acids, particularly leucine. In the absence of leucine, TORC1-dependent proteins of the translation machinery are inactivated. The sensing of amino acids by mTORC1 initiates from within the lysosomal lumen and requires a signaling complex associated with the lysosomal membrane. The complex contains the Rag GTPase, the so-called regulator complex, and the vacuolar ATPase (V-ATPase). Through a V-ATPase-dependent process, luminal amino acids activate the GEF (guanine nucleotide exchange factor) activity of regulator toward Rag. GTP-binding Rag recruits mTORC1 to the cytosolic lysosomal surface. A protein complex named GATOR1 (GAP activity toward Rag 1) stimulates the GTPases activity of Rag, thereby inhibiting the mTORC1 pathway (Bar-Peled *et al.*, 2013).

8.5.2 mTORC2: Inputs

Growth factors such as IGF1 stimulate mTORC2 by a poorly defined mechanism that requires PI3K. Thus, PI3K is involved in the stimulation of both mTORC1 and mTORC2 (Figure 8.8).

8.5.3 mTORC1: Outputs

The activated TORC1 kinase has several major substrates that effect several biological processes, as illustrated in Figure 8.9 and described as follows.

Protein synthesis. A major activity of mTORC1 is the activation of protein synthesis. The 5′ cap of an mRNA to be translated is bound by the eukaryotic translation initiation factor 4 (eIF4), which consists of different subunits. The subunit eIF4E binds to the 5′ cap structure of the mRNA. The eIF4E-binding protein (4E-BP1) blocks this activity. mTOR directly phosphorylates 4E-BP1, in this way enabling the binding of eIF4E to the 5′ cap of the mRNA and the initiation of translation. mTORC1 also phosphorylates and activates the p70S6 kinase (S6K), which phosphorylates the small ribosomal subunit protein S6, leading to a further increase in the rate of protein synthesis. mTORC1 particularly stimulates the translation of a subset of mRNAs, which have a string of pyrimidines in their 5′ translated region. Among the mTORC1 target proteins are cyclin D1, Myc, and the hypoxia-inducible factor 1 (HIF1) (Manning and Cantley, 2007).

Lipid synthesis. mTORC1 stimulates the transcription factor sterol-regulatory element-binding protein1/2 (SREB1/2), which controls the expression of numerous genes involved in the biosynthesis of fatty acids and cholesterol (Laplante and Sabatini, 2012).

Glucose uptake and glycolysis. In most cell types, Glut1 is the main glucose transporter. The activation of mTORC1 leads to a significant increase in the concentration of HIF1. HIF1 stimulates the transcription of the *SLC2A1* gene, which encodes the Glut1 glucose transporter. By stimulating the expression of genes involved in glycolysis, HIF1 executes major metabolic programs (see Chapter 9).

Autophagy. The mTORC1 pathway promotes growth by negatively regulating autophagy. Autophagy denotes the controlled intracellular degradation of proteins and cellular organelles. The degradation may be limited to a single or a few organelles, but it may also proceed to complete self-digestion and cell death. mTORC1 directly phosphorylates and inhibits the ULK1/Atg13 members of a kinase complex required to initiate autophagy (see Chapter 4).

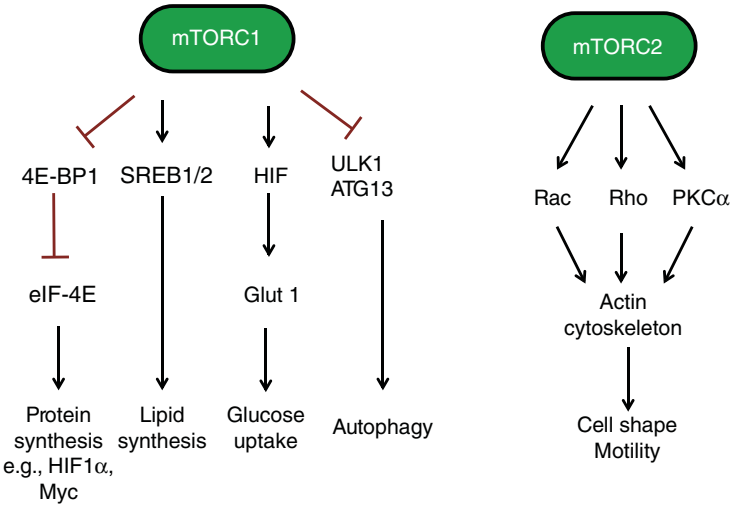

Figure 8.9 Major substrates and effects of mTORC1 and mTORC2.

8.5.4
mTORC2: Outputs

mTORC2 phosphorylates and activates AKT. mTORC2 activates PKCα and the small GTPases Rho and Rac. These proteins affect the actin cytoskeleton. Thus, mTORC2 regulates cell shape and cell movement via the actin cytoskeleton (Figure 8.8).

8.5.5
Feedback Controls

Upon activation, both mTORC1 and S6K can phosphorylate IRS1, which targets IRS1 for degradation. In addition, mTORC1 attenuates receptor protein tyrosine kinase (RTK) signaling by phosphorylating the adapter protein GRB10 (growth factor receptor-bound protein 10). Furthermore, mTORC1 inhibits RTKs such as EGF receptor, ErbB2, and the IGF1/insulin receptor (Fruman and Rommel, 2014; Rodrik-Outmezguine *et al.*, 2011).

8.6
PTEN

(Video: The tumor suppressor PTEN – open website: https://www.youtube.com/watch?v=_EGX8VZFwyo*)*

By dephosphorylating PIP3, PTEN negatively regulates the activity of AKT. However, PTEN has additional function. The four nuclear localization signal (NLS) motifs of PTEN indicate that the protein executes important functions in

the nucleus. Nuclear PTEN dephosphorylates ERK1/2 kinases. As major effectors of the MAPK pathway, ERK1/2 are responsible for activation of several transcription factors. In this way, PTEN inhibits the MAPK pathway. In addition, PTEN assists the repair of DNA double-strand breaks by increasing the concentration of RAD51 in the nucleus. Furthermore, PTEN interacts with the centromere protein C (CENP-C). In summary, PTEN negatively regulates proliferation and cell growth and positively contributes to genomic stability. It is these properties of the PTEN protein that make it a major tumor suppressor gene (Song, Salmena, and Pandolfi, 2012).

8.7
Activation of the PI3K/AKT/mTOR Pathway in Cancer

8.7.1
Sporadic Carcinomas

"The Cancer Genome Atlas (TCGA)" initiative has performed a genome-wide analysis of point mutations and small insertions/deletions across 12 major cancer types (Table 8.2) (Kandoth *et al.*, 2013). The *PIK3CA* gene was the second most commonly mutated gene, right behind *TP53.* Genes of the PI3K/AKT/mTOR pathway were mutated most frequently in endometrial carcinomas of the uterus. Mutations of the *PTEN* gene were most frequent in the brain tumor glioblastoma multiforme. For the AKT1 gene, mutation frequencies were generally low, with the highest fraction in breast cancer (2.5%).

Among the genes of the PI3K/AKT/mTOR pathway, *PIK3CA* is the gene most frequently mutated in human cancer. The gene *PI3KCA* encodes the catalytic

Table 8.2 Relative mutation frequencies[a] (%) of genes encoding proteins of the PI3K/AKT/mTOR pathway.[b]

	PIK3CA	*PTEN*	*PIK3R1*	*PIK3CG*	*MTOR*	*STK11*[c]
Bladder	**17.4**[d]	<5	<5	<5	<5	<5
Breast	**33.6**	<5	<5	<5	<5	<5
Colorectal	**17.6**	<5	<5	<5	<5	<5
Glioblastoma m.	**11.0**	**30.7**	**11.4**	<5	<5	<5
Head and neck squamous	**20.6**	<5	<5	<5	<5	<5
Lung squamous	**14.9**	**8.1**	<5	**7.5**	<5	<5
Lung adeno	<5	<5	<5	**5.3**	**7.5**	**8.8**
Renal clear cell	<5	<5	—	<5	**6.0**	<5
Uterine endometrial	**52.2**	**63.5**	**30.9**	<5	5.2	<5
All	**17.8**	**9.7**	<5	<5	<5	<5

a) Point mutations and small insertions/deletions.
b) Kandoth *et al.*, 2013.
c) Encoding liver kinase B1 (LKB1).
d) Bold print denote incidences above 5%.

p110α subunit of class 1 PI3Ks. The great majority of point mutations affect the catalytic or the helical domain. The point mutations increase the lipid kinase activity of the enzyme (Figure 8.2).

The STK11 gene encodes the LKB1. Inactivating mutations of LKB1 activate mTOR. Additionally, inactivating mutations of genes encoding components of the GATOR1 complex (DEPDC5, NPRL2) activate mTORC1 (Bar-Peled *et al.*, 2013).

In addition to point mutations and small insertions/deletions, genes of the PI3K/AKT/mTOR pathway may be affected by gene amplifications or gene deletions. Amplifications of the *PIK3CA* gene have been observed in major carcinomas such as breast, lung, ovarian, prostate, and thyroid cancer at significant frequencies. Amplification of the *PIK3CB* gene has also been reported. Amplification of the AKT2 gene has been found in 12% of ovarian carcinomas. In contrast, amplifications of the *AKT1* gene are rare (Bellacosa *et al.*, 2005; Thorpe, Yuzugullu, and Zhao, 2015).

8.7.2 Hamartoma Syndromes

(Video: Activation of mechanistic Target OF Rapamycin Complex 1 (mTORC1) in hamartoma syndromes and spontaneous tumors – enhanced ebook and closed website: mTOR_HIF_02_ebook.mp4)

As described earlier, the complex of TSC1 and TSC2 acts as GAP for the small GTPase Rheb. The GTP-binding form of Rheb activates mTORC1. In its GAP function, the TSC1/2 complex inactivates Rheb and, consequently, inactivates mTOR. mTORC1 stimulates the synthesis of major oncogenic proteins such as Myc, cyclin D1, and HIF1.

TSC1 is also known as *hamartin*, and TSC2 as *tuberin*. The designation "hamartin" indicates that the protein plays a role in syndromes associated with the growth of hamartomas. A hamartoma is a tumor that is composed of the tissue elements present in the tissue of origin. Pathways leading to the TSC1/2 complex and the components of the complex themselves have been implicated in syndromes characterized by the growth of hamartomas (Figure 8.10). It has been suggested that the expression of HIF1α mediated by mTORC1 plays a particularly important role in the pathogenesis of hamartomas.

The *STK11* gene, encoding the AMPK inhibitor LKB1, is inactivated in the Peutz–Jeghers syndrome. The Peutz–Jeghers syndrome, also known as *hereditary intestinal polyposis syndrome*, is an autosomal dominant genetic disease characterized by the development of multiple hamartomatous polyps in the gastrointestinal tract. Although hamartomas are benign tumors with an extraordinarily low potential for malignancy, patients with Peutz–Jeghers syndrome have an increased risk to develop malignant tumors including those of breast, thyroid, and uterus. Consequent to the loss of LKB1 activity, the inhibitory action of the TSC 1/2 complex on the synthesis of HIF1α, Myc, and cyclin D1 is alleviated. Inactivating mutations in the *PTEN* tumor suppressor gene lead to the Cowden syndrome. Besides other tumors, such as benign skin

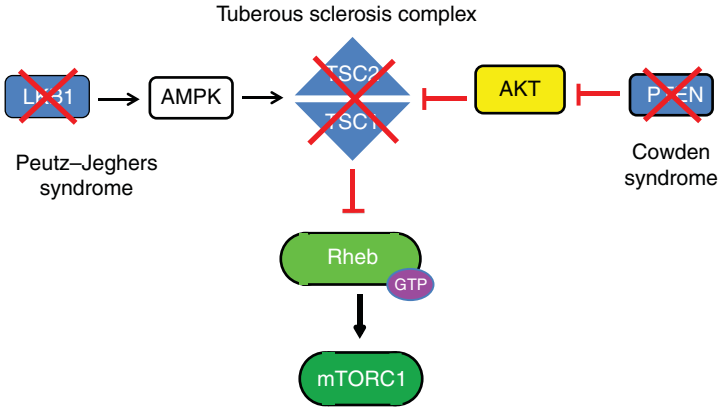

Figure 8.10 Hamartoma syndromes associated with tumor suppressor genes involved in the regulation of mTORC1.

tumors and dysplastic gangliocytoma of the cerebellum, patients with Cowden syndrome develop multiple hamartomas in the gastrointestinal tract. Similar to patients with Peutz–Jeghers syndrome, patients with Cowden syndrome show a significantly increased incidence for different cancers. When PTEN activity is reduced or lost, the activity of AKT increases. Consequently, the inhibitory activity of the TSC 1/2 complex on the synthesis of oncogenic mTORC1 target proteins is blocked. Mutations in either *TSC1* or *TSC2* cause tuberous sclerosis complex, a syndrome also accompanied by hamartomas. The inactivation of either TSC1 or TSC2 leads to increased concentrations of oncogenic mTORC1 targets (Brugarolas and Kaelin, 2004; Laplante and Sabatini, 2012).

8.8 PKC in Cancer

More than three decades of research on PKC have not yet clarified the exact role of PKC in tumorigenesis. In contrast to the hyperactivation of other kinases such as BRaf or AKT, which induces proliferation and tumor growth, PKC hyperactivation might even decrease the risk of tumor development. The question whether kinases of the PKC family are oncoproteins or tumor suppressors can only be answered knowing the isoform and the cellular context. For example, PKCα was found to be upregulated in some tumor types, such as those of bladder and breast, and down-regulated or genetically deleted in others such as colorectal and malignant renal cell carcinoma. As an effector of HER2/Neu in breast cancer cells, PKCα activates proliferation and tumor growth. In contrast, activation of PKCα in non-small-cell lung cancer (NSCLC) cells leads to upregulation of $p21^{CIP1}$ and to inhibition of cell growth and senescence. Results from research on the effects of PKCδ on tumorigenesis are also diverse. Whereas overexpression of the PKCδ encoding gene *PRKCD* inhibits tumor development in a murine skin tumor model, tumor incidence is

reduced by *PRKCD* knockout in a murine lung tumor model. The ambiguous and diversified role of PKC is the main reason why a PKC-inhibiting anticancer drug has not yet reached clinical applications.

8.9 Therapy

Within the PI3K/AKT/mTOR pathway, the following general classes of inhibitors can be distinguished (Fruman and Rommel, 2014):

Pan class I PI3K inhibitors. Pan class I inhibitors inhibit each of class I catalytic subunits. The broad specificity of the kinases implies off-target effects on other kinase families. In addition, therapeutically effective doses may not be tolerable.

Isoform selective PI3K inhibitors. Since the p110α subunit is mutated most frequently in human tumors, the development of inhibitors of this isoform is most advanced. Since the p100α subunit contains mutational hotspots, it may be possible to specifically target this protein in its mutated form, comparable to the BRaf oncoprotein. Since p110α is a major mediator of insulin signaling, the inhibition of p110α is associated with hyperinsulinemia and glucose intolerance. Convincing progress has been made with an inhibitor of the delta subunit (see the following text).

Rapamycin analogs (rapalogs). Rapamycin donated its name to mTOR. Following their use as immunosuppressants, rapamycin and rapalogs (everolimus, temsirolimus, deforolimus) were tested in the treatment of cancer. In most tissues and tumors, rapalogs do not directly inhibit mTORC2. Thus, mTORC2 is still able to activate AKT. Monotherapy with rapalogs extended patients' survival time with renal cell carcinoma, mantle cell lymphoma, and neuroendocrine tumors. The FDA approved rapalogs for the use against these tumor entities. In patients with hormone-positive breast cancer, the combination of everolimus with an aromatase inhibitor showed a statistically significant survival benefit.

pan-PI3K-mTOR inhibitors. Since there is considerable structural homology between the ATP binding sites of PI3Ks and mTOR, compounds can inhibit both kinases. For example, wortmannin, which has been widely used as PI3K inhibitor in cell culture experiments, also inhibits mTOR.

Active-site mTOR inhibitors. Active-site inhibitors are compounds that compete for ATP binding in both mTOR complexes. In this setting, mTORC2 can no longer activate AKT.

AKT inhibitors. AKT inhibitors have been developed that inhibit its activity by competing for ATP binding.

Drugs of each class have been tested or are being tested in clinical trials (www.clinicaltrial.gov). Examples are presented in Table 8.3. An extended list of compounds that affect the PI3K/AKT/mTOR pathway has been published (Fruman and Rommel, 2014).

Table 8.3 Summary of anticancer drugs and exemplified compounds with anticancer potency affecting the PI3K/AKT/mTOR pathway.

Compounds	Target	Tumor
Rapalogs (everolimus, temsirolimus, deforolimus)	mTOR in mTORC1	Renal cell carcinoma, mantle cell lymphoma, neuroendocrine tumors, hormone-positive breast tumor
Ibrutinib	Bruton's kinase	Diffuse large B-cell lymphoma (DLBCL)
Idelalisib + CD20 antibody	p110δ subunit of PI3K	Chronic lymphocytic leukemia (CLL)
Antibody LJM716 + BYL719	erbB3 + p110α	HER2/Neu-positive breast cancer xenograft
NVP-BEZ235 + ARRY-142886	pan-PI3K/mTOR + MEK	Murine lung tumor
Chloroquine + NVP-BEZ235	Autophagosome formation + PI3K/mTOR	Glioma
Exemestane + everolimus	Aromatase + mTOR	Breast cancer

Considering the importance of the PI3K/AKT/mTOR pathway, one would assume that drugs interfering with the pathway are highly efficient in the treatment of major human malignancies. However, this is not the case. Results of initial clinical trials of monotherapies attacking central nodes of the pathway such as PI3-kinases, AKT, or mTOR were disappointing. However, the surprising and long-lasting effects of an inhibitor of the catalytic p100δ PI3K subunit indicate that successful cancer treatment by PI3K inhibition may be possible. Surprisingly, this success has been achieved with a PI3K isoenzyme, which is not mutated in human tumors.

As outlined in Chapter 10, CD19 is an important accessory receptor molecule in B-cell signaling. After antigen binding, the Src kinase Lyn phosphorylates CD19 on tyrosine residues, followed by the binding of the p85/p110δ PI3K isoenzyme to phosphotyrosine-containing peptide motifs. p110δ converts PIP2 to PIP3, which associates with Bruton's kinase (Figure 10.6). Ibrutinib, an inhibitor of Bruton's kinase, is effective in the treatment of diffuse large B-cell lymphoma (DLBCL) and has been certified by the FDA. The PI3K inhibitor idelalisib effectively inhibits the catalytic subunit p110δ. Idelalisib was tested as monotherapy in patients with indolent non-Hodgkin's lymphoma. The efficacy of idelalisib was similar or superior to those of other treatment options. In relapsed chronic lymphocytic leukemia, idelalisib in combination with a CD20 monoclonal antibody was compared to the application of the antibody alone. The overall response rate with the idelalisib combination was 81% versus 13% in the control group (Fruman and Cantley, 2014).

Outside of B-cell tumors, trials testing single inhibitors have delivered disappointing results. Why is this the case? So far, we used the term pathway for the

PI3K/AKT/mTOR system. However, as outlined earlier, the system is rather a network than a pathway, with many inputs, extensive cross talks, feedback circuits, and redundancies. In order to overcome the intricacies of the network, rational combination therapies have to be designed that are based on specific molecular properties of the tumor to be attacked.

In preclinical trials, combination therapies effectively inhibited tumor growth:

- As outlined earlier, PI3K/AKT/mTORC1 signaling mediates a negative feedback on RTK activity and signaling. Inhibiting p110α will relieve this block. Consequently, a combination of the ErbB3-neutralizing antibody LJM716 and the p110α-selective inhibitor BYL719 inhibited the growth of ErbB2 (HER2/Neu)-positive breast cancer xenografts (Figure 8.11a) (Garrett *et al.*, 2013).
- Ras activates PI3K/AKT/mTORC1 signaling via the MAPK pathway and the PI3K pathways. Murine lung cancer driven by mutant KRas did not substantially respond to a dual-pan-PI3K/mTOR inhibitor (NVP-BEZ235). However, in combination with a MEK inhibitor (ARRY-142886), the tumors shrank significantly (Figure 8.11b) (Engelman *et al.*, 2008).
- As outlined earlier, mTORC1 inhibits the formation of autophagosomes. The antimalarial agent chloroquine impairs the degradation of proteins in the phagosome. Single-agent therapy with either the dual PI3K/mTOR inhibitor NVP-BEZ235 or chloroquine slowed tumor growth. However, the combination of both agents caused tumor regression because of apoptosis of the tumor cells (Figure 8.11c) (Fan *et al.*, 2010).

Meanwhile, combination therapy has entered the clinic. In a phase 3 trial, the aromatase inhibitor exemestane was applied as single agent or in combination with the rapalog everolimus in patients with breast cancer. Everolimus combined with the aromatase inhibitor significantly increased progression-free survival (Baselga *et al.*, 2012).

8.10 Outlook

Because of the many inputs and outputs of the PI3K-AKT-mTOR pathway, monotherapies that target the pathway in major human malignancies have not been successful in the past. However, there are examples of successful combinatorial therapies, both in preclinical and clinical settings. For a successful therapy, the cross talk between the PI3K-AKT-mTOR pathway and other tumorigenic pathways should be analyzed in the patient's tumor sample prior to beginning therapy. This diagnostic approach may lead to the selection of highly effective individualized drug combinations.

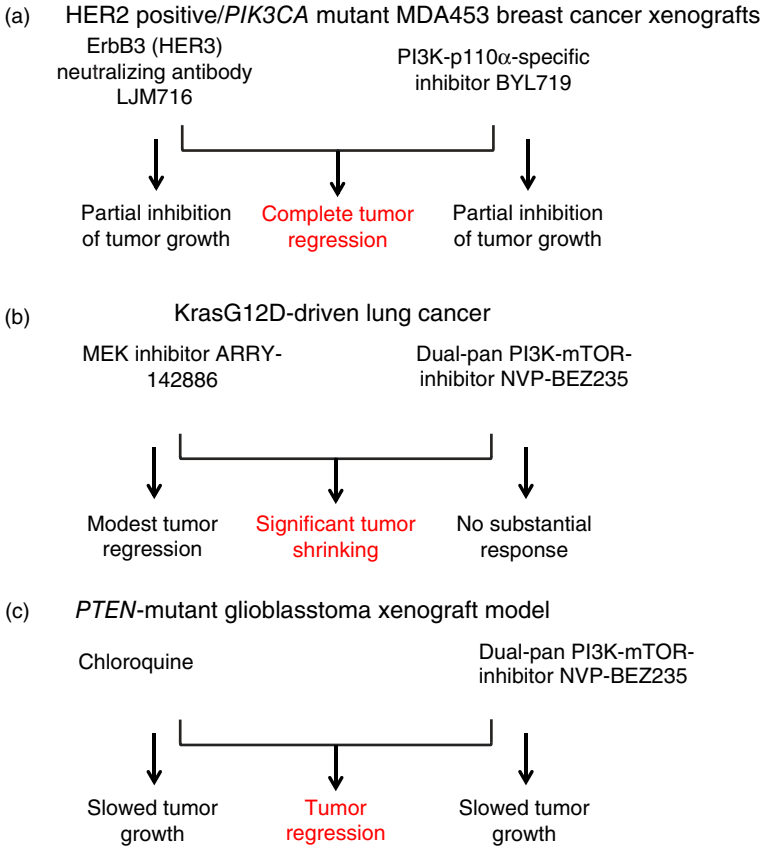

Figure 8.11 Preclinical models of combinatorial inhibition of the PI3K/AKT/mTOR pathway. (a) Trastuzumab-resistant HER2/Neu-positive/*PIK3CA* mutant MDA453 xenografts regressed completely after 3 weeks of combinatorial therapy with the ErbB3-inhibiting monoclonal antibody LJM716 and the PI3K p110α-specific inhibitor BYL719, whereas monotherapies inhibited growth only partially (Garrett *et al.*, 2013). (b) In a KRasG12D-driven lung cancer mouse model, inhibitors of the MAPK and PI3K/AKT/mTOR pathways were applied either as single agent or in combination. Whereas treatment of KRas-mutant mice with the MEK inhibitor ARRY-142886 led to only modest tumor regression and the dual-pan-PI3K-mTOR inhibitor NVP-BEZ235 did not lead to a substantial response at all, there was marked synergy when both drugs were combined (Engelman *et al.*, 2008). (c) The antimalarial agent chloroquine inhibits autophagosome maturation, and the dual-pan-PI3K-mTOR inhibitor NVP-BEZ235 induces autophagy. In a xenotransplant model of the PTEN-mutant GS2 glioblastoma cell line, single-agent therapy slowed tumor growth. When the agents were combined, tumor cells underwent apoptosis and the tumor regressed. (Based on results published by Fan *et al.* (2010).)

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9
Hypoxia-Inducible Factor (HIF)

Summary

Hypoxia-inducible factor (HIF) is a protein dimer, in which one of three different α chains combines with one of two β chains. Among HIFs, HIFs containing the HIF1α and HIF1β chains are the best characterized. Under normoxic conditions, members of the prolyl hydroxylase domain (PHD) family of dioxygenases hydroxylate two critical proline residues in the HIFα chains. When either a single or both

Cancer Signaling: From Molecular Biology to Targeted Therapy, First Edition.
Christoph Wagener, Carol Stocking, and Oliver Müller.

Companion Website:www.wiley.com/go/wagener/cancersignaling

of the critical proline residues have been hydroxylated, the von Hippel–Lindau protein (pVHL) binds to HIFα. The pVHL associates with an ubiquitin ligase complex, which induces the attachment of ubiquitin to HIFα. Polyubiquitinated HIFα is degraded by the 26S proteasome. In addition to the proline residues, HIFα proteins contain a critical arginine residue in the C-terminal transactivation domain. The residue is hydroxylated by a dioxygenase, designated "factor inhibiting HIF1α" (FIH1). The hydroxylated arginine residue inhibits the association of the p300 and CBP transcriptional coactivators with HIFα.

Under hypoxic conditions, the α chain is no longer hydroxylated and HIFα is stabilized. In addition to oxygen, oncogenic pathways can upregulate and activate HIF. Spontaneous or inherited mutations leading to the inactivation of the *VHL* tumor suppressor gene are often associated with clear cell renal cell carcinoma (CCRCC). In the absence of pVHL, HIF is constitutively active. HIF is also upregulated in many other types of human carcinomas in response to hypoxia and/or active oncogenic pathways. HIF contributes to tumor progression by assisting cell survival, angiogenesis, connective tissue remodeling, and invasive tumor growth.

9.1 Responses of HIF to Hypoxia and Oncogenic Pathways

(Video: Hypoxia-Inducible Factors (HIFs). Part 1: Tumor hypoxia – only closed website: HIF_01_01_ebook.mp4)

Cells within growing tissues need a constant supply of oxygen. Under physiological conditions, the delivery of oxygen is well regulated; under pathological conditions, however, the availability of oxygen may be limited. For example, severe hypoxia may develop in rapidly growing tumors. In small tumors with a diameter of less than 1 mm, oxygen may be supplied by diffusion, thus the fraction of oxygen in small tumors corresponds to the fraction in normal tissues. When tumor growth continues, the oxygen levels drop in the tumor areas distant from blood vessels. When oxygen levels drop below 0.02%, tumor cells experience severe hypoxia and die. In Figure 9.1, a stained section of a tumor is shown. The tumor contains vital tissue areas. However, in other areas of the tumor, the tissue became necrotic because of insufficient supply of oxygen and nutrients.

When cells experience shortage of oxygen, they produce a family of transcription factors classified under the term hypoxia-induced factor (HIF). HIF takes measures to guarantee the survival of cells at low oxygen pressure. HIF induces the growth of new blood vessels from existing blood vessels (angiogenesis), which deliver oxygen and nutrients. For growth of new blood vessels, the extracellular matrix (ECM) is remodeled. HIF may assist the invasion of tissue areas with more favorable oxygen conditions. Furthermore, HIF directs the cellular metabolism to adapt to the limiting oxygen pressure.

Many effects of HIF such as angiogenesis, ECM remodeling, and invasive growth are properties of malignant cells. Oncogenic pathways may induce these

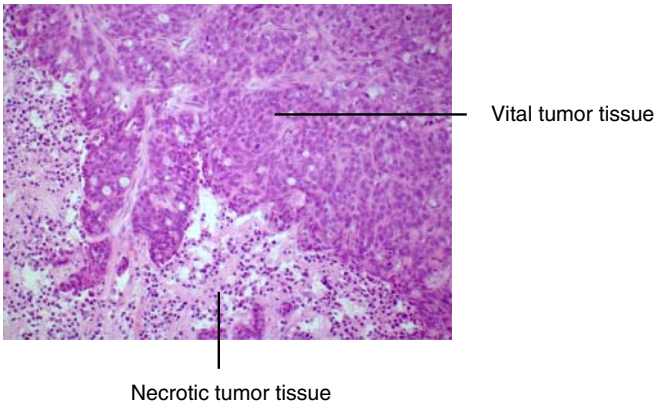

Figure 9.1 Tumor necrosis. Both vital and necrotic areas of tumor tissue are observed in a subcutaneous xenotransplant of a human colonic carcinoma cell line. (Courtesy of Professor Udo Schumacher, Hamburg.)

malignant traits by activating HIF. However, some effects of HIF such as the inhibition of proliferation by HIF1α contradict the malignant phenotype. In these situations, oncogenic pathways override the antitumorigenic effect of HIF. In order to understand the contribution of HIF to the malignant phenotype, its action has to be considered in the context of oncogenic pathways.

9.2 HIF Functional Domains

The HIF is a transcription factor, which consists of an α subunit and a β subunit. One of three α subunits dimerizes with one of two β subunits. The α subunits are sensitive to oxygen, whereas the β subunits are not. Among the α subunits, the functions of HIF1α and HIF2α are best understood. Alternatively spliced mRNA species encode a number of HIF3α isoforms. The fact that all three HIFα isoforms are conserved in metazoans indicates that the different isoforms exert distinct functions. This is supported by the observation that targeted deletion of the *Hif1a* gene in mice results in embryonic death, whereas Hif2α deficiency causes embryonic and perinatal lethality or severe developmental abnormalities (Keith, Johnson, and Simon, 2012). In the following, the term HIFα will be used when the mentioned properties apply to both HIF1α and HIF2α (EPAS1). In referring to a particular property of an α subunit, the designations HIF1α or HIF2α will be used.

In Figure 9.2a, the domain structure of an HIF1α subunit is shown. The subunit contains an N-terminal basic helix-loop-helix motif that associates with the DNA. Toward the C-terminus, two transactivation domains can be distinguished. These domains guide the transcription of HIF target genes into mRNA. Besides the helix-loop-helix domain, an adjacent domain toward the C-terminus is essential for

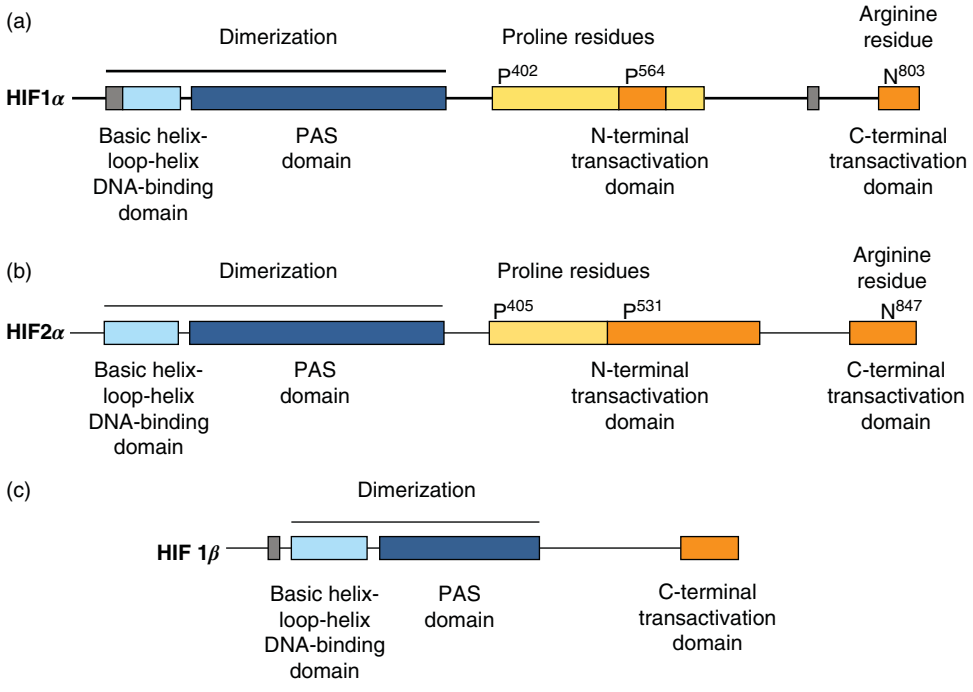

Figure 9.2 (a) Domain structure of HIF1α. The PAS domain is an evolutionary conserved dimerization motif. (b) Domain structure of HIF2α. (c) Domain structure of HIF1β.

dimerization with a HIFβ subunit. The proline residues indicated in the figure are important for the stability of the protein, and the arginine residue regulates transcriptional activity. The HIF2α subunit shares the same domains as HIF1α (Figure 9.2b). HIF1β (ARNT) contains the basic helix-loop-helix domain, which is part of the dimerization motif. The protein contains only one transactivation domain (Figure 9.2c).

9.3 Regulation of HIF

9.3.1 Regulation of HIF under Normoxic Conditions

(Video: Hypoxia-Inducible Factors (HIFs). Part 2: Regulation of HIF under normoxic and hypoxic conditions – only closed website: HIF_01_02_ebook.mp4)

HIFα subunits contain two proline residues that are critical for the stability of proteins. For simplicity, one instead of two residues is shown in Figure 9.3. Normally, the concentration of molecular oxygen in most human tissues is in the

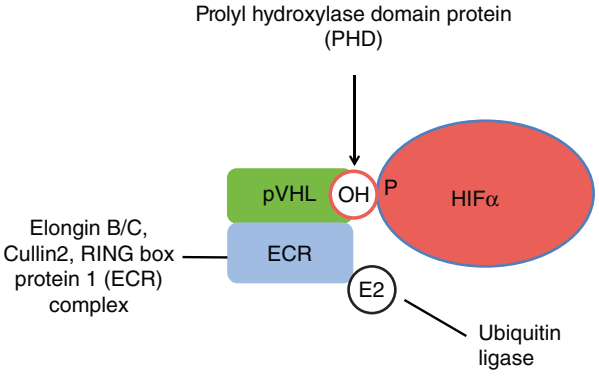

Figure 9.3 Hydroxylation of HIF1α by prolyl hydroxylase domain (PHD) dioxygenases. A PHD dioxygenase hydroxylates one or two prolyl residues in HIFα. pVHL associates with the hydroxy-prolyl residue. The pVHLα domain assembles the so-called ECR complex consisting of Elongin B and C, Cullin 2, RING box protein 1 and an E2 ubiquitin ligase, resulting in ubiquitination and proteasomal degradation of HIFα (Greer *et al.*, 2012).

range of 10–30 μM. Under these normoxic conditions, a member of the prolyl hydroxylase domain family (PHD) hydroxylates one or both of the critical proline residues in HIFα proteins. PHD2 (encoded by the EGLN1 gene) is the major hydroxylase of HIF1α, whereas PHD3 (encoded by the EGLN3 gene) is relatively more effective in hydroxylating HIF2α. The PHDs are dioxygenases. These are enzymes that incorporate both atoms of molecular oxygen into their products. One oxygen is incorporated into a proline residue, and the second oxygen atom is used in the oxidative decarboxylation of α-ketoglutarate, an intermediate of the tricarboxylic acid (TCA) cycle (Figure 9.4). PHD dioxygenases depend on bivalent iron. The products of the reaction are a hydroxyproline residue, carbon dioxide, and succinate, also an intermediate of the TCA cycle. The K_m values of PHD dioxygenases are considerably higher than the concentration of intracellular pO_2. Thus, cellular pO_2 affects PHD enzymes across the entire physiological range. Fumarate and succinate, both metabolites of the tricarboxylic cycle, inhibit the activity of PHD enzymes (Kaelin and Ratcliffe, 2008; Majmundar, Wong, and Simon, 2010).

When a single or both of the critical proline residues have been hydroxylated, the von Hippel–Lindau protein (pVHL) binds to HIFα (Figure 9.3). The *VHL* gene is a tumor suppressor gene. Inactivating germline mutations of the gene predispose individuals to tumors such as clear cell renal cell carcinomas (CCRCCs) and hemangioblastoma (see the following text). The pVHL associates with a ubiquitin ligase complex that causes the attachment of ubiquitin to HIFα. Polyubiquitinated HIFα is degraded by the 26S proteasome (Greer *et al.*, 2012).

In addition to the proline residues, HIFα proteins contain a critical arginine residue in the C-terminal transactivation domain. The residue is hydroxylated by a dioxygenase designated factor inhibiting HIF1α (FIH1) (Figure 9.5). Similar

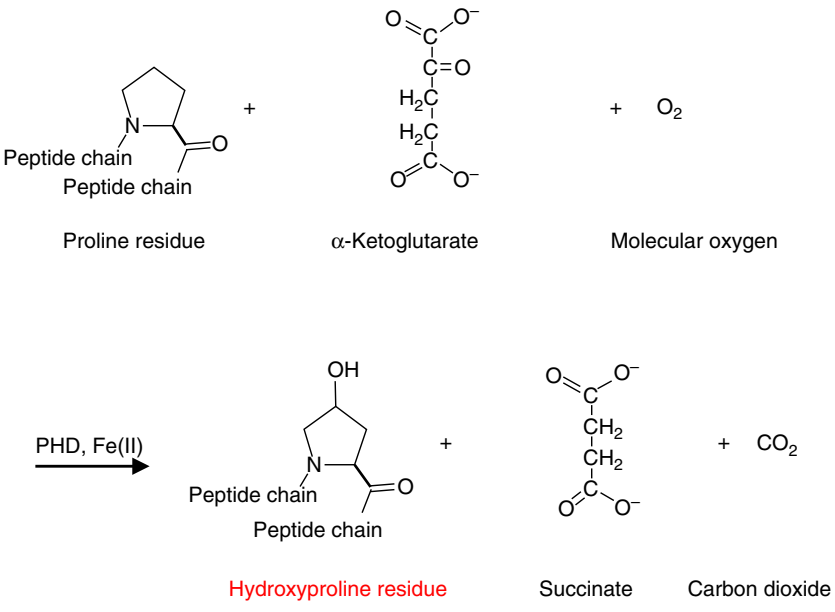

Figure 9.4 Reaction catalyzed by the prolyl hydroxylase domain (PHD) dioxygenases.

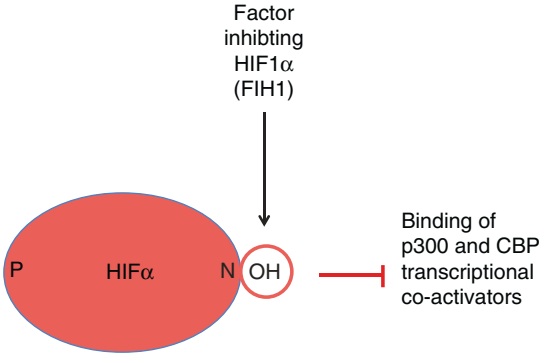

Figure 9.5 Hydroxylation of an arginine residue in the C-terminal transactivation domain of HIF1α by factor inhibiting HIF1α (FIH1). Hydroxylation blocks the binding of the transcriptional coactivators p300 and CBP.

to the PHDs, FIH1 incorporates both atoms of molecular oxygen into its product. One oxygen atom is used in the oxidative decarboxylation of α-ketoglutarate, and the second atom is incorporated into the arginine residue. The products of the reaction are a hydroxyarginine residue, carbon dioxide, and succinate. The hydroxylated arginine residue inhibits the association of the p300 and CBP transcriptional coactivators with HIFα.

9.3.2 Regulation of HIF under Hypoxic Conditions

(Video: Hypoxia-Inducible Factors (HIFs). Part 2: Regulation of HIF under normoxic and hypoxic conditions – only closed website: HIF_01_02_ebook.mp4)

Under hypoxic conditions, the activity of PHD dioxygenases decreases. As a consequence, the proline residues of HIF are no longer hydroxylated. A HIFα subunit associates with a HIFβ subunit. The heterodimer binds to the consensus sequence C/ACGTG in DNA. Since FIH1 no longer hydroxylates the arginine residue in the C-terminal transactivation domain of HIFα, the p300 and CBP coactivators are able to bind. The heterodimer activates the transcription of target genes (Figure 9.6). HIFα proteins are stabilized at different oxygen concentrations. HIF2α is stabilized at moderate concentrations, whereas HIF1α accumulates only at lower concentrations of O_2.

9.3.3 Oxygen-Independent Regulation of HIF

As detailed in Chapter 8, the mechanistic target of rapamycin (mTOR) pathway coordinates a variety of environmental cues to regulate growth and homeostasis. mTOR interacts with several proteins to form two distinct complexes, mTOR complex 1 (mTORC1) and mTORC2. Major inputs sensed by mTORC1 are cellular stress, energy level, and amino acid content. mTORC1 particularly stimulates the translation of a subset of mRNAs that have a string of pyrimidines in their 5′ translated region. One of the major mTORC1 target proteins is the HIF1 (Manning and Cantley, 2007).

In addition to oxygen, metabolites regulate the activity of PHD family hydroxylases (Figure 9.7). Succinate and fumarate, both metabolic intermediates of the TCA cycle, inhibit the activity of all three PHDs. Fumarate is metabolized by fumarate hydratase and succinate by succinate hydrogenase. Inactivation of these enzymes leads to high cellular levels of fumarate or succinate, respectively.

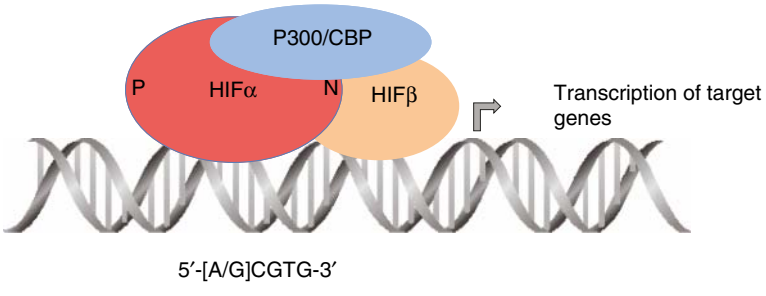

Figure 9.6 Binding of a HIFα/HIFβ dimer to consensus sequences in the DNA in the absence of oxygen. After association with transcriptional coactivators, the HIF dimer activates transcription of target genes.

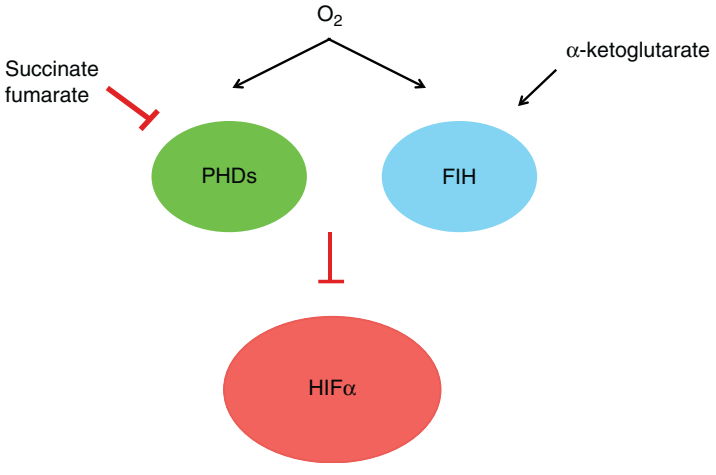

Figure 9.7 Regulation of prolyl hydroxylase domain (PHD) family dioxygenases and factor inhibiting HIF1α (FIH1) by metabolites of the tricarboxylic acid (TCA) cycle.

9.3.4 Context-Dependence of HIF Regulation

All human cells require a constant supply of oxygen to carry out oxidative phosphorylation in mitochondria. As pointed out by Gregg L. Semenza, who first described HIF, cells generally respond in one of three ways when oxygen is reduced (Semenza, 2013):

- Cell proliferation is inhibited to prevent any further increase in the number of O_2-consuming cells.
- The rate of oxidative phosphorylation is decreased and the rate of glycolysis is increased in order to decrease O_2 consumption per cell.
- The production of angiogenic factors is increased in order to increase O_2 delivery.

In the context of malignant disease, two additional responses should be mentioned:

- Tumor cells activate antiapoptotic signals to escape death.
- Tumor cells invade the surrounding tissue to escape O_2 deficiency.

Some of the responses mentioned are compatible with tumor growth. However, inhibition of proliferation contradicts the behavior of tumor cells. In addition, the increase of glycolysis at the expense of oxidative phosphorylation poses problems to the generation of new cells, which need biomass in the form of proteins, nucleotides, and lipids.

In principle, the increase in HIF concentration and activity can be a consequence or a cause of tumor growth. Hypoxia in tumors induces the expression of

HIF. In this case, the increased activity of HIF is the consequence of malignancy. Tumors may contain well-oxygenated areas, which are close to blood vessels, as well as hypoxic areas further apart from blood supply. Thus, the regulation of HIF is often heterogeneous within tumors.

On the other hand, oncogenic pathways activate HIF. In this scenario, HIF may execute programs that lead to tumor growth. Thus, there may be a causal relationship between HIF activity and malignant transformation. As pointed out in Chapter 2, tumor cell clones within a solid tumor may be heterogeneous. In some clones, pathways may be activated that activate HIF. In other clones, pathways may be activated that circumvent the need for HIF.

In summary, the expression and function of HIF in the progression of malignant disease are cell and context dependent. Conclusions that apply to a particular tumor and situation may not apply to other tumors and other situations.

9.4
Regulation of HIF in Malignant Disease

9.4.1
Expression of HIF in Human Tumors

The presence of HIF1α and HIF2α can be observed in tumor sections by immunohistochemical methods. In many tumor types, upregulation of HIF1α, HIF2α, or both of these factors correlates with a poor prognosis of patients. These observations hold true for major carcinomas, such as carcinoma of the breast, bladder, colorectum, pancreas, and prostate. In breast cancer, the expression of HIF1α in early stage diseases is accompanied by a significantly increased mortality rate compared to tumors with low expression levels (Semenza, 2010). In certain cancers such as non-small-cell lung cancer, head and neck squamous cell carcinoma, and neuroblastoma, however, HIF1α expression correlates with lower cancer stage or decreased patient mortality (Bertout, Patel, and Simon, 2008).

9.4.2
von Hippel–Lindau Disease

There are few, if any, indications that genes encoding HIF transcription factors are mutated in human malignant tumors. However, the protein VHL, which mediates the proteasomal degradation of HIFα, acts as a tumor suppressor. Germline mutations predispose to the von Hippel–Lindau (VHL) disease, which is associated with CCRCC and other tumors (Kaelin, 2007). In addition, somatic mutations of the *VHL* gene have been identified as drivers of CCRCC (Gerlinger *et al.*, 2012).

Eugen von Hippel was a German ophthalmologist who first described angiomas of the retina; the Swedish physician Lindau found similar angiomas

in the spinal cord. The hereditary disease of patients presenting with these and additional symptoms was designated VHL disease. In addition to CCRCC, VHL patients are at an increased risk for hemangioblastomas, pheochromocytomas, pancreatic islet cell tumors, and additional rare tumors. Linkage analysis in families with VHL disease led to the identification of the *VHL* gene in 1993 (Latif *et al.*, 1993). Clinically, the VHL disease is transmitted in an autosomal dominant manner. However, following the Knudson hypothesis of tumor suppressor genes, both alleles of the tumor suppressor gene are inactivated in CCRCC.

The VHL protein contains one α domain and one β domain. The β domain binds HIFα transcription factors and the α domain nucleates a multiprotein complex containing elongin B, elongin C, cullin2, and the RING box protein 1 (Rbx1). Based on these properties, the VHL protein complex targets HIFα for proteasomal degradation (Figure 9.3).

Several lines of evidence indicate that the lack of proteasomal degradation of HIFα, particularly HIF2α, contributes to the pathogenesis of CCRCC (Kaelin, 2008):

- $VHL^{-/-}$ CCRCC regularly exhibits high levels of either both HIF1α and HIF2α or HIF2α alone.
- In xenograft studies, overproduction of HIF2α (but not HIF1α) is sufficient to override the tumor suppressor function of VHL.
- Silencing of the *EPAS1* gene, which encodes HIF2α, suppresses the growth of $VHL^{-/-}$ tumor cells.

Some properties of HIF2α may explain its higher contribution to tumorigenesis. In contrast to HIF2α, HIF1α remains susceptible to proteasomal degradation in $VHL^{-/-}$ cells, possibly by interacting with ubiquitin ligases other than the pVHL protein complex. In addition, HIF2α cooperates with the Myc oncoprotein, whereas HIF1α acts as Myc antagonist (see the following text).

The aforementioned findings indicate that HIFα, particularly HIF2α, are essential in the pathogenesis of $VHL^{-/-}$ tumors. However, functions of pVHL independent of HIF, such as maintenance of the primary cilium and regulation of apoptosis, may contribute to tumorigenesis (Kaelin, 2007).

9.5 HIF Targets in Cancer

9.5.1 Target Genes of HIF1α and HIF2α

HIFs interact with a number of signaling pathways such as Notch, Wnt, and Myc. With respect to the Myc signaling network, HIF1α and HIF2α act differently. HIF1α, but not HIF2α, inhibits Myc by displacing Myc from promoters. As a consequence, the inhibition of Myc-repressed genes such as genes encoding cyclin-dependent kinase inhibitors p21 and p27 is relieved and Myc-activated

Table 9.1 Target genes of HIF1α and HIF2α.[a),b)]

Gene (protein)	Function	HIF1α target gene	HIF2α target gene
SLC2A1 (GLUT1)	Glucose transporter	+	+
PLIN2 (ADRP)	Lipid metabolism	+	+
VEGFA (VEGFA)	Angiogenesis	+	+
LOX (LOX)	Cross-linking of collagen	+	+
BCL2 (BCL2)	Antiapoptotic	+	−
BIRC5 (BIRC5)	Antiapoptotic	+	−
HK2 (hexokinase 2)	Glycolysis	+	−
LDHA (LDHA)	Glycolysis	+	−
MCT4 (monocarboxylate transporter 4)	lactate excretion	+	−
LOX (LOX)	Cross-linking of collagen	+	−
TWIST1 (TWIST1)	EMT[c)]	+	−
EPO (erythropoietin)	Erythropoiesis	−	+
TGFA (TGFα)	Growth factor	−	+
CCND1 (cyclinD1)	Cell cycle progression	−	+
DLL4 (DLL4)	NOTCH signaling, EC[c)] branching	−	+
*POU5F1 (*OCT4)	Stem cell pluripotency	−	+

a) Expression of EPO was tested in kidney and liver cells, the expression of DLL4 in endothelial cells. The expression of the remaining genes was tested in renal cell carcinoma cells.
b) Keith, Johnson, and Simon (2012) and Pawlus, Wang, and Hu (2014).
c) EMT, epithelial–mesenchymal transition and ECs, endothelial cells.

genes encoding cyclin D2 and E2F are downregulated. This may be the reason that HIF1α exerts an antiproliferative effect. In contrast, HIF2α affects Myc activity agonistically.

In addition to intervening in other signaling pathways to regulate gene activity, HIFs also bind DNA directly to activate gene transcription. Some genes can be activated by both HIF1α and HIF2α, whereas others are activated by either HIF1α or HIF2α (Table 9.1). Chromatin immunoprecipitation, followed by high-throughput DNA sequencing (ChIP-Seq technique), revealed approximately 500 high-affinity sites for HIF1α and/or HIF2α.

9.5.2 HIF Target Genes Affecting Tumor Growth

HIF target genes affect various aspects of the behavior of malignant cells and tumors, as outlined as follows (Figure 9.8) (Keith, Johnson, and Simon, 2012).

The products of several HIF target genes regulate *cell proliferation and survival.* HIF2α increases oxygen supply by stimulating the production of erythropoietin (EPO), an important growth and survival factor for red blood cells. The

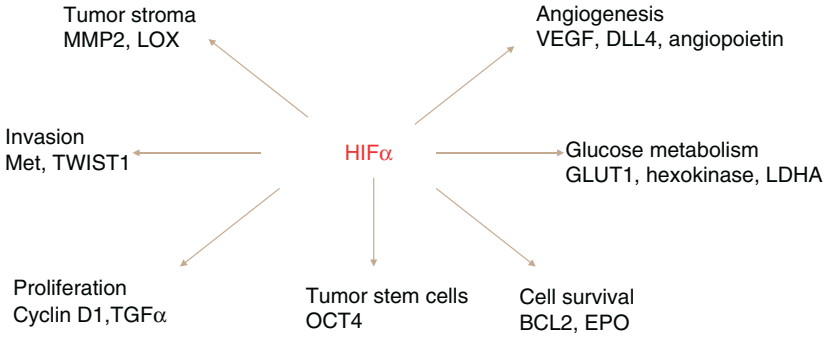

Figure 9.8 Products of HIFα-regulated target genes.

antiapoptotic *BCL2* gene is a target of HIF2α. The EGF receptor and its ligand TGFα are central players in the growth of carcinomas. HIF2α activates the *EGFR* and *TGFA* genes that encode these factors respectively.

As explicated earlier, tumors need blood vessels for the supply of oxygen and nutrients when their diameter exceeds 1 mm. Both HIF1α and HIF2α activate the transcription of the gene encoding VEGF (vascular endothelial growth factor) (*VEGFA*). By binding to its receptor, VEGF stimulates *angiogenesis*, which means the growth of new blood vessels branching from existing blood vessels. HIF2α stimulates the expression of the NOTCH ligand DLL, which induces the branching of endothelial cells (see Chapter 12).

In order to escape low oxygen pressure, tumor cells invade the surrounding tissue. HIF stimulates the *invasive growth* of tumor cells. Often, epithelial cells acquire a fibroblastoid phenotype, a phenomenon known as *epithelial–mesenchymal transition (EMT)*. One of the HIF target genes encodes MET, the receptor of hepatocyte growth factor (HGF). HGF is also known as *scatter factor*, since it reduces the adhesive properties of cells organized in epithelia. HIF2α stimulates the transcription of the *TWIST1* gene, the product of which induces epithelial–mesenchymal transition by downregulating E-cadherin.

In solid tumors, blood vessels need a connective tissue scaffold, the so-called *tumor stroma* to reach the tumor cells. Growing tumors permanently reorganize their stroma in order to guarantee their blood supply. *MMP2*, a HIF1α target gene, encodes matrix metalloproteinase 2, which degrades collagens, laminin, fibronectin, and other component of the ECM. In the reconstitution of collagenous matrix, collagen fibers are cross-linked by lysyl oxidase (LOX), which is also a product of a HIF1α target gene (Keith, Johnson, and Simon, 2012; Semenza, 2003, 2010).

HIF2α stimulates the expression of the gene *POU5F1*, which encodes a transcription factor involved in the *maintenance of stem cell pluripotency.* These and other observations led to the hypothesis that HIF2α is involved in the regulation of tumor stem cells. Indeed, HIF depletion in $CD133^+$ glioblastoma cells, which are enriched in tumor stem cell, reduced their tumorigenic and angiogenic potential (Li *et al.*, 2009).

9.5.3 HIF Target Genes Affecting Metabolism

9.5.3.1 Glucose Uptake and Metabolism

(Video: Hypoxia-Inducible Factors (HIFs). Part 3: Effects of Hypoxia Inducible Factors (HIFs) – only closed website: HIF_03_ebook.mp4)

The *SCLC2A1* gene, which encodes a type 1 glucose transporter (GLUT1), responds to both HIF1α and HIF2α. Thus, both factors increase the cellular uptake of glucose (Figure 9.9).

HIF1α activates the transcription of genes encoding enzymes involved in glycolysis (Figure 9.10). HIF1α stimulates the synthesis of hexokinases 1 and 2. Hexokinase is one of the key enzymes in the glycolytic pathway. Because of its low K_m value, glucose that has entered the cell is phosphorylated with maximum speed. Under physiological conditions, the reaction is irreversible. The next enzyme stimulated by HIF1α is phosphofructokinase. Since phosphofructokinase regulates the throughput of glucose, it acts as a pacesetter of glycolysis. The formation of fructose-1,6-bisphosphate from fructose-6-phosphate catalyzed by phosphofructokinase is irreversible. Fructose-1,6-bisphosphate aldolase is the next glycolytic enzyme stimulated by HIF1α. Finally, HIF1α stimulates the synthesis of phosphoglycerate kinase. In the reaction catalyzed by phosphoglycerate kinase, ATP is generated from ADP without the consumption of oxygen. It is evident that HIF1α regulates the synthesis of essential glycolytic enzymes.

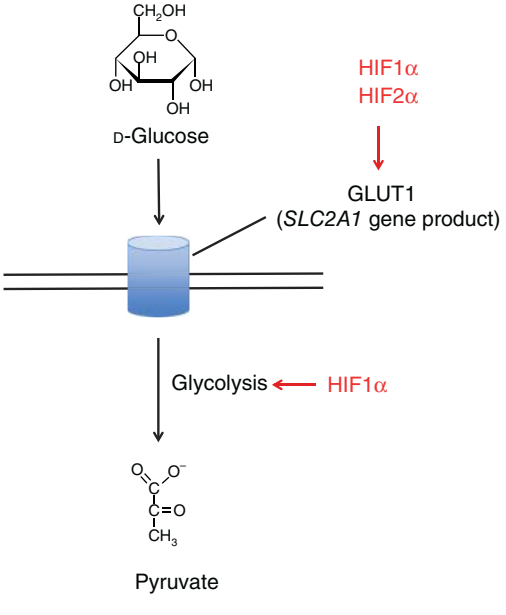

Figure 9.9 Regulation of glucose uptake and glycolysis by HIF.

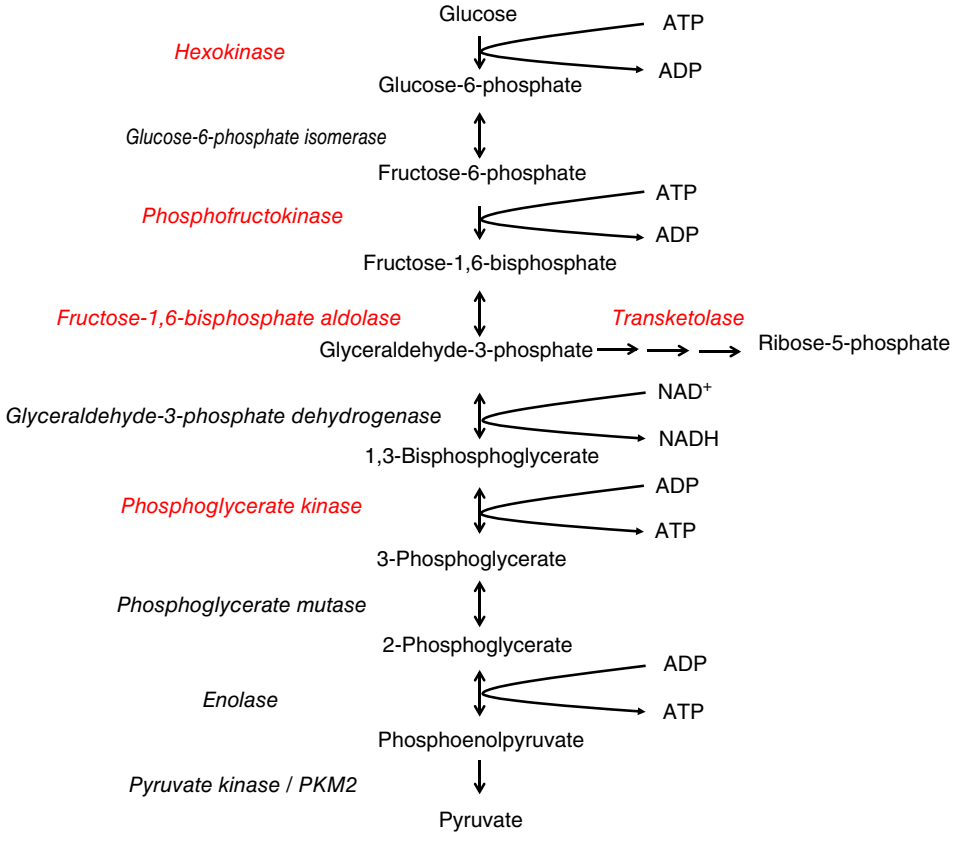

Figure 9.10 Effect of HIF1α on the glycolytic and pentose phosphate pathways. HIF1α stimulates the transcription of genes that encode enzymes of the glycolytic and pentose phosphate pathways (red).

The lactate dehydrogenase A (*LDHA*) gene encodes an isoenzyme of lactate dehydrogenase, and the pyruvate dehydrogenase kinase 1 (*PDK1*) gene encodes an isoenzyme of the pyruvate dehydrogenase kinase. Under aerobic conditions, pyruvate is decarboxylated and oxidized by pyruvate dehydrogenase to generate acetyl coenzyme A (ac-CoA). Most of the pyruvate is completely oxidized to carbon dioxide through the TCA cycle. Pyruvate dehydrogenase kinase phosphorylates and inhibits pyruvate dehydrogenase. HIF1α stimulates the synthesis of pyruvate dehydrogenase kinase and, in this way, reduces the mitochondrial oxidative metabolism of pyruvate. Under anaerobic conditions, lactate dehydrogenase generates lactate from pyruvate. HIF1α stimulates the synthesis of lactate dehydrogenase and consequently the synthesis of lactate. This pathway is known as anaerobic glycolysis, since it is preferred at oxygen shortage (Figure 9.11).

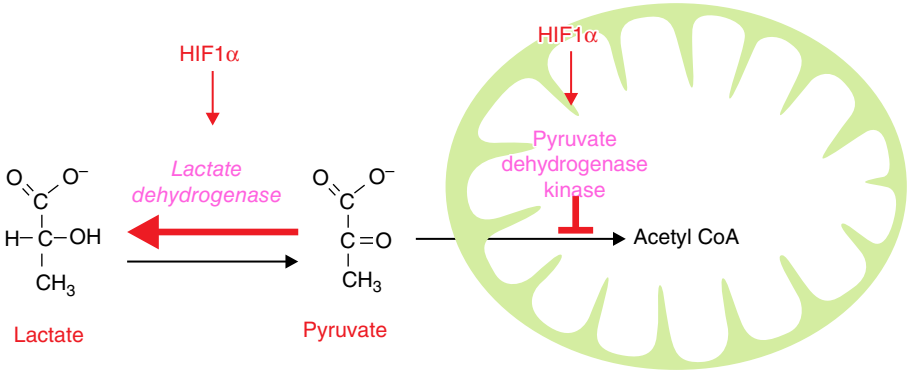

Figure 9.11 Inhibition of oxidative phosphorylation by HIF1α. Under hypoxic conditions, HIF1α activates the transcription of the genes *LDHA* (lactate dehydrogenase A) and *PDK1* (pyruvate dehydrogenase kinase 1). This deviates the metabolism of pyruvate from the mitochondrial TCA cycle and oxidative phosphorylation to the production of lactate. Many tumor cells prefer this pathway even under aerobic conditions (aerobic glycolysis), an effect known as *Warburg effect*.

9.5.3.2 HIF1α and the Warburg Effect

In the thirties of the last century, the German biochemist and Nobel laureate Otto Warburg reported that tumor cells prefer the production of lactate instead of metabolizing pyruvate in the mitochondrial TCA cycle, even in the presence of oxygen. This metabolic pathway has been named aerobic glycolysis since it takes place in proliferating cells under aerobic conditions. The phenomenon is known as the *Warburg effect*. Warburg hypothesized that the decreased aerobic phosphorylation in tumor cells is due to a decreased number of mitochondria. Indeed, HIF1 transactivates the *BNIP3* gene, encoding a protein that promotes mitochondrial-selective autophagy, as a means to reduce oxidative metabolism, as observed in hypoxic mouse embryonic fibroblasts.

The HIF1α target genes *LDHA* and *PDK1* are also central to the regulation of aerobic glycolysis. Growth-promoting pathways such as the PI3K/AKT and MAPK pathways upregulate and activate HIF1α in proliferating normal cells and tumor cells.

9.5.3.3 The Warburg Paradox

Oxidative phosphorylation yields up to 38 mol ATP per mol glucose, whereas anaerobic glycolysis yields 2 mol ATP/mol glucose. With regard to ATP production, anaerobic glycolysis is inefficient. It makes sense that a cell switches to anaerobic glycolysis when oxygen is limiting. However, tumor cells tend to circumvent the TCA cycle and oxidative phosphorylation and convert pyruvate into lactate even in the presence of oxygen.

A cell destined to divide needs amino acids, nucleotides, and fatty acids for building new daughter cells (see Chapter 8). For this reason, metabolism shifts to the generation of biomass at the expense of ATP production (Vander Heiden,

Cantley, and Thompson, 2009; Ward and Thompson, 2012). The effect of HIF1α on glucose metabolism as outlined earlier contradicts the demand of proliferating cells. Acetyl CoA is needed for the synthesis of fatty acids, cholesterol, and isoprenoids, lipids required for the synthesis of cellular membranes. Since HIF1α keeps pyruvate away from mitochondria, it does not support the synthesis of lipids required for membrane synthesis.

There are a number of explanations for the "Warburg paradox":

Pyruvate Kinase M (PKM)

Pyruvate kinase catalyzes the conversion of phosphoenolpyruvate to pyruvate. The *PKM* gene encodes two differentially spliced mRNAs, which encode the PKM isoforms PKM1 and PKM2. The PKM1 isoform is expressed in differentiated cells, whereas the PKM2 isoform predominates in cells of embryonic tissues and tumors. When wild-type and HIF1α knockout mouse embryonic fibroblasts were cultivated under hypoxic conditions, both PKM1 and PKM2 mRNA levels were significantly increased in the wild-type, but not in the knockout cells. Under hypoxic conditions, the protein concentrations of PKM2 were nearly double as high as under normoxic conditions. These findings indicate that the PKM2 isoenzyme is a target of HIF1α (Luo *et al.*, 2011).

Counterintuitively, the PKM2 isoenzyme exhibits a lower enzymatic activity than the PKM1 isoenzyme does. Because of the lower enzymatic activity, glycolytic intermediates may accumulate upstream of phosphoenolpyruvate and may be shunted into anabolic pathways, such as the synthesis of glycine, serine, and threonine from glyceraldehyde-3-phosphate. The transketolase isoenzymes 1 and 2 are targets of HIF1α. Since transketolase catalyzes fully reversible transfer reactions, HIF1α may assist the synthesis of ribose-5 phosphate as a precursor of nucleotides at increased concentrations of glycerolaldehyde-3 phosphate (Figure 9.10). Observations of the metabolic pathway in imatinib-resistant cells in chronic myeloid leukemia (CML) support this theory (see Chapter 6). A nonhypoxic induction of HIF1α was observed in imatinib-resistant, BCR-ABL transformed CML cells. HIF1α induced an enhanced rate of glycolysis; however, glucose flux through the TCA cycle and the oxidative arm of the pentose phosphate pathway (PPP) was reduced. In contrast, the nonoxidative PPP enzyme transketolase was activated. Thus, in the resistant cells, HIF1α redirected the metabolism of glucose both as an energy source and for the increase of biomass necessary for the generation of new cells. Importantly, the use of oxythiamine, which can inhibit both the pyruvate dehydrogenase complex and transketolase, enhanced Imatinib sensitivity, both in cultured cells and in xenografts (Zhao *et al.*, 2010).

Glutamine Metabolism

In tumor cells, aerobic glycolysis is accompanied by an increase in cellular uptake and metabolism of the amino acid glutamine. As mentioned earlier, HIF2α

stimulates the activity of Myc. In a number of Myc-transformed cells, it has been shown that the cells are "addicted" to glutamine. On withdrawal of glutamine from the cell culture medium, such cells undergo apoptosis, and the pharmacological inhibition of glutaminase impaires the growth of tumor xenografts from Myc-expressing B cells (Le *et al.*, 2012). Oncogenic Myc promotes the utilization of glutamine by enhancing the expression of glutaminase. Glutaminase deaminates glutamine to glutamate. In the mitochondrion, glutamate is converted into α-ketoglutarate. The mitochondrial isocitrate dehydrogenase (IDH2) then converts α-ketoglutarate into isocitrate. Aconitase converts isocitrate into citrate, which can leave the mitochondrion via the citrate carrier into the cytosol. Here, citrate is the starting point for the synthesis of fatty acids, cholesterol, and isoprenoids (Figure 9.12). Glutamate can also be converted into the amino acids proline, arginine, and glutamine (Ward and Thompson, 2012).

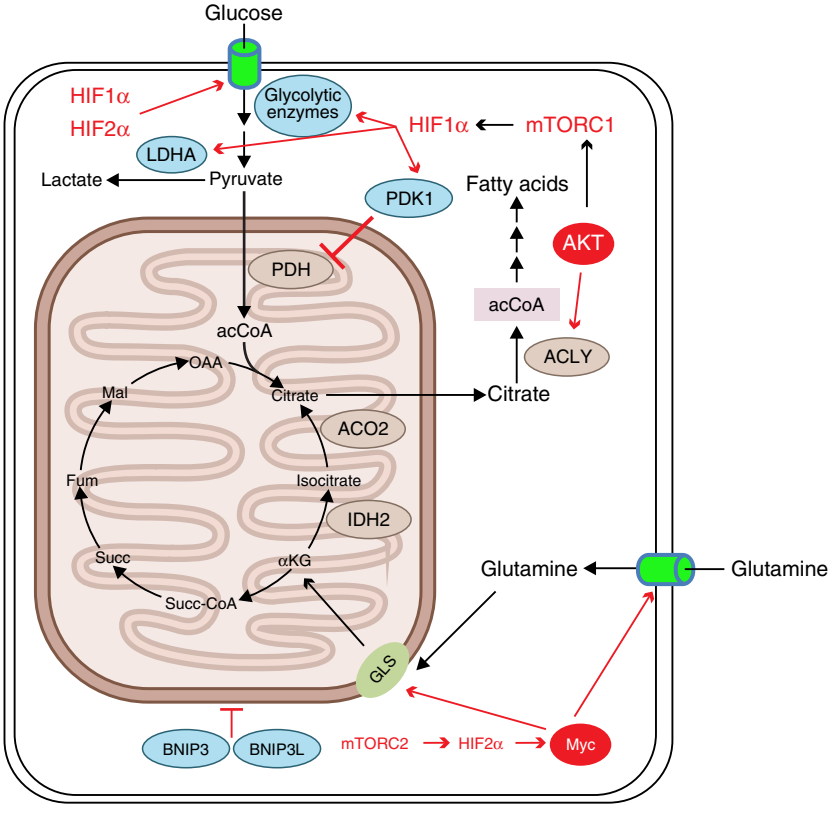

Figure 9.12 Solving the Warburg paradox. The PI3K/AKT pathway stimulates the synthesis of HIF1α via mTORC1. HIF1α stimulates glucose uptake and glycolysis and favors the production of lactate. AKT phosphorylates and activates the ATP-dependent citrate lyase, in this way stimulating the synthesis of fatty acids. As a Myc agonist, HIF2α supports the metabolism of glutamine.

PI3K/AKT Pathway

After export of citrate into the cytosol, ATP citrate lyase (ACL) catalyzes the conversion of citrate into acetyl-CoA. AKT phosphorylates and activates ACL, in this way stimulating the synthesis of essential lipids needed for the generation of biological membranes. In AKT-driven tumors, ACL knockdown slows tumor growth in vivo, supporting the importance of the activation of ACL by AKT (Figure 9.12) (Ward and Thompson, 2012).

Reverse Warburg Effect

Solid tumors contain richly oxygenated areas close to blood vessels as well as poorly oxygenated areas distant from blood vessels. Well-oxygenated cancer cells can generate energy via the TCA cycle and oxidative phosphorylation, whereas cancer cells in hypoxic regions may feed cancer cells in well-oxygenated regions. The *MCT4* gene, which encodes the monocarboxylate transporter 4, is a HIF1α target gene. Excess lactate produced under anaerobic conditions may be exported from the hypoxic cell via the MTC4 transporter. Tumor cells in the well-oxygenated tumor area express the MCT1 transporter allowing the uptake of lactate, which is converted into pyruvate by the LDHB isoenzyme. Pyruvate is then metabolized in the mitochondrial TCA cycle (Figure 9.13). A-cyano-4-hydroxycinnamate (CHC) is a compound that inhibits the MCT1 transporter. In both a mouse model of lung carcinoma and a xenotransplant model of human colorectal carcinoma cells, the CHC compound induced a switch from lactate-fueled respiration to glycolysis. CHC retarded tumor growth, since the hypoxic/glycolytic tumor cells died from glucose starvation (Semenza, 2008; Sonveaux *et al.*, 2008). The phenomenon has been termed *reverse Warburg effect* (Pavlides *et al.*, 2009).

9.6
TCA Cycle Intermediates and Tumor Syndromes

As mentioned earlier, the TCA cycle intermediates fumarate and succinate inhibit all three PHDs (Figure 9.7). Heterozygous germline mutations of the *FH* gene encoding fumarate hydratase predispose to leiomyomas and renal cell cancer. The enzyme succinate dehydrogenase consists of four subunits encoded by the genes *SDHA*, *SDHB*, *SDHC*, and *SDHD*. Germline loss-of-function mutations in any of these genes or a gene coding an assembly factor (*SDAHF2*) causes a syndrome characterized by paragangliomas and pheochromocytomas (Kaelin and Ratcliffe, 2008).

9.7
Drugs Targeting HIFs

HIF1α and HIF2α may exert different effects. In addition, the impact of the transcription factors on tumor growth and tumor progression is context dependent.

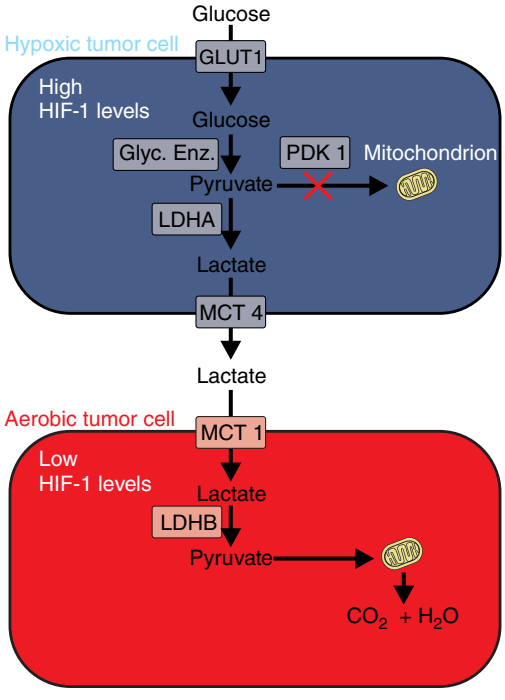

Figure 9.13 Reverse Warburg effect. Lactate produced in the hypoxic tumor cell is exported into the extracellular space via the monocarboxylate transporter 4 (MCT4). Tumor cells in the well-oxygenated area of the tumor take up lactate via MCT1. The lactate dehydrogenase B isoenzyme, which prevails under aerobic conditions, converts lactate to pyruvate, which is converted to acetyl-CoA in the mitochondria and metabolized in the TCA cycle. When the MCT1 transporter is blocked, tumor cells in well-oxygenated tumor areas consume glucose. This leads to glucose starvation of tumor cells. (According to data by Sonveaux *et al.* (2008).)

These conditions should be kept in mind when therapeutic approaches are considered that affect the concentration and/or activity of HIF transcription factors.

With the exception of hormone receptors, transcription factors are difficult to target. This applies also to HIFs. However, the diverse regulatory circuits of HIF mRNA expression as well as protein translation, stabilization, and degradation provide several routes to target HIFs by pharmacological agents (Table 9.2) (Semenza, 2012).

mRNA expression. Aminoflavone, the active compound of the prodrug AFP-464, has been reported to inhibit the expression of HIF1α on both the mRNA and protein levels. EZN-2968 is an antisense oligonucleotide that specifically targets HIF1α. A clinical trial provided preliminary proof of concept for the modulation of HIF1α mRNA and protein expression in tumor biopsies after administration of the drug (Jeong *et al.*, 2014).

Protein translation. mTOR inhibitors such as rapamycin and everolimus inhibit the translation of HIF1α. A high-throughput screen of drugs approved

Table 9.2 HIF1α inhibiting drugs: molecular mechanisms of action.[a)]

mRNA	Synthesis	Protein stability	Dimerization with HIF1β	Transactivation
Aminoflavone EZN-2968	mTOR inhibitors Cardiac glycosides	HSP90 inhibitors Antioxidants Berberine YC1	Acriflavine	Bortezomib

a) Semenza (2012).

by the Food and Drug Administration of the United States revealed that digoxin and other cardiac glycosides inhibit HIFα translation and the growth of xenografts. As mentioned earlier, the PI3K/AKT pathway increases the translation of HIF1α via mTOR. Since a variety of tyrosine kinases activate this pathway, tyrosine kinase inhibitors such as inhibitors of BCR/ABL, HER2/Neu, and the EGF receptor inhibit the synthesis of HIF1α. Synthetic oligonucleotides have been developed that inhibit translation after binding to HIF1α mRNA.

HIF1α stability. Several drugs induce the degradation of HIF1α. Inhibitors of heat shock protein 90 (HSP90) cause the VHL-independent degradation of HIF1α. Other agents such as antioxidants, a thioredoxin inhibitor, class II histone deacetylases, the natural product berberine, and the YC-1 guanylate cyclase inhibitor are additional agents that decrease the stability of HIF1α by a variety of mechanism.

HIF1α-dependent transactivation. The C-terminal transactivation domain of HIF1α interacts with the coactivator p300. This interaction is targeted by bortezomib, a proteasome inhibitor approved by the FDA for the therapy of multiple myeloma and mantle cell lymphoma (see Chapter 14).

Other drugs affect HIF heterodimerization and DNA-binding activities of HIFα.

9.8 Outlook

The pVHL-HIFα pathway is essential for the growth of CCRCC. It has been suggested that the tumor cells are addicted to the pathway. This observation led to the concept of "synthetic lethality." Two genes are synthetically lethal if inhibition of either gene is compatible with viability, but inhibition of both leads to cell death. Compounds have been screened in CCRCC, in which the *VHL* gene was either active or silenced. Drugs were identified that inhibited proliferation and xenograft growth only in the absence of *VHL* gene transcription. This may be a rewarding approach to specifically target tumors with functional inactivation of the *VHL* tumor suppressor gene (Kaelin and Ratcliffe, 2008; Majmundar, Wong, and Simon, 2010).

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10
NF-κB Pathways

Cancer Signaling: From Molecular Biology to Targeted Therapy, First Edition.
Christoph Wagener, Carol Stocking, and Oliver Müller.

Companion Website:www.wiley.com/go/wagener/cancersignaling

Summary

Nuclear factor kappa B (NF-κB) transcription factors affect fundamental cellular responses such as proliferation, differentiation, survival, and metabolism. Many different receptors activate the NF-κB pathway. Signaling through these receptors involves several sets of proteins, which only partly overlap. Many signaling proteins of the NF-κB pathway are ubiquitinylated, thereby adding an additional level of complexity to NF-κB signaling. NF-κB signaling is involved in, but not restricted to, central aspects of innate and adaptive immunity. Gene mutations affecting the NF-κB pathway occur mainly in malignancies of the B-cell lineage, however, mutations are also found in subsets of common cancers, for example, in breast cancer. Anti-inflammatory drugs such as aspirin or corticoids are effective in the prevention or treatment of cancer. Since NF-κB regulates inflammatory reactions, NF-κB signaling might be affected by these drugs. However, only a few drugs that specifically target the pathway in cancer patients are currently available.

10.1 NF-κB Signaling in Inflammation, Growth Control, and Cancer

Nuclear factor kappa B, abbreviated NF-κB, was first described as a transcription factor that regulates the expression of the κ (kappa) light chain of immunoglobulins (components of the B-cell receptor). The family of NF-κB factors consists of five members. These factors combine to form various combinations of homo- and heterodimers and regulate the transcription of target genes.

Components of the NF-κB pathway are highly conserved in evolution and are even present in lower metazoans, such as sponges, hydra, and jellyfish. A conserved and important function of this pathway is control of the inflammatory response, an essential manifestation of innate immunity. The inflammatory response requires tightly regulated mechanisms of growth controls; thus, the NF-κB pathway has evolved to mediate a wide range of cellular functions, including replication and growth, programmed cell death, cell fate decisions, and differentiation. Furthermore, NF-κB regulation of these diverse functions is not limited to hematopoietic cells of the innate or adaptive immune system, but is also found in many cells of epithelial origin (Ben-Neriah and Karin, 2011). Perturbations in NF-κB signaling can affect each of these cellular functions in the genesis and growth of malignant tumors. The first molecular demonstration that disruption of the NF-κB pathway could be linked to oncogenesis came with the realization that the v-*rel* oncogene of an acute transforming retrovirus causing reticuloendotheliosis in turkey encoded a homolog of a NF-κB subunit. This finding confirmed the complex relationship of inflammation, growth control, and cancer.

The NF-κB pathway is activated by soluble and membrane-bound ligands that bind to cell membrane receptors, notably members of the family of TNF (tumor necrosis factor) receptors, Toll-like receptors, the IL1 receptor, and members of

the antigen receptor superfamily. In addition to extracellular ligands, the NF-κB pathway may be activated by intracellular pathogens, which are recognized by intracellular pattern recognition proteins belonging to the NOD (nucleotide oligomerization domain)-like receptor (NLR) or retinoic acid inducible gene (RIG)-I-like receptor families. Additional intracellular stimuli of the NF-κB pathway are DNA damage and reactive oxygen species (ROS). Depending on the type of cell membrane receptor and the intracellular stimuli, different proteins take part in the activation of NF-κB target genes.

10.2 The Core of NF-κB Signaling

Mammals harbor five members of the NF-κB family: RelA/p65, RelB, c-Rel, NF-κB1 (p105), and NF-κB2 (p100). NF-κB1 and NF-κB2 are processed to the DNA-binding subunits p50 and p52, respectively (Figure 10.1). NF-κB proteins form homodimers or heterodimers, which bind to κB binding sites in the DNA with the sequence motif 5′-GGGRNWYYCC-3′ (N: any base; R: purine; W: adenine or thymine; Y: pyrimidine). All members of the NF-κB family contain Rel homology

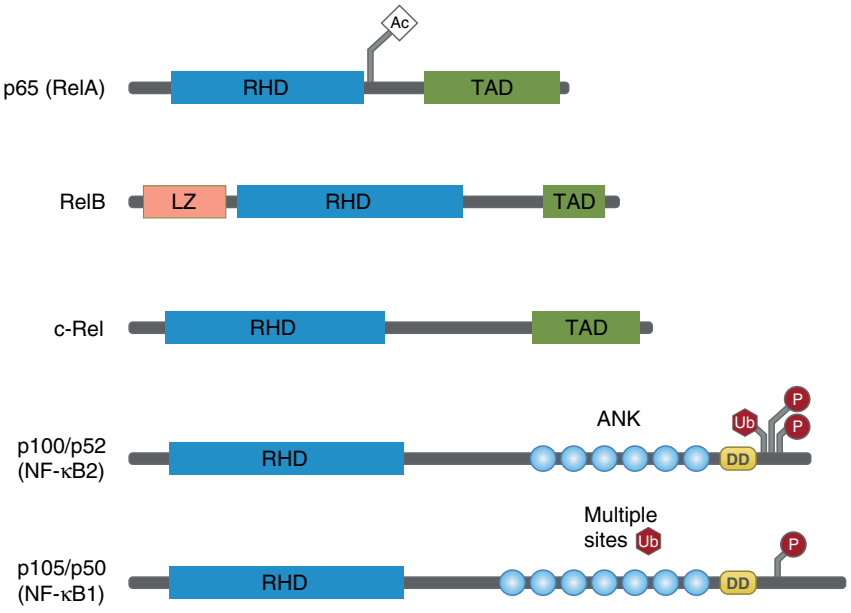


Figure 10.1 NF-κB family. Phosphorylation (P) and ubiquitinylation (Ub) sites that mediate proteasomal degradation are indicated. ANK, ankyrin repeats; DD, death domain; LZ, leucine zipper; RHD, Rel homology domain; and TAD, transactivation domain. Ankyrin repeats and the leucine zipper mediate protein–protein interactions, the transactivation domain binds to regulators of transcription. (According to Hayden and Ghosh (2008), with permission, modified.)

domains (RHDs). The RHDs mediate dimerization, DNA binding, and interaction with inhibitory IκB proteins (see the following text). Three members (p65, RelB, c-Rel) can activate transcription via their transactivation domains (TADs). Depending on the composition of the dimers, they can either activate or inhibit the transcription of target genes. The p65/p50 heterodimer is regarded as the prototypical NF-κB complex.

In the absence of activating stimuli, NF-κB dimers are held inactive in the cytoplasm through association with IκB proteins (Figure 10.2). Before NF-κB dimers can enter the nucleus and bind to DNA, the factors have to be freed from

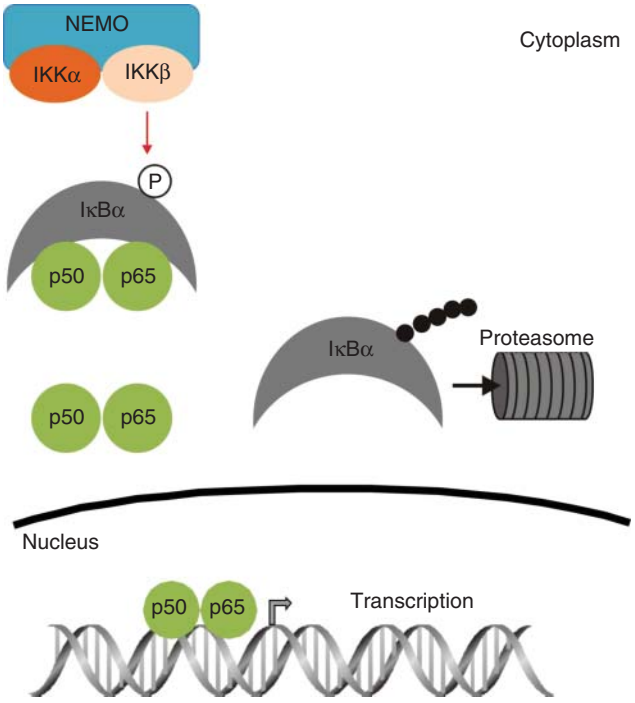


Figure 10.2 The core of NF-κB signaling. In the absence of an external stimulus, NF-κB dimers are held in an inactive state in the cytoplasm. In the present example, the major NF-κB dimer of the canonical pathway, the p50/p65 dimer, is shown. The dimer associates with an inhibitory protein, which prevents the NF-κB dimer from entering the nucleus. Here, the IκBα protein is shown. Before NF-κB dimers can enter the nucleus and bind to DNA, the factors have to be freed from IκB binding. This is initiated by phosphorylation of serine residues in the IκB destruction box. The phosphorylation is mediated by a kinase integrated in a trimeric complex of IκB kinases, abbreviated IKK. The IKK complex is activated after binding of ligands to their receptors, for example, of the TNF-receptor family. In the canonical pathway, the complex of IκB kinases consists of IκB kinase α and β and of NEMO (NF-κB essential modulator). IKKβ phosphorylates the serine residues in the destruction box of IκB, in the present example of IκBα. Phosphorylation of serine residues in the destruction box of IκBα initiates ubiquitinylation and proteasomal degradation of the protein. The NF-κB dimer enters the nucleus, binds to NF-κB binding sites in the DNA, and regulates transcription of NF-κB response genes.

IκB binding. This is initiated by phosphorylation of serine residues in an amino acid sequence motif conserved in IκB proteins, the so-called *destruction box*. The kinases phosphorylating the serine residues are known as *IκB kinases (IKKs)*. In the canonical NF-κB pathway, the IκB kinases IKKα and IKKβ build a complex with NEMO (NF-κB essential modulator). Phosphorylation of IκB leads to its association with the βTrCP ubiquitin ligase and thereby initiates ubiquitinylation of IκB. Ubiquitinylated IκB proteins are directed to the cytosolic proteasome and degraded.

The NF-κB dimer is thus freed to enter the nucleus, to bind to its binding sites in the DNA, and to regulate transcription.

10.3 Family of IκB Proteins

There are eight IκB proteins in mammals (Figure 10.3): IκBα, IκBβ, IκBε, IκBζ, BCL3, IκBNS (IκBδ), and the precursor proteins p105 (NF-κB1) and p100 (NF-κB2). IκB proteins possess ankyrin repeat domains, which mediate protein–protein interactions. The function of IκB proteins is not limited to sequestration of NF-κB in the cytoplasm. For example, IκBβ, IκBζ, and BCL3 can associate with DNA-bound NF-κB dimers and, in this way, regulate the transcriptional response.

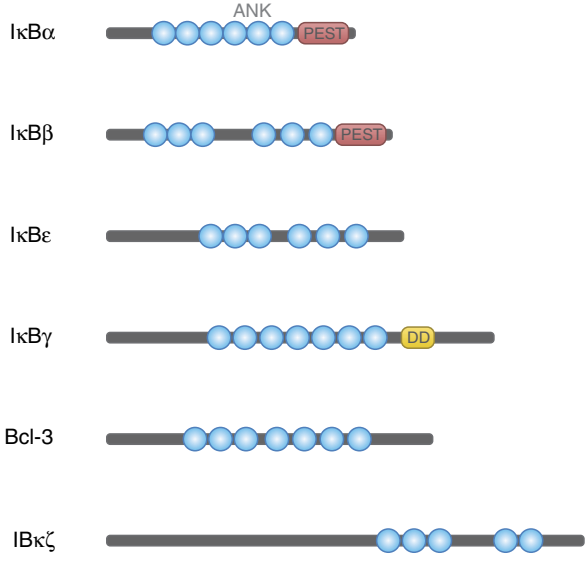


Figure 10.3 IκB family. ANK, ankyrin repeats; DD, death domain; and PEST, domain rich in proline (P), glutamic acid (E), serine (S), and threonine (T). Ankyrin repeats mediate protein–protein interactions, PEST domains are involved in protein degradation. (According to Hayden and Ghosh (2008), with permission, modified.)

IκBα and IκBβ are expressed in all tissues, whereas the expression of IκBε is restricted to hematopoietic cells. The expression patterns of BCL3, IκBζ, and IκBNS are more limited. Since these IκB family members are upregulated following activation of NF-κB, they act at a later timepoint during transcriptional response. The precursor proteins p105 (NF-κB1) and p100 (NF-κB2) can act as NF-κB inhibitors, can be processed to form the transcription factors p50 or p52, respectively, or can be degraded.

IκBα, IκBβ, and IκBε are regarded as classical members of the IκB family. When all three of these IκB proteins are removed, inducible NF-κB activation by TNFα is completely abolished. The prototypical member of the IκB family, IκBα, is the main regulator of the p65/p50 heterodimer. It masks the nuclear localization sequence of p65 and sequesters p65/p50 dimers in the resting state. In the signaling state, IκBα ubiquitinylation and degradation by the proteasome release the NF-κB dimer to enter the nucleus and to bind to DNA. IκBε is assumed to selectively regulate p65 homodimers and c-REL/p65 heterodimers. In comparison to IκBα, degradation and resynthesis of IκBε is delayed, which may affect later stages of NF-κB signaling. Similarly to IκBε, IκBβ undergoes slow degradation and resynthesis.

10.4 Canonical NF-κB Signaling from TNF Receptor 1

(Video: NF-κB pathways.Part 1: The canonical pathway of NF-κB activation – only closed website: NF_κB_Video_01_ebook.mp4)

Many signaling proteins of the canonical NF-κB pathway were first described in tumor necrosis factor receptor 1 (TNFR1) signaling. For this reason, TNFR will serve as an example for the canonical pathway. In addition to NF-κB signaling, ligation of TNFR1 by soluble TNF induces the mitogen-activated protein kinase (MAPK) pathway, as well as pathways of apoptosis and necroptosis. The NF-κB pathway counteracts apoptotic TNFR1 pathways by activating antiapoptotic transcriptional programs.

TNFR1 belongs to the subgroup of receptors of the TNF receptor family containing an intracellular death domain (DD). Interaction of the receptor with cytoplasmic adapter proteins occurs through the DDs found on both proteins. The DD of TNFR1 is essential for signaling through the NF-κB pathway (Figure 10.4).

Upon binding with a TNFα trimer, TNFR1 forms oligomeric complexes. This leads to the binding of the cytoplasmic adapter protein TRADD (TNF receptor-associated protein with a death domain) to the cytoplasmic part of the receptor containing the DD. The DD of TRADD gives rise to a proapoptotic signaling complex through the association with the adapter protein FADD (complex II). The survival and inflammatory signals of the TNFα receptor are mediated by complex I, comprising the proteins TRADD, the receptor interacting protein (RIP1), and TRAF2 (TNF receptor-associated factor 2). RIP1 contains a DD, which associates with the TNFR1 DD. Based on sequence similarities, mode of regulation, and

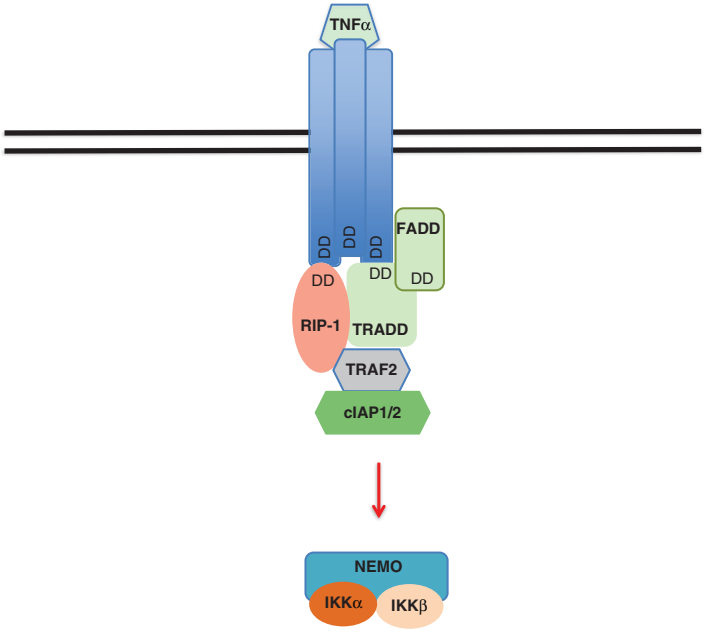


Figure 10.4 Proteins associated with the TNF receptor 1 (TNFR1). Upon binding of a TNFα trimer, oligomeric complexes of TNFR1 form. This leads to the binding of the cytoplasmic adapter protein TRADD (TNF receptor-associated protein with a death domain). TRADD contains a death domain, which associates with the death domain of the receptor. The death domain of TRADD can give rise to a proapoptotic signaling complex through the association with the adaptor protein FADD (complex II). The survival and inflammatory signals of the TNF receptor are mediated by TRADD, the receptor-interacting protein (RIP1) and TRAF2 (complex I). TRADD recruits RIP1, which acts as a scaffold protein and recruits the IKK complex by binding to NEMO. TRADD contains a special TRAF-binding domain, which recruits TRAF2 (TNF receptor-associated factor 2). TRAF2 binds cellular inhibitors of apoptosis (cIAP1 and cIAP2), which are essential for IKK activation. Induced proximity and conformational changes activate IKKs.

substrate specificities of their catalytic domain, RIP kinases are classified as serine/threonine kinases. However, in TNFR1 signaling, the kinase activity of RIP1 appears to be dispensable, and RIP1 acts primarily as a scaffold protein.

Upon assembly of the survival/proinflammatory complex, kinases are activated that phosphorylate the serine residues in the destruction box of IκB proteins. Mammals harbor two enzymatically active IκB kinases, IKKα and IKKβ (Figure 10.5). IKKα and IKKβ share 52% overall sequence identity, with the highest degree of homology in the catalytic domains. IKKα and IKKβ form a complex with NEMO. NEMO does not contain a kinase domain and is structurally unrelated to IKKα or IKKβ. The C-terminal region of NEMO mediates activation of IKK and interaction with upstream signaling adapters, whereas the N-terminus interacts with IKKs (Hayden and Ghosh, 2012).

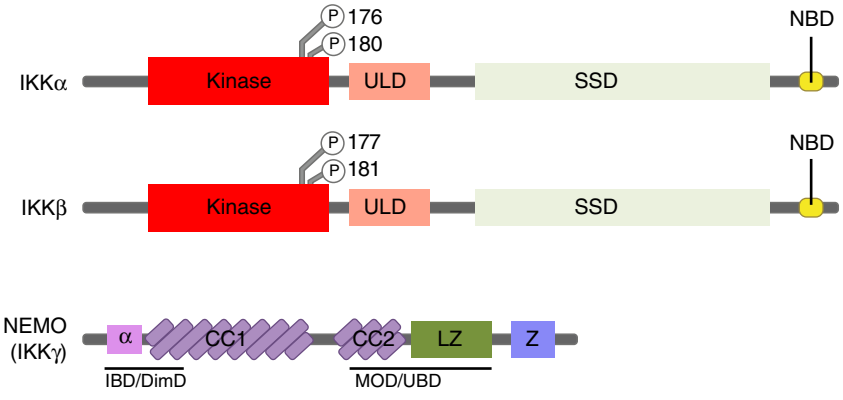


Figure 10.5 IκB kinases (IKKs). (P), phosphorylated serine residues in active kinases. α, α-helical domain; CC, coiled-coil domain; NBD, NEMO-binding domain; LZ, leucine zipper; SDD, scaffolding and dimerization domain; ULD, ubiquitin-like domain; Z, zinc finger domain; IBD/DimD, IKK-binding domain/dimerization domain; and MOD/UBD, minimal oligomerization domain/ubiquitin-binding domain. (According to Hayden and Ghosh (2012), with permission, modified.)

Activation of the IKK complex requires phosphorylation of two serine residues in the activation loop of at least one of the IKK subunits. Despite decades of research, the mechanism underlying the phosphorylation and activation of IKKs is still elusive. Two alternative theories are currently debatted. According to one theory, one IKK transphosphorylates and activates the other IKK in the complex. According to a second theory, an upstream IKK-kinase, most probably the IKK homologous TAK1 (transforming growth factor activated kinase-1), phosphorylates and activates the IKKs.

In cells overexpressing IKKs, dimerization is necessary and sufficient to activate enzymatic activity. Under physiological conditions, however, dimerization is mediated by NEMO. Consistent with this function, NEMO with a mutated oligomerization domain or a recombinant oligomerization domain alone prevents IKK activation and NF-κB signaling (Hayden and Ghosh, 2012).

The signalosome recruited to the TNFR1 is composed of an oligomeric complex of adaptor proteins devoid of enzymatic activity. Based on this observation, it is thought that higher order structures are necessary for the induction of IKK activity. However, how exactly oligomerization regulates IKK activities has not yet been established. RIP1 appears to be key to build a higher order structure including the IκB kinases–NF-κB essential modulator (IKK-NEMO) complex. RIP1 binds to NEMO and requires NEMO in order to activate IKK. The role of RIP1 is stressed by the finding that targeted disruption of the RIP1 gene severely compromises NF-κB signaling. Ubiquitinylation of RIP1 may assist its function in recruiting and activating IκB kinases.

10.5 B-Cell Receptor Signaling

NF-κB is also a downstream target of the B-cell receptor (BCR). As the BCR is constitutively activated in various malignancies of the B-cell lineage, its signaling pathway will be described in detail. The BCR consists of four immunoglobulin chains with antibody specificities anchored in the plasma membrane. In order to signal, the immunoglobulin chains associate with the coreceptors CD79A, CD79B, and CD19. CD79 proteins activate primarily the MAPK and mTOR pathways, whereas CD19 initiates NF-κB signaling. Antigen-induced aggregation of BCR activates nonreceptor protein kinases of the SRC family of tyrosine kinases, which phosphorylate the ITAMs (immunoreceptor tyrosine-based activation motifs) in the cytoplasmic part of CD79A and CD79B. ITAMs contain critical tyrosine residues, which, upon phosphorylation, associate with Src homology 2 (SH2) domains of binding proteins. The two phosphorylated tyrosine residues in the ITAM motif associate with the tandem SH2 domains of the tyrosine kinase SYK. SYK recruits the B-cell linker protein (BLNK), which coordinates the phosphorylation and activation of Bruton's tyrosine kinase.

After antigen binding to BCR, the coreceptor CD19 is phosphorylated by LYN, a nonreceptor tyrosine kinase of the SRC family. The phosphotyrosine residues of CD19 associate with the p85 subunit of phosphatidylinositol-4,5-bisphosphate 3-kinase (PI3K) (Figure 10.6). The associated catalytic subunit generates phosphatidylinositol-3,4,5-trisphosphate (PIP3) in the plasma membrane (see Chapter 8). PIP3 recruits the nonreceptor phosphotyrosine kinase, Bruton's tyrosine kinase (BTK), to the plasma membrane. BTK phosphorylates and activates phospholipase Cγ (PLCγ), which cleaves phosphatidylinositol-4,5-bisphosphate (PIP2) to inositoltrisphosphate (IP3) and diacylglycerol (DAG). DAG activates PKCβ. Upon PKCβ activation, we enter the path specifically leading to NF-κB signaling. PKCβ phosphorylates the protein CARD11 (caspase-associated recruitment domain), which transfers the protein from a closed into an open conformation. CARD11, a member of a family of proteins carrying a CARD, acts as a scaffold protein, which, after translocation to the plasma membrane, recruits the proteins BCL10 and MALT1 (CBM signalosome). The BCL10 (B-cell lymphoma 10) and the mucosa-associated lymphoid tissue 1 MALT1 genes were identified by their translocation in mucosa-associated lymphoid tissue (MALT) lymphomas. MALT1 contains a caspase (cysteinyl-aspartate-cleaving protease) like domain, which acts as a protease. MALT1 binds TNF receptor-associated factor 6 (TRAF6), which catalyzes the K63 ubiquitinylation (see following text) of NEMO, MALT1, and of itself. The protein TAB2 recognizes the ubiquitin chains of TRAF6 and enables the phosphorylation and activation of IKKβ by the TAK1 kinase. The protein A20 terminates NF-κB signaling by removing K63-linked ubiquitin chains from NEMO, TRAF6, and MALT1 (Staudt, 2010; Young and Staudt, 2013).

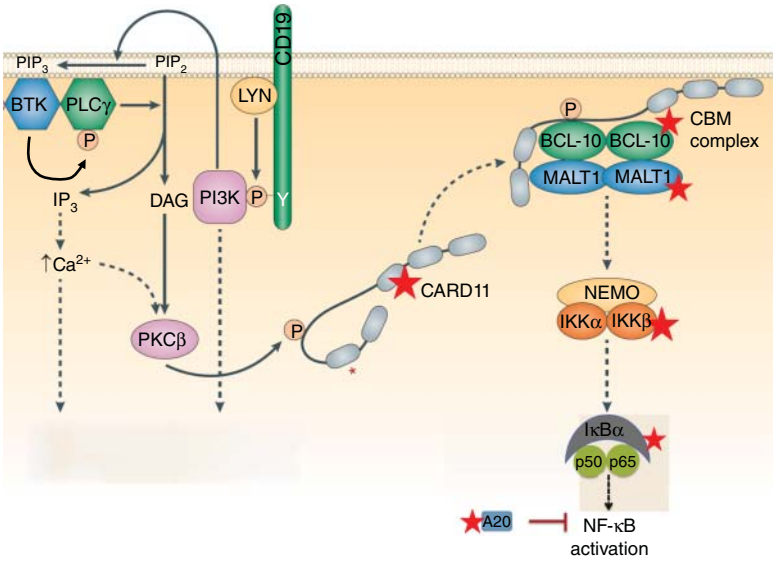


Figure 10.6 Signaling from CD19, coreceptor of the B-cell receptor. Upon antigen binding, the SRC family kinase LYN tyrosine phosphorylates CD19. This leads to the binding and activation of PI3-kinase (PI3K) and the synthesis of PIP3. The PH (pleckstrin homology) domain of Bruton's tyrosine kinase (BTK) binds to phosphatidylinositol-1,4,5-trisphosphate (PIP3) and phosphorylates phospholipase Cγ (PLCγ). PLCγ releases diacylglycerol (DAG) and inositol trisphosphate (IP3) from PIP2. DAG in concert with an elevated cytoplasmic Ca^{2+} level activates protein kinase Cβ (PKCβ), which phosphorylates CARD11. Upon phosphorylation, CARD11 is transferred from a closed into an open conformation. CARD11 then forms the CBM complex, which activates the canonical NF-κB pathway. Proteins that are mutated in malignancies of the B-cell lineage are marked by red asterisks. (According to Young and Staudt (2013), with permission, modified.)

10.6 Other Receptors Activating the Canonical Pathway

In addition to TNFR1 and the B-cell receptor, several other receptors, including the T-cell receptor, the Toll-like receptors, and the IL-1 receptor, activate the canonical NF-κB pathway. The proteins leading to the NEMO-IKK complex in these activated signaling pathways differ somewhat from the proteins mediating TLR1 or BCR signaling. However, the NEMO-IKK complex constitutes a central switch in each of these pathways.

10.7 Alternative NF-κB Pathway

(Video: NF-κB pathways. Part 2: Alternative NF-κB pathway – only closed website: NF_κB_Video_02_ebook.mp4)

Canonical pathways of NF-κB strictly depend on NEMO, whereas the alternative pathway does not. In the absence of stimuli of the alternative NF-κB pathway (steady state), a receptor-independent complex, composed of cellular inhibitors of apoptosis (cIAP1/2) and the proteins TRAF2 and TRAF3, is formed (Figure 10.7a). TRAF3 binds constitutively to the NF-κB inducing kinase (NIK). cIAP1 and cIAP2 possess activities both for ubiquitin binding and ubiquitin ligation. When the protein kinase NIK associates with the complex, ubiquitin residues are attached to NIK. Subsequently, the ubiquitinylated NIK kinase is directed to the proteasome and degraded. This mechanism explains the very low cellular levels of NIK during steady state.

The alternative NF-κB pathway is activated by a variety of ligands such as CD40 ligand (CD40L), B-cell activating factor (BAF), and lymphotoxin β (LTβ). Signaling by CD40 will be used as an example of the alternative signaling pathway (Figure 10.7b). Originally, CD40 was identified as a surface marker of B cells. CD40L is expressed by activated T helper cells and is essential for the induction of class-switch recombination of the B-cell immunoglobulin in response to T-cell-dependent antigens.

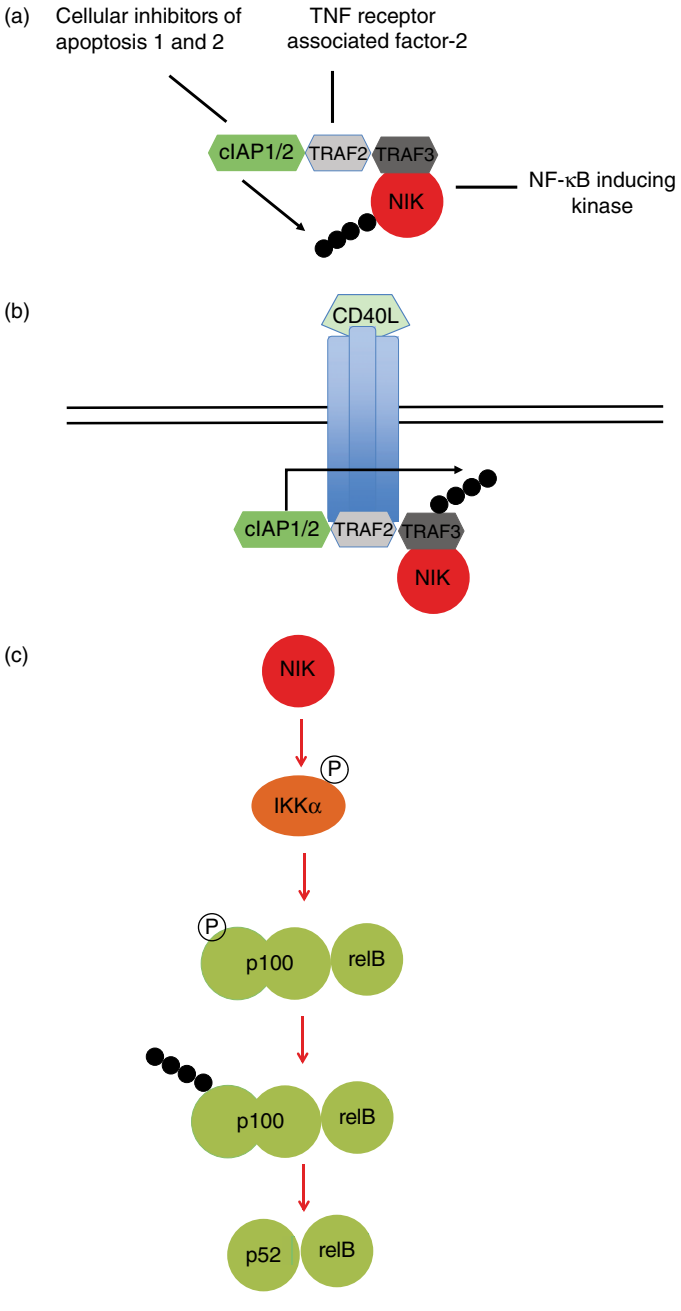
CD40 forms ligand-independent homodimers. Since CD40L exists as a trimer, receptor oligomers form after binding of CD40L to CD40. CD40 possesses three TRAF-binding motifs in the cytoplasmic part of the receptor. Among the TRAF proteins, TRAF2 exhibits the highest affinity to the cytoplasmic tail. TRAF2, in association with cIA1/2 and TRAF3, is recruited to the oligomeric receptor. In this situation, cIAP ubiquitinylates TRAF3, which leads to its proteolytic degradation. There is experimental evidence that ubiquitinylation of cIAP itself is a prerequisite for cIAP1/2-dependent TRAF3 ubiquitinylation and degradation (Hayden and Ghosh, 2014).

In the absence of a TRAF2/cIAP/TRAF3 complex, constitutively active protein kinase NIK phosphorylates and activates IKKα (Figure 10.7c). IKKα phosphorylates p100, the precursor of the p52 partner of RelB, both members of the family of NF-κB proteins. p100 is ubiquitinylated and, upon partial proteasomal degradation, the p52/RelB dimer is formed. After entering the nucleus, the dimer binds to consensus sequences in the DNA and activates the transcription of chemokine and cytokine genes.

10.8 Terminating the NF-κB Response

Terminating the NF-κB response is an essential aspect of NF-κB regulation, since a sustained and elevated NF-κB response is associated with chronic inflammation and malignancy. A number of negative feedback loops exist to terminate signaling, including downregulation of the stimulating receptor after ligand binding or activation of genes encoding inhibitors, as illustrated as follows.

Among the target genes of NF-κB transcription factors are genes coding for IκB proteins. The gene encoding IκBα (*NFKBIA*) is rapidly induced following NF-κB activation and is essential for shutting down the NF-κB response. Mice with


(a)
Cellular inhibitors of apoptosis 1 and 2
TNF receptor associated factor-2
cIAP1/2
TRAF2
TRAF3
NIK
NF-κB inducing kinase
(b)
CD40L
cIAP1/2
TRAF2
TRAF3
NIK
(c)
NIK
P
IKKα
P
p100
relB
p100
relB
p52
relB

targeted deletion of the *NFKBIA* gene develop systemic inflammation, since they fail to terminate TNF-dependent responses appropriately (Hayden and Ghosh, 2014).

The protein A20 also counteracts canonical and alternative NF-κB signaling. In most cell types, A20 levels rapidly increase upon NF-κB activation due to the presence of two NF-κB binding sites in the TNFAIP3 gene, which encodes the A20 protein. Mice deficient for A20 die prematurely due to severe multiorgan inflammation. In genome-wide association studies, *TNFAIP3* has been identified as susceptibility locus for human inflammatory and autoimmune diseases such as rheumatoid arthritis and inflammatory bowel disease. Somatic mutations and deletions in *TNFAIP3* have been identified in various B-cell malignancies. A20 is highly expressed in estrogen-resistant breast cancer cell lines and in aggressive breast carcinomas. Mutations in the adenomatous polyposis coli (*APC*) gene induce intestinal tumors, both in man and mice. In mice, the tumorigenic potential of *APC* mutations is aggravated by the specific deletion of A20 in intestinal epithelial cells (Catrysse *et al.*, 2014).

10.9
Ubiquitinylation in NF-κB Signaling

Attachment of ubiquitin to proteins constitutes an important posttranslational modification. Ubiquitinylation not only labels proteins for degradation but also mediates and fine-tunes the interaction of proteins. Ubiquitin is a protein of 76 amino acids, which is expressed ubiquitously. Three classes of enzymes are involved in ubiquitinylation of proteins: E1 ubiquitin activating enzymes, E2 ubiquitin conjugating enzymes, and E3 ubiquitin ligases. There are two mammalian E1 enzymes, 35 E2 enzymes, and more than 600 E3 ubiquitin ligases. Once the first ubiquitin is attached to the target protein, additional ubiquitins are sequentially conjugated to the carboxy terminal glycine of the bound ubiquitin to

Figure 10.7 (a) Steady state of the alternative NF-κB pathway. A receptor-independent complex of cellular inhibitors of apoptosis (cIAP1 and cIAP2) and the proteins TRAF2 (TNF receptor-associated factor 2) and TRAF3 is formed. cIAP1 and cIAP2 possess activities both for ubiquitin binding and for ubiquitin ligation. When the protein kinase NIK associates with the complex, ubiquitin residues are attached to NIK. Subsequently, the ubiquitinylated NIK kinase is directed to the proteasome and degraded. (b) Activation of the alternative pathway of NF-κB signaling. The activation of NF-κB via CD40 is shown as an example. Upon binding of CD40 ligand (CD40L), a receptor trimer is formed. The cytoplasmic part of the receptor binds to TRAF2 in association with cellular inhibitors of apoptosis (cIAP1/2) and TRAF3, which keeps the protein kinase NIK inactivated. Via their ubiquitin binding and ligating activities, the cellular inhibitors of apoptosis attach ubiquitin residues to TRAF3. This leads to the proteolytic degradation of TRAF3. (c). NIK freed from TRAF3 binding phosphorylates and activates IKKα. IKKα phosphorylates p100, thereby initiating its ubiquitinylation and partial degradation. The p52/RelB dimer enters the nucleus and binds to kB sites in the DNA.

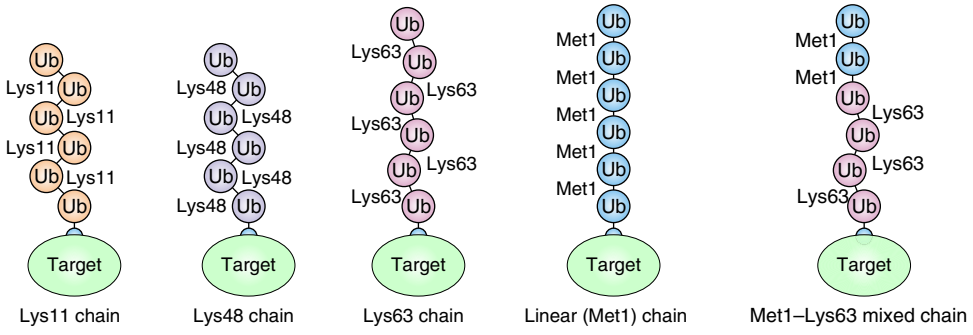


Figure 10.8 Ubiquitin chains. Chains in which ubiquitin is conjugated to lysine 11 (K11) or lysine 48 (K48) signal degradation. Lysine 63 (K63) ubiquitin chains are involved in signaling. The chains are assembled by the formation of isopeptide bonds between the C-terminal carboxyl group of one ubiquitin and an ε-amino group of one of seven lysine residues of another ubiquitin. In linear ubiquitin chains, a peptide bond is formed between the carboxyl group of the C-terminal glycine of one ubiquitin and the α-amino group of the methionine 1 residue in another ubiquitin. (According to Iwai, Fujita, and Sasaki (2014), with permission, modified.)

form polyubiquitin chains (Figure 10.8). The polyubiquitin chains are assembled by isopeptide bonds between the C-terminal carboxyl group of glycine of one ubiquitin and an ε-amino group of one of seven lysine (K) residues in another ubiquitin. Polyubiquitin chains conjugated to K11 or K48 lead to proteasomal degradation of the proteins, to which the ubiquitin chains have been attached. In addition to the chains with isopeptide bonds between C-terminal carboxyl groups and lysine residues, a peptide bond can be formed between the carboxyl group of the C-terminal glycine of one ubiquitin and the α-amino group of the N-terminal methionine (Met1) of another ubiquitin. These ubiquitin chains are known as *linear* or *M1 chains*. K63 chains and linear chains associate with binding proteins and are found to be attached to protein components of signalosomes (Iwai, Fujita, and Sasaki, 2014).

Ubiquitin-mediated proteasomal degradation is of prime importance in regulating NF-κB signaling. Ubiquitin K11 and/or K48 chains guide members of the IκB family to the proteasome, and ubiquitinylation of p100 leads to the partial degradation and release of p52. During the steady state of the alternative signaling pathway, ubiquitinylated NIC is degraded in the proteasome, and in the active state, TRAF3 is ubiquitinylated and degraded.

In addition to ubiquitin chains mediating proteasomal proteolysis, K63 and linear ubiquitin chains have been found to be attached to proteins of the NF-κB signaling cascade. K63 and linear ubiquitin chains are attached to a fraction of RIP1 proteins. Similarly, K63 and linear ubiquitin chains decorate NEMO. It has been suggested that the protein A20 terminates NF-κB signaling by cleaving and thereby deubiquitinylating (DUB) the K63 chains of RIP1 and NEMO. Ubiquitinylation also affects the regulation of the transcriptional response, as shown in the case of the atypical IκB protein BCL3.

The A20 protein inhibits NF-κB signaling. A20 contains an amino-terminal DUB activity and a C-terminal zinc finger domain, which supports E3 ubiquitin ligase activity. Major substrates of the DUB activity of A20 are the K63 chains of RIP1 and NEMO. In B-cell receptor signaling, A20 terminates NF-κB activity by removing K63-linked ubiquitin chains from NEMO, TRAF6, and MALT1 (Hayden and Ghosh, 2012; Staudt, 2010).

The role of ubiquitin chains and of DUB enzymes in NF-κB pathways is complex and appears to depend on particular receptors and specific states of signaling.

10.10 Transcriptional Regulation

The transcriptional response to NF-κB transcription factors is regulated at different levels. Firstly, NF-κB proteins act as dimers (Figure 10.9) and the composition of the dimers likely determines the affinity to specific DNA sequences, either

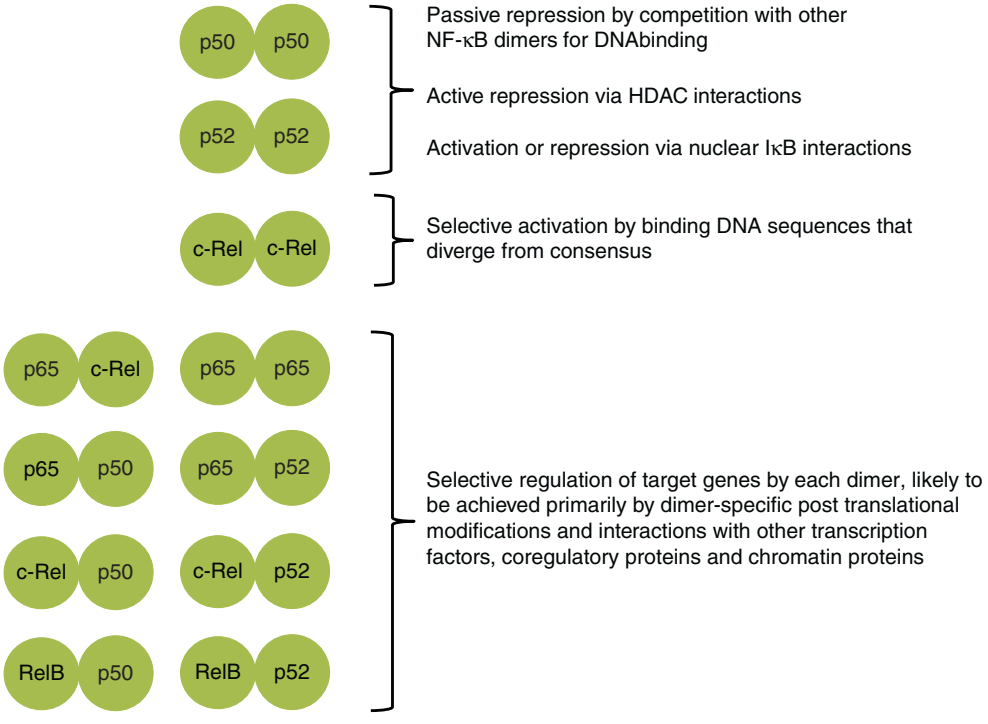


Figure 10.9 Model by which different NF-κB dimers contribute to selectivity of transcriptional responses to NF-κB-inducing stimuli. The selective function of all dimers may be influenced by the bound DNA sequence and its effect on dimer conformation and coregulatory interactions. (According to Smale (2012), with permission, modified.)

directly or through interactions with cooperating binding partners. Thus, the NF-κB DNA recognition motifs are quite degenerate, contributing to the known fact that different NF-κB activating stimuli lead to the transcription of different sets of genes.

Secondly, whereas three members of the NF-κB family contain TADs, two members do not (Figure 10.1). Since NF-κB proteins regulate transcription as dimers, the presence or absence of TADs in individual dimers affects the transcriptional response. Thus, dimers with TADs activate transcription, whereas dimers lacking TAD act as transcriptional repressors.

The transcriptional response also depends on the association of DNA-bound NF-κB dimers with transcriptional coregulators. Depending on its phosphorylation status, IκBβ binds to DNA together with NF-κB complexes, particularly p65/c-Rel heterodimers, promoting continuous binding to specific kB sites and, in this way, augmenting the late transcription of target genes. The IκB protein BCL3 has been found in association with p50 and p52 homodimers in the nucleus. Since BCL3 is able to activate transcription, it may confer transactivating potential to dimers, which otherwise are transcriptional repressors. BCL3 is ubiquitinylated, and transcriptional activity appears to depend on its ubiquitin chains. Consequently, the deubiquitinase CYLD is a negative regulator of NF-κB signaling in particular settings.

Finally, the status of chromatin affects the accessibility of NF-κB-binding sequences in the DNA. It is assumed that the cell-type-specific responses to NF-κB transcription factors are partly explained by the differences in chromatin organization. NF-κB family members affect the organization of chromatin once bound to DNA. NF-κB dimers associate with proteins that regulate the status of chromatin (Figure 10.10). It has been shown that acetylation of histones leads to an open chromatin conformation, which is reversed by their deacetylation. Consequently, genes recruiting NF-κB subunits early in the signal response display higher levels of histone acetylation, whereas genes bound later have low basal acetylation levels that increase upon stimulation. NF-κB family members are able to recruit both histone acetylases (HATs) such as CBP and p300, and

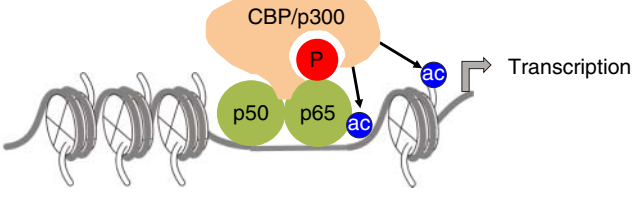


Figure 10.10 Regulation of chromatin state and transcription by the NF-κB p50/p65 dimer. Phosphorylation of p65 leads to the interaction of the dimer with the CBP/p300 coactivator complex. CBP and p300 contain histone acyltransferase domains. Upon association of CBP/p300 with the p50/p65 dimer, p65 is acetylated (ac). In addition, surrounding histones are acetylated, leading to an open configuration of chromatin.

histone deacetylases (HDACs). Posttranslational modifications of NF-κB proteins such as phosphorylation and ubiquitinylation regulate the association with transcriptional coregulators (Bhatt and Ghosh, 2014; Smale, 2012).

10.11 Physiological Role of NF-κB Transcription Factors

NF-κB transcription affects fundamental cellular responses such as proliferation, differentiation, survival, and metabolism. There are only a few, if any, organ systems, in which NF-κB signaling is not involved. Historically, most information has been obtained in cells and organs involved in inflammation and immunity, where NF-κB is a central player. NF-κB regulates lymphoid organogenesis and many steps in the differentiation, expansion, and survival of lymphoid and myeloid cells. In innate immunity, NF-κB regulates both the initiation and the execution of the immune response. NF-κB is responsible for transcription of the genes encoding many proinflammatory cytokines and chemokines. Receptors of inflammatory chemokines such as TNF receptors and Toll-like receptors signal via NF-κB. In the adaptive immune system, activation, differentiation, proliferation, and effector functions of B and T cells are affected by NF-κB signaling. As a central regulator of inflammation, NF-κB also mediates anti-inflammatory responses (Hayden and Ghosh, 2011).

The role of NF-κB signaling extends beyond the hematopoietic cells that mediate innate and adaptive immune responses. When the gene encoding p65, IKKβ, or NEMO is disrupted in mice, massive apoptosis of hepatocytes occurs leading to embryonic lethality. This observation not only demonstrates that the canonical NF-κB pathway is active in epithelial cells of the liver but that NF-κB signaling prevents apoptosis of these cells. Mice lacking NEMO or both IKKα and IKKβ in intestinal epithelial cells develop severe chronic colitis, characterized by epithelial ulceration, elevated expression of proinflammatory mediators, and infiltration of immune cells. Thus, the lack of NEMO sensitizes intestinal epithelial cells to apoptosis, comparable to the deficiency of canonical NF-κB signaling in hepatocytes. Because NEMO is an X-chromosomal gene, loss of NEMO causes embryonic lethality in male mice. Heterozygous females exhibit abnormalities of the skin that closely resembles the rare human disease Incontinentia pigmenti, which is caused by *NEMO* gene mutations. A characteristic feature of Incontinentia pigmenti is the increased rate of keratinocyte apoptosis. As discussed in the following text, the antiapoptotic effect of NF-κB is also the main driver in oncogenesis (Wullaert, Bonnet, and Pasparakis, 2011).

Knockout studies have also demonstrated that NF-κB signaling is involved in bone development, bone homeostasis, and bone remodeling. Mice with targeted deletions of both the gene encoding IKKα and that for IKKβ develop osteopetrosis due to a defect in osteoclast differentiation (Iotsova *et al.*, 1997). The bone remodeling process is regulated by the concerted action of bone-resorbing osteoclasts and bone-forming osteoblasts. The receptor activator of NF-κB

(RANK) and its ligand (RANKL) regulate the interplay of these cells. RANKL, a member of the TNF superfamily, is produced by osteoblasts. RANKL binds to the osteoclast receptor RANK, which activates NF-κB signaling (Anandarajah, 2009).

10.12 Mutational Activation of NF-κB Pathways in Malignant Disease

(Video: NF-κB pathways. Part 3: Activation of the NF-κB pathway in malignant disease – only closed website: NF_κB_Video_03_ebook.mp4)

10.12.1 B-Cell Lymphomas

Lymphomas are tumors that develop from lymphocytes. About 90% of human lymphomas are B-cell lymphomas. The various types of lymphoma reflect different stages of B-cell differentiation. Hodgkin's lymphoma and non-Hodgkin's lymphoma constitute two general classes of B-cell lymphoma. Among the non-Hodgkin's lymphomas, diffuse large B-cell lymphoma (DLBCL) is most prevalent, comprising about 40% of the cases. Based on different stages of differentiation and genomic lesions, three entities of DLBCL are distinguished: activated B-cell like diffuse large B-cell lymphoma (ABC DLBCL), germinal center B-cell like diffuse large B-cell lymphoma (GCB DLBCL), and primary mediastinal B-cell lymphoma (PMBL).

The rapid phosphorylation and turnover of IκBα and the nuclear localization of p50/p65 heterodimers are characteristic for the ABC subtype of DLBCL. Thus, the NF-κB pathway is almost always constitutively activated in this entity of DLBCL (Figure 10.6).

In about 10% of ABC DLBCL, the constitutive activation of the NF-κB pathway can be attributed to mutations in CARD11. In contrast to wild-type CARD11, which localizes diffusely in the cytoplasm, mutant CARD11 forms complexes with other component of the CBM (CARD11/BCL-10/Malt1) signalosome, suggesting active signaling. In about one-quarter of ABC DLBCL, the A20 protein is inactivated by nonsense mutations or genomic deletions of the *TNFAIP3* gene. Such mutations are rarely found in other subtypes of DLBCL. Since A20 serves as a negative regulator, its absence leads to augmented and deregulated NF-κB signaling.

NF-κB signaling is also activated in Hodgkin's lymphoma. Hodgkin's lymphoma contains the characteristic Hodgkin Reed–Sternberg (HRS) cells, which are surrounded by inflammatory cells. About half of Hodgkin's lymphomas harbor the Epstein–Barr virus (EBV). The virus encodes the LMP1 protein, which activates the NF-κB pathway. However, also in EBV negative cases, p50/p65 heterodimers are constitutively found in the nucleus, indicating active NF-κB signaling. In about 20% of Hodgkin's lymphoma cases, HRS cells harbor inactivating mutations in the *NFKBIA* gene, which encodes IκBα. Occasionally, the *NFKBIE* gene

(encoding IκBε) is affected by inactivating mutations. Genetic inactivation of the *NFKBIA* or *NFKBIE* genes occurs preferentially in EBV negative cases, supporting the oncogenic function of active NF-κB signaling. Finally, HRS cells from 44% of Hodgkin lymphomas, mostly EBV negative, harbor mutations and deletions in *TNFAIP3*, leading to the inactivation of A20.

MALT lymphoma is a B-cell lymphoma that involves the mucosa-associated lymphoid tissues. It often affects the stomach, but can be found in other mucosal sites as well. In the majority of cases, gastric MALT lymphoma is associated with persistent infection with *Helicobacter pylori.* In many of these cases, eradication of *H. pylori* infection by antibiotic treatment causes sustained complete remission.

In about one-quarter of gastric MALT lymphomas, a t(11;18) translocation is found. The translocation leads to the production of a fusion protein derived from *BIRC3* (encoding cIAP2) and *MALT1* sequences. These cases do not respond to antibiotic therapy. Less often, the *MALT1* gene is amplified or translocated from chromosome 18 into the immunoglobulin heavy-chain locus (*IGH*) on chromosome 14 [t(14;18)]. As a consequence, the *MALT1* gene is overexpressed. All of these genomic mutations lead to activation of the NF-κB pathway (Staudt, 2010; Niemann and Wiestner, 2013).

10.12.2 Multiple Myeloma

Multiple myeloma is an incurable malignancy of mature B-lymphoid cells. The uncontrolled proliferation of plasma cells in the bone marrow leads to characteristic "punched-out" bone lesions. About 95% of transformed plasma cells retain the ability to secrete antibodies, which produce a slender peak in the electropherogram of plasma proteins due to their clonality (see Chapter 2). Circulating monoclonal immunoglobulins give rise to progressive renal insufficiency, particularly when isolated light chains are secreted.

Gene expression analyses revealed that about 80% of primary samples of multiple myelomas displayed a gene signature characteristic for NF-κB signaling. Both the canonical and alternative pathways of NF-κB signaling can be involved, indicated by the nuclear localization of p65 (canonical pathway) or nuclear p52 and RelB (alternative pathway), respectively.

The NF-κB gene signature may be explained by signals from the bone marrow microenvironment to plasma cells. Cells of the bone marrow microenvironment produce the TNF family members BAFF (B-cell activating factor of tumor necrosis factor family) and APRIL (a proliferation-inducing ligand). Plasma cells express BCMA (B-cell maturation antigen), the receptor for BAFF, and TACI (transmembrane activator and calcium modulator and cyclophilin ligand interactor), the receptor for APRIL. However, in many multiple myelomas, genomic mutations activate the NF-κB signaling pathway.

As described earlier and depicted in Figure 10.11, NIK is an important enzyme, which can activate canonical and alternative NF-κB signaling. Many of the gene

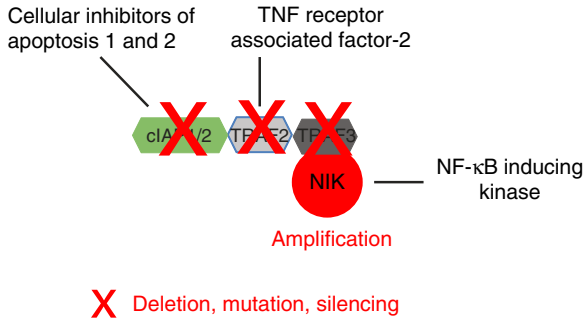


Figure 10.11 Mechanisms leading to the activation of NIK in multiple myeloma. Cellular inhibitors of apoptosis, TRAF2 or TRAF3 can be inactivated by deletion, mutation, and/or silencing of the coding genes. In addition, the *MAP3K14* gene, which encodes NIK, can be amplified. NIK can also be activated by the amplification of genes, which, under physiological conditions, signal via the alternative NF-κB pathway (not shown).

mutations identified in multiple myelomas free NIK from negative regulation: (i) deletion of genes coding for cellular inhibitors of apoptosis (*BIRC2*, *BIRC3*), (ii) deletion, mutation, or silencing of either the *TRAF2* of *TRAF3* gene, or (iii) amplification of the *MAP3K14* gene, which encodes NIK. When overexpressed, NIK can activate NF-κB signaling. NIK can also be activated by the amplification of genes, which, under physiological conditions, signal via the alternative NF-κB pathway. One of these genes encodes CD40. Another gene (*TNFRSF13B*), which may be amplified in myeloma, encodes the receptor TACI. Furthermore, the gene encoding the Lymphotoxin-beta (LTβ) receptor (LTBR) may be amplified. The alternative pathway can also be activated downstream of NIK. For instance, mutations in the *NFKB2* gene lead to a truncated p100 protein resembling the p52 transcription factor, which, under normal conditions, is generated from the p100 precursor by proteolytic cleavage.

Massive parallel sequencing of malignant plasma cells in myeloma patients identified additional mutated genes of the NF-κB pathway (Chapman *et al.*, 2011). Mutations affect the genes encoding IKKβ (*IKBKB*) and an IKKβ-binding protein (*IKBIP*), as well as a member of the RIP family of protein kinases (*RIPK4*). Mutations of the *CARD11* gene are observed not only in ABC DLBCL but also in multiple myeloma. Other mutant genes encode tumor necrosis factor receptor superfamily member 1A (*TNFRSF1A*) and Toll-like receptor 4 (*TLR4*). Three genes code for proteins that are involved in ubiquitinylation/deubiquitinylation. CYLD acts as deubiquitinase, and βTrCP (BTRC) and MEKK1 (MAP3K1) are E3 ubiquitin protein ligases. Genes and proteins related to NF-κB signaling that are mutated in multiple myeloma, are summarized in Table 10.1.

Table 10.1 Genes and proteins involved in NF-κB signaling that are mutated in multiple myeloma.

Gene	Encoded protein	Function
BIRC2	cIAP1	Ubiquitin binding and ligation
BIRC3	cIAP2	Ubiquitin binding and ligation
TRAF2	TRAF2	Scaffold protein
TRAF3	TRAF3	Scaffold protein
MAP3K14	NIK	Phosphorylation of IκB
IKBKB	IKKβ	Phosphorylation of IκB
IKBIP	IKK-interacting protein	Proapoptotic
RIPK4	RIPK4	Proapoptotic
CARD11	CARD11	Member of the CBM complex
TNFRSF1A	TNFR 1	TNFα receptor
TNFRSF13B	TACI	Member of TNF receptor family
CD40, (TNFRSF5)	CD40	Member of TNF receptor family
LTBR (TNFRSF3)	Lymphotoxin β-receptor	Member of TNF receptor family
NFKB2	P100/p52	Member of NF-κB family
TLR4	TLR4	Toll-like receptor 4
BTRC	βTrCP	E3 ubiquitin ligase
MAP3K1	MEKK1	E3 ubiquitin ligase
CYLD	CYLD	Deubiquinylating enzyme

10.12.3 Activation of NF-κB Pathways by Polycomb-Mediated Loss of microRNA-31 in Adult T-Cell Leukemia/Lymphoma

Adult T-cell leukemia/lymphoma (ATL) is a rare aggressive neoplasm with a very poor prognosis. Human T-cell leukemia virus type I (HTLV-I) has been recognized as an etiological agent. The viral protein Tax activates the NF-κB pathway. However, constitutive activation of NF-κB has also been observed in ATL in the absence of HTLV-I. In ATL cells, the deregulated activity of NIK may cause aberrant NF-κB signaling. However, the gene encoding NIK (*MAP3K14*) appears not to be amplified.

An epigenetic mechanism has been identified that may underlie the increased activity of NIK in ATL cells. Epigenetic silencing involves proteins of the Polycomb group (PcG). In vertebrates, PcG proteins form four well-characterized Polycomb repressive complexes (PRC 1–4). The complexes 2–4 contain the catalytically active protein EZH2, which trimethylates the lysine residue in position 27 of histone 3, thereby establishing the H3K27me3 mark. The polycomb protein in PRC1 associates with the H3K27me3 mark, leading to a compaction of chromatin and a repression of transcription (Figure 10.12).

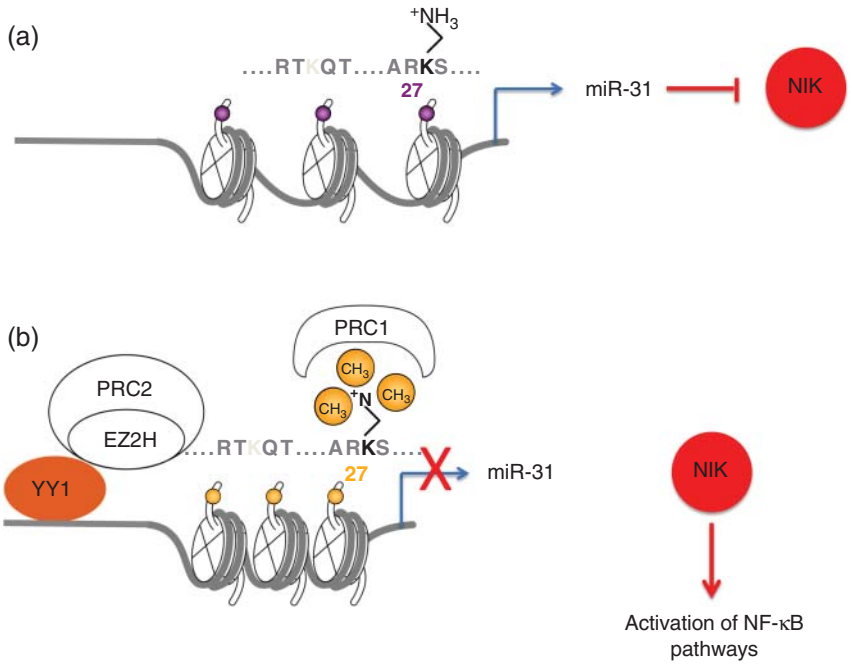


Figure 10.12 Activation of NF-κ B pathways by polycomb-mediated loss of microRNA-31 in adult T-cell leukemia. (a) MicroRNA-31 (miR-31) can silence the *MAP3K14* gene, which encodes the NIK kinase. (b) In human adult T-cell leukemia, synthesis miR-31 can be inhibited by Polycomb group proteins. Upstream of the miR-31 region, binding motifs for the YY1 transcription factor are present. The YY1 transcription factor can recruit the Polycomb repressor complex 2 (PRC2), which contains the methyl transferase EZH2. Upon binding of the PRC2 repressor complex to the YY1 transcription factor, the lysine residue (K) in position 27 of histone 3 is trimethylated. It is known that trimethylated lysine in position 27 of histone 3 recruits the polycomb repressor complex 1 (PRC1). This leads to a compaction of chromatin. As a consequence, the transcription of the gene encoding miR-31 is repressed, the NIK gene is no longer silenced, and the NIK kinase can activate NF-κB pathways. (According to Yamagishi *et al.* (2012), with permission, modified.)

In a screen of microRNAs in ATL cells, the expression of microRNA-31 (miR-31) was profoundly repressed in all ATL patients tested. Since miR-31 silences the *MAP3K14* gene encoding NIK, the loss of miR-31 leads to increased concentration and activity of NIC. In a small proportion of leukemias, the genomic region containing the *hsa-miR-31* coding region is deleted. In the vast majority of cases, however, genomic mutations cannot explain the downregulation of miR-31. Upstream of the miR-31 region, binding motifs for the YY1 transcription factor are present. The YY1 transcription factor can recruit the PRC2, which is significantly upregulated in ATL. Upon binding of the PRC2 repressor complex to the YY1 transcription factor, the lysine residue in position 27 of histone 3 is trimethylated.

The resulting H3K27me3 mark recruits the PRC1 complex, which leads to a compaction of chromatin. As a consequence, the transcription of the miR-31 gene is repressed, the NIK gene is no longer silenced, and the NIK kinase can activate NF-κB pathways (Yamagishi *et al.*, 2012).

10.12.4 Carcinomas

Among the human carcinomas occurring with a high frequency, mutations of genes involved in the NF-κB pathway have been identified in breast cancer. In the *NFKBIE* (encoding IκBε) and *NFKBIA* (encoding IκBα) genes, truncating and frameshift mutations were identified that lead to loss of function of the encoded proteins. Multiple nonsynonymous mutations in *IKKB* (encoding IKKβ) are compatible with activating mutations. In addition, splice-site mutations were found in the *NFKB1* gene (encoding p105). Mutations were also identified in the AGAP1 and AGAP2 genes. Downregulation of the GTPase-activating proteins AGAP1 or AGAP2 has been shown to enhance NF-κB signaling, which provides a plausible explanation for the inactivating splice-site and frameshift mutations. The gene and mutation spectrum observed in breast cancer specimens indicates that the NF-κB pathway is activated by several different mechanisms in breast cancer.

The prevalence of mutations in genes involved in NF-κB signaling differs between breast cancer subtypes. The highest frequency was found in prognostically unfavorable carcinomas with amplifications of the *ERBB2* (*HER2/NEU*) gene or in the "triple-negative" (lacking mutations in *ERBB2* and the receptor genes for progesterone and estrogen) breast cancers. Genes involved in NF-κB signaling are mutated in 22% of carcinomas in this group (Wood *et al.*, 2007; Jiao *et al.*, 2012).

The *CYLD* gene acts as a classical tumor suppressor gene in familial cylindromatosis. Patients with cylindromatosis display multiple benign tumors of skin adnexa, for example, of the sweat glands. CYLD is a thioesterase, which deubiquitinylates a number of proteins involved in NF-κB signaling, particularly NEMO. The deubiquitinylation of NEMO and other proteins of the NF-κB signaling pathway suggests that CYLD negatively regulates NF-κB signaling. Thus, loss of CYLD function activates the pathway (Staudt, 2010).

10.13 Cross Talk between Mutant KRas and NF-κB

In various carcinomas, members of the NF-κB family have been found to be upregulated, but mutations in genes of the NF-κB pathway have not been identified. In these tumors, mutations may activate pathways that feed into the NF-κB pathway.

KRAS is the oncogene most frequently activated in human carcinomas. About three-quarters of human lung carcinomas with *KRAS* mutations coexpress KRas

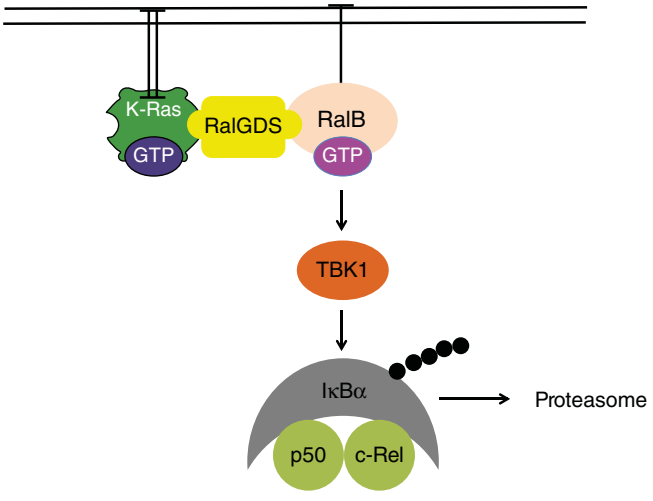


Figure 10.13 Activation of NF-κB signaling by mutant KRas. Mutant KRas associates with RalGDS, a guanine nucleotide exchange factor of the Ral-GEF family. RalGDS assists in the exchange of GDP, bound to RalB, against GTP. This leads to the activation of the RalB protein. Active RalB activates TANK binding kinase 1 (TBK1), which phosphorylates serine residues of the destruction box of IκBα. IκBα is ubiquitinylated and directed to the proteasome. As a consequence, the p50/c-Rel dimer is released.

and NF-κB gene signatures. In a mouse model of lung cancer caused by overexpression of mutant KRas and targeted deletion of *TP53*, the canonical NF-κB pathway was activated. When these mice were treated with an IκBα suppressor, the growth of tumors slowed down (Meylan *et al.*, 2009). As described in Chapter 7, KRas stimulates the MAPK and PI3K pathways. In addition to these pathways, the KRas-associated RAL nucleotide exchange factors mediate the binding of GTP to the RAL GTPase RALB (Figure 10.13). RALB activates the kinase TBK1 (TANK binding kinase 1), which is involved in TLR signaling to NF-κB. The importance of TBK1 in transformation is supported by the experimental finding that silencing of the *TBK1* gene killed cells transformed with mutant KRAS, but not with wild-type KRAS (Barbie *et al.*, 2009).

10.14 Inflammation, NF-κB, and Cancer

Since the identification of the first oncogene, *SRC*, cancer has been regarded to be essentially a genetic disease (Vogelstein *et al.*, 2013). However, the conditions that led to the discovery of the oncogene have often been overlooked (Figure 10.14). *SRC* is the transforming gene of the Rous sarcoma virus (RSV). In chicks infected with the RSV, tumors grow at the locations where wounds have occurred. Similarly, the local injection of factors such as TGFβ or FGF2 initiated the growth

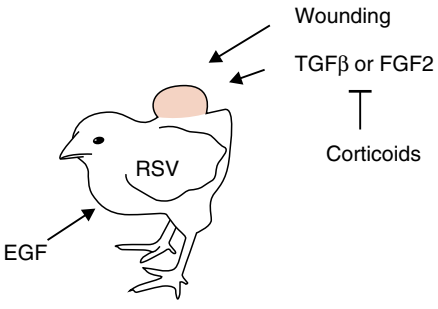


Figure 10.14 Wounding and inflammation in Rous sarcoma virus (RSV) induced tumorigenesis. Infection of chicks with RSV does not suffice to induce tumors. Tumors are induced only after wounding or after local injection of TGFβ and FGF2. Anti-inflammatory corticoids inhibit tumor formation. Locally injected EGF does not induce tumors. (Based on data by Martins-Green, Boudreau, and Bissell (1994).) (Wagener and Müller, 2009), with permission.

Table 10.2 Inflammatory conditions associated with cancer.

Inflammatory condition	Cancer
Bronchitis	Lung cancer
Ulcerative colitis	Colonic cancer
Reflux esophagitis	Esophageal cancer
Gastritis (*Helicobacter pylori*)	Gastric cancer
Viral hepatitis (B,C)	Liver cancer
Cystitis (Schistosoma)	Bladder cancer

of tumors in RSV-infected chicks. Both factors induce inflammatory reactions. Growth of tumors could be inhibited by the application of corticoids. Corticoids suppress inflammatory reactions. In contrast to TGFβ and FGF2, EGF did not give rise to tumors, since it does not induce inflammation (Martins-Green, Boudreau, and Bissell, 1994). Since wound healing and inflammation share many pathophysiological aspects, these findings suggest that inflammation is an essential feature of malignant tumors. In fact, inflammation is regarded as one of the hallmarks of cancer (Hanahan and Weinberg, 2011).

Inflammation can be both a cause and a consequence of cancer. In both situations, NF-κB plays a central role. Chronic inflammation is a risk factor for the formation of malignant tumors. According to estimates, chronic inflammation causes about 15% of human cancers. In Table 10.2, chronic inflammatory conditions that are associated with human carcinomas are listed.

Colitis-associated colorectal cancer (CAC) is an inflammatory condition, which predisposes to colorectal cancer. CAC accounts for 5% of sporadic colorectal carcinomas. The interplay between NF-κB activation in intestinal epithelial cells and inflammatory myeloid cells has been analyzed in a colitis-associated cancer model.

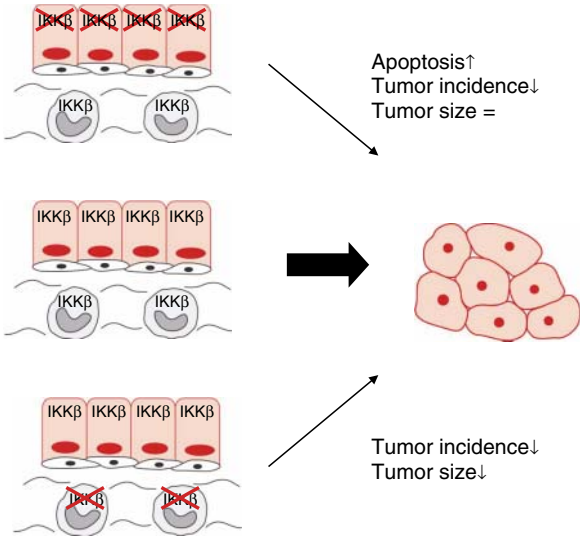


Figure 10.15 NF-κB signaling in a model of colitis-associated cancer. Mice injected with the carcinogen azoxymethane and fed with dextran sulfate sodium (DSS) salt develop colitis-associated cancer. Targeted deletion of the IKKβ encoding *IKBKB* gene leads to an increased rate of apoptosis upon DSS feeding. Consequently, tumor incidence is lower compared to wild-type mice. However, the size of tumors, which develop, does not differ. Targeted deletion of the *IKBKB* gene in myeloid cells is accompanied by a reduced production of cytokines. In this setting, both number and size of tumors are reduced. (Based on data by Greten *et al.* (2004).) (Wagener and Müller, 2009), with permission.

Mice fed with a single dose of the carcinogen azoxymethane and repeated doses of dextran sulfate sodium salt (DSS) develop chronic inflammation, which greatly enhances the incidence of azoxymethane-induced tumors. In mice, in which IKKβ was shut off specifically in intestinal epithelial cells by targeted deletion of the *Ikbkb* gene, the rate of apoptosis increased upon treatment with DSS. This was accompanied by a decrease in the incidence of tumors. However, the size of those tumors that developed was not different from the tumors in wild-type mice. When the *Ikbkb* gene was deleted in myeloid cells, both tumor incidence and tumor size were reduced, since less proinflammatory cytokines were produced. This experiment demonstrated that NF-κB signaling both in epithelial and myeloid cells can contribute to tumorigenesis (Figure 10.15) (Greten *et al.*, 2004).

10.15
Activation of Osteoclasts in Multiple Myeloma and Breast Cancer Metastases

One of the typical clinical features of multiple myelomas are "punched-out" bone lesions. As described earlier, bone resorption is mediated by osteoclasts.

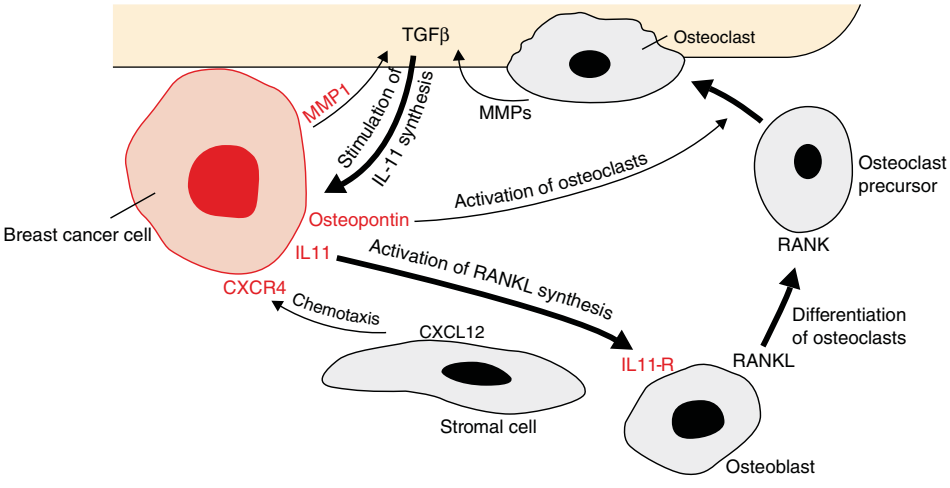


Figure 10.16 Vicious cycle of osteolytic breast cancer metastases. A clone of a breast cancer cell line preferentially metastasized to bone. This cell clone produces the factors indicated in red, the identification of which led to the deduction of a vicious cycle. Bone marrow stromal cells produce the chemokine CXCL12, which binds to the CXCR4 receptor of tumor cells. The tumor cells invade the bone marrow along the chemokine gradient. IL-11 produced by tumor cells stimulates the synthesis of RANKL in osteoblasts. RANKL binds to RANK in osteoclast precursors, thereby stimulating their differentiation to osteoclasts. Osteopontin supports differentiation and activation of osteoclasts. MMP1 produced by osteoclasts and tumor cells liberates TGFβ from the bone matrix. TGFβ stimulates the synthesis of IL-11 in the tumor cells, thereby closing the vicious cycle. (Based on data by Kang *et al.* (2003).)

Osteoclasts are activated by the binding of RANKL to its receptor RANK. RANK, a member of the TNF receptor superfamily, activates the NF-κB pathway. Etodolac (SDX-308) is a nonsteroidal anti-inflammatory drug employed in clinical trials for the treatment of osteoarthritis. In experimental systems, etodolac effectively inhibited multiple myeloma cell proliferation and osteoclast activity. Etodolac was shown to suppress TNFα-induced NEMO and IκBα phosphorylation and degradation and the subsequent activation of NF-κB in human multiple myeloma cells. These data suggest a causal relationship between aberrant NF-κB signaling and the development of bone lesions in multiple myeloma (Feng *et al.*, 2007).

Bone is a preferred tissue for distant metastases of mammary carcinomas. The metastases are lytic, which means that bone tissue is destroyed. As described earlier, NF-κB signaling is involved in bone development, bone homeostasis, and bone remodeling. The activation of osteoclasts by the binding of RANKL to the osteoclast receptor RANK appears to be central to the generation of lytic bone metastases. Experimental results suggest a vicious cycle in the generation of bone metastases (Figure 10.16) (Kang *et al.*, 2003).

Table 10.3 Compounds affecting targets, which are involved in NF-κB signaling.

Compound	Target	Tumors
Fostamatinib	Inhibition of SYK	DLBCL
Ibrutinib	Inhibition of Bruton's tyrosine kinase	DLBCL
Bortezomib	26S proteasome inhibitor	Multiple myeloma
Non steroidal anti-inflammatory drugs (aspirin, sulindac)	Cyclooxygenases I and II	Various
Glucocorticoids	NF-κB pathway inhibition	Various

10.16 Targeting NF-κB Pathways

Although NF-κB-activating signal transduction pathways and negative feedback mechanisms have been studied in great detail, there are only few examples of successful therapies that directly target components of the pathways (Table 10.3).

10.16.1 B-Cell Malignancies

In B-cell lymphomas, successful targeted therapies have been developed and applied. After binding of antigen to the BCR, the tyrosine kinase SYK initiates BCR signaling. Besides NF-κB, SYK affects other signaling pathways such as the MAPK pathway. The SYK inhibitor Fostamatinib has been tested in clinical trials. In addition to SYK, Fostamatinib inhibits other tyrosine kinases, for example, FLT3 (FMS-like tyrosine kinase 3), KIT, and JAKs (Janus kinases). In a phase II clinical trial, an objective response was observed in 24% of DLBCL cases. Because of the many targets of the drug, the role of NF-κB pathway inhibition in the therapeutic response is uncertain (Young and Staudt, 2013).

Ibrutinib is an inhibitor that forms a covalent bond with a cysteine residue near the active site of Bruton's tyrosine kinase. Only nine tyrosine kinases contain a cysteine residue in a similar position, which lends Ibrutinib a high specificity. In a phase II clinical trial, an objective response was achieved in 40% of ABC DLBCL patients. As explicated earlier, the activation of NF-κB signaling is essential in this lymphoma entity. Since CARD11 acts downstream of BTK, lymphomas with CARD11 mutations are insensitive to Ibrutinib.

Inhibition of the proteolytic activity of MALT1 affected growth and survival of ABC DLBCL, but not of GCB DLBCL cell lines. This finding indicates that MALT1 may be a specific therapeutic target in MALT lymphomas (Hailfinger *et al.*, 2009).

Bortezomib is a 26S proteasome inhibitor, which has been approved for the treatment of multiple myeloma. Since IκB proteins are degraded via the 26S proteasome, it has been suggested that the effect of bortezomib is explained partly by the inhibition of NF-κB signaling. This assumption is supported by

the finding that the subgroup of myeloma cells with a NF-κB signature is more sensitive to bortezomib (Mulligan *et al.*, 2007).

10.16.2 Carcinomas

Triple-negative breast cancer has a poor prognosis. In this entity of breast cancer, NF-κB signaling is activated in a significant number of patients. Thus, members of the NF-κB pathway are potential targets in mammary carcinomas with an active NF-κB pathway.

In other common carcinomas, mutations of genes of the NF-κB pathway are rare. However, as demonstrated for colorectal and lung cancer, the transcription of NF-κB responsive genes may be activated by cross talk with other pathways.

Future clinical trials will show if the NF-κB pathway will be targetable in carcinomas.

10.16.3 Anti-Inflammatory Drugs

Anti-inflammatory drugs are applied both for the prevention and the treatment of cancer.

The incidence of colorectal cancer can be decreased by the anti-inflammatory drug aspirin. In clinical trials, daily use of aspirin reduced risk of colorectal cancer by 24% and mortality from colorectal cancer by 35% after a delay of 8–10 years. Data from clinical trials indicate that aspirin use can reduce the mortality of other cancers as well (Garcia-Albeniz and Chan, 2011). Aspirin is an irreversible inhibitor of the cyclooxygenase (COX) isoenzymes I and II. The transcription of the COX-2 gene is activated by NF-κB. However, aspirin preferentially blocks the COX-I isoenzyme.

In patients with familial adenomatous polyposis (FAP) of the colon, therapy with the nonsteroidal anti-inflammatory drug sulindac reduced the number of newly acquired polyps and induced the regression of established polyps (Ulrich, Bigler, and Potter, 2006).

With respect to the treatment of malignant disease, glucocorticoids are most effective among anti-inflammatory drugs. Glucocorticoids are commonly used in the treatment of leukemias, lymphomas, and myelomas. Glucocorticoids exert a strong anti-inflammatory effect by downregulating proinflammatory cytokines and by inhibiting the NF-κB pathway (Erstad and Cusack, 2013).

10.17 Outlook

A number of different pathways feed into the NF-κB response. In the respective pathways, different signaling proteins are involved. The complexity of NF-κB

signaling is further increased by the formation of various NK-κB dimers, which regulate different genes and may act either as activators or as inhibitors of transcription. So far, differential targeting of NF-κB transcription factors is not feasible. Therefore, therapeutic drugs will target incoming pathways. However, in order to inhibit a particular pathway, compounds must be developed that specifically interfere with signaling factors of the targeted pathway. The drugs may be successful in those tumors, in which the targeted pathway is active. In order to identify the group of patients that potentially benefit from the inhibitor, a predictive test must be available. If these preconditions are fulfilled, NF-κB inhibitors may be effective in well-defined groups of tumor patients.

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11
Wnt Signaling

Summary

The Wnt pathway is the pathway with the most mutations in human tumors. Tumor suppressor genes and oncogenes of the Wnt pathway are mutated in 90% of all colorectal carcinomas, 80% of pancreas carcinomas, 30% of stomach carcinomas, 25% of all skin tumors, and many hepatocellular and prostate carcinomas. Furthermore, overactivation of the pathway has been found in more than 90% of all metaplastic breast carcinomas. The genes that are most often mutated encode the proteins β-catenin, APC (adenomatous polyposis coli protein) and axin. In

Cancer Signaling: From Molecular Biology to Targeted Therapy, First Edition.
Christoph Wagener, Carol Stocking, and Oliver Müller.

Companion Website:www.wiley.com/go/wagener/cancersignaling

this chapter, we first present the simplified and linear version of the canonical Wnt pathway before we take a look at its complex branches, intersections, and connections with other pathways. In the third part, we provide some examples of compounds with therapeutic potential that interfere with Wnt pathway activity.

11.1 The History of Wnt

The eponymous growth factor Wnt (pronounced "wint"), which represents the first extracellular level of the Wnt pathway, derives its name from two genes. In the early 1980s, Roel Nusse and Harold Varmus searched for new genes causing mammary tumors. They infected mice with the mouse mammary tumor virus (MMTV) and analyzed the viral integration sites in the tumor genomes. Interestingly, the integration sites in independent tumors clustered prominently in a specific genomic position. In proximity to the integration site was a proto-oncogene activated by the virus, which was named int-1 (Nusse *et al.*, 1984). The search for genes with homologous sequences revealed that int-1 is a member of a gene family that is highly conserved over species. The ortholog of int-1 in the fruit fly was found to be the segment polarity gene wingless *wg*. This gene was originally discovered by the Nobel laureates Christiane Nüsslein-Volhard and Eric Wieschaus as a gene responsible for wing formation in Drosophila melanogaster (Nusslein-Volhard and Wieschaus, 1980). With the discovery of other orthologs and homologs, it became obvious that the members of the int/Wg family have various functions in different species. Thus, the name int/Wg was no longer adequate and was changed to Wnt, as a fusion of the gene names Wg and int-1 and as an abbreviation for wingless-related integration site (Table 11.1).

11.2 The Canonical Wnt Pathway

Based on the participating proteins and cellular effects, three different Wnt pathways can be distinguished: the canonical pathway, the planar cell polarity pathway, and the Wnt/calcium pathway. The proto-oncoprotein β-catenin is the key protein of the canonical variant, whereas the two other pathways operate independently of it. Here, only the canonical Wnt pathway will be outlined because it is the most important one in carcinogenesis and also the best characterized variant of the three versions.

Three different states of the canonical Wnt pathway can be differentiated (Clevers and Nusse, 2012): the nonactivated state, the activated state in the presence of the extracellular Wnt factor, and the nonphysiologically activated state.

Table 11.1 Genes and the corresponding proteins of the Wnt pathway that show tumor specific alterations and are involved in cancer development.

Gene	Protein	Function in Wnt pathway	Alteration in tumor tissue	Cancer
WNT(s)	Wnt(s)	Activating hormone	Overexpression	Breast, melanoma
WIF1	Wnt-inhibiting factor 1	Inhibitory hormone	Downregulation	Lung
SFRP(s)	Secreted frizzled-related protein(s)	Inhibitory hormone	Deletion, downregulation	Bladder, cervix, colon
FZD(s)	Frizzled(s)	Hormone receptor	Overexpression	Kidney
LRP5	Low-density lipoprotein receptor-related protein 5	Coreceptor	Overexpression	Osteosarcoma
DVL	Disheveled	Scaffolding	Overexpression	Breast
GSK3B	Glycogen synthase kinase-3 β	Serine/threonine kinase	Deregulated	Colon
FRAT1	FRAT-1	GSK3β inhibitor	Overexpression	T-cell lymphoma
AXIN(s)	Axin(s)	Scaffolding	LOF	Ovary, liver
APC	Adenomatous polyposis coli protein	Various	Deletion, downregulation, LOF	Colon and others
CTNNB1	β-catenin	Transactivator	Overexpression, GOF	Various
TCF-4	Transcription factor 4	Transcription factor	LOF	Colon
BTRC	β transducin repeat-containing protein	Substrate recognition component of ubiquitin ligase	Downregulation	Prostate

GOF, gain-of-function mutation and LOF, loss-of-function mutation.
(s) Indicates more than one homologous gene or protein.

11.2.1 The Nonactivated Wnt Pathway

(Video: The Wnt pathway in a normal and in a tumor cell – only open website: https://www.youtube.com/watch?v=YuVkRTOYOlQ*)*

In the normal cell, the pathway is inactive in the absence of the Wnt factor or if the binding of Wnt to its receptors is prevented by WIF (Wnt-inhibiting factor) or SFRP (secreted frizzled-related protein) (Figure 11.1). Both extracellular proteins are able to capture the Wnt protein before it interacts with the receptor. In this inactive state, several proteins in the cytosol are associated to form the so-called destruction complex, which regulates the Wnt-pathway effector β-catenin. The

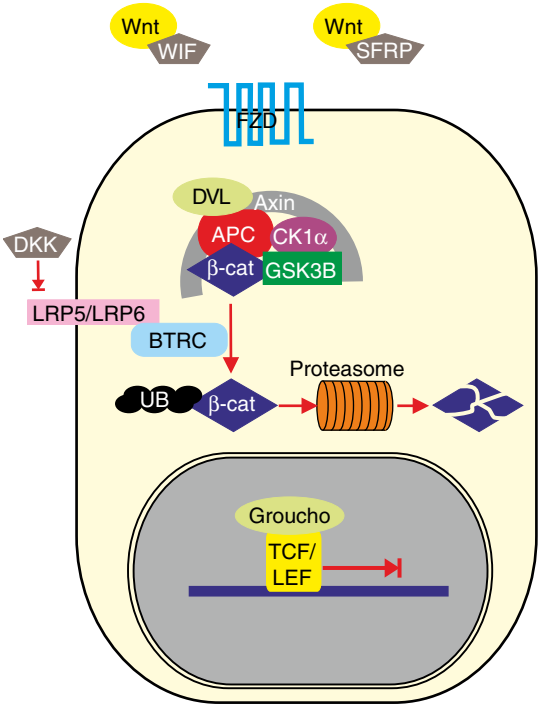

Figure 11.1 The Wnt pathway in its inactive state. There is no extracellular Wnt factor present or it is captured by WIF or SFRP. The destruction complex phosphorylates β-catenin (β-cat), which is ubiquitinylated and degraded by the proteasome. In the nucleus TCF/LEF inhibits gene expression. (Wagener and Müller, 2009), with permission.

destruction complex includes the tumor suppressor proteins APC (adenomatous polyposis coli protein) and axin, the serine/threonine kinases casein-kinase-1α (CK1α) and glycogen-synthase-kinase-3 β (GSK3B), the protein phosphatase 2A (PP2A), and the E3-ubiquitin ligase BTRC (β-transducin repeat-containing protein). As members of the destruction complex, the kinases CK1α and GSK3B phosphorylate β-catenin at several serine and threonine residues in the so-called degron motif at its amino terminus. Phosphorylated β-catenin is recognized by BTRC. BTRC is activated by the coreceptor LRP5 (LDL receptor-related protein 5) or LRP6, both of which can be inhibited by the tumor suppressor protein DKK (Dickkopf-related protein). Activated BTRC transfers ubiquitin peptides onto β-catenin to mark it for subsequent degradation in the 26S proteasome. This biochemical sequence ensures low β-catenin concentrations, both in the cytosol and in the nucleus. In the absence of nuclear β-catenin, transcription factors of the TCF/LEF (T-cell factor/lymphoid-enhancing factor) family occupy and repress promoters of its target genes, assisted by transcriptional corepressors such as Groucho. Gene expression and resulting biological effects are blocked.

11.2.2 The Physiologically Activated Wnt Pathway

The pathway is physiologically activated by the extracellular Wnt factor via autocrine and paracrine mechanisms. The 19 homologous Wnt genes, which have been identified both in murine and in human cells (see Wnt homepage: http://www.stanford.edu/~rnusse/wntgenes/humanwnt.html), code for proteins that are posttranslationally modified by palmitoylation and glycosylation. Both modifications are necessary for proper secretion of the proteins. The lipid modification is necessary for its interaction with the membrane-bound receptor frizzled (FZD) and the coreceptor LRP5/6 (Figure 11.2). The binding of Wnt to its receptors leads to heterodimerization of FZD with LRP5/6 and to the phosphorylation of LRP5/6 by CK1 homologs (e.g. CK1α, γ, and ε). Axin binds to phosphorylated LRP5/6 and disheveled (DVL) binds to FZD. Earlier models suggested that the destruction

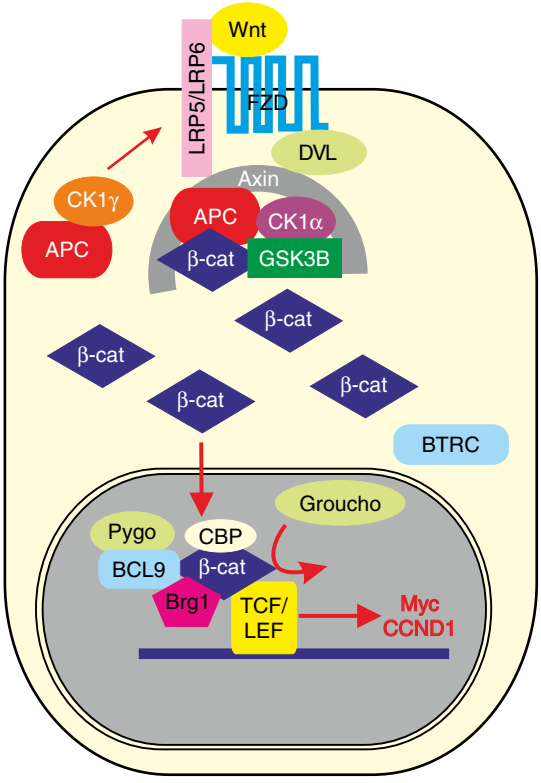

Figure 11.2 The Wnt pathway in its active state. The extracellular Wnt factor binds to the membrane-bound heterodimerized receptor. The destruction complex phosphorylates β-catenin (β-cat), which prevents ubiquitinylation. The levels of cytosolic and nuclear β-catenin rise. In the nucleus, β-catenin releases inhibition of gene expression. Exemplified target genes are *MYC* and *CCND1*. (Wagener and Müller, 2009), with permission.

complex dissociates as a consequence of Axin being recruited to the LRP/FZD receptor complex, in which case β-catenin is no longer phosphorylated. However, more recent data show that the intact destruction complex binds to FZD and LRP5/6 and that β-catenin is still phosphorylated (Li *et al.*, 2012). In the presence of the Wnt signal, β-catenin is not ubiquitinylated. Subsequently, the complex becomes saturated by phosphorylated β-catenin, leading to the accumulation of newly synthesized β-catenin and its translocation into the nucleus. In the nucleus, β-catenin replaces the transcription repressor and Groucho-homologous protein TLE1 (transducin-like enhancer protein 1) from TCF/LEF and recruits transcriptional coactivators and histone modifiers such as Brg1, CBP, Bcl9, and Pygo. Subsequently, the expression of target genes is induced. Major target genes are *CCND1* and MYC, encoding the cell cycle activator cyclin D1 and the MYC transcription factor, respectively.

11.2.3 The Nonphysiologically Activated Wnt Pathway in the Absence of the Wnt Signal

In normal cells, several tumor suppressor proteins are expressed that inhibit the Wnt pathway. Among them are WIF, SFRP, and Wnt5A, which prevent the binding

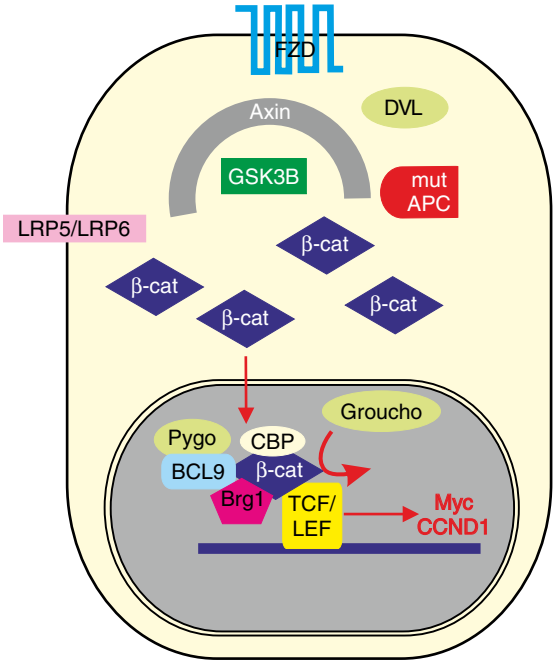

Figure 11.3 The Wnt pathway activated by a mutation in the APC gene. The mutationally truncated APC protein is not able to associate with other proteins in the destruction complex. As a consequence, the levels of cytosolic and nuclear β-catenin (β-cat) rise. In the nucleus, β-catenin releases inhibition of gene expression. (Wagener and Müller, 2009), with permission.

of the Wnt factor to its receptor, and axin and APC, which are necessary for the proper formation of the destruction complex. Inactivating mutations in the corresponding genes lead to an increase in the β-catenin concentration and to the activation of downstream signaling independently of activation by the Wnt factor (Figure 11.3). A similar effect is observed through activating mutations or overexpression of the oncogene *CTNNB1*, encoding β-catenin, or overexpression of the *WNT* gene itself.

11.3 The Wnt Network

During the early years of Wnt research, the pathway was regarded as a linear arrangement of intracellular signaling proteins that connected the extracellular Wnt factor with intracellular gene expression. Today, we know that the scenario is much more complicated. The pathway is highly branched and connected with several other biochemical pathways, creating a multidimensional, multilevel network. The complexity of the network finds its basis at two levels. First, some proteins of the Wnt pathway also function in other pathways or in other contexts. Secondly, the pathway controls a multifaceted targetome with several levels of transcription. Some of the target genes themselves are able to affect not only the Wnt pathway but also other pathways.

11.4 Proteins of the Wnt Pathway with Diverse Functions

11.4.1 APC (Adenomatous Polyposis Coli Protein)

The APC protein is the largest protein with the most variable functions of all Wnt pathway proteins. The discovery of the corresponding tumor suppressor gene was a result of a search for genes that are responsible for the inherited cancer predisposition FAP (familial adenomatous polyposis) (Kinzler *et al.*, 1991). Shortly thereafter, inactivating mutations in the *APC* gene were also discovered in more than two thirds of all noninherited (sporadic) colorectal carcinomas (Powell *et al.*, 1992). Because *APC* mutations are already present at very early tumor stages, they are assumed to be the first and initiating events in the development of sporadic colorectal tumors. The question of how the inactivation of a single gene can initiate such a fatal cascade of transforming events can be answered by looking at the manifold roles the APC protein plays within the cell. The biochemical bases for its effects can be traced to the many functional domains that were identified within the 2843 amino acid APC protein (Figure 11.4). APC interacts with several proteins including β-catenin, axin, tubulin, and EB1 (end-binding protein 1). Most tumor-relevant *APC* mutations lead to premature translation stops and

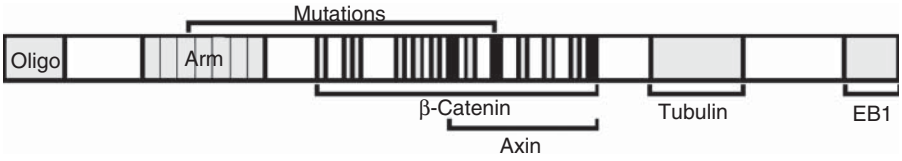

Figure 11.4 The APC protein with sequence motifs and functional domains. The protein comprises an amino terminal oligomerization domain (oligo), 7 armadillo repeats (Arm), 10 repetitive β-catenin-binding motifs (gray), 3 repetitive axin binding boxes (black), a tubulin-binding domain and a carboxyl terminal EB1-binding domain. Most mutations are located within the amino terminal half and lead to a truncated protein. (Wagener and Müller, 2009), with permission.

to truncated proteins. APC proteins lacking the carboxyl terminal domain cannot interact with tubulin, axin, or EB1. The interactions of APC with tubulin, as a monomer of microtubules, and with EB1, which itself regulates microtubule dynamics, are important for its function to stimulate cell migration. The interaction between APC and axin is essential for the proper formation of the destruction complex and thus for the downregulation of β-catenin concentration.

The important roles of the multifunctional APC protein came to light through the analysis of cells carrying an inactivated *APC* gene (Aoki and Taketo, 2007). Cells with inactive APC show increased proliferation rates, defects in the dynamics of the cytoskeleton, disordered cell migration, and unequally segregated chromosomes. These effects can be regarded as major starting points of tumorigenesis and are based on the four main functions of the APC protein (Figure 11.5). (i) First of all, APC regulates the level of β-catenin. In the absence of intact APC, the level of the proto-oncoprotein β-catenin is increased in the cytosol and also in the nucleus. Additionally, APC exports β-catenin out of the nucleus and thus prevents the accumulation of β-catenin in the nucleus, the induction of target genes, and the activation of cell cycle and mitosis. (ii) Thirdly, APC controls apoptosis. Cells with mutated APC are insensitive toward apoptotic signals. The increased expression of intact APC leads to apoptosis. (iii) Fourth, the APC protein is necessary for the regular segregation of chromosomes during mitosis (Rusan and Peifer, 2008). It is assumed that APC is necessary for the intact connection between microtubules and chromosomes at the kinetochores. Without this connection, the chromosomes do not properly segregate to the daughter cells. As a consequence, *APC* negative cells show

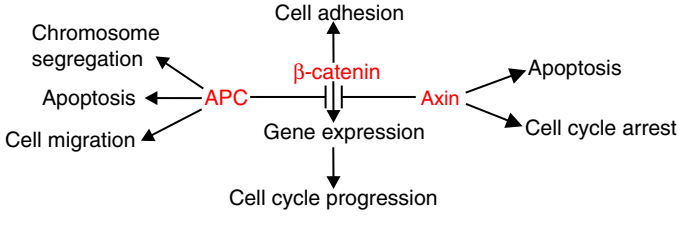

Figure 11.5 Three proteins of the Wnt pathway (red) with diverse functions and their interplay with other functions and pathways.

chromosomal instability (CIN). In fact, the proportion of CIN positive colon tumors is with 85% in a similar range as the proportion of colon tumors with *APC* mutations. (iv) APC controls dynamics of microtubules and has thus an influence on cell migration and the formation of the mitotic spindle apparatus.

11.4.2 β-Catenin

Catenins, which were named after the Latin word *catena* (chain), were originally identified as indirect links between cell adhesion proteins and the cytoskeleton. Beta-catenin and its homolog plakoglobin (γ-catenin) bind to the cytosolic domain of the membrane-anchored E-cadherin. In addition, β-catenin binds to α-catenin, vinculin, and Eplin, forming a bridge between the adherens junction and the contractile actin–myosin cytoskeleton. In addition to this structural function, the proto-oncoprotein β-catenin has a dynamic signaling function in the canonical Wnt pathway. Its elevated concentration is equivalent to the activated state of the pathway. In the nonactivated state, β-catenin is degraded by the proteasome after interaction with the destruction complex, phosphorylation, and ubiquitinylation. Activation of the pathway leads to a more stable protein. The level of β-catenin in the nucleus increases in parallel to its level in the cytosol. Nuclear β-catenin releases transcriptional repression performed by TCF/LEF.

Most mutations in the β-catenin coding gene *CTNNB1* lead to the loss of phosphorylation sites for the kinases CK1α and GSK3B (Polakis, 2007). Mutated β-catenin cannot be phosphorylated. Nonphosphorylated β-catenin is more stable because it is not ubiquitinylated and thus it is not recognized by the proteasome. *CTNNB1* mutations have been identified in many tumor types including brain, skin, stomach, and liver tumors. The majority of colorectal carcinomas with intact *APC*, which amount to approximately 10%, carry mutations in *CTNNB1*. Mutations in *APC* and *CTNNB1* have not been identified within the same tumor. These findings support the model that inactivating *APC* mutations and activating *CTNNB1* mutations have similar biochemical consequences. Nevertheless, there are some important differences: the progression risk of an adenoma with *CTNNB1* mutation is lower than the risk of an adenoma with *APC* mutation. Additionally, in contrast to *APC* mutations, germline mutations in *CTNNB1* have never been reported.

11.4.3 Axin

Axin was named after its ability to induce a second body axis in mouse embryos (Salahshor and Woodgett, 2005). There are two axin coding genes in human cells with 45% homology: AXIN1 and AXIN2 (also called *axil for axin-like protein*) (Figure 11.6). The two gene products are involved in at least three different pathways. In the stress-activated pathway, axin activates the kinase MEKK1, which phosphorylates the kinase SAPK (stress-activated protein kinase), possibly leading

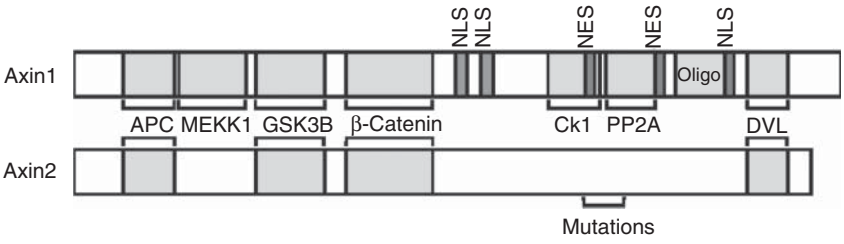

Figure 11.6 Structures of Axin1 and Axin2. Axin1 comprises 862 amino acids, Axin2 comprises 843 amino acids. Mutations in the *AXIN1* gene are distributed over the entire sequence, whereas mutations in *AXIN2* are found between codon 637 and 714. Binding domains for the proteins APC, MEKK1, GSK3B, β-catenin, Ck1, PP2A, and DVL are shown. NLS, nuclear localization sequence; NES, nuclear export sequence; PP2A, protein phosphatase 2A; Oligo, oligomerization domain and MEKK1, MEK kinase 1. (Wagener and Müller, 2009), with permission.

to apoptosis. In the TGFβ (transforming growth factor β) pathway, axin promotes the phosphorylation of SMAD3, which is able to block the cell cycle. In the inactive Wnt pathway, products of the axin coding genes are essential for the degradation of β-catenin.

The cellular concentrations of the proteins Axin1 and β-catenin correlate inversely. In the absence of the Wnt signal, a homodimer of Axin1 forms a scaffold for the proteins of the destruction complex. Inactivation of the *AXIN* genes by mutation leads to an elevated β-catenin concentration. Mutations in the *AXIN1* gene are distributed over all exons. Nevertheless, there are differences in the mutation spectra between different tumor types. Large deletions have mainly been found in medulloblastoma, whereas point mutations are more frequent in liver tumors. Most mutations affect the protein regions that are responsible for its interaction with APC, β-catenin, or GSK3B. Mutated Axin1 is not able to form a scaffold for the proteins of the destruction complex. As a consequence, the intracellular level of β-catenin increases. Most mutations in *AXIN2* lead to a premature translation stop and to the loss of the DVL-binding domain, which is located at the carboxy-terminal region of the protein.

11.5 The Wnt Targetome

The Wnt pathway controls a high number of target genes, which in its entirety is called the *Wnt targetome* (Vlad *et al.*, 2008). Besides its multifunctional proteins, the multifaceted targetome of the Wnt pathway is the basis for its manifold functions and its connections with other pathways. The evaluation of data from microarray studies that have been performed to identify Wnt target genes in different cell types and organisms has yielded more than 2000 candidate target genes (http://www.stanford.edu/group/nusselab/cgi-bin/wnt/target_genes_microarray). How is it possible that a single pathway that regulates only a few transcription factors controls so many target genes? The answer comes from a closer

look at the hierarchy of gene activation. In fact, only the minority of target genes is regulated by promoters that contain binding motifs for the TCF/LEF transcription factor. Instead, most target genes are regulated by products of other target genes or by target pathways. This multilevel character of the targetome is also illustrated by a closer look at the functions of the target genes. Many targets operate as transcription factors, which regulate the expression of other genes, or as autocrine and paracrine factors, which regulate other pathways. Such indirect targets can be understood as regulators or amplifiers of the original signal executed by Wnt.

11.5.1 The Three Levels of the Wnt Targetome

Three levels of the Wnt targetome can be distinguished (Figure 11.7). Target genes on the primary level contain TCF/LEF binding motifs in their promoters and are directly controlled by TCF/LEF. These primary or direct targets include effectors with direct biological effects, such as the proteolytic enzyme MMP-7, transcription regulators such as MYC, and pathway regulators such as VEGF (vascular endothelial growth factor). The secondary level includes two types of targets: indirectly regulated genes (e.g., p21), which are regulated by transcription regulators, and "target pathways" (e.g., VEGF receptor tyrosine kinase pathway), which are regulated by pathway regulators. On the tertiary level, effectors such as the VEGF target gene DSCR1 (Down syndrome critical region protein 1) are regulated by the target pathways. Thus, the Wnt pathway can be considered as

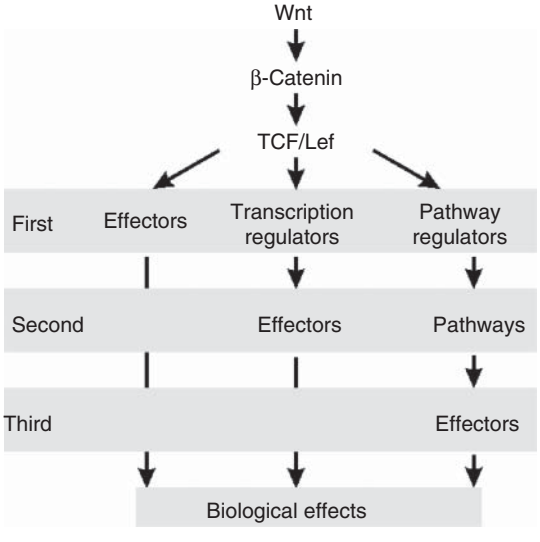

Figure 11.7 The three levels of the targetome of the Wnt pathway. Target genes on the first level are directly regulated by TCF/LEF. Genes and pathways on the second and third levels are regulated by target genes on the first or the second level, respectively. (Vlad *et al.*, 2008).

Table 11.2 Target pathways and corresponding functions affected by the activated Wnt pathway.

Target pathway	Function
G-protein-MAPK activation, CREB signaling, EGF signaling, ERK-PI3K (collagen) signaling, erythropoietin signaling, hedgehog signaling, JAKSTAT signaling, MAPK signaling, NGF signaling, PDGF signaling, PTEN signaling, SAPK-JNK signaling, VEGF signaling, Wnt signaling (calcium)	Proliferation
ACH-R apoptosis signaling, antiapoptotic pathway, CD40L signaling, death receptor signaling, FAS signaling, mitochondrial apoptosis control, NF-κB signaling, p53 signaling, PTEN signaling	Apoptosis
AKT signaling, p38 signaling, p53 signaling, Rb signaling	Cell cycle
Regulation of myogenesis	Differentiation
Insulin signaling, lipid signaling	Metabolism
IFNα signaling, IL-1 and IL-6 signaling, IL-2 signaling, IL-3 signaling, TGFβ signaling, toll-like receptor signaling	Inflammation, immune response
Integrin signaling	Cell adhesion

Confirmed target genes from microarray studies were classified using the software Pathway Assist.

a higher-ranked "master pathway," which regulates genes with direct biological effects, transcription regulators, and other signaling cascades.

Potential Wnt targets are involved in at least 36 different pathways controlling apoptosis, inflammation, proliferation, and metabolism (Table 11.2).

The allocation of the differentially expressed genes to different target pathways shows that each of the target pathways is regulated by up to 14 target genes (Table 11.3). The number of targets involved in a pathway indicates the impact of Wnt signaling on this pathway.

Surprisingly, the pathway that includes the highest number of target genes is the Wnt pathway itself. The high investment into feedback regulation indicates the importance of a tight control of the Wnt pathway by positive and negative feedback loops (Table 11.4). In addition, various temporal and functional levels of regulation assure that the cellular metabolism of a Wnt activated cell does not get out of control. For example, DKK1 modulates the formation of the ternary complex LRP5/6, frizzled, and Wnt ligand (Semenov *et al.*, 2001). DKK1 is a target and a negative modulator of the canonical Wnt pathway at the same time resulting in a negative-feedback loop.

11.5.2 Biological Effects of Wnt Target Genes

Products of target genes encompass a large variety of functions, including cell cycle kinase regulation, cell adhesion, hormone signaling, and transcriptional regulation (Table 11.2). The plurality and diversity of these functions correlate with

Table 11.3 Number of target genes of the Wnt pathway and the pathways, in which the genes are involved. (Vlad *et al.*, 2008).

Pathway	Number of target genes involved in the pathway
ACH-R apoptosis signaling, CREB signaling, IFNα signaling, mitochondrial apoptosis control, Rb signaling	1
CD40L signaling, lipid signaling, PTEN signaling, regulation of myogenesis	2
Antiapoptotic pathway, VEGF signaling	3
AKT signaling, death receptor signaling, G-protein-MAPK activation, NF-κB signaling	4
Erythropoietin signaling, FAS signaling, IL-1 and IL-6 signaling, IL-2 signaling, IL-3 signaling, NGF signaling, TGFβ signaling, toll-like receptor signaling	5
Insulin signaling	6
MAPK signaling, SAPK-JNK signaling	7
Integrin signaling, JAK-STAT signaling	8
EGF signaling, ERK-PI3K (collagen) signaling	9
p38 signaling, p53 signaling, PDGF signaling	10
Hedgehog signaling	11
Wnt signaling	14

The number of genes correlates with the impact of the Wnt pathway on the corresponding pathway.

Table 11.4 Target genes of the Wnt pathway, which themselves play a role in the Wnt pathway (http://www.stanford.edu/group/nusselab/cgi-bin/wnt/).

Target gene	Expression trend in cells with activated Wnt pathway	Effect of gene product on Wnt pathway	Gene product interacts with
FZD	Down	Inactivate	Wnt
DFZ2	Down	Inactivate	Wnt
DFZ3	Up	Activate	Wnt
FZD7	Up	Activate	Wnt
Arrow/LRP	Down	Inactivate	Wnt
DKK	Up	Inactivate	LRP
Dally (HSPG)	Down	Inactivate	Wnt
Wingful/notum	Up	Inactivate	HSPG
Naked	Up	Inactivate	Disheveled
Nemo	Up	Inactivate	TCF
AXIN2	Up	Inactivate	β-Catenin
BTRC	Up	Inactivate	β-Catenin
TCF1	Up	Inactivate	TCF
LEF1	Down	Activate	β-Catenin

the variety of different biological effects of the Wnt pathway, including activation of cell cycle progression and proliferation, inhibition of apoptosis, regulation of embryonic development, cell differentiation, cell growth, and cell migration. In cancer cells with a mutationally activated Wnt pathway, at least 20 target genes have been identified, which encode proteins that activate the cell cycle and/or proliferation, such as MYC, cyclin D1, c-Jun, Fra-1, EphB/ephrin-B, FGF18, Met, WISP, survivin, PTTG1 protein, VEGF, and endothelin-1. The multigene-based proliferation signal provides an explanation for the strong effects of the Wnt pathway on proliferation in tumor cells.

11.6 The Wnt Pathway as Therapeutic Target

The central role of a hyperactivated Wnt pathway in tumors makes it a pathway of high therapeutical interest. Since 2007, several pharmaceutical companies have concentrated their research on inhibiting Wnt signaling, announcing their collaboration to find and to develop modulators of the Wnt signaling as new therapeutics (Zimmerman, Moon, and Chien, 2012) (Table 11.5). Principal molecules include small molecules, as well as antibodies and small therapeutic proteins, the latter of which are to enter phase I clinical tests soon. An optimal anti-Wnt drug would interfere with a well-defined and clear-cut biochemical activity of one single protein in the Wnt pathway without affecting other functions of the target protein or other pathways. Based on today's knowledge, such a drug is not realistic for two reasons. First, the Wnt pathway lacks a specific enzymatic activity that can be easily and specifically targeted. Secondly, the Wnt pathway is a highly branched, multidimensional network with many functions. Thus, targeting the Wnt pathway means targeting many intracellular and intercellular biological activities.

11.6.1 Strategies to Identify Anti-Wnt Drugs

A common strategy for screening drugs that affect the Wnt pathway is a cell-based reporter gene assay. Reporter genes under control of a promoter containing TCF/LEF binding motifs, such as the TOPflash system, can be used to quantify relative transcriptional activity as a readout for Wnt pathway activity (Korinek *et al.*, 1997). In addition, bioinformatic approaches, such as molecular modeling and virtual screening, can be useful for detecting inhibitors that interfere with pivotal amino acids of the β-catenin sequence that interact with other molecules (e.g., TCF) (Trosset *et al.*, 2006). Any drug candidate identified in such a screen has to be further evaluated in biochemical experiments to identify the exact target, followed by biological experiments to explore cross-reactivities and toxicity. The $Apc^{-/-}$ mouse, which develops intestinal carcinomas, is a good example of a suitable animal model for further characterization of drug candidates (Su *et al.*, 1992).

Table 11.5 Examples of compounds that interfere with Wnt pathway activity. (Röhrs and Müller, 2007).

Compound	Experiment for identification	Molecular target	Effects
Interference site 1			
Frizzled8CRD-hFc	Various	Extracellular Wnt factor	Inhibits growth of teratocarcinomas
Wnt1 antibody	Growth of tumor cells	Extracellular Wnt factor	Induces apoptosis in tumor cells
Inhibitors of Wnt production (IWPs)	TOPflash reporter gene assay	Wnt secretion, Porcupine acyltransferase	Inhibit aberrant cell growth
	Zebrafish tail regeneration studies		Promote β-catenin degradation
Interference site 2			
NSC668036	Structure-based virtual ligand screen	Dsh PDZ Domain	Inhibits interaction of DVL with FZD Inhibits Wnt signaling *in vivo*
ICG-001	TOPflash reporter gene assay	CBP/β-catenin interaction	Inhibits CBP/β-catenin interaction
XAV939	TOPflash reporter gene assay	Axin	Activates Axin Inhibits Wnt induced gene expression
Interference site 3			
Imatinib (Gleevec)	Screen for BCR-ABL1-kinase inhibitor	Tyrosine kinase PDGFR, abl-kinase, c-kit	Inhibits activation of β-catenin
SU5416	Cellular tyrosine kinase assay	VEGF receptor	Inhibits angiogenesis Inhibits VEGF-dependent mitogenesis of endothelial cells Inhibits tumor growth
Interference site 4			
PKF118-310 and others	ELISA of TCF/β-catenin interaction	TCF/β-catenin interaction	Inhibits TCF/β-catenin interaction
PNU-74654	Molecular modeling and virtual docking	TCF/β-catenin interaction	Inhibits TCF/β-catenin interaction
Interference site 5			
10058-F4	Yeast two-hybrid screen for inhibitors of Myc-Max interaction	Myc-Max interaction	Inhibits cell cycle progression Inhibits tumor growth
Flavopiridol	Cell proliferation screen	Cyclin-dependent kinases	Inhibits cell cycle progression Inhibits cyclin D1 expression

Table 11.5 *Continued*

Compound	Experiment for identification	Molecular target	Effects
Unknown or various interference sites			
Quercetin	Various	Various	Inhibits inflammation via affecting several processes Inhibits TCF/β-catenin signaling Changes expression patterns of Wnt target genes
Sulindac sulfone (Exisulind, Aptosyn)	Various tests for anti-tumorigenic activity	cGMP phosphodiesterase, various	Induces and activates protein kinase G Induces apoptosis Increases β-catenin phosphorylation promoting its degradation
NO-ASA	Molecular design of anti-inflammatory and anti-thrombic drugs	COX	Inhibits COX pathway
		TCF/β-catenin interaction	Activates guanylate cyclase Inhibits TCF/β-catenin interaction
SMAF-1	TOPflash reporter gene assay	COX, APC	Inhibits COX pathway Simulates APC function
NSAIDs	various	COX, various	Various effects on Wnt pathway, for example, increase in β-catenin phosphorylation
Hexachlorophene	TOPflash reporter gene assay	Siah-1	Promotes β-catenin degradation Inhibits tumor growth
Murrayafoline A	TOPflash reporter gene assay	β-catenin response transcription	Represses expression of *CCND1* and *MYC* Promotes degradation of intracellular β-catenin

Interference sites correlate to Figure 11.8.

In some cases, subsequent chemical modifications of the drug candidate might improve the specificity or the biological activity.

11.6.2 Molecules Interfering with the Wnt Pathway

Potential drug targets are found throughout the entire Wnt signaling cascade (Table 11.5). Figure 11.8 shows the five main sites of the pathway where anti-cancer drugs might interfere. The first targeting level is the Wnt factor itself, which can be attacked during its synthesis, secretion, or interaction with its membrane-bound receptors. Small molecules, called inhibitors of Wnt production (IWPs), have been identified that specifically target the membrane-bound acyltransferase porcupine, a protein important for Wnt secretion (Mo *et al.*, 2013). In addition, a soluble Wnt receptor fused to the immunoglobulin Fc domain, called *Frizzled8CRD-hFc*, effectively sequesters the extracellular Wnt factor and is able to inhibit the growth of teratocarcinomas *in vivo* (DeAlmeida *et al.*, 2007). Finally, several monoclonal antibodies against the Wnt factor with promising effects against cancer cells have been introduced (He *et al.*, 2004).

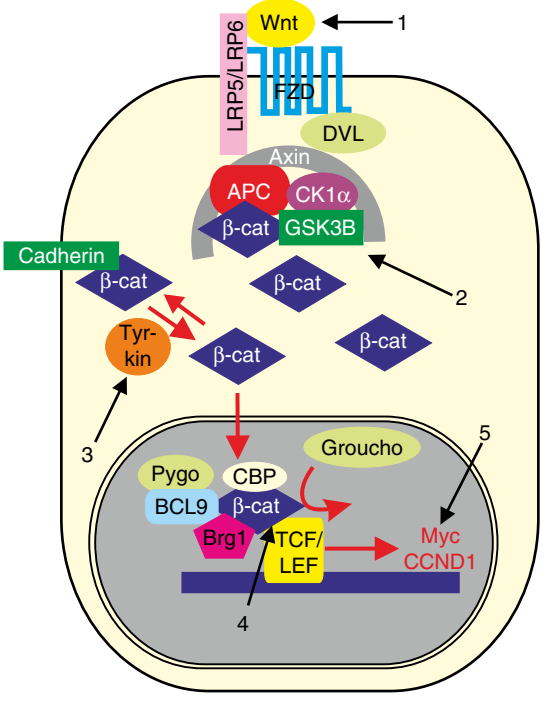

Figure 11.8 Major sites where compounds with therapeutic potential might interfere with Wnt pathway activity. (Röhrs and Müller, 2007).

Proteins of the destruction complex, its assembly, and its interaction with the Wnt receptors represent the second level. An exemplicative compound interfering at this level is NSC668036, which inhibits the interaction of DVL with the intracellular domain of the receptor and thereby the transfer of downstream signals (Shan *et al.*, 2005).

Among the approved drugs for cancer treatment that could target Wnt activation is imatinib (Gleevec). Imatinib is a small-molecule inhibitor of protein tyrosine kinases and was originally identified in a screen for molecules inhibiting the oncogenic kinase BCR-ABL1 (Chapter 6). Imatinib was connected to the Wnt pathway by the suggestion that it may downregulate the cytosolic β-catenin pool by inhibiting the interaction between β-catenin and cadherin, which is regulated by tyrosine phosphorylation. Indeed, imatinib is able to downregulate Wnt signaling in human colorectal cancer cells with overactivated Wnt signaling and in Wnt-1-induced cancer cells (Zhou *et al.*, 2003).

The interaction between β-catenin and TCF-4 provides a target that is specific for the activated Wnt pathway. The search for such interaction inhibitors in a high-throughput ELISA screening study yielded several small molecules with promising features, including PKF118-310 and PKF118-744 (Lepourcelet *et al.*, 2004). These two compounds belong to the rare examples of molecules that specifically affect a protein–protein interaction.

The fifth and lowest level of interference is the Wnt targetome. Interfering with the activities of Wnt target gene products and other downstream modulators of the Wnt pathway, which are not exclusive Wnt target genes, includes the risk of unspecific side effects. For example, flavopiridol inhibits the target gene product cyclin D1, which activates the cyclin-dependent kinase 1 (CDK1) and consequently the progression of the cell cycle (Carlson *et al.*, 1999). Thus, cyclin D1 inhibition might lead to general inhibition of cellular proliferation.

Finally, some compounds with various or uncharacterized effects on the Wnt pathway should be mentioned. These include nonsteroidal anti-inflammatory drugs (NSAIDs), such as acetylsalicylic acid (ASA), sulindac, and indomethacin that inhibit cyclooxygenases and can lower the risk of colorectal cancer development (Herendeen and Lindley, 2003). Particularly, sulindac inhibits nuclear β-catenin localization (Boon *et al.*, 2004) and ASA activates N-terminal CK1α/GSK3B phosphorylation of β-catenin promoting its degradation (Dihlmann, Klein, and Doeberitz Mv, 2003).

11.7 Outlook

Based on the fact that the Wnt pathway is the signaling pathway with the highest mutation rates in human tumors, the search for new drugs that interfere with the activities of this pathway will continue. The pathway neither transfers the signal in a linear one-dimensional manner, nor depends on a single specific enzymatic activity. Thus a multidimensional and multilevel therapeutic approach is needed

to repress the multifaceted Wnt signaling network in tumor cells. Such a combined therapy might consist of a therapeutic antibody against an extracellular Wnt factor, a small molecule inhibitor of a cytosolic kinase and an inhibitor of a major Wnt target gene.

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12
Notch Signaling

Summary

Notch signaling plays an important role in embryonic development and lineage decisions. Since Notch receptors and their ligands Dll and Jagged are membrane-bound proteins, Notch signaling is mediated via direct cell–cell contact. Depending on the physiological role in lineage decisions, *NOTCH* genes may act as oncogenes or tumor suppressor gene. In acute T cell leukemia (T-ALL) and in chronic

Cancer Signaling: From Molecular Biology to Targeted Therapy, First Edition.
Christoph Wagener, Carol Stocking, and Oliver Müller.

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lymphocytic leukemia (CLL), Notch signaling fosters tumor growth. In contrast, Notch signaling is compromised in squamous cell carcinomas of the skin, the lung, and the head and neck. General inhibitors of Notch signaling harbor toxic side effects. In addition, prolonged inhibition of Notch signaling may support the growth of squamous cell carcinomas. Side effects may be reduced by the specific inhibition of ligand–receptor interactions.

12.1 Introduction

The observation of a notch in the wings of the fruit fly *Drosophila melanogaster* by John S. Dexter in 1914, followed by mapping the trait to a specific genetic allele by Thomas Hunt Morgan in 1917, marked the first recognition of this important developmental pathway (Wang, 2011). Notch signaling is essential for embryonic development and tissue homeostasis. Aberrant gain or loss of components of the Notch signaling pathway has been linked to a number of malformations such as Alagille syndrome, Tetralogy of Fallot, and syndactyly. In the adult, disturbances of the Notch pathway can cause the so-called CADASIL (cerebral autosomal dominant arteriopathy with subcortical infarcts and leukoencephalopathy) syndrome and cancer (Kopan and Ilagan, 2009).

12.2 Determination of Cell Fate Decisions

Notch and its ligands are type I transmembrane proteins that are important regulators of cell fate, proliferation, and survival in a large number of developmental processes. As integral proteins of the cell membrane, their signaling is restricted to neighboring cells. Different scenarios of cell fate determination by Notch signaling have been discerned in studies of *D. melanogaster* and are depicted in Figure 12.1. The Drosophila eye is composed of units called *ommatidia*. An ommatidium contains a cluster of photoreceptor cells surrounded by support cells and pigment cells. In a developing ommatidium, Notch activity can be detected in both photoreceptors present at early stages of development. In later stages, lateral inhibition leads to the suppression of Notch signaling in one of the two cells (Figure 12.1a). Notch signaling can also regulate decisions regarding the differentiation of stem cells into different lineages (Figure 12.1b). In the example shown, the stem cell contains a Notch-inhibiting protein (Numb), which is asymmetrically distributed. During cell division, the inhibiting protein is distributed to one of the daughter cells, which leads to Notch signaling from the inhibited daughter cell to the noninhibited daughter cell. Notch signaling can also define cell boundaries (Figure 12.1c) (Bray, 2006).

The Notch signaling pathway is in itself quite simple, with no secondary messengers needed to relay a signal to regulate transcription. However, the complexity

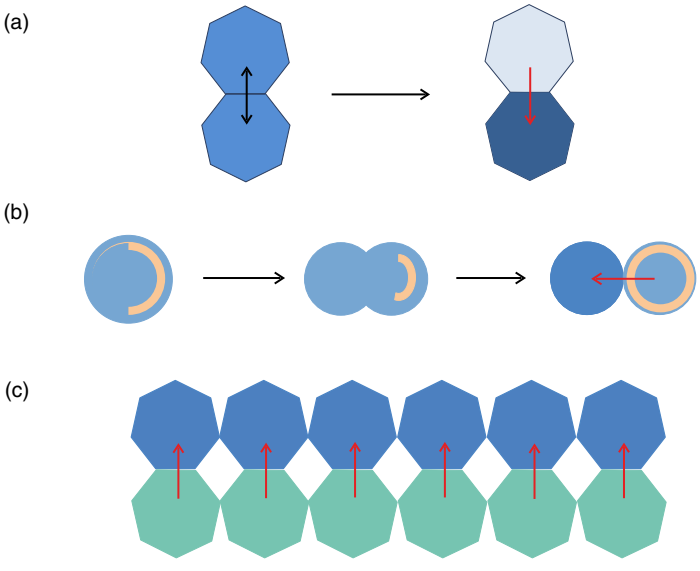

Figure 12.1 (a) Lineage decision by amplification of differences in Notch signaling. In the developing eye of *Drosophila melanogaster*, both posterior photoreceptors show Notch signaling at early stages of development. Small differences in Notch signal intensities in the same cell type are amplified by lateral inhibition. At later stages, Notch signaling is only detected in one cell, and thus, cells undergo differential cell fate decision. Narrow arrows indicate direction of Notch activation; dark blue cells denote cells with have high levels of Notch activity. (b) Lineage decision by asymmetric distribution of a Notch inhibitor. During division of a putative stem cell, the Notch inhibitor (e.g., Numb) is distributed to only one of the daughter cells. This leads to unidirectional Notch signaling and asymmetric cell division. One daughter cell remains stem cell, the second becomes a progenitor cell, which enters the differentiation program (c) Cell fate decisions at cellular boundaries. Unidirectional signaling guarantees Notch activity in only one of the two adjacent cell lineages. Intrinsic differences between two cell populations (e.g., restricted expression of ligands) determine the direction of signaling and Notch activation. (Bray (2006), with permission, modified.)

of the system lies in a number of posttranslational regulators and feedback mechanisms that determine whether a ligand–receptor interaction leads to productive signaling. A short summary is presented next, followed by examples in which altered Notch signaling has been implicated in cancer.

12.3 Notch Proteins and Notch Ligands

The mammalian genome encodes four highly homologous Notch proteins, Notch1, Notch2, Notch3, and Notch4. Notch proteins are cleaved in a number of distinct steps during processing and trafficking to the membrane and after ligand binding, a particular characteristic of the Notch signaling pathway. The

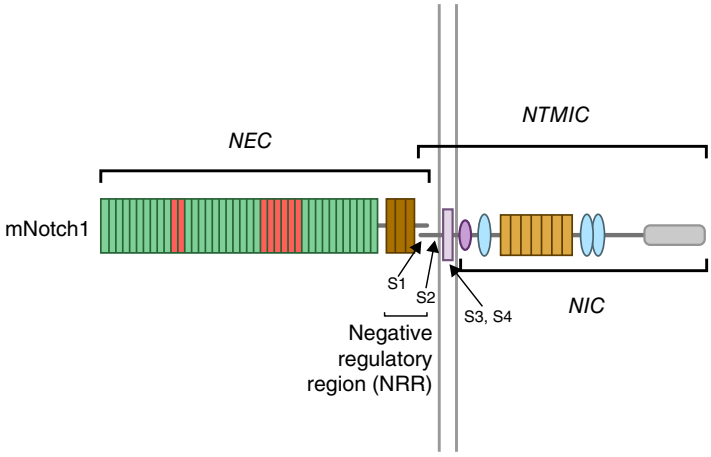

Figure 12.2 Depiction of Notch1 fragments generated by proteolytic cleavage. Proteolytic cleavages play important roles in Notch signaling. The Notch precursor is cleaved during biosynthesis at the S1 site. S2 marks the cleavage site by ADAM metalloproteases. S3 and S4 are the sites within the transmembrane domain that are cleaved by γ-secretase. The composite negative regulatory region (NRR) protects cleavage at the S2 domain and is composed of the heterodimerization domain (HD) and three Lin12/Notch repeats (LNR). NEC, Notch extracellular fragment; NIC, Notch intracellular fragment; and NTMIC, Notch transmembrane and intracellular fragment.

mammalian Notch precursor is initially cleaved in the Golgi apparatus at the S1 site during protein processing, resulting in two fragments held together by noncovalent linkages: the Notch extracellular fragment, abbreviated NEC fragment, and the Notch transmembrane and intracellular fragment, abbreviated NTMIC fragment (Figure 12.2). The N-terminus of the NEC fragment contains a number of EGF repeats, which interact with Notch ligands. The so-called Notch negative regulatory region (NRR) encompasses three Lin12/Notch repeats (LNR) and a heterodimerization (HD) domain, which spans the C-terminal amino acids of the NEC fragment and the N-terminal amino acids of the NTMIC fragment. During Notch signaling, the NTMIC fragment is cleaved at the N-terminus (S2 site) by proteases belonging to the ADAM (a disintegrin and metalloprotease) family (ADAM10 and ADAM17) (Bray, 2006). As explained in the following text, cleavage at the S2 site is a prerequisite for further cleavage and activation of Notch. Since the S2 site is protected against cleavage in the absence of ligand binding, the NRR maintains the receptor in an "off" position. The X-ray structure of human Notch2 has demonstrated that the NRR adopts an autoinhibitory conformation. Extensive interdomain interactions within the NRR bury the metalloprotease site. Thus, substantial conformational movement is necessary to expose this site during ligand activation (Gordon *et al.*, 2009). Considering the importance of the NRR, it is not surprising that many of the Notch-activating mutations in T-cell leukemia affect this region, exposing the S2 protease site (see the following text). The NIC fragment, which is released after ligand binding

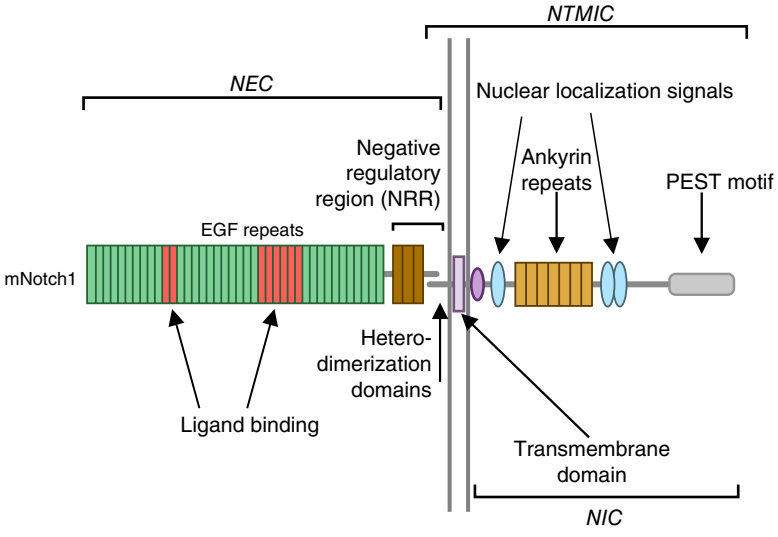

Figure 12.3 Functional and structural domains of Notch1.

and cleavage as outlined as follows, contains nuclear localization domains that guide the intracellular fragment into the nucleus. The ankyrin repeats mediate interactions with other proteins. Most C-terminal, the NIC fragment contains a so-called PEST domain, which harbors degradation signals (degrons) regulating the stability of the intracellular Notch fragment (Figure 12.3).

Mammals produce two groups of classical Notch ligands, the ligands Jagged1 and Jagged2, and the Delta-like proteins Dll1, Dll3, and Dll4. The domain organizations of Jagged1 and Dll1 are shown in Figure 12.4. The DSL domains, as well as the specialized tandem EGF repeats, called DOS domains are involved in receptor binding. Jagged ligands, but not Delta-like ligands, contain a cysteine-rich motif, which has partial homology to the von Willebrand factor type-C domain. Ligands of both groups are anchored in the plasma membrane by their transmembrane domains (D'Souza, Miyamoto, and Weinmaster, 2008).

12.4 Notch Signaling

12.4.1 The Notch Signaling Pathway

(Video: Notch signaling – enhanced ebook and closed website: Notch_ebook.mp4)

Notch signaling is initiated by binding of a Notch ligand to the Notch receptor, which are expressed by opposing cells. It is thought that ligand binding to Notch, combined with the initiation of ligand endocytosis, induces a conformational change in the NRR of Notch. The conformational change may

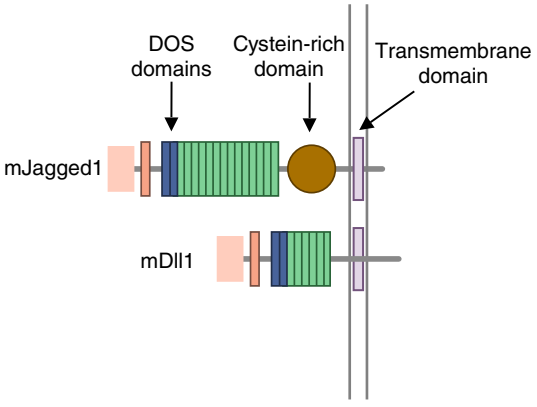

Figure 12.4 Notch ligands and their functional domains.

be induced by mechanical force generated by *trans* endocytosis of a Notch ligand that would expose the S2 cleavage site for ADAM metalloproteases ("lift and cut") (Figure 12.5) (Gordon *et al.*, 2009). The complex of Notch ligand and the NEC is endocytosed by the ligand-expressing cell. After shedding of the Notch ectodomain, the transmembrane domain of the remaining Notch fragment (NTMIC) is attacked by γ-secretase, a multicomponent member of intramembrane-cleaving proteases. γ-Secretase progressively cleaves the transmembrane domain of the NTMIC fragment, most probably starting at the inner plasma membrane leaflet at site 3 (S3) and ending near the middle of the transmembrane domain at site 4 (S4) (Figure 12.6) (Kopan and Ilagan, 2009). As a result of cleavage, the Notch intracellular (NIC) fragment is released into the cytoplasm and enters the nucleus. Significantly, different amino acids may be present at the N-terminus, but only NIC fragments with an N-terminal valine residue escape N-end rule degradation by the proteasome (Tasaki *et al.*, 2012). Cleavage then proceeds to site 4. As a result, a small N-beta peptide is released from the lipid bilayer.

The NIC polypeptide does not bind to DNA on its own. Instead, Notch regulates transcription by binding to its partner, the CSL protein (Figure 12.7). The abbreviation CSL refers to the homologs in different species, namely CBF1 in mammals, Su(H) in the fruit fly, and Lag-1 in *Caenorhabditis elegans.* The abbreviation used for the human gene *RBPJ* is based on an alternative designation for the human protein: "recombining binding protein suppressor of hairless (abbreviated RBP_{jk})." In the following, the designation CBF1 (Cp-binding factor 1) will be used for the mammalian transcription factor. In the absence of Notch, CBF1 recruits transcriptional corepressors (Figure 12.7a). Upon Notch binding, the corepressor proteins are released from CBF1. The complex of CBF1 and Notch binds the coactivator Mastermind (Mastermind-like protein 1 (Mam1) in mammals) via the interface of the Notch ankyrin domain and the CBF1 protein (Figure 12.7b). After recruitment of coactivators such as histone acetyltransferases and chromatin remodeling

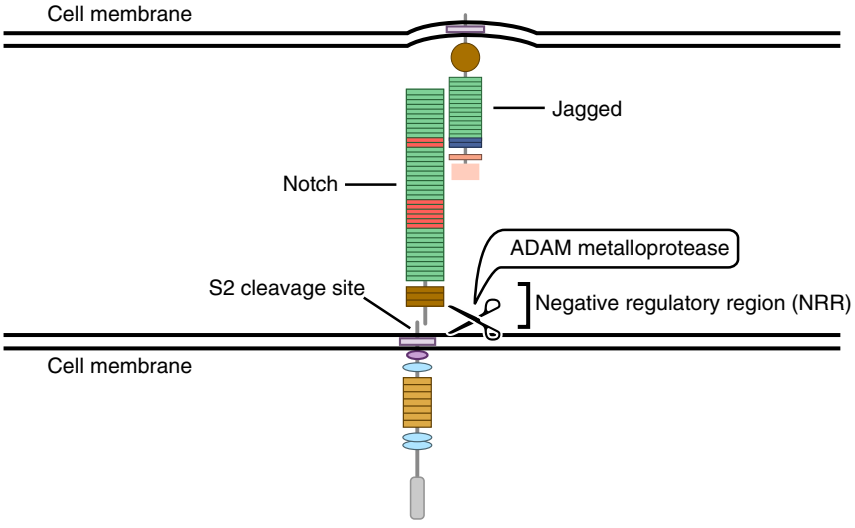

Figure 12.5 Mechanism of S2 cleavage of Notch. Endocytosis of the Notch ligand induces a conformational change in the negative regulatory region, which is now open for cleavage by ADAM proteases ("lift and cut").

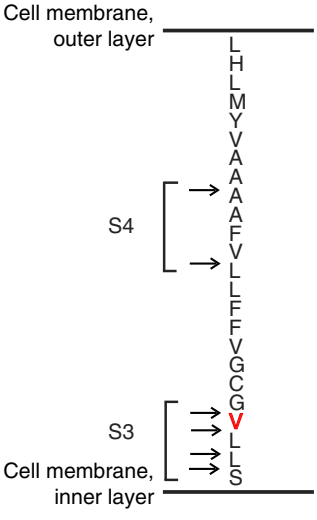

Figure 12.6 γ-secretase cleavage sites in the transmembrane domain of murine Notch 1 (Kopan and Ilagan, 2009). S3 cleavage releases the Notch intracellular fragment (NIC), but only cleavage products that posses the N-terminal valine (denoted in red) are stable, as they escape proteasomal degradation mediated by the N-end rule.

factors to the complex, the multiprotein complex induces the transcription of Notch target genes (Kopan and Ilagan, 2009).

Which genes are transcribed in response to Notch signaling is dependent on the specific Notch paralog activated and on the cellular and developmental context. Notch target genes are involved in the regulation of differentiation, proliferation, and apoptosis. Individual target genes will be described in chapters on different tumor entities.

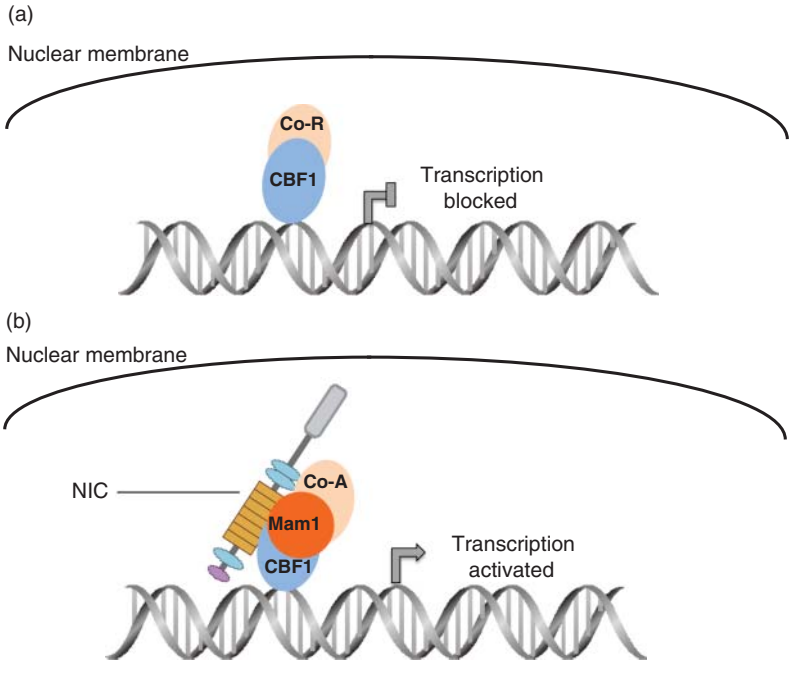

Figure 12.7 (a) Inhibition of transcription by the association of CBF1 (RBP_{jk}) with corepressors in the absence of Notch. (b) Transcription of Notch-responsive genes. After association of Notch with CBF1 (RBP_{jk}), Mam1 (mastermind-like protein 1) and coactivators are recruited into the complex. The complex activates transcription.

12.4.2 Regulation of Notch Signaling by Posttranslational Modification

In addition to the essential proteolytic cleavage steps for activating Notch signaling, a number of other posttranslational events regulate Notch activity, including ubiquitinylation and glycosylation.

12.4.2.1 Ubiquitinylation

Ubiquitinylation plays an important role in the regulation of Notch and Notch ligands (Le Bras, Loyer, and Le Borgne, 2011). In Table 12.1, E3 ubiquitin ligases involved in Notch signaling are listed. As mentioned earlier, it has been proposed that exposure of the S2 cleavage site in the NTMIC fragment is induced by pulling forces generated by *trans* endocytosis of a Notch ligand. If this hypothesis is true, endocytosis of Notch ligands should be critical for Notch signaling. Indeed, it has been shown that regulators of DSL ligand endocytosis are required for signaling activity. Among these regulators are two E3 ubiquitin-protein ligases (NEURL1B, the homolog of "neutralized" in Drosophila, and MIB1, the homolog of Mind

Table 12.1 E3 ubiquitin ligases in Notch signaling.

E3 ubiquitin ligase	Functional role of ubiquitination
Mind bomb family (Mib 1/2)	Dll1 internalization
Neutralized family	
Neur1	Notch1 lysosomal degradation
Neur2	Notch1 (proteasomal?) degradation
Deltex family (Deltex1)	Notch proteasomal degradation
Suppressor of deltex family	
AIP4	Notch1 lysosomal degradation
Itch	Notch1 (proteasomal?) degradation
Nedd4	NEXT-fragment endocytosis prior to gamma secretase cleavage
Fbw7	Notch (proteasomal?) degradation
c-Cbl	Notch1 ligand-independent lysosomal degradation

Dll4, Delta-like 4; AIP4, ADP-ribosylation factor-like 6 interacting protein 4; Nedd4, neural precursor cell expressed, developmentally downregulated gene 4-like; NEXT, Notch extracellular fragment; and Fbw7, F-box and WD repeat domain containing 7. Hrs is an endosome-associated protein. Question marks refer to a presumed function. (Le Bras, Loyer, and Le Borgne, 2011).

bomb in Drosophila). Monoubiquitination of DSL is thought to serve as a signal for DSL internalization by promoting their interaction with cytosolic adaptor proteins, which contain ubiquitin-binding domains (Le Borgne, 2006).

Ubiquitination is also involved in the regulation of Notch receptors. Several ubiquitin ligases can direct Notch receptor trafficking toward lysosomal degradation or toward recycling, thereby regulating the half-lives of Notch receptors. Proteolytic cleavage by γ-secretase results in three main NIC fragments with different N-termini, namely valine, lysine, and serine. According to the N-end rule in protein degradation, the half-lives of proteins are determined by the N-terminal amino acids and their susceptibility to ubiquitinylation (Tasaki *et al.*, 2012). According to this rule, the NIC fragments with serine or lysine as N-terminal amino acids are rapidly degraded by the 26S proteasome. Because of its stability, the Notch fragment with an N-terminal valine is the major mediator of Notch signals (Kopan and Ilagan, 2009).

12.4.2.2 **Glycosylation of Notch**

The extracellular portion of Notch is extensively glycosylated. After translation, the Notch protein is O-fucosylated on EGF repeats by the peptide O-fucosyltransferase 1 (O-FucT-1), encoded by the *POFUT1* gene. Studies in Drosophila indicate that O-FucT-1 also acts as chaperone to promote folding and transport of Notch from the endoplasmic reticulum to the cell membrane, thus it is currently unclear if the chaperone activity or fucosylation is essential for Notch activity. In favor of the latter hypothesis, it has been demonstrated that the fucosyl residues can be extended by the so-called Fringe glycosyltransferases. In Drosophila, Fringe (*O*-fucosylpeptide 3-β-*N*-acetylglucosaminyltransferase)

regulates the differential response to different Notch ligands. Fringe-mediated addition of a single *N*-acetylglucosamine residue on EGF repeat 12 is sufficient to enhance the binding to Delta and to reduce binding to the Jagged homolog Serrate. In mice, a substitution in an analogous position generates a hypomorphic allele unable to mediate Dll-dependent processes in T-cells. In combination with a null allele, it is embryonic lethal. Whether the glycosyltransferase, the chaperone, or both activities of glycosyl transferases regulate the activities of Notch receptors has yet to be resolved (Kopan and Ilagan, 2009).

12.5 Notch Signaling in Malignant Disease

(Video: Notch signaling – enhanced ebook and closed website: Notch_ebook.mp4)

NOTCH genes may act as oncogenes and as tumor suppressor genes. The decision if Notch signaling fosters or suppresses tumor growth largely depends on the normal functions of Notch signaling in lineage decisions, survival, and proliferation in the specific tissue. Evidence of aberrant activation or inactivation of the Notch signaling pathway in cancers of many different tissue types has accumulated over the past decade. Examples of the contrasting tumor suppressor and oncogenic roles for Notch signaling in cancer are highlighted next in the context of what is currently known about its normal function.

12.5.1 Acute T-Cell Leukemia (T-ALL)

Notch signaling plays a critical role in the cell fate of early thymocytes in T-cell development. In addition to specifying T-cell fate by suppressing B-cell and myeloid transcription programs, it plays a key role in expanding T-cell clones that have passed β selection, the first checkpoint in T-cell development. The first chain of the T-cell receptor (TCR) to be rearranged is the β chain. The rearranged β chain forms a heterodimer with a surrogate Tα chain. During β selection, only those T cells survive that have successfully rearranged the β locus to produce a functional β chain. Transition through this checkpoint requires two signals, one generated by the pre-TCR and a second generated by Notch1. Once β selection is completed, Notch1 expression and Notch signaling are downregulated and cell division ceases (Aster, Pear, and Blacklow, 2008).

The important role Notch1 plays in T-cell acute lymphoblastic leukemia (T-ALL) was first discovered by the analysis of a reciprocal translocation between chromosomes 7 and 9 (t(7;9)(q34;q34.3)), leading to the translocation of the *NOTCH1* gene into the T-cell receptor beta (*TCRβ*) gene locus. As a result of the translocation, enhancer/promoter sequences of the TCRβ locus drive the transcription of the *NOTCH1* allele (Ellisen *et al.*, 1991). The translocation t(7;9) is rare, being observed in less than 1% of human T-ALL. However, recent results have determined that Notch1 is activated by point mutations in over

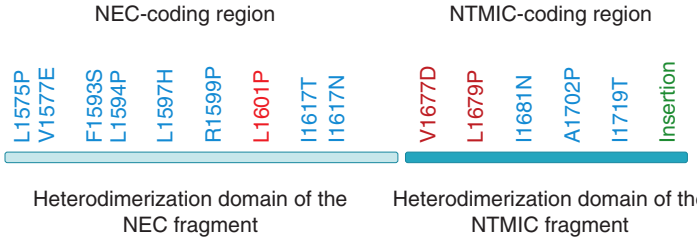

Figure 12.8 Activating leukemia-associated mutations within the Notch1 heterodimerization domain in T-ALL. Red color: heterodimer dissociation under native conditions (about 20% of mutations); blue color: intrinsically less stable heterodimers (about 80% of mutations); and green color: unveiling of the S2 cleavage site without a reduction in heterodimer stability (rare). NEC, Notch extracellular fragment and NTMIC, Notch transmembrane and intracellular fragment. (According to Aster, Pear, and Blacklow (2008), with permission, modified.)

half of all T-ALL patients. Two major "hot spots" of mutations have been characterized in T-ALL: mutations in the heterodimerization domains that induce ligand-independent activation and mutations in the PEST domain that increase stability of the NIC fragment. Mutations in the heterodimerization domains, which reside within the negative regulatory region (NRR), are the most common type of mutations in T-ALL, occurring in approximately half of the tumors. Mutations cluster in exons 26 or 27 encoding the heterodimerization domains of the NEC- and NTMIC-fragments, respectively, and consist of either single amino acid substitutions or short in-frame deletions or insertions (Figure 12.8). More rarely, tandem insertions of 12–15 amino acids at the C-terminus of the NTMIC-heterodimerization domain, which results in the duplication of the S2 cleavage site, are also observed. Mutations in the heterodimerization domain render Notch1 susceptible to ligand-independent cleavage at the S2 site. As explained earlier, cleavage at the S2 site is a prerequisite for subsequent cleavage at the S3 sites by γ-secretase, which liberates the intracellular Notch1 fragment (Aster, Pear, and Blacklow, 2008).

About one third of the Notch1 mutations in T-ALL affect the intracellular PEST domain encoded by exon 34 of the *NOTCH1* gene. The mutations are either point mutations that introduce stop codons or deletions/insertions that cause shifts in the reading frame. The most common mutation is a deletion spanning amino acid residues 2524–2556. Commonly deleted amino acid sequences are similar to a particular c-Myc sequence, which is recognized by E3 ubiquitin ligase complexes containing the F-box/WD repeat-containing protein 7 (Fbw7). Interestingly, T-ALLs that lack mutations of the Notch1 PEST domain often have loss-of-function mutations in the *FBXW7* gene. These and other findings indicate that the mutations in the PEST domain increase the stability and activity of the intracellular Notch1 fragment by preventing its ubiquitination and proteasomal degradation (Aster, Pear, and Blacklow, 2008).

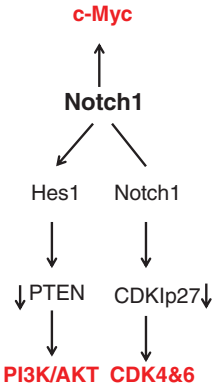

Figure 12.9 Transcription of Notch-responsive genes in T-ALL.

Insight into the critical target genes of Notch activity in human T-ALL has come from cell lines in which Notch can be inactivated by inhibiting γ-secretase. These studies have identified the *MYC* and *HES* genes as major targets of Notch1 signaling (Figure 12.9). *MYC* is a major human oncogene, which regulates cell cycle progression, apoptosis, and malignant transformation (see Chapter 3). *HES1* encodes a helix-basic-loop-helix transcriptional repressor. HES1 represses the expression of PTEN, which acts as a negative regulator of the PI3K/AKT pathway. In turn, PI3K/AKT activates the mTOR pathway, which is a regulator of protein synthesis and cell size (see Chapter 8). In contrast, Notch negatively regulates levels of the cyclin-dependent kinase (CDK) inhibitor p27. Taken together, these findings indicate that the activation of Notch1 in T-ALL favors cell cycle progression by decreasing the activity of the CDK inhibitor p27 and maintaining high MYC levels, but also fosters cell growth by activation of the mTOR pathway (Figure 12.9)

12.5.2 Chronic Lymphocytic Leukemia

Chronic lymphocytic leukemia (CLL) is the most frequent form of leukemia in adults in Western countries and is a clonal lymphoproliferative B-cell neoplasm. The absence or presence of somatic hypermutations in the gene encoding the immunoglobulin heavy chain (IgV_H) is used to subtype CLL. The unmutated subtype (with no or few hypermutations) is thought to originate from pregerminal naïve B-lymphocytes, whereas the subtype with extensive mutations likely originates from postgerminal memory B-cells. Patients in the unmutated subtype with more immature lymphocytes have a poor prognosis.

Mutations in the *NOTCH1* gene have been identified in 12% of CLL patients. The mutations generate a premature stop codon, resulting in Notch1 proteins with truncated PEST domains. As described earlier, truncation of the PEST domain stabilizes the NIC fragment and, in this way, sustains Notch signaling. Thus, in CLL, *NOTCH1* also acts as an oncogene.

Interestingly, *NOTCH1* mutations are mainly detected in patients with unmutated immunoglobulin heavy chains. Accordingly, patients with *NOTCH1* mutations have a more advanced clinical stage at diagnosis, more adverse biological features, and poorer prognosis than patients with wild-type *NOTCH1* genes (Puente *et al.*, 2011).

12.5.3 Chronic Myelomonocytic Leukemia (CMML)

Chronic myelomonocytic leukemia (CMML) is characterized by increased fractions of monocytes and blasts in the bone marrow and the peripheral blood. The first indication that Notch signaling may contribute to this disease came from a mouse model, in which Notch signaling was inactivated in hematopoietic stem cells. To study hematopoiesis in the absence of any Notch signaling, researchers targeted Nicastrin, a nonredundant member of the γ-secretase complex (Klinakis *et al.*, 2011). Inactivation of Notch signaling resulted in an aberrant accumulation of granulocyte/monocyte progenitors, extramedullary hematopoiesis, and the induction of CMML-like disease. These findings indicate that Notch signaling acts as tumor suppressor in this experimental setting.

When human hematopoietic progenitor cells were cultured on stroma cells expressing different Dll and Jagged ligands, Notch ligands efficiently suppressed differentiation of the progenitors toward granulocyte and monocyte lineages. This finding indicates that Notch signaling may contribute to the pathogenesis of human CMML. Indeed, it was found that a substantial fraction of CMML patients harbored mutations in Notch pathway genes such as *NCSTN* encoding Nicastrin, *MAML*, and *NOTCH2*. Some of the mutant alleles were tested regarding the effect on Notch signaling. Each of the mutant alleles tested affected Notch signaling negatively as either dominant negative or null alleles. Thus, in CMML, genes of the Notch pathway act as tumor suppressor genes (Klinakis *et al.*, 2011).

12.5.4 Breast Cancer

The analysis of retroviral integration sites in the DNA of tumor cells of diverse hosts, such as rodents, cats, or birds, has contributed significantly to the identification of oncogenes. Retroviral proviruses integrate randomly into the host genome. The integrated proviral genomes can lead to the upregulation or dysregulation of genes that contribute to carcinogenesis. One of the cellular oncogenes identified upon the integration of the mouse mammary tumor virus (MMTV) was the *Notch4* gene. Proviral insertion led to the expression of a truncated Notch protein lacking most of the extracellular portion, but retaining the Notch4 transmembrane and intracellular domains (N4TMIC fragment). When this N4TMIC fragment is expressed under the control of the MMTV promoter in transgenic mice, mammary gland development arrests and poorly differentiated adenocarcinomas develop. In contrast to other oncogenes identified by retroviral integration,

the *NOTCH4* gene appears not to be mutated in human breast cancer. However, h-Numb-mediated negative regulation of Notch signaling has been reported to be lost in about half of human breast cancer specimens, indicating that Notch signaling is enhanced in these mammary carcinomas. In tissue sections of human breast cancer, a high expression of Jagged 1 and Notch1 was associated with a poor prognosis of the patients, indicating that an upregulation of Notch1 signaling may be associated with tumor progression (Bolos, Grego-Bessa, and de la Pompa, 2007).

12.5.5 Cholangiocellular Carcinoma (CCC)

Evidence for the importance of Notch signaling for liver development and function has come from a genetic disorder, Alagille syndrome, associated with bile duct anomalies, ranging from fewer bile ducts as normal to complete bile duct atresia. The disease is caused by a deletion of the *JAG1* locus on chromosome 20p12. The *JAG1* gene encodes the Notch ligand Jagged1. It has also been shown that Notch signaling induces fate decisions in hepatoblasts and terminally differentiated hepatocytes, allowing them to differentiate toward the biliary lineage (Zong *et al.*, 2009).

Cholangiocellular carcinoma (CCC), which accounts for up to 15% of all primary liver cancers, is a liver cancer with biliary differentiation. In contrast to cell lines from hepatocellular carcinoma, cell lines derived from CCC specimens expressed Notch1 and the Notch1 target HES1. Notch proteins are also expressed in CCC, as shown by the binding of a specific antibody to tumor sections (Figure 12.10). The hypothesis that Notch signaling is involved in CCC is supported by transgenic mice that express the Notch1 intracellular fragment (NIC) under the control of albumin regulatory elements and alpha-fetoprotein enhancers. At as early as 8 months of age, mice develop changes in the nuclear morphology of their liver tissue. When primary tissues of livers at that stage are transplanted into immunodeficient mice, tumors develop that show many features of human CCC. The CCCs induced in this manner express high levels of cyclin E, which drives the cell cycle in late G1- and early S-phases of the cell cycle. Bipotential hepatic progenitor cells expressing the Notch1 intracellular (N1IC) fragment formed tumors when transplanted into immunodeficient mice. However, no or only small tumors developed when the cells were cotransfected with shRNAs against cyclin E. These experiments indicate that, at least in the experimental setting described, the upregulation of cyclin E by Notch1 is causally involved in the genesis of CCC (Zender *et al.*, 2013).

NOTCH gene mutations that activate the Notch signaling pathway are rare in human CCC cases. However, the *FBXW7* gene is mutated in about one third of CCCs (Akhoondi *et al.*, 2007). As described earlier, the genet product Fbw7 is a component of an ubiquitin ligase guiding the intracellular Notch1 fragment to proteasomal degradation. Thus, the increase in Notch1 activity may be due to a reduction in proteasomal degradation, at least in a fraction of human CCCs.

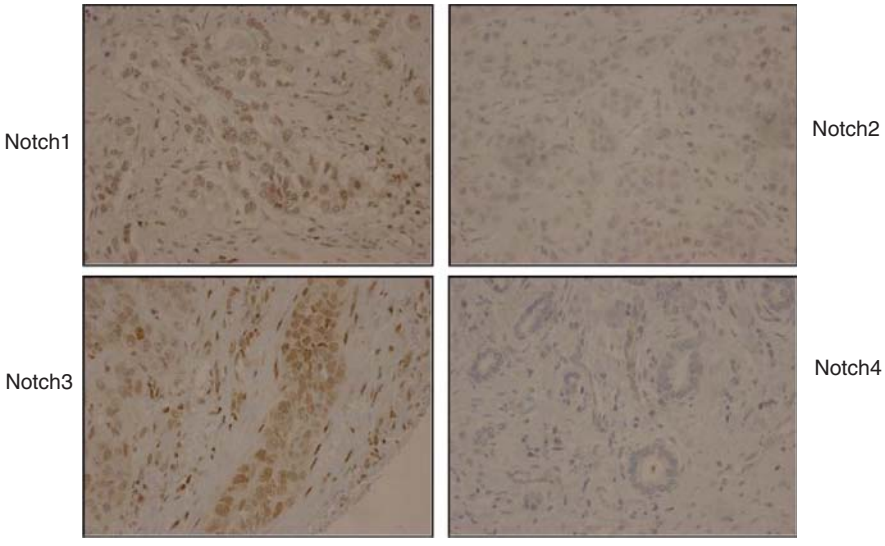

Figure 12.10 Expression of Notch family members in human cholangiocellular carcinomas. (Zender *et al.* (2013), with permission.)

12.5.6 Squamous Cell Carcinomas (SCCs)

Squamous epithelia consisting of renewable epithelial cells create an environmental barrier in the skin, esophagus, lung, and cervix. In the mouse skin, Notch signaling promotes the exit of squamous keratinocytes from the cell cycle and the entry into differentiation (Bolos, Grego-Bessa, and de la Pompa, 2007). In view of the similarity in squamous epithelia at different locations, one can assume that this activity of Notch signaling is a general property of squamous cell epithelia. Ablation of Notch1 in the mouse epidermis results in epidermal hyperplasia followed by the development of skin tumors and facilitates chemical-induced skin carcinogenesis (Nicolas *et al.*, 2003). In this model, Notch1 acts as tumor suppressor.

NOTCH alleles are mutated in different types of human squamous cell carcinomas (SCCs). *NOTCH1* or *NOTCH2* mutations were identified in about 75% of human cutaneous SCCs and also in SCCs of the lung (Wang *et al.*, 2011). Mutations in *NOTCH1*, *NOTCH2*, or *NOTCH3* genes were also detected in SCCs of the head and neck (Stransky *et al.*, 2011). *NOTCH* mutations included nonsense and frameshift mutations leading to receptor truncations as well as point mutations in important functional domains, indicating that loss-of-function mutations drive these SCCs (Figure 12.11). Mutant *NOTCH* genes have also been described in about one third of Chinese patients with esophageal cancer (Song *et al.*, 2014).

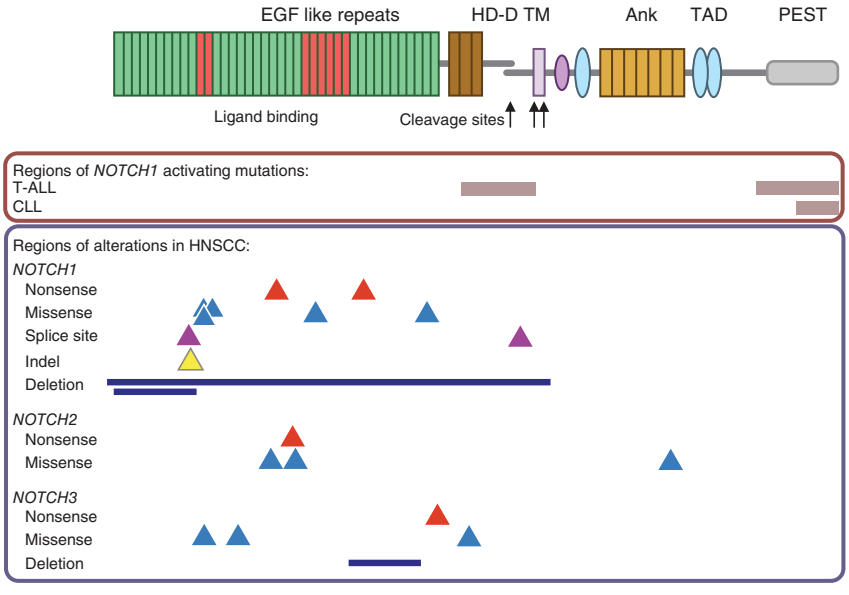

Figure 12.11 *NOTCH* gene mutations identified in head and neck squamous cell carcinomas. HD-Ds, heterodimerization domains; TMs, transmembrane domains; Ank, ankyrin repeats; TAD, transactivation domain; and PEST, PEST domain. Note that all nonsense mutations observed in head and neck squamous cell carcinomas (HNSCC) occur upstream of the transactivation domain, required for transcription. Arrowheads indicate point mutations of the class indicated to the left. The mutational clusters observed in HNSCC on the one hand and T-ALL and CLL on the other hand differ from each other. (Stransky *et al.* (2011), with permission, modified.)

12.5.7 Small-Cell Lung Cancer (SCLC)

In a comprehensive DNA sequence analysis of small-cell lung cancers, inactivating mutations of *NOTCH* family genes were identified in 25% of the cases (George *et al.*, 2015).

12.5.8 Angiogenesis

Tumor growth and metastasis depend on angiogenesis (blood vessel formation) to obtain an adequate supply of oxygen and nutrients. The importance of Notch signaling in this process is demonstrated in the autosomal dominant inherited disease CADASIL (cerebral autosomal dominant arteriopathy with subcortical infarcts and leukoencephalopathy). CADASIL is a microangiopathy of cerebral blood vessels, which leads to multiple cerebral infarctions. The disease is

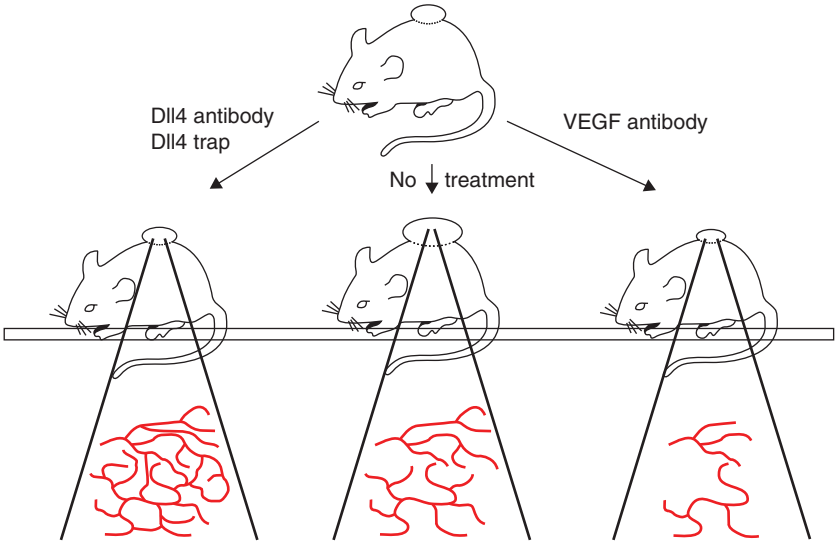

Figure 12.12 Xenotransplants treated with inhibitors of Dll4 and VEGF. Inhibition of Dll4 increased sprouting and branching of blood vessels, whereas inhibition of VEGF decreased sprouting and branching. Both treatments blocked tumor growth. Dll4 traps are soluble recombinant Dll4 proteins, which inhibit the binding of cell-bound Dll4 to Notch. (Based on data published by Noguera-Troise *et al.* (2006) and Ridgway *et al.* (2006).)

caused by a mutation in the *NOTCH3* gene (Louvi, Arboleda-Velasquez, and Artavanis-Tsakonas, 2006). Among the Notch ligands, DLL4 is known to play a distinct role in angiogenesis. DLL4 is expressed by endothelial tip cells, which bud out of preexisting vessels. The tip cells are followed by motile proliferative endothelial tube cells that express Notch and form the lumina of new vessels. Notch signaling in the tube cells downregulates the VEGF receptor 2 and, in this way, decreases the VEGF-induced sprouting and branching of newly formed blood vessels. *In vivo* mouse studies have demonstrated that the inhibition of Dll4 binding to Notch increases sprouting and branching of blood vessels, increasing vessel formation. However, the blood vessels were nonfunctional, thus Dll4 antibodies actually inhibited tumor growth in mouse models (Figure 12.12) (Noguera-Troise *et al.*, 2006; Ridgway *et al.*, 2006).

12.6 Drugs Targeting the Notch Pathway

If Notch pathway inhibitors are to be used in the therapy of malignant disease, one must consider that, depending on the tumor type, Notch may act as either a tumor promoter or a tumor suppressor. In tumors such as T-ALL and CLL, Notch

signaling supports tumor growth. Since Notch signaling is frequently abrogated in SCCs, inhibition of Notch may foster unwanted growth of SCC (Extance, 2010). A second concern regards the toxicity of Notch inhibitors. Since the Notch pathway is central to the regulation of stem cells and lineage decisions, many tissues may be affected by Notch inhibition. For example, Notch signaling favors the differentiation of gastrointestinal precursor cells toward an epithelial fate and away from secretory goblet cells. Notch inhibitors favor the differentiation of secretory goblet cells, which leads to massive diarrhea.

γ-*Secretase inhibitors (GSIs).* GSIs were first developed as potential therapy for Alzheimer's disease. Only after it was recognized that Notch signaling is involved in T-ALL, a phase I clinical trial with a GSI (Merck MK-0752) was performed. The major side effects of first-line Notch-inhibiting drugs are gastrointestinal toxicity and diarrhea. This is due to the inhibition of Notch signaling in the gut epithelial stem and progenitor cells, which preferentially adopt a goblet cell fate at the expense of the enterocyte cell fate. Other potential side effects to these inhibitors may be attributable to the fact that Notch proteins are not the only targets of γ-secretase. Additional targets are Notch ligands Dll and Jagged, as well as the amyloid precursor protein, CD44, ErbB4, and E-cadherin, among others. Upon longer exposure to Notch inhibitors, these targets may cause additional unwanted side effects (Purow, 2012). It should be noted that a clinical trial using a GSI (Semagacestat) for the treatment of Alzheimer's disease was halted, partly because treatment was associated with an increased risk of skin cancer (Extance, 2010).

Antibodies. In contrast to GSIs, antibodies promise high target specificities. By targeting the binding sites of Notch receptors or Notch ligands, Notch signaling can be blocked. This approach has been taken in murine tumor models. Blockade of Dll4 resulted in increased tumor vascularity. However, because the new blood vessels were nonfunctional, the tumors were poorly perfused, resulting in decreased tumor growth (Noguera-Troise *et al.*, 2006; Ridgway *et al.*, 2006). Antibodies were developed that specifically target the negative regulatory regions (NRR) of Notch1 or Notch2. Since none of the known T-ALL mutations fell within the Notch1 antibody epitope, the antibody was effective also against T-ALL cell lines harboring activating mutations in the NRR. As found with Dll4 antibodies, the Notch1 antibody induced nonfunctional angiogenesis and inhibited the growth of tumor xenografts. When Notch1 and Notch2 antibodies were combined, severe goblet cell metaplasia was induced. Goblet cell metaplasia was significantly milder using Notch1 antibody alone and absent with Notch2 antibody alone. Thus, targeting Notch receptors individually may decrease side effects significantly, when compared to GSIs, which do not discriminate between Notch receptors and which hit additional targets (Wu *et al.*, 2010).

12.7 Outlook

NOTCH1 gene mutations activate Notch signaling in malignancies of T-cells (T-ALL) and B-cells (CLL), whereas inactivating mutations in *NOTCH* family genes occur in significant fractions of common cancers, such as SCCs of the lung, esophagus, and skin, as well as in small-cell lung cancer. In T-ALL and CLL, the tumorigenic mechanism underlying *NOTCH1* gene activation is fairly well understood. However, the effect of *NOTCH* family gene inactivation in carcinomas is still unclear. Inhibition of NOTCH signaling will be an attractive therapeutic option for the treatment of these two subtypes of leukemia once side effects can be controlled. It is anticipated that many tumor-promoting pathways are activated by inactivation of *NOTCH* family genes in major human carcinomas. Thus, understanding the underlying molecular mechanisms is a prerequisite for therapeutic interventions.

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13
Hedgehog Signaling

Summary

The hedgehog (Hh) pathway is central to embryonic development and tissue regeneration. In mammals, three hedgehog ligands exist, sonic hedgehog (sHh),

Cancer Signaling: From Molecular Biology to Targeted Therapy, First Edition.
Christoph Wagener, Carol Stocking, and Oliver Müller.

Companion Website:www.wiley.com/go/wagener/cancersignaling

Indian hedgehog (iHh), and desert hedgehog (dHh). In vertebrates, the hedgehog signaling pathway is closely associated with the primary cilium. Two proteins, patched (Ptch) and smoothened (Smo), are essential in initiating Hh signaling. In the absence of hedgehog, Ptch prevents Smo from entering the cilium, Smo remains embedded in cytoplasmic vesicles. Upon binding of Hh to Ptch, Ptch is internalized and Smo enters the membrane of the cilium. Smo acts on the transcription factors of the canonical Hh pathway, Gli1, Gli2, and Gli3. In basal cell carcinomas of the skin and in medulloblastomas of the cerebellum, the canonical Hh pathway is activated by mutations of signaling proteins. A number of noncanonical pathways assist the activation of Gli proteins in several carcinomas, such as pancreatic, esophageal, and lung carcinomas. Drugs inhibiting the canonical Hh pathway, particularly inhibitors of Smo, are being employed in the treatment of patients with basal cell carcinoma or medulloblastoma.

13.1 Overview of Hedgehog Signaling

(Video: The hedgehog signaling pathway – Part 1: Normal situation – enhanced ebook and closed website: Hedgehog_Video_01_ebook.mp4)

In 1980, the Nobel laureates Christiane Nüsslein-Volhard and Eric Wieschaus first described the hedgehog gene in their analysis of embryos of the fruit fly *Drosophila melanogaster* with mutational defects. The designation "hedgehog" was derived from their observation that Drosophila embryos were covered with denticle hair when the hedgehog (*hh*) gene was defective (Nüsslein-Volhard and Wieschaus, 1980) (Figure 13.1). In humans, disruption of hedgehog signaling can result in developmental disorders such as incomplete cleavage of the forebrain during embryogenesis (holoprosencephaly), short-ended phalanges (brachydactyly), and limb defects.

The main players in the mammalian hedgehog signaling pathway are the hedgehog ligands (Hh), the hedgehog receptors patched (Ptch), the seven transmembrane protein smoothened (Smo), and the transcription factors Gli1, Gli2, and Gli3. Gli proteins are part of a complex that contains a kinesin-family member (Kif7) and Sufu (suppressor of fused). In vertebrates, hedgehog signaling is associated with the primary cilium, a structure present in most vertebrate cells.

Ptch is a multipass membrane protein located at the base of the cilium. In the absence of Hedgehog binding to Ptch, Smo is embedded in the membrane of cellular vesicles (Figure 13.2a). In this location, Smo is unable to signal. In the absence of signaling by Smo, Gli3 is phosphorylated by different protein kinases (Figure 13.2b). Phosphorylated Gli3 binds the βTrCP (β transducin repeat-containing E3 ubiquitin protein ligase) subunit of the stem cell factor (SCF)-type E3 ubiquitin ligase, which directs Gli3 to the proteasome. Partial degradation in the proteasome generates the transcriptional repressor Gli3R, which represses the transcription of Hh-responsive genes (Figure 13.2c).

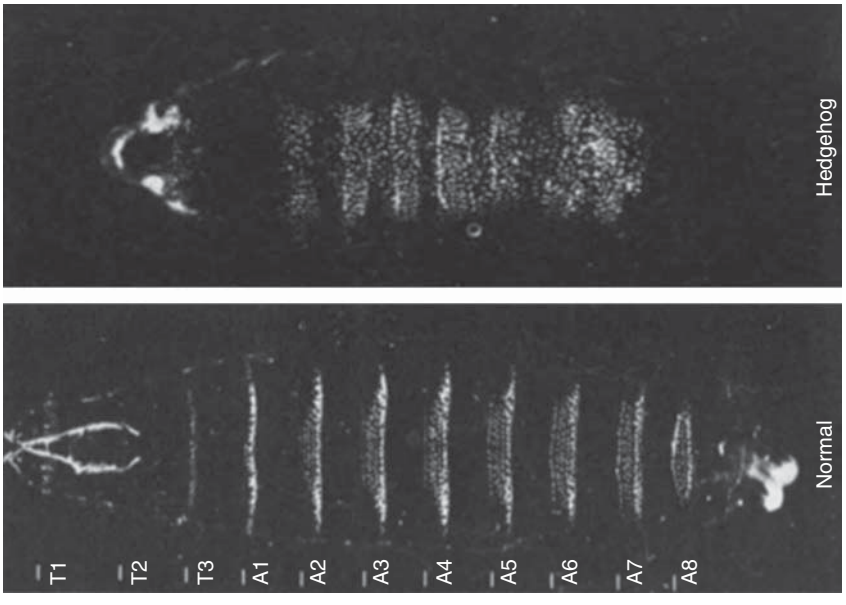


Figure 13.1 Hedgehog mutant of a *Drosophila melanogaster* embryo. In the upper chart, the Hedgehog mutant is shown. The designation "Hedgehog" was derived from coverage by denticle hair. A normal embryo is depicted in the lower chart. (Nüsslein-Volhard and Wieschaus (1980), with permission.)

Active hedgehog signaling is initiated by binding of Hh to Ptch (Figure 13.3a). After binding, the complex of Hh and Ptch is internalized and directed to the lysosome. In this situation, the vesicular location of Smo is relieved and Smo enters the primary cilium (Figure 13.3b). Smo moves up to the tip of the cilium, where it signals through an inhibitory G protein to a complex of Gli2, Kif7, and SuFu. After Smo signaling, Gli2 leaves the complex and enters the nucleus. Upon binding to Hh response elements, Gli2 activates transcription (Figure 13.3c).

13.2 Hedgehog Ligands

Whereas there is only one hedgehog gene in *D. melanogaster* (*hh*), vertebrate genomes contain three homologous genes named sonic hedgehog (SHH), desert hedgehog (DHH), and Indian hedgehog (IHH). If not specifically addressed, the encoded proteins will be collectively referred to as Hedgehog (Hh) or Hh ligand. As a major morphogen, the concentration gradients of Hh must be fine-tuned and precisely controlled. This is done by posttranslational modifications that modulate the way Hh is secreted. After entering the secretory pathway, Hh proteins undergo

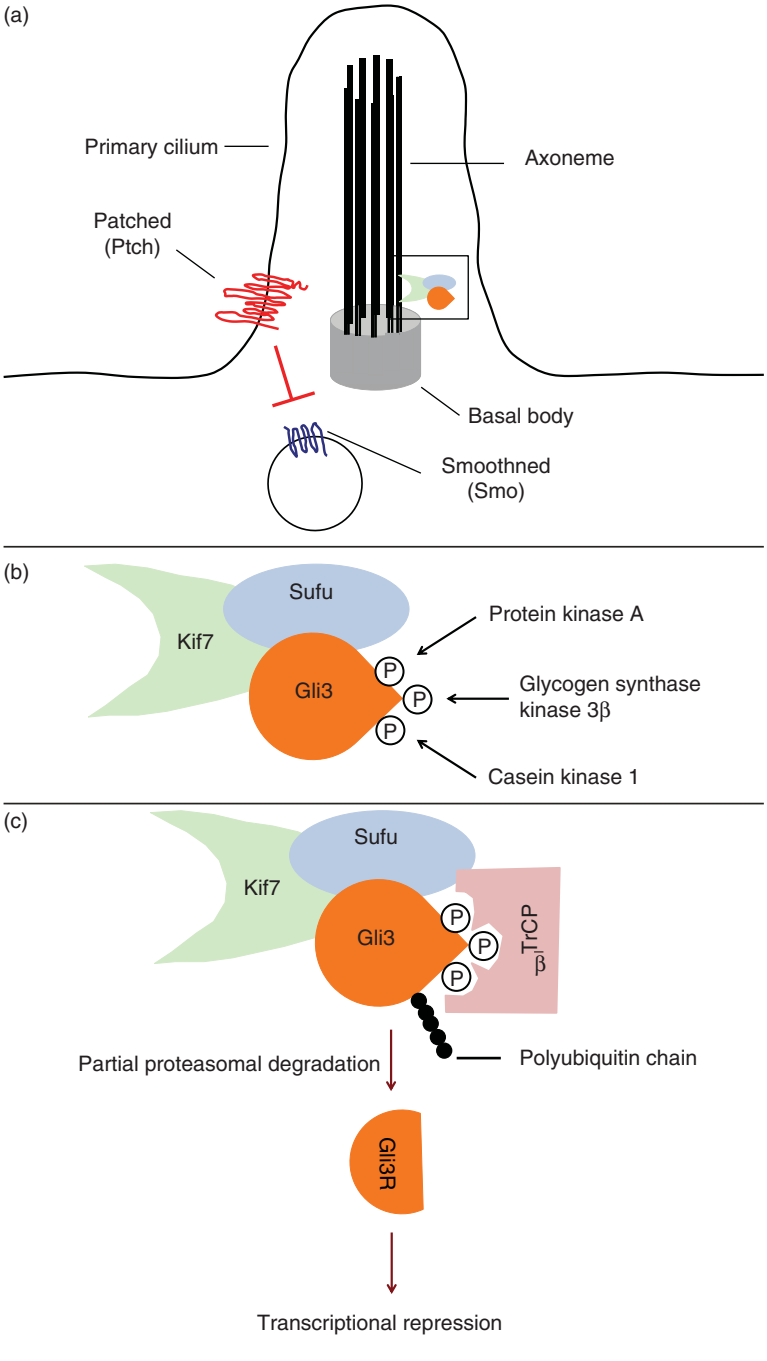
autoproteolytic cleavage, generating an N-terminal and a C-terminal peptide. The N-terminal peptide is covalently linked to two hydrophobic compounds, namely cholesterol and palmitic acid. The C-terminal fragment is degraded by the proteasome, whereas the posttranslationally modified N-terminal fragment acts as ligand (Figure 13.4). In the following, the abbreviation Hh refers to the lipid-modified N-terminal fragment.

Before being released from the secreting cell, the lipid-modified Hh is associated with the plasma membrane. Dispatched, a multipass protein related to Ptch, cooperates with a secreted glycoprotein (Cegp1) to release sHh from the plasma membrane. Both proteins bind to the cholesterol moiety of sHh. Lipid-modified Hh can form soluble multimers or can be integrated into lipoprotein particles. Alternatively, Hh may be integrated into exovesicles (Briscoe and Therond, 2013).

13.3 The Primary Cilium

In mammals, the hedgehog signaling pathway is closely associated with the primary cilium, a nonmotile structure present on the apical pole of most vertebrate cells (Figure 13.2a). In general, cilia are present in noncycling differentiated cells or in stem cells in the G_0 phase. Primary cilia are involved in important processes during development and tissue homeostasis, such as sensing cell topology, migration, differentiation, mechanical stimulation, and chemosensation, among others. After completion of the cell cycle, the cilium is built on the older (mother) centriole attached to a Golgi vesicle. The mother centriole is part of the basal body, from which the microtubule of the axoneme extends into the cilium. Primary cilia contain the so-called intraflagellar transport machinery (IFT). Components of the IFT and associated proteins are transported along axonemal microtubules by kinesin 2 motor proteins in the anterograde (base-to-tip) direction and by cytoplasmic dynein 2 in the retrograde (tip-to-base) direction. In mutants affecting building of primary cilia or IFT, hedgehog signaling is severely disturbed. These and other findings indicate that the primary cilium is essential for hedgehog signaling (Satir, Pedersen, and Christensen, 2010).

Figure 13.2 Signaling in the absence of hedgehog (Hh). (a) In the absence of Hh, the multipass membrane protein Patched (Ptch) is located at the base of the primary cilium. Through signaling by Ptch, the seven-pass protein Smoothened (Smo) remains embedded in cytoplasmic vesicles. A complex containing kinesin-family member 7 (Kif7), Sufu, and the transcription factor Gli3 (rectangle) is located at the base of the axoneme, which is anchored in the basal body. (b) In association with Kif7 and Sufu, Gli3 is phosphorylated by different protein kinases. (c) Phosphorylated Gli3 binds the beta-TrCP subunit of an E3 ubiquitin ligase, which leads to partial degradation of Gli3 by the proteasome. The resulting Gli3R protein suppresses the transcription of Hh-responsive genes.


(a)
Primary cilium
Axoneme
Patched
(Ptch)
Basal body
Smoothned
(Smo)
(b)
Sufu
Kif7
Gli3
P
P
P
Protein kinase A
Glycogen synthase
kinase 3β
Casein kinase 1
(c)
Sufu
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P
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Partial proteasomal degradation
Polyubiquitin chain
Gli3R
Transcriptional repression

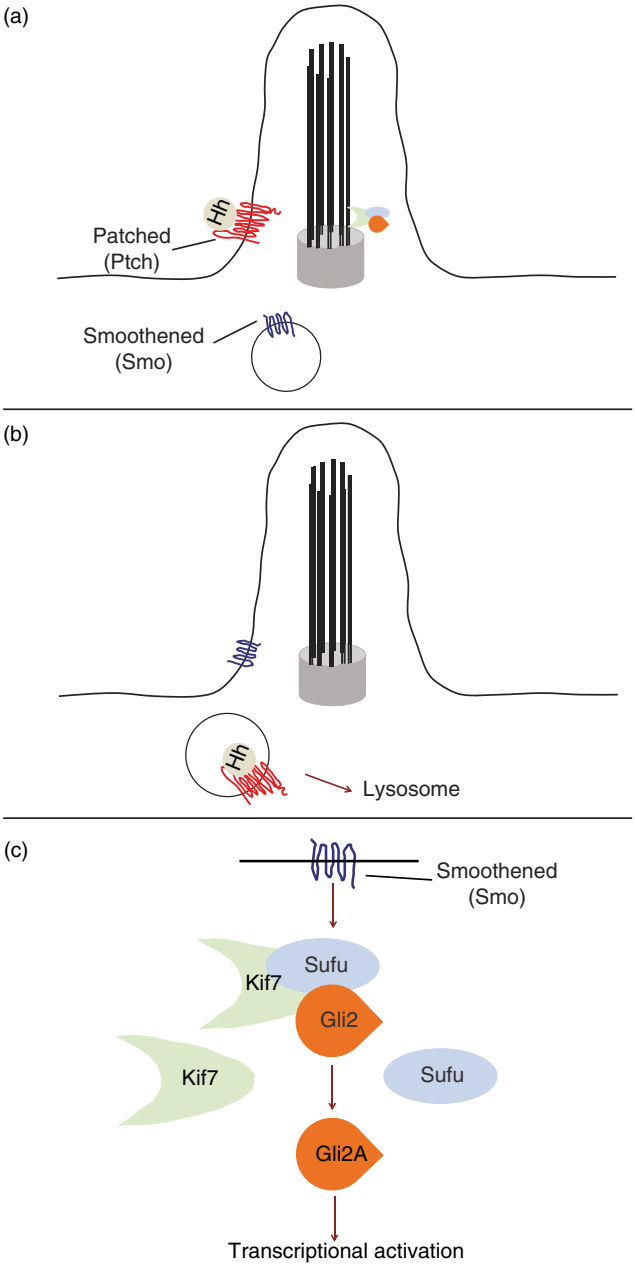

Figure 13.3 Signaling after binding of Hh to patched (Ptch). (a) Hh binds to its receptor Ptch. (b) The complex of Hh and Ptch is internalized and directed to the lysosome. Ptch no longer signals to keep Smo in intracellular vesicles and off the cilium. Smo enters the membrane of the primary cilium. (c) Smo moves to the top of the cilium. Here, Smo signals through an inhibitory G-protein to a complex of Gli2, kinesin-family member 7 (Kif7), and Sufu. Activated Gli2 (Gli2A) leaves the complex, moves into the nucleus, and stimulates transcription after binding to recognition sequences in the DNA.

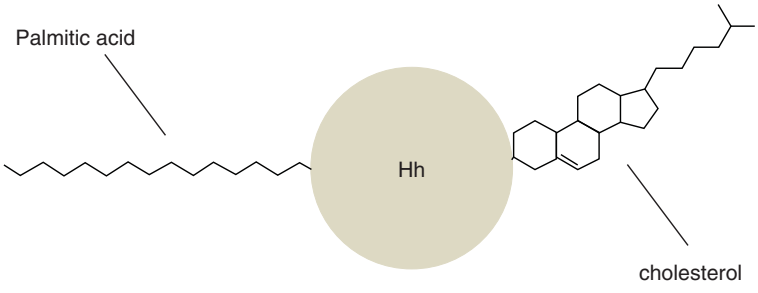

Figure 13.4 Schematic view of the hedgehog protein, posttranslationally modified by palmitic acid and cholesterol.

13.4
Patched (Ptch) and Smoothened (Smo)

Secreted Hh ligands signal through their canonical receptor Ptch, a multipass transmembrane protein. In turn, Ptch regulates the cellular localization of a seven-pass transmembrane protein Smo, a member of the G-protein-coupled receptors (GPCR) (Figures 13.2 and 13.3). The Ptch-Smo regulation is central to the Hh signaling pathway. In the absence of Hedgehog ligand, Smo remains embedded in the membrane of cellular vesicles. This cellular location is dependent on signals derived from Ptch, the nature of which is poorly understood. Hh ligand binding to Ptch leads to its displacement from the cilium and concomitant relocalization of Smo to the cilium membrane. This localization of Smo is critical for activation of the downstream effectors of Hh signaling (Teglund and Toftgard, 2010).

13.5
Gli Transcription Factors

The Gli transcription factors are the major effectors of the Hh signaling pathway. Whereas the fruit fly *D. melanogaster* harbors only one homologous gene, named Cubitus interruptus (*ci*), mammals harbor three *GLI* genes, numbered *GLI1*, *GLI2*, and *GLI3*. The designation "GLI" is derived from a putative oncogene, the *GLI1* gene, amplified in human GLIoma (Kinzler *et al.*, 1987). All Gli proteins contain a zinc-finger DNA-binding motif and transactivation motifs. Gli2 and Gli3, but not Gli1, harbor N-terminal repressor domains (Figure 13.5).

Gli2 and Gli3 are the essential mediators of signaling by sHh. Gli2 acts mainly as an inducer of hedgehog-responsive genes, whereas Gli3 acts mainly as a repressor. Gli1 amplifies hedgehog signaling through its transactivation domain (Hui and Angers, 2011).

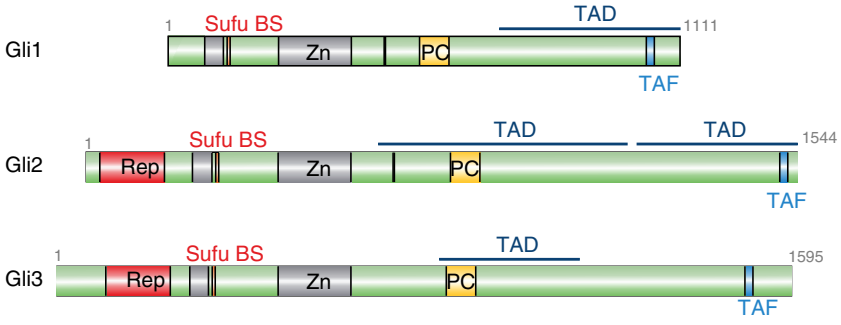

Figure 13.5 Schematic view of domains and motifs in Gli proteins. Rep, repressor domain; Sufu BS, Sufu-binding site; Zn, Zinc-finger DNA-binding domain; TAD, transcription activation domain; PC, phosphorylation cluster; and TAF, binding site for TATA box-binding protein-associated factor. (Hui and Angers (2011), with permission.)

13.6 Signaling in the Absence of Hedgehog

(Video: The hedgehog signaling pathway – Part 1: Normal situation – enhanced ebook and closed website: Hedgehog_Video_01_ebook.mp4)

In the absence of Hh binding to Ptch, Gli proteins actively suppress the activation of Hh-responsive genes. Gli proteins are part of a complex of proteins that contains Kif7 and Sufu. Sufu was identified in Drosophila as a genetic suppressor of the phenotype resulting from decreased *fu* (fused) function. In mice, genetic ablation of the *Sufu* gene leads to constitutive Gli-mediated activation of the hedgehog pathway, demonstrating that this complex is essential for negatively regulating Gli transcription factors. The transcriptional activity of the Gli transcription factors is regulated by proteolytic cleavage and posttranslational modifications. Gli3 is thought to be the primary mediator of suppressor activity, and in the absence of Hh signaling, Gli3 is sequentially phosphorylated at its C-terminus by protein kinase A, glycogen synthase kinase 3β, and casein kinase 1 (Figure 13.2b). Phosphorylation of Gli3 leads to the recruitment of the βTrCP subunit of the SCF-type E3 ubiquitin ligase. Subsequently, Gli3 is ubiquitinylated and directed to the proteasome. In the proteasome, the C-terminal part of Gli3 is degraded. The resultant fragment, abbreviated Gli3R, acts as a transcriptional repressor. After entering the nucleus, Gli3R binds to the DNA, where it represses hedgehog-responsive genes (Figure 13.2c).

13.7 Signaling after Binding of Hedgehog to Patched

(Video: The hedgehog signaling pathway – Part 1: Normal situation – enhanced ebook and closed website: Hedgehog_Video_01_ebook.mp4)

Hh binds to its receptor Ptch, localized at the base of the primary cilium. Upon binding of Hh to Ptch, the ligand–receptor complex is internalized and directed to the lysosome. The inhibition of Smo is relieved, and Smo is directed to the primary cilium. The mechanism that leads to the activation of Smo is not yet fully understood. In response to hedgehog binding, Smo moves up the primary cilium. In parallel, Gli proteins in association with Sufu translocate to the tip of the cilium. This movement depends on the molecular motor Kif7.

Smo belongs to the superfamily of GPCR. Experimental findings indicate that Smoothened signals via an inhibitory G protein, abbreviated G_i. The molecular details of the potential interaction between Smo and the inhibitory G protein have not yet been firmly established. It is thought that G_i mediates the release of Gli proteins from the complex with Sufu. In response to Hh stimulation, Gli2 acts as principal activator of Hh-responsive genes. Active Gli2 translocates from the primary cilium into the cytoplasm and subsequently into the nucleus. After binding to DNA, hedgehog-responsive target genes are expressed. Among these genes are proto-oncogenes such as *MYC* and the cyclinD1-encoding gene *CCND1.* Interestingly, *GLI1* is among the Hh-responsive genes, indicating the presence of a positive-feedback loop (Hui and Angers, 2011).

13.8 Activation of the Canonical Hedgehog Pathway in Basal Cell Carcinoma and Medulloblastoma

(Video: The hedgehog signaling pathway – Part 2: Activation of the Hedgehog pathway in basal cell carcinoma and medulloblastoma – only closed website: Hedgehog_Video_02_ebook.mp4)

Mutations in genes encoding signaling proteins of the hedgehog pathway are most frequently observed among human tumors such as basal cell carcinomas (BCC) and desmoplastic medulloblastomas (MB). An example of BCC is shown in Figure 13.6, in which the patient harbors several BCCs in areas exposed to sunlight, as typically observed. Locally, the tumors behave like true carcinomas, invading the surrounding tissue. Fortunately, the tumors rarely metastasize. Nevoid BCC syndrome (NBCCS), also known as *Gorlin syndrome*, is a rare condition inherited in an autosomal dominant manner. Diagnostic criteria of Gorlin syndrome are lamellar calcification of the falx cerebri, jaw keratocysts, palmar or plantar pits, and multiple BCCs. The existence of a first-degree relative with NBCCS would confirm the diagnosis (Table 13.1) (Gorlin, 1995).

Medulloblastoma, a primitive neuroectodermal tumor of the cerebellum, is the most common malignant brain tumor in children. In the tissue section shown in Figure 13.7, the tumor invades the molecular layer of the cerebellum. When the tumor abuts the meninges, a connective tissue response takes place. This variant is called *desmoplastic medulloblastoma*.

It was in Gorlin syndrome that the causal role of the hedgehog pathway in human tumors was first recognized. Inactivation of a *PTCH1* allele in the

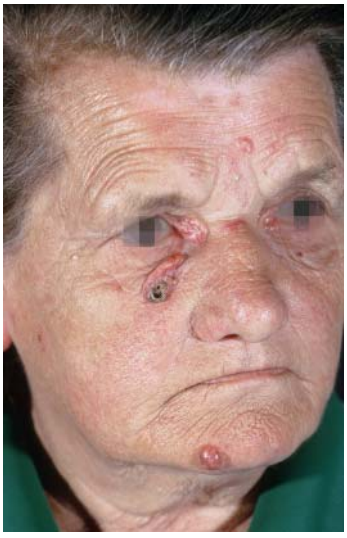

Figure 13.6 Clinical picture of basal cell carcinomas. The patient shown here harbors several basal cell carcinomas, which typically are located in areas exposed to sunlight. (By courtesy of Prof. Dr Ingrid Moll, University Medical Center Hamburg-Eppendorf, Germany.)

Table 13.1 Diagnostic criteria of nevoid basal cell carcinoma syndrome (NBCCS, Gorlin syndrome).

Lamellar calcification of the falx cerebri
Jaw keratocysts
Palmar or plantar pits (two or more)
Multiple basal cell carcinomas (>5 in a lifetime) or a basal cell carcinoma before age of 30 years
Evidence of familial syndrome: first-degree relative with NBCCS

germline disposes to Gorlin syndrome (Figure 13.8). In BCC of Gorlin patients, the second *PTCH1* allele is inactivated by somatic mutations. Thus, *PTCH1* behaves as a classical tumor suppressor gene. In a family with Gorlin syndrome lacking a germline *PTCH1* mutation, a loss-of-function mutation of the *SUFU* gene has been described.

In sporadic BCC, the mutational inactivation of both *PTCH1* alleles is frequently found (Figure 13.8). The inactivation of both alleles leads to the inactivation of the Ptch1 receptor. Smoothened is no longer excluded from the primary cilium, and the hedgehog signaling pathway is active. Somatic mutations of *PTCH1* have been identified in more than 90% of sporadic BCCs. In those sporadic BCCs that do not harbor *PTCH1* mutations, activating mutations of the *SMO* gene are frequently found. It is thought that BCC is addicted to an active hedgehog pathway.

In patients with medulloblastoma, a correlation between the specific gene within the canonical Hh pathway that harbors mutations and the age of the patient is found. Inactivating mutations of the *PTCH1* gene dominate in all

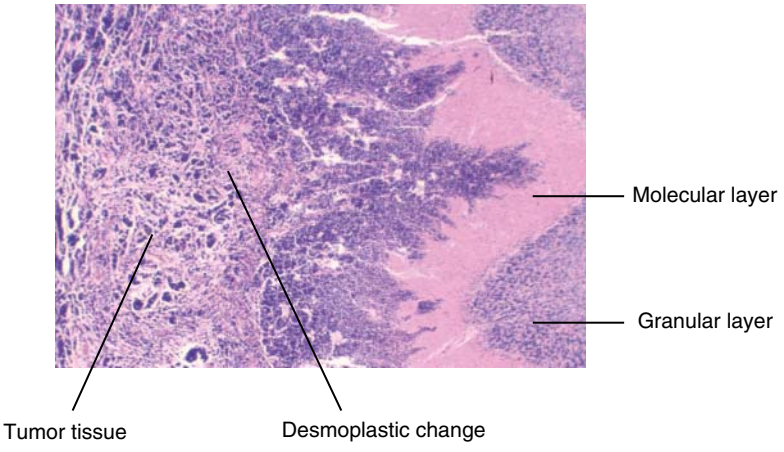

Figure 13.7 Tissue section of a desmoplastic medulloblastoma. The tumor invades the molecular layer of the cerebellum. (By courtesy of Prof. Dr Markus Glatzel, University Medical Center Hamburg-Eppendorf, Germany.)

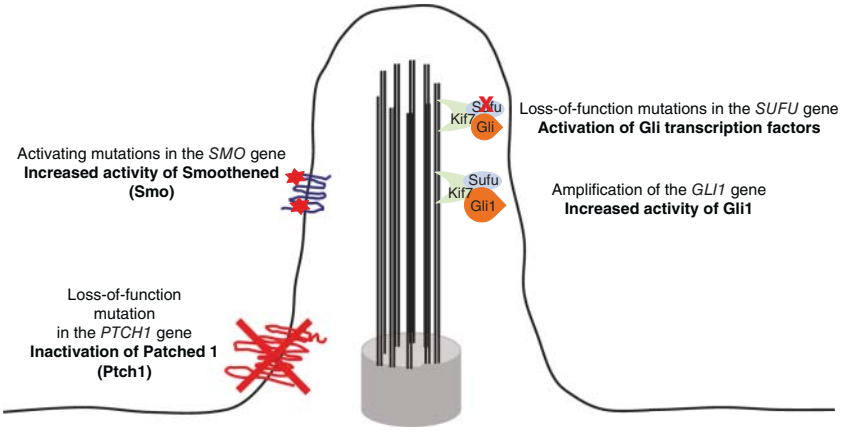

Figure 13.8 Mechanisms leading to the activation of the hedgehog pathway in basal cell carcinoma, medulloblastoma, and rhabdomyosarcoma.

patients. Mutations of the *SUFU* genes are frequently found in infants, but only rarely in older patients. In adults, but not in younger patients, activating mutations of *SMO* are second in frequency to mutations of *PTCH1* (Figure 13.8) (Kool *et al.*, 2014).

In addition to BCC and medulloblastoma, genes of the Hedgehog pathway are mutated in rhabdomyosarcoma. Mutations inactivating *PTCH1* and *SUFU* genes or *GLI1* amplifications have been described.

Whole-Genome Sequencing approaches indicate that somatic *PTCH1* mutations are present in other cancer types, such as ovarian and endometrial cancer, albeit infrequently (http://cancer.sanger.ac.uk/cancergenome/projects/cosmic/).

13.9
Noncanonical Activation of Hedgehog-Responsive Genes

The canonical pathway of hedgehog signaling involves the Ptch receptor and Smo, resulting in the activation of Gli transcription factors. In noncanonical pathways, Gli factors are activated by pathways that circumvent Ptch and Smo.

13.9.1
KRas

The best example for the noncanonical activation of Gli transcription factors is the activation in cells of pancreatic ductal adenocarcinoma (PDAC). Among human malignancies, PDAC displays the highest fraction with mutant *KRAS* oncogenes. *KRAS* mutations are an early event in PDAC, as evidenced by their presence in early pancreatic intraepithelial neoplasia (PanIN) lesions. It has been shown that oncogenic KRas activates Gli1 in cultured PDAC cells (Ji *et al.*, 2007) and that silencing of *KRAS* leads to the downregulation of Gli1 (Nolan-Stevaux *et al.*, 2009). Murine loss-of-function and gain-of-function models further corroborate the interplay of mutant KRas protein and Gli transcription factors. Mice that express a mutant *Tp53* allele in addition to the mutant *Kras* allele in their pancreas develop carcinomas of the pancreas rapidly, with a median latency of 120 days. Mice expressing the inhibitory *Gli3T* transgene, which encodes a truncated Gli3 protein, develop carcinomas with a significantly longer latency (Figure 13.9a). In a gain-of-function model, Gli1 was ectopically expressed in pancreatic epithelial cells. Similarly to ectopic *Gli3T*, *Gli1* expression did not initiate pancreatic tumors on its own. In conjunction with mutant *Kras*, however, *Gli1*-expressing mice had widespread formation of stage 3 PanIN lesions, typical of high-grade dysplasia, resulting in almost complete disruption of normal pancreatic architecture (Figure 13.9b) (Rajurkar *et al.*, 2012). Thus, although the mechanism by which KRas activates Gli1 expression is not yet fully understood, this appears to be a necessary step in the transforming potential of Kras in the pancreas.

13.9.2
Atypical Protein Kinase-Lambda/Iota (aPKCι)

Atypical protein kinase C-lambda/iota (aPKCι) is an enzyme that is involved in the determination of cell polarity. The enzyme partners with proteins that are associated with the centrosome and promotes the assembly of primary cilia. The enzyme phosphorylates the zinc-finger domain of Gli1 to activate DNA binding and transcriptional activity (Atwood and Oro, 2014). Since Gli1 creates a positive-feedback loop by activating the transcription of the aPKCι, the enzyme amplifies Gli1 activity independently of the canonical Hh pathway. In cultured BCC cells, blocking aPKCι function inhibits Hh signaling and proliferation (Atwood *et al.*, 2013). aPKCι phosphorylates the transcription factor SOX2, which regulates

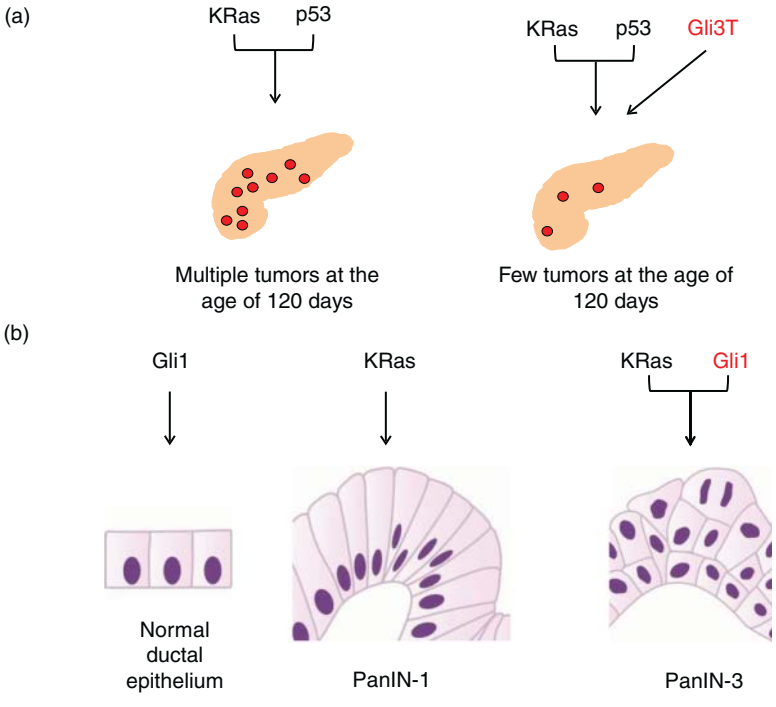

Figure 13.9 (a) A dominant suppressive Gli3 construct (GliT3) increases the latency in the development of pancreatic carcinomas in mice. In *Pfa1a-Cre*; *LSL-Kras*^{G12D}; *Trp53*$^{flox/wt}$ mice, multiple carcinomas developed at the age of 120 days. At this time point, few tumors were present in *Pfa1a-Cre*; *LSL-Kras*^{G12D}; *Trp53*$^{flox/wt}$; *R26-Gli3T* mice (based on results published by Rajurkar *et al.* (2012)), (b) Gli1 accelerates *Kras*-initiated pancreatic tumorigenesis in mice. In *Pfa1a-Cre*; *R26-Gli1* mice, the pancreas was normal. At 2 months of age, *Pfa1a-Cre; LSL-Kras*^{G12D} mice demonstrated only a few early PanIN lesions. In *Pfa1a-Cre;LSL-Kras*^{G12D}; *R26-Gli1* mice, however, multiple PanIN lesions developed, some corresponding to human PanIN-3 lesions. (Based on results published by Rajurkar *et al.* (2012).)

maintenance of stem cell properties. Phosphorylated SOX2 is recruited to the promoter of Hh acyltransferase, which mediates the palmitoylation of Hh. Palmitoylation is a prerequisite of Hh activity (Justilien *et al.*, 2014). In this way, aPKCι appears to support the population of tumor stem-cell-like cells, both in ovarian carcinoma (Wang, Hill, and Fields, 2013) and in squamous cell lung carcinoma (NSCLC, non-small-cell lung cancer) (Justilien *et al.*, 2014) (Figure 13.10).

13.9.3 PI3-Kinase-AKT (PI3K-AKT)

In the studies on the neuronal fate of chicken neural explants, it was shown that stimulation of the PI3-Kinase-AKT (PI3K-AKT) pathway by insulin-like growth factor I potentiates Gli activation induced by low levels of sHh (Riobo *et al.*, 2006).

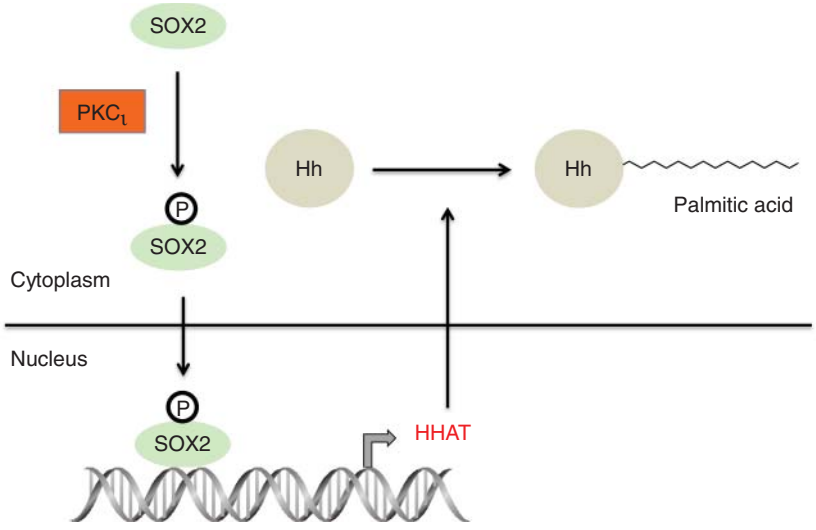

Figure 13.10 Schematic diagram on the roles of aPKCι and SOX2 in the activation of *HHAT* (hedgehog acyltransferase) transcription and Hh activation by palmitoylation. (Based on results published by Justilien *et al.* (2014).)

This finding stimulated studies on the interplay between hedgehog signaling and PI3K-AKT signaling. In mouse xenografts of human gastric carcinoma specimens, enforced expression of sHh significantly enhanced the incidence of lung metastases. Considering epithelial–mesenchymal transition (EMT) as a potential basis for this observation, EMT was studied in further detail. Interestingly, PI3K-AKT inhibition blocked sHh-induced EMT, indicating the PI3K-AKT pathway as an effector of sHh (Yoo *et al.*, 2011).

13.9.4 mTOR

The mechanistic target of rapamycin complex 1 (mTORC1) regulates cell size and proliferation, the synthesis of proteins and lipids, energy metabolism, and autophagy. A number of oncoproteins, tumor suppressors, growth factors, and cytokines feed into the activity of the complex (see Chapter 8). mTOR phosphorylates and activates the translational regulator S6K1 (S6 kinase 1). In esophageal adenocarcinoma, tumor necrosis factor α (TNFα) stimulates the development and progression of the tumor by activating the mTOR pathway. In cultures of different esophageal adenocarcinoma cell lines, it has been shown that TNFα activates the transcription of Gli1-dependent genes. Cyclopamine (11-deoxojervine), a natural inhibitor of the canonical hedgehog pathway, did not interfere with Gli1 activation, indicating that the signaling from TNFα to Gli1 circumvents the canonical pathway. When inhibitors of mTOR were applied, however, activation of Gli1 was blocked. The activation of Gli1 is mediated by the

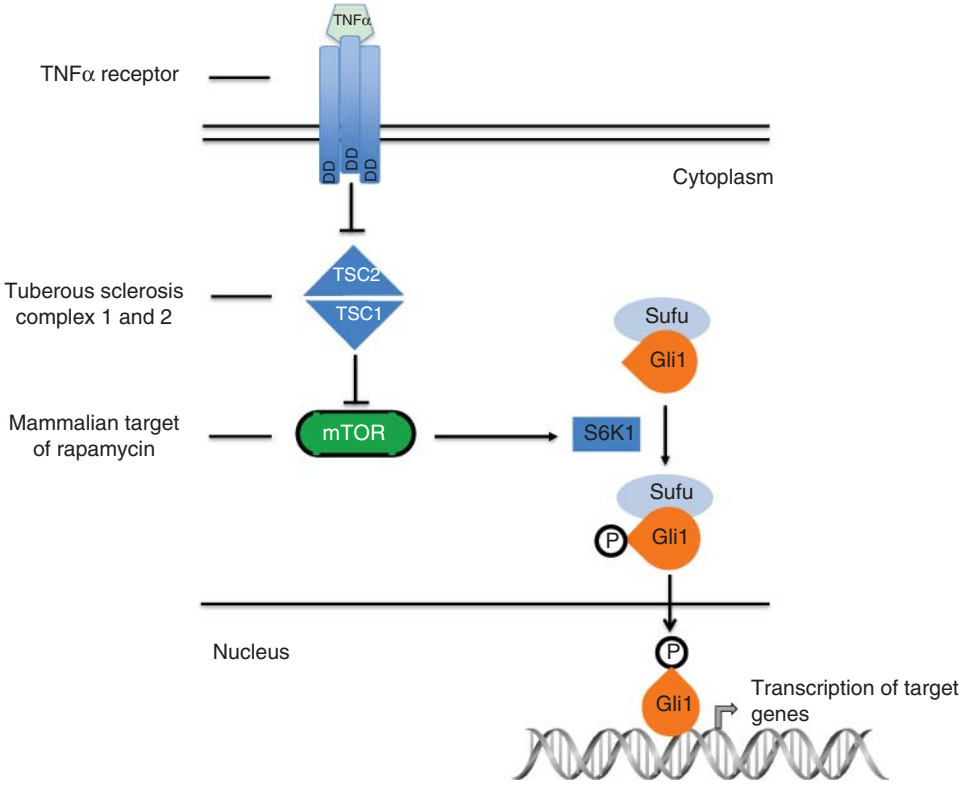

Figure 13.11 Schematic diagram of the noncanonical mTOR/Gli1 pathway. (Based on results published by Wang *et al.* (2012).)

phosphorylation of a serine residue by S6K1, leading to the release of Gli1 from its inhibitor Sufu (Figure 13.11) (Wang *et al.*, 2012).

13.10
Paracrine Activation of Hedgehog Signaling

Hedgehog acts as a classical morphogen to control cell patterning and differentiation in embryonic tissues by mediating paracrine effects on mesenchymal cells. It has been hypothesized that Hh secreted by epithelial tumor cells could similarly act on cells of the tumor microenvironment in a paracrine manner. The most convincing data on a potential paracrine effect of sHh were derived from PDAC and its precursor lesion, PanIN.

sHh is expressed in PDAC and in its precursor lesion PanIN, but is absent in normal pancreatic ducts (Thayer *et al.*, 2003). When the expression of Gli1 was

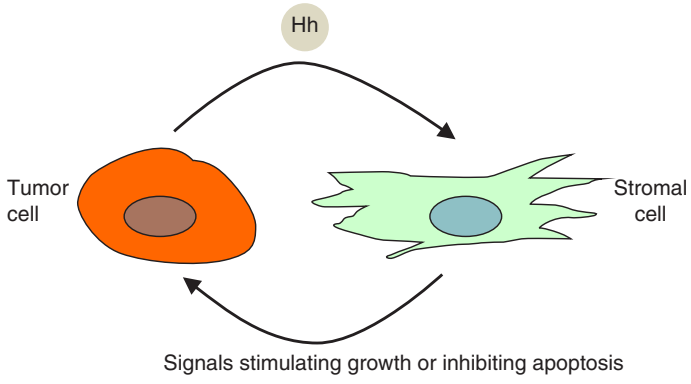

Figure 13.12 Model of the paracrine stimulation of tumor growth by hedgehog.

analyzed separately in the epithelial and stromal compartment of PDAC specimens, Gli1 expression turned out to be 40–120-fold higher in the stromal cells than in the epithelium (Tian *et al.*, 2009). Considering the expression of sHh by epithelial tumor cells, this finding indicates a paracrine effect of sHh on the stromal tumor compartment (Figure 13.12). Xenotransplantation experiments further corroborate this assumption. Fresh human tumor tissue was transplanted into immunodeficient mice. In the growing tumors, the host provided the cells of the tumor microenvironment. Using probes specific for murine transcripts, a significant correlation between the transcription of human *SHH* and *IHH* hedgehog genes on the one hand and murine *Gli1* gene on the other hand was observed. To confirm the activity of the hedgehog pathway in stromal cells, the *Smo* gene was ablated from mouse fibroblasts that were then coinjected with epithelial tumor cells to generate a stromal support. Reduced Smo levels resulted in much smaller tumors. Reminiscent of the function of Hh in embryonic development, stromal cell expressed the *Wnt* gene upon stimulation by Hh. These experiments suggest that tumor cells secrete Hh, thereby activating Hh signaling in cells of the tumor microenvironment. When Hh signaling is blocked, the interaction of the tumor stroma with tumor cells is disturbed, leading to impaired tumor growth (Figure 13.12) (Yauch *et al.*, 2008).

13.11 Pharmacological Inhibition of the Hedgehog Pathway

(Video: The hedgehog signaling pathway – Part 3: Inhibition of the Hedgehog pathway by drugs binding to Smoothened – only closed website: Hedgehog_Video_03_ebook.mp4)

Different approaches to inhibit the hedgehog signaling pathway have been applied in preclinical investigations, as well as in clinical trials, including

Table 13.2 Effects of drugs blocking canonical Hedgehog signaling.

Compound	Effect
MAb5E1, Robotnikinin	Inhibition of Hedgehog binding to Ptch
Cyclopamine, Vismodegib	Direct inhibition of Smo
Itraconazole	Blocking translocation of Smo into cilium
Gant 51 and 58, arsenic trioxide	Inhibition of Gli transcription factors

patients with BCC and medulloblastoma, as well as with other malignant tumors (Table 13.2). As explicated before, the effectors of Hh signaling, namely the Gli transcription factors, can be activated by either the canonical Hh pathway or by other incoming signals. The canonical pathway itself can be activated either by Hh ligands or by mutations of proteins within the pathway. Accordingly, different levels of inhibition of Hh signaling can be distinguished (Amakye, Jagani, and Dorsch, 2013).

13.11.1 Inhibition of Hh Binding to Ptch

The monoclonal antibody 5E1 inhibits the binding of Hh ligands to their receptor Ptch. The antibody has been used in preclinical experiments, but not in clinical trials so far. Other approaches to inhibit sHh ligands include the drug Robotnikinin, which inhibits interaction with Ptch by binding to sHh. Inhibitors of Hh acyltransferase prevent the attachment of the palmitoyl residue to Hh. Since palmitoylation is essential for the activity of Hh ligands, activation of Ptch is blocked.

13.11.2 Inhibitors of Smoothened

In 1959, Binns and coworkers reported on congenital cyclopian-type malformations in lambs, which were caused by maternal ingestion of the range plant *Veratrum californicum* (Babbott, Binns, and Ingalls, 1962). Ingestion of the plant by ewes during the first 10 days of gestation led to the cyclopian-type malformation of the lamb. Only in 1998, it was found that the active compound in *V. californicum*, the alkaloid cyclopamine, inhibits the hedgehog pathway (Cooper *et al.*, 1998). The antagonistic effect of cyclopamine is based on direct binding to Smoothened. As a consequence, Smo does not enter the primary cilium and signaling is blocked. As a therapeutic drug, cyclopamine has a number of drawbacks such as low water solubility, low affinity to Smo, poor bioavailability, and suboptimal pharmacokinetics (Athar *et al.*, 2014). Particularly troublesome are off-target effect, which obscured experimental results in the past.

Because of the limitations of cyclopamine as a therapeutic drug, other Smo inhibitors including synthetic cyclopamine derivatives have been developed.

Meanwhile, the Smoothened inhibitor vismodegib (11-deoxojervine) has been approved by the Food and Drug Administration of the United States for the treatment of locally advanced and metastatic BCC (http://www.accessdata.fda.gov/drugsatfda_docs/labe
Additionally, a report has been published on spectacular treatment effects of vismodegib in a patient with medulloblastoma (Rudin *et al.*, 2009). However, the effect did not last longer than several months, and eventually, resistance against the drug developed.

Following the application of vismodegib, a number of other alternative Smoothened inhibitors have been developed and have been or are currently being tested in clinical trials (http://www.clinicaltrials.gov). Although the Hh pathway is disturbed by mutant components of the pathway in more than 90% of BCCs, the fraction of responders to Smo inhibitors is lower than expected. In a vismodegib study, for example, more than half of the patients with locally advanced BCC did not respond (Sekulic *et al.*, 2012). In general, Smo inhibitors are well tolerated, and the most common side effects include taste alterations, muscle spasm, alopecia, anorexia, and fatigue. For the different compounds, the dose-limiting toxicities are quite variable. Smo inhibitors were also tested in carcinomas such as colorectal, ovarian, and pancreatic cancer. The results of clinical trials have been disappointing so far (Amakye, Jagani, and Dorsch, 2013).

13.11.3 Inhibition of Cilial Trafficking

The assembly of cilia and the cilial location of Hh signaling proteins are essential for the activity of the pathway in vertebrates. Several Hh-inhibiting compounds have been shown to affect cilial location or cilial trafficking of the signaling proteins. Inhibitors such as vismodegib and sonidegib block translocation of Smo into the cilial membrane. Some recently identified inhibitors affect the assembly of cilia. Depending on structural features, glucocorticoids may either promote or inhibit the translocation of Smo (Amakye, Jagani, and Dorsch, 2013). The Food and Drug Administration of the United States approved Itraconazole as an antifungal agent. Unexpectedly, Itraconazole prevents the translocation of Smo into the cilium. In preclinical models, Itraconazole was effective against BCC and MB, when the tumors were dependent on Hh signaling. Remarkably, the agent inhibited Hh pathway activation and tumor growth associated with acquired resistance to Smoothened antagonists (Kim *et al.*, 2013). Itraconazole also showed some antitumor effect in patients with metastatic prostate cancer (Antonarakis *et al.*, 2013).

13.11.4 Inhibition of Gli

Mutations downstream of Smo such as mutations in Sufu or amplification of the *GLI1* gene can activate Gli transcription factors. In addition, noncanonical pathways affect the activity of Gli. For these reasons, compounds that affect the activation of Gli transcription factors or their effector functions are candidates

for antitumor agents. Two molecules, GANT58 and GANT51, were selected in a screening approach for compounds that interfere with the effect of Gli transcription factors and inhibit tumor growth. Other Hh pathway inhibitors affect Gli processing or Gli activation. Arsenic trioxide (ATO) is a drug approved by the Food and Drug Administration of the United States for the treatment of acute promyelocytic leukemia. ATO acts as an inhibitor of Gli transcription factors by blocking the hedgehog-induced accumulation of Gli2 in the primary cilium. ATO was effective against medulloblastoma allografts and Ewing sarcoma xenografts with high levels of Gli1 expression (Beauchamp *et al.*, 2011). Similarly to Itraconazole, ATO inhibited Hh pathway activation and tumor growth associated with acquired resistance to Smo antagonists (Kim *et al.*, 2013).

13.11.5 Resistance against Direct Inhibitors of Smoothened

(Video: The hedgehog signaling pathway – Part 3: Inhibition of the Hedgehog pathway by drugs binding to Smoothened – only closed website: Hedgehog_Video_03_ebook.mp4)

Several mechanisms have been described that underlie resistance against direct Smo inhibitors. Most of these mechanisms are restricted to preclinical tumor therapy models. By binding to Smoothened, direct inhibitors such as cyclopamine or vismodegib block the hedgehog pathway. During therapy with direct Smoothened inhibitors, cell clones may expand that harbor constitutionally active, point-mutated Smoothened proteins. In the first medulloblastoma patient responding to vismodegib, a point mutation in the *SMO* gene (D473H) most probably mediated resistance against the drug (Yauch *et al.*, 2009). Alternatively, the pathway may be activated downstream of Smoothened by amplification of the *GLI2* gene. In addition, amplification of the *CCND1* gene has been observed in resistant cell clones. Since the cyclinD1-encoding *CCND1* gene is one of the target genes activated by the hedgehog pathway, transcription of the *CCND1* gene may have become independent of the hedgehog signal (Amakye, Jagani, and Dorsch, 2013; Hui and Angers, 2011).

It is assumed that noncanonical pathways contribute to resistance of tumors against drugs targeting the Hh pathway. As explicated earlier, Gli1 may be activated via the mTOR pathway (Wang *et al.*, 2012). One of the main upstream regulators of mTOR is the PI3K-AKT pathway (see Chapter 8). In a mouse model of medulloblastoma, a pathway gene expression signature indicated that PI3K signaling was upregulated in resistant tumors. The addition of a PI3K inhibitor or the combination of this inhibitor with an mTOR inhibitor with the Smo antagonist in the initial treatment markedly delayed the development of resistance (Buonamici *et al.*, 2010).

As mentioned before, the atypical PKCι leads to the phosphorylation and activation of Gli1 via S6K1. It was shown that an aPKCι-specific peptide inhibitor blocked the growth of Smo inhibitor-resistant BCC cell lines (Atwood *et al.*, 2013).

13.12 Outlook

So far, inhibitors of the Hh pathway have been shown to be effective only in those patients whose tumors harbor mutations in genes of the canonical pathway. As stated in a review by Amayake *et al.*: "Tumors that have shown clinically meaningful responses are either very rare (such as medulloblastoma) or rarely require systemic therapy (such as BCC)" (Amakye, Jagani, and Dorsch, 2013). When Hh pathway mutations are absent, Smo inhibitors generally failed in clinical trials. This experience applies to tumors such as ovarian cancer, pancreatic adenocarcinoma, and colorectal carcinoma.

In order to improve therapeutic efficacy, several measures could be taken.

- As explicated earlier, noncanonical pathways such as PI3K-AKT-mTor or aPKCι may activate Gli transcription factors. Blocking these pathways may inhibit the activation of Gli. The use of potential Gli inhibitors should be based on predictive biomarkers such as a Gli transcription signature. In medulloblastoma patients, for example, a gene signature predicted the response to the Smo inhibitor sonidegib with high reliability (Amakye, Jagani, and Dorsch, 2013).
- The monotherapy of tumors by agents blocking defined signaling pathways is effective in those tumors that are addicted to the respective pathway. However, such tumor entities are rather the exception than the rule. In the majority of tumors, different pathways cooperate to achieve malignant transformation. In these cases, combinatorial therapies must be designed to block the pathways involved. In preclinical tumor models, the combinations of Hh pathway inhibitors with inhibitors of either the PI3K-AKT pathway or the MAPK pathway have proven to be effective. In a mouse model of chronic myeloid leukemia (CML), cyclopamine enhanced the effect of the ABL1 inhibitor nilotinib (Dierks *et al.*, 2008).
- Inhibition of either the Hh pathway alone or together with other signaling pathways may be combined with conventional chemotherapy regimens. For example, the addition of the Hh inhibitor cyclopamine and a secretase inhibitor, which blocks the Notch pathway, to antimitotic chemotherapeutic drugs reduced growth of docetaxel-resistant prostate cancer by eliminating tumor-initiating cells (Domingo-Domenech *et al.*, 2012).

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14
TGFβ Signaling

Summary

Members of the transforming growth factor beta (TGFβ) superfamily regulate cell fate, differentiation and proliferation in embryonic development, and tissue regeneration. TGFβ superfamily members bind to a cellular receptor complex comprised of both a pair of type 1 and a pair of type 2 receptors. TGFβ receptors signal through Smad proteins, which regulate the transcription of target genes. Depending on the particular context, TGFβ signaling may either inhibit or foster tumor growth. The gene encoding the TGFβ type 2 receptor (*TGFBR2*) contains a sequence stretch of repetitive sequences that are prone to deletions or insertions in patients with defects of the mismatch repair (MMR) system of DNA. Germline mutations in genes of the MMR system cause the hereditary nonpolyposis colorectal cancer (HNPCC) syndrome. The *SMAD4* gene is a tumor suppressor gene inactivated in major sporadic carcinomas, for example,

Cancer Signaling: From Molecular Biology to Targeted Therapy, First Edition.
Christoph Wagener, Carol Stocking, and Oliver Müller.

Companion Website:www.wiley.com/go/wagener/cancersignaling

pancreatic cancer. On the other hand, TGFβ may support tumor progression by inducing an epithelial–mesenchymal transition (EMT). In patients with advanced malignancies, members of the TGFβ superfamily may be responsible for weight loss and cachexia.

14.1 The TGFβ Superfamily

The protein family of transforming growth factor beta (TGFβ) emerged with the evolution of multicellular animals. In multicellular organisms, the regulation of cell fate is central to embryonic development and tissue regeneration and a major task fulfilled by TGFβ superfamily members. Ligands and receptors as well as their cellular signaling pathways are highly conserved during evolution.

Members of the TGFβ superfamily of morphogens comprise TGFβs, activins, Nodal, bone morphogenetic proteins (BMPs), growth and differentiations factors (GDFs), and the anti-Müllerian hormone. In a strict sense, three TGFβs are present in vertebrates, namely TGFβ1, TGFβ2, and TGFβ3. In the following, the term *TGFβ* covers all three members of the superfamily. Based on their function in early vertebrate development, the BMPs, activins, Nodal, and GDFs are sometimes summarized as "BANGs" (Wakefield and Hill, 2013).

Members of the TGFβ superfamily, particularly the BANGs, are involved in the earliest stages of embryonic development. At the blastocyst stage, Nodal regulates transcription factors such as Oct4, which determine the pluripotency of stem and progenitor cells (Wu and Hill, 2009). Later in development, members of the Nodal/activin and BMP subfamilies are key regulators of body axes and tissue patterning. Nodal is also required for the induction of the three germ layers: endoderm, mesoderm, and, indirectly, ectoderm. Members of the TGFβ superfamily are central players in organogenesis. In the context of tumor progression, the involvement in epithelial–mesenchymal transition (EMT) is of particular interest. A prominent example in embryogenesis is the invasion of the heart cushion by endocardial cells from the atrioventricular canals, which eventually give rise to heart valves (Mercado-Pimentel and Runyan, 2007).

In the fully developed organism, the regulation of tissue homeostasis is one of the major functions of TGFβ superfamily members. The role of TGFβs in scarring will be given as an example. The example has a counterpart in solid tumors, in which the deposition of structural elements into the connective stroma tissue is central to tumor progression. Myofibroblasts respond to cues from TGFβs in order to fulfill their function in wound healing and tissue repair. When TGFβ signaling is inappropriate, the deposition of extracellular matrix (ECM) may be disturbed, resulting in excessive scarring. The importance of TGFβ in ECM remodeling is also evident in the congenital disorder Marfan syndrome, which is caused by defects of connective tissue. One particular type of Marfan syndrome, Marfan syndrome II, and a related disease, Loeys–Dietz syndrome, are caused by mutations in TGFβ receptors (Gordon and Blobe, 2008).

14.2
Structure and Processing of TGFβ Superfamily Members

A striking feature of the protein sequences of members of the TGFβ superfamily is the so-called cysteine knot motif, with seven invariant cysteine residues within a mature subunit (Sun and Davies, 1995). Six cysteine residues form three disulfide bonds. The unpaired seventh cysteine residue makes an intermolecular cysteine bridge between two monomers to form a dimer (Figure 14.1). Members of the TGFβ superfamily are synthesized as pre-pro-proteins, which contain a signal peptide, a prodomain, and the mature domain. A proteolytic cleavage site is located between the prodomain and the mature domain (Bottner, Krieglstein, and Unsicker, 2000).

The TGFβs and other members of the TGFβ superfamily are synthesized as dimers. The TGFβ propeptide, known as latency-associated protein, LAP, remains bound to the mature TGFβ peptide after the bond between the prodomain and the mature domain has been cleaved by a furin-type protease (Figure 14.2). Via a

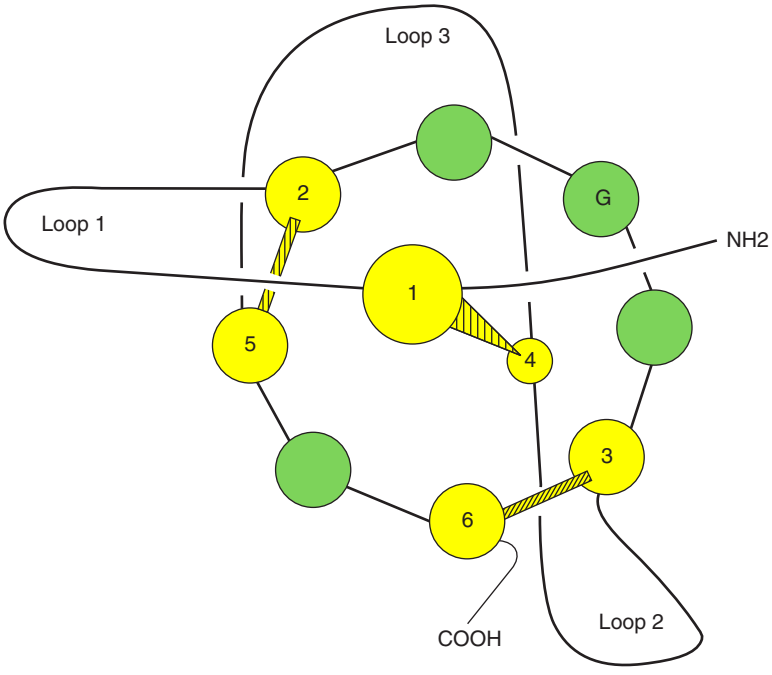

Figure 14.1 Schematic view of the structure of 8-amino-acid-ring cystine-knot proteins. The six cysteines belonging to the cystine knot are shown in yellow. The remaining 4 amino acids (aa's) belonging to the 8-aa-ring are shown in green. G stands for the glycine residue that is conserved in all 8-aa-rings of cystine-knot proteins. The black and yellow segments represent the three disulfide bridges forming the cystine knot. The size of the ring is exaggerated compared to that of the loops for the sake of clarity (Alvarez, Cohareau, and Combarnous, 2009) (Creative Common License for unrestricted use).

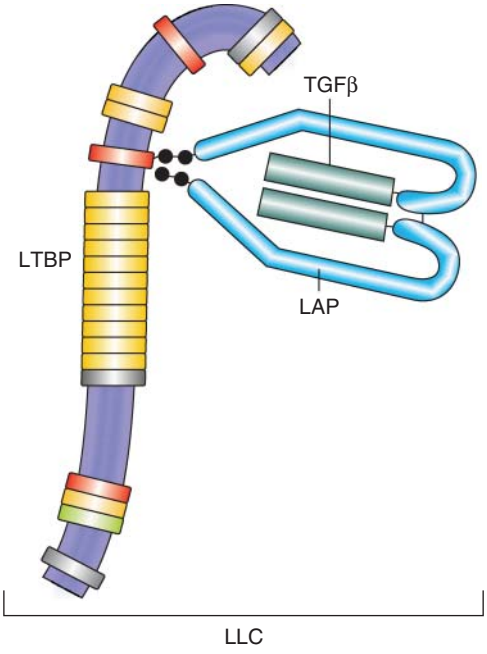

Figure 14.2 Large latent complex (LLC). After proteolytic cleavage of the proprotein, TGFβ1 remains associated with the prodomain (latency-associated complex, LAP). In the complex, TGFβ1 is inactive. LAP is covalently bound to the latent TGFβ-binding protein (LTBP). The complex of TGFβ, LAP, and LTBP is designated "large latent complex (LLC)." Latent TGFβ is activated by proteolysis, integrins, thrombospondin-1, reactive oxygen species (ROS), and low pH. (Lafyatis (2014), with permission, modified.)

cystine bridge, the LAP is bound to the so-called latent TGFβ-binding protein, abbreviated LTBP. The complex of mature TGFβ, LAP, and LTBP, known as *large latent complex*, is deposited in the extracellular matrix. Active TGFβ can be liberated from this complex by the binding of integrins to the Arg-Gly-Asp (RGD) sequence of LAP, which induces a conformational change and the release of active TGFβ from LAP. In addition to integrin binding, TGFβ can be activated by a variety of other mechanisms or molecules such as proteolytic cleavage, thrombospondin-1, reactive oxygen species, and low pH (Annes, Munger, and Rifkin, 2003; Herpin, Lelong, and Favrel, 2004). For some BMPs and other family members, prodomain association does not affect activity (Sengle *et al.*, 2011).

14.3
The TGFβ Signaling Pathway

(Video: TGFβ signaling – enhanced ebook and closed website: TGFbeta_ebook.mp4)

Members of the TGFβ superfamily bind to a receptor complex composed of two pairs of cellular receptors: one pair belonging to the type 1 and the other

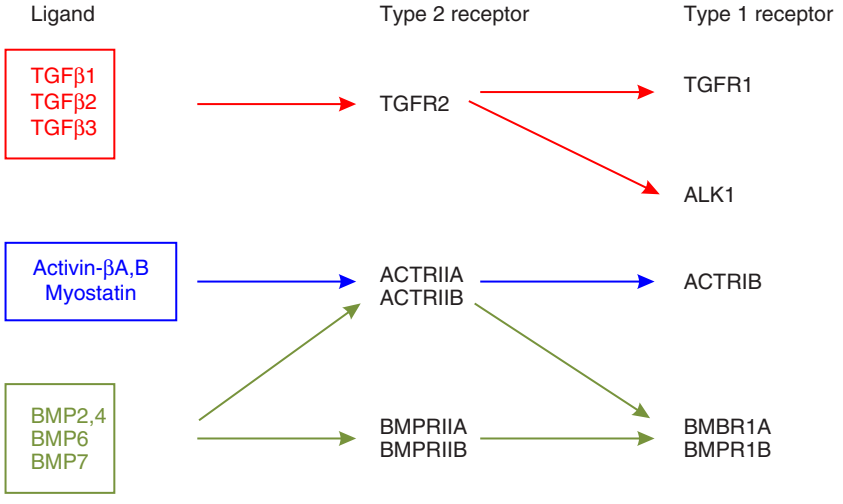

Figure 14.3 Some combinations of type 2 and type 1 receptors in signaling by members of the TGFβ superfamily. (Based on data published by Wakefield and Hill (2013).)

pair to type 2 receptors (TGFR1 and TGFR2, respectively). The two structurally related receptor types are transmembrane serine/threonine kinases. The receptor system for the entire TGFβ superfamily is created from seven type 1 to five type 2 receptors paired in different combinations. The high flexibility of the ligand–receptor–signaling systems is a result of both the differential pairing of type 1 and type 2 receptor dimers and the binding of different TGFβ superfamily ligands to these various receptor combinations, in addition to the usage of a wide spectrum of signal transduction modules. In Figure 14.3, the binding of members of the TGFβ superfamily to different receptor combinations is shown.

The mode of ligand binding to receptor ectodomains differs for different members of the TGFβ superfamily. TGFβ1 and TGFβ3 bind first to the type 2 receptor, whereas TGFβ2 requires both type 2 and type 1 receptor ectodomains for initial binding. In contrast, BMP2 and BMP4 do not bind well to the type 2 receptor BMPR2, but bind efficiently to the type 1 receptor BMPR1A. In addition to the type 1 and 2 receptors, additional surface proteins fine-tune the cellular response to TGFβ superfamily ligands. Betaglycan, a proteoglycan containing chondroitin sulfate and heparan sulfate side chains, enhances the binding of TGFβ2 to the TGFR2 receptor, and endoglin, a single-pass type 1 transmembrane glycoprotein, is required for efficient TGFβ signaling through the TGFR1-dimer ALK1-dimer pair in endothelial cells (Feng and Derynck, 2005).

After TGFβ binding to the ectodomains of the two receptor pairs, a stable receptor complex is formed. Following complex formation, the type 2 receptor kinase phosphorylates a conserved glycine- and serine-rich motif (GS sequence) in the cytoplasmic domain of the type 1 receptor kinase (Figure 14.4). This phosphorylation activates the type 1 receptor kinase, leading to autophosphorylation of

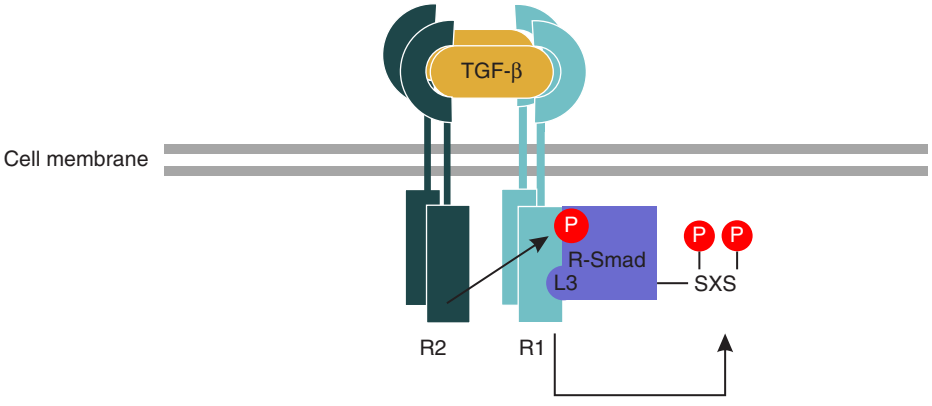

Figure 14.4 After binding of a TGFβ dimer to dimers of type 1 and type 2 receptors, the kinase of the type 2 receptor phosphorylates the type 1 receptor. After phosphorylation, R-Smad (Receptor-Smad) binds to the type 1 receptor. The type 1 receptor kinase phosphorylates the SXS motif in the R-Smad.

the type 1 receptor and to the phosphorylation of the Smad proteins. The so-called Smad proteins transduce signals from the cell membrane to the nuclear DNA. The designation "Smad" stems from the gene *MAD* (mothers of decapentaplegic) of *Drosophila melanogaster* and the *Sma* (small) gene of *Caenorhabditis elegans.* In vertebrates, three groups of Smads can be distinguished: the receptor-binding R-Smads, common Smads, and inhibitory Smads. In vertebrates, eight different genes code for Smads. TGFβs and activins activate the R-Smads Smad2 and Smad3, whereas BMPs activate the R-Smads Smad1, Smad5, and Smad8. After binding of an R-Smad to the receptor complex, two serine residues in the SXS motif of the R-Smad are phosphorylated. Within the kinase domain, type 1 receptors harbor a loop sequence, the so-called L45 loop. The L45 loop interacts with the L3 loop of an R-Smad. Binding and signaling specificities reside in the L45 structure of the type 1 receptor and the L3 loops of R-Smads (Figure 14.4) (Feng and Derynck, 2005).

R-Smads contain a C-terminal SXS motif, in which both serine residues are phosphorylated by the type 1 receptor kinase. This leads to a conformational change in the R-Smad and the dissociation from the receptor. Subsequently, two R-Smads form a trimeric complex with a common Smad, which is Smad4 in vertebrates. The trimeric complex, consisting of two R-Smads and the common Smad, is shuttled into the nucleus (Feng and Derynck, 2005).

The inhibitory Smads compete for binding of R-Smads to the type 1 receptor. Both, the inhibitory Smad6 and the inhibitory Smad7, are involved in negative-feedback loops. BMP signaling via Smad1 and Smad5 induces the expression of Smad6, whereas TGFβ signaling via Smad3 induces the expression of Smad7. However, BMPs and TGFβ can also induce Smad7 and Smad6 expression, respectively. Besides inhibition of receptor binding, a second mechanism exists, whereby inhibitory Smads block signal transduction by type 1 receptors. Both

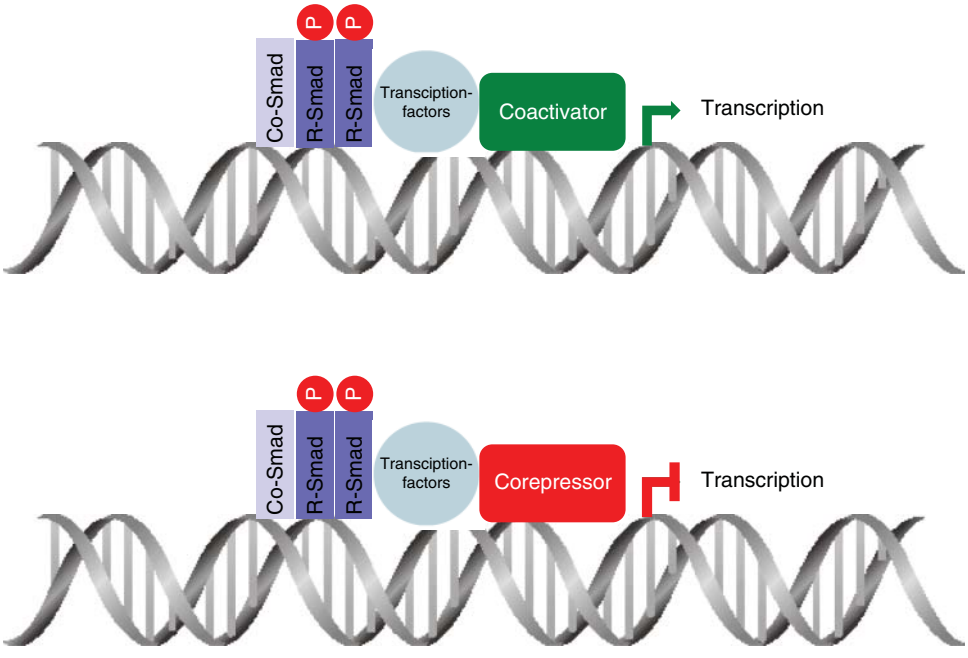

Figure 14.5 Smads bound to the DNA associate with transcription factors. When the complex binds to coactivators, transcription of responsive genes is induced. In contrast, association with a corepressor leads to transcriptional inhibition.

inhibitory Smads bind to the Smurf E3 ubiquitin ligase. By binding of inhibitory Smads to the type 1 receptor, the ubiquitin ligase is translocated to the receptor and leads to its degradation by the proteasome (Feng and Derynck, 2005).

14.4 Transcriptional Regulation by TGFβ Superfamily Members

The trimeric protein complex of two R-Smads and Smad4 associates with specific sequence motifs in the DNA (Figure 14.5). Smad4 and all of the R-Smads except Smad2 bind DNA directly. Smad3 and Smad4 recognize the sequence AGAC or its reverse complement, and Smad1, Smad5, and Smad8 seem to prefer GC-rich elements with the sequence GRCGNC for binding (Wakefield and Hill, 2013). The binding affinity of Smads to their recognition motifs is rather low. DNA-binding affinity increases significantly by association of Smads with transcription factors. The variable association with a great number of different transcription factors explains the pleiotropic effects of TGFβ superfamily members.

Some of the Smad-binding transcription factors that are relevant for the functions of TGFβ superfamily members in carcinogenesis and cancer progression are

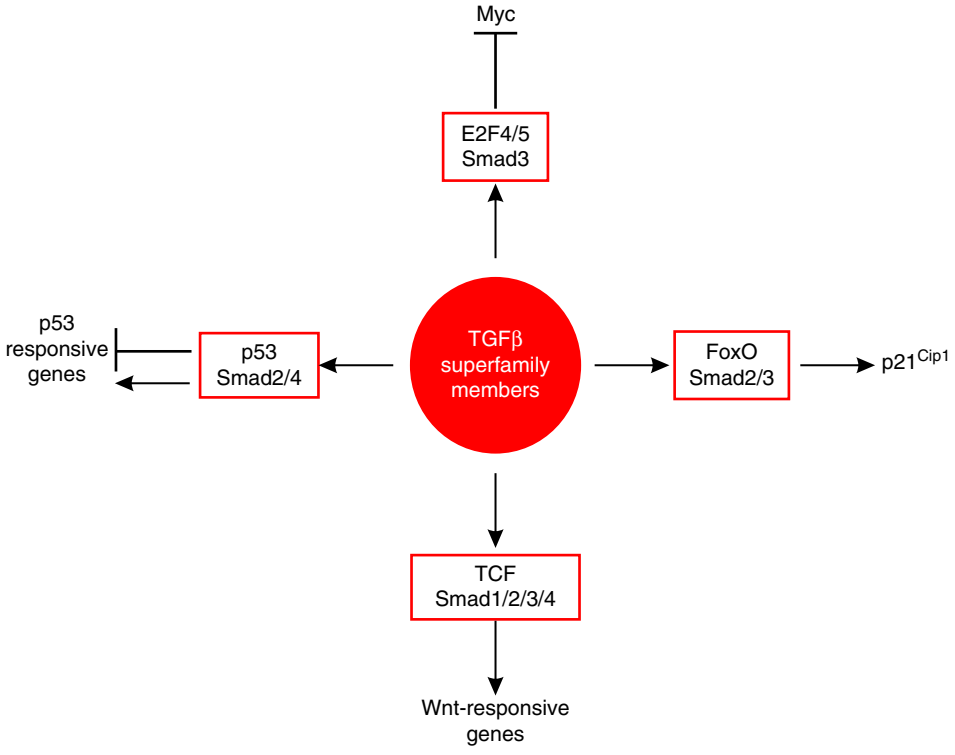

Figure 14.6 The association of R-Smads with transcription factors leads to the activation or inhibition of pathways relevant in cancer.

depicted in Figure 14.6. In epithelial tissues, TGFβ inhibits proliferation by inducing the transcription of the cyclin-dependent kinase (CDK) inhibitors $p21^{Cip1}$ and $p15^{Ink4b}$ on the one hand and repressing the transcription of the *MYC* gene on the other. The Smad3 and Smad4 complex interacts with the forkhead transcription factor FOXO (forkhead box O) at the promoter of the CDKN1A gene encoding $p21^{Cip1}$ to induce its transcription. SP1, a transcription factor of the Zinc finger family of transcription factors, drives the transcription of both the *CDKN1A* gene and the gene encoding $p15^{Ink4b}$ (CDKN1B). Complexes of Smad 2, 3, and 4 at promoters of both genes induce transcription by association with SP1. Smad3 and Smad4 interact with transcription factors that bind to recognition sites at the *MYC* promoter. Smad3 forms a complex with the factors E2F4/DP1/p107 in the cytoplasm. In response to TGFβ, the complex associates with Smad4 and translocates to the *MYC* promoter. Binding of the complex to the *MYC* promoter is essential for the repression of transcription from the *MYC* gene. Downregulation of Myc is a prerequisite for growth inhibition by TGFβs (Feng and Derynck, 2005).

Smad3 and Smad4 can associate with the transcription factor TCF (T-cell-specific transcription factor), the major effector of the Wnt signaling pathway.

Smad3 also binds to Axin, which is a negative regulator of the Wnt pathway. Smad3 interacts with the p52 NF-kB subunit at adjacent NF-kB and Smad-binding sites. This leads to an activation of NF-kB signaling. Finally, signaling by members of the TGFβ superfamily may be accompanied by the formation of p53/Smad complexes, which bind to adjacent p53 and Smad binding sites in promoters of responsive genes.

Smads can associate with both inducers and repressors of transcription (Figure 14.5). CBP/p300 is one of the major transcriptional coactivators that associate with Smad complexes. On the other hand, their interaction with corepressors can inhibit the transcriptional response.

In the nucleus, phosphatases dephosphorylate the R-Smads. Dephosphorylated R-Smads can be relocated back to the cytoplasm. Thus, R-Smads constantly shuffle between the cytoplasm and the nucleus, independently of a signal. It has been suggested that the constant shuttling of Smads, as well as the quasilinear signaling pathway that lacks amplification steps, is particularly well suited for the interpretation of gradients of ligand concentrations (Wakefield and Hill, 2013).

14.5
Regulation of Stem Cells by TGFβ Superfamily Members

Members of the TGFβ superfamily regulate the renewal and lineage specification of embryonic stem cells, tissue specific stem cells, and cancer stem cells (Oshimori and Fuchs, 2012). Nodal/activin signaling is critical for self-renewal and pluripotency of both human and murine embryonic stem cells. In human embryonic stem cells, Nodal signaling and the inhibition of glycogen synthase kinase 3β (GSK3β) are sufficient to maintain the undifferentiated state.

In the developed organism, the renewal and repair of tissues depend on tissue stem cells. In some tissues such as the intestine, the renewal of the epithelium follows mainly an intrinsic program. In other tissues such as the blood, however, cell renewal is highly flexible and responds to external cues, such as infection or replenishment after bleeding. There seems to be hardly any tissue, in which tissue stem cells do not depend on members of the TGFβ superfamily to regulate the balance between quiescence, renewal, and differentiation. Typical for morphogens, the effects of TGFβs are highly dependent on concentration. In the hematopoietic system, for example, high concentrations of TGFβ inhibit proliferation of both myeloid-biased and lymphoid-biased hematopoietic stem cells. At lower concentrations, however, the block in proliferation of myeloid-biased hematopoietic stem cells is relieved, whereas the proliferation of lymphoid-biased stem cells is still inhibited (Challen *et al.*, 2010). In stem cells of the hair follicle, BMPs maintain quiescence. When the gene encoding the BMP type 1-receptor BMPR1A is conditionally ablated, the stem cells become active and produce tumor-like structures (Kobielak *et al.*, 2003).

As outlined in Chapter 2, tumor stem cells (TSCs) are cells within a tumor, which, when transplanted as single cells, give rise to a new tumor with the

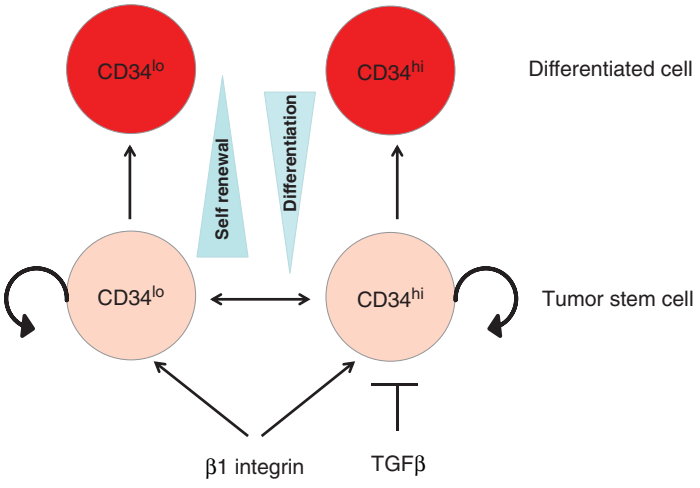

Figure 14.7 The impact of TGFβ on self-renewal and differentiation. Tumor stem cells were isolated based on the expression of the CD34 marker from the tumor–stroma interface of squamous cell carcinomas (SCCs) of the mouse skin. Activated β1 integrin promotes self-renewal of both tumor stem cell populations, whereas active TGFβ/TGFβ receptor II signaling primarily influences CD34hi tumor stem cells, restricting their self-renewal and expansion (Schober and Fuchs, 2011).

characteristics of the parental tumor. Similarly to embryonic and tissue stem cells, TSCs self-renew and give rise to nonstem tumor cells, which proliferate and differentiate. The ability of members of the TGFβ superfamily to regulate the self-renewal and differentiation of TSCs has been shown in different experimental systems. Stem cells from a murine squamous cell carcinoma (SCC) serve as an example (Figure 14.7) (Schober and Fuchs, 2011). The murine skin is a well-established model for tumorigenesis. SCCs can be induced by treatment with the mutagen 7,12-dimethylbenz[*a*]anthracene (DMBA) and the tumor promoter 12-*O*-tetradecanoylphorbol-13-acetate (TPA). In mice lacking a functional TGFβ receptor II (*Tgfbr2* k.o.) in the skin, the development of SCCs is accelerated, and the carcinomas are highly aggressive. The cell surface protein CD34 is a marker of hair follicle stem cells. This marker was used to isolate TSCs from SCCs induced by treatment with DMBA and TPA. Two populations of stem cells were isolated from the tumor–stroma interface, which differed in the expression of the CD34 marker. Both populations expressed high levels of the integrin α6β1. Those TSCs, which lacked TGFR2, gave rise to highly aggressive SCCs, which showed a greatly enhanced ability to give rise to secondary tumors. Interestingly, these properties were compromised when signaling by the integrin was blocked. Thus, in this model, the α6β1integrin increases the malignant potential of TSCs. This increase is reversed by TGFβ.

14.6 TGFβ Superfamily Members as Tumor Suppressors in Human Cancer

(Video: TGFβ signaling – enhanced ebook and closed website: TGFbeta_ebook.mp4)

Biallelic inactivating mutations of the *TGFBR2* gene, which encodes the type 2 receptor for the TGFβs, are frequently observed in colorectal, gastric, biliary, pulmonary, ovarian, esophageal, and head and neck carcinomas. The *TGFBR2* gene contains a sequence stretch of 10 consecutive adenine nucleotides. Repetitive sequences such as the sequence found in the *TGFBR2* gene are prone to deletions or insertions in patients with defects of the mismatch repair (MMR) system of the DNA. Germline mutations in genes of the MMR system cause the so-called hereditary nonpolyposis colorectal cancer (HNPCC) syndrome. In HNPCC tumors, poly(A) errors are found in the majority of the patients. If adenine insertion or deletion leads to an altered reading frame, the encoded receptor is truncated and nonfunctional. Similarly, defects of the MMR system in sporadic gastrointestinal tumors frequently lead to the inactivation of the TGFβ type 2 receptor.

The ACVR2 gene encodes the activin type II receptor, which binds activin and BMPs. The *ACVR2* gene contains two sequence stretches of eight consecutive adenine nucleotides. Mutations in one of the two polyadenine tracts have been identified in gastrointestinal tumors with MMR defects, such as gastric and colorectal cancer (Levy and Hill, 2006) (Table 14.1).

In pancreatic cancers, chromosome 18q21 deletions are frequently observed. The deletions regularly encompass the *SMAD4* gene. The second allele is also inactivated, either by deletions or by mutations. Thus, the *SMAD4* gene behaves as a classical tumor suppressor gene (Levy and Hill, 2006). Interestingly, biallelic inactivation is also observed in colorectal cancer, but only when the MMR system is intact. This observation demonstrates that for the initiation and progression of carcinomas, it is not a single protein that needs to be inactivated but the TGFβ pathway itself.

Table 14.1 Polyadenine tract mutations of genes encoding type II receptors of the TGFβ superfamily in tumors with microsatellite instability (MSI^+).[a)]

Carcinoma	Frequency (%)
$Poly(A)_{10}$ tract mutations of *TGFBR2*	
Biliary tract	~50
Colon	60–90
Gastric	~70
$Poly(A)_8$ tract mutations of *ACVR2*	
Colon	58–90
Gastric	~44

a) Levy and Hill (2006).

Table 14.2 Juvenile polyposis syndrome (JPS): inactivating mutations in the TGFβ signaling pathway.[a)]

Gene	Mutation frequency (%)
BMPR1A	24–50
SMAD4	25–56

a) Levy and Hill (2006)

In a screen for point mutations and deletions in pancreatic cancer, mutations in the BMPR2 and *SMAD3* genes were observed in addition to the known mutations of the *TGFBR2* and *SMAD4* genes. The TGFβ signaling pathway was affected in each of the 24 tumors investigated (Jones *et al.*, 2008).

The Juvenile Polyposis Syndrome (JPS) is an autosomal dominant disorder, in which hamartous polyps develop in the intestine (see Chapter 8). The polyps are associated with an increased risk of adenocarcinomas. In subsets of JPS patients, the disease is caused by germline mutations in either the ALK3 (*BMBR1A*) gene or the *SMAD4* gene (Levy and Hill, 2006) (Table 14.2).

In general, the exclusive inactivation of the signaling pathways of TGFβ superfamily members is not sufficient to induce spontaneous tumor formation in mouse models. Tumors result only after additional carcinogenic events. For example, in mice with an inactivated *Apc* gene, the additional disruption of the *Tgfbr2* gene favors the progression of polyps to carcinomas (Munoz *et al.*, 2006). Similarly, ablation of the *Tgfbr2* gene favors the conversion of premalignant into malignant lesions when a mutant *Kras* gene is expressed in the pancreatic epithelium (Ijichi *et al.*, 2006) (Figure 14.8).

14.7 Active role of TGFβ in Tumor Progression

During embryonic development, TGFβs can induce EMT. As mentioned in the introduction, the invasion of the heart cushion by endocardial cells from the atrioventricular canals is a prominent example (Mercado-Pimentel and Runyan, 2007). A similar effect can be exerted by TGFβs during tumor progression. The dual role played by TGFβ in tumor progression is exemplified by the experiment shown in Figure 14.9. A constitutively active *Tgfbr1* gene attenuates the formation of mammary carcinomas induced by the *Erbb2/Her2-neu* oncogene. However, an active TGFβ pathway favors the formation of extravascular lung metastases (Siegel *et al.*, 2003).

It appears counterintuitive that TGFβs act as a tumor suppressor on the one hand, but promote tumor progression on the other. This apparent contradiction may be resolved by the fact that the tumor suppressive pathway is attenuated in

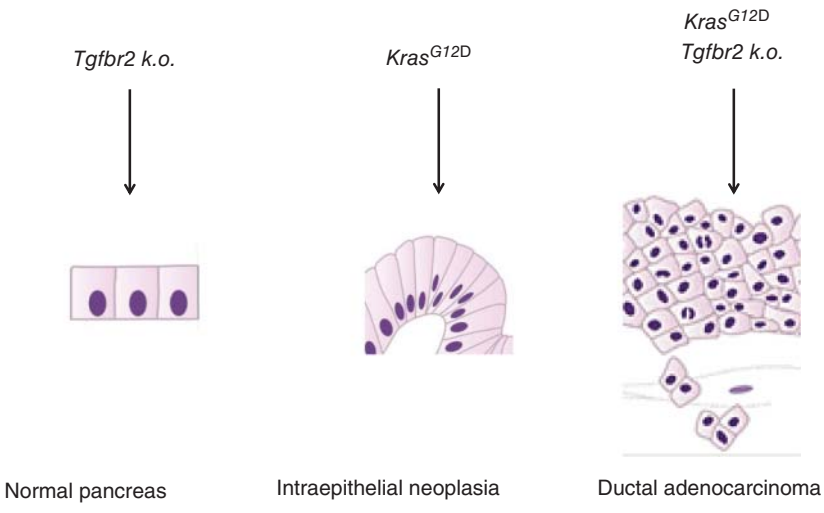

Figure 14.8 Interplay of tumor suppressor activity of the TGFβ pathway and an oncogene in tumor development. Expression of a mutant *Kras* gene (*Kras*^{G12D}) in ductal cells of the mouse pancreas led to intraepithelial neoplasia (PanIN). When a pancreas-selective *Tgfbr2* knockout was combined with the expression of a mutant *Kras* gene, 100% of the animals developed pancreatic adenocarcinomas. A pancreas-selective *Tgfbr2* knockout alone gave no discernible phenotype. (Based on data by Ijichi *et al.* (2006).)

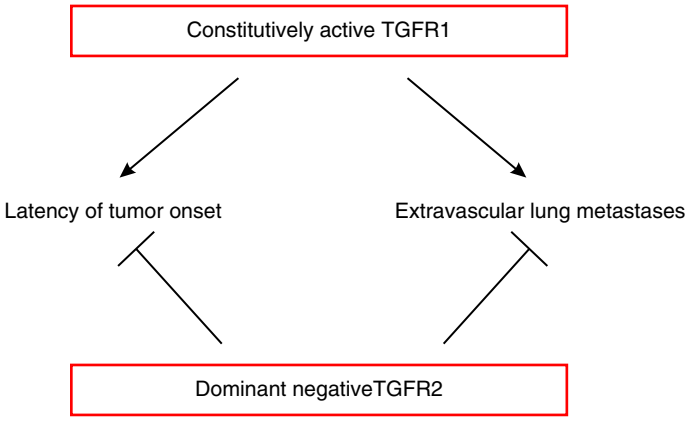

Figure 14.9 Dual roles of the TGFβ pathway in tumorigenesis. Transgenic mice that express the *Erbb2* oncogene under the control of the mouse mammary tumor virus (MMTV) promoter develop mammary carcinomas. The expression of a constitutively active TGFβ receptor type 1 (TGFR1) inhibits proliferation and increases the time to the onset of tumor growth; however, it also increases the fraction of animals with lung metastases. Expression of a dominant negative TGFβ receptor type 2 (TGFR2) has the opposite effects. (Based on data published by Siegel *et al.* (2003)).

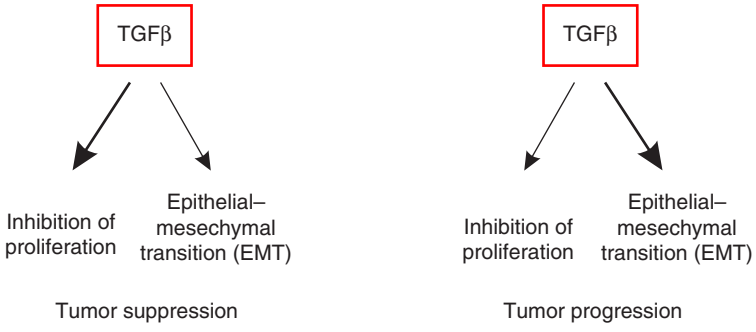

Figure 14.10 Depending on context, TGFβ inhibits proliferation and suppresses tumor growth, or TGFβ induces epithelial mesenchymal transition and supports tumor progression.

those cells, in which EMT is induced (Figure 14.10). Indeed, it has been shown that TGFβ induced EMT in epithelial progenitor cells, in which tumor-propagating properties were already present (Mani *et al.*, 2008).

14.8 Drugs Interfering with TGFβ Signaling

Pleiotropy and context dependence are cardinal features of biological effects exerted by members of the TGFβ superfamily (Massague, 2008). In early phases of carcinogenesis, TGFβ suppresses proliferation by inhibiting CDK and the Myc oncoprotein. However, when tumors progress, the inhibitory arm of TGFβ actions may be attenuated, and TGFβ assists tumor progression (Figure 14.10). These dual aspects of TGFβ action impede the development of efficacious antitumor compounds. For instance, TGFβs may foster EMT in tumor progression, but when drugs block this effect of TGFβs, the inhibitory effect of TGFβ on epithelial cell proliferation will also be negated. Conversely, the administration of TGFβ agonists to inhibit proliferation may boost tumor progression (Akhurst and Hata, 2012).

TGFβ signaling can be inhibited at different levels: (i) inhibition of TGFβ synthesis, (ii) blocking of ligand binding to their receptors, and (iii) inhibition of receptor kinase activity. At each level, clinical trials have been reported that showed some antitumor activity. Some examples are cited as follows.

Inhibition of TGFβ synthesis. Trabedersen (AP12009) is an antisense oligonucleotide that targets TGFβ2. In a phase IIb clinical trial, the drug was tested in patients with refractory glioblastoma and anaplastic astrocytoma. Six months after the start of treatment, the drug had no effect on tumor growth. However, delayed responses were reported after the end of treatment (Neuzillet *et al.*, 2015).

Blocking of ligand binding to their receptors. The TGFβ antibody fresolimumab (GC1008) has been tested in a phase I clinical trial in the treatment of malignant melanoma and renal cell carcinoma. There was preliminary evidence of antitumor activity (Morris *et al.*, 2014).

Inhibition of receptor kinase activity. Galunisertib (LY2157299) is a small-molecule inhibitor of TGFβRI kinase activity. The drug showed antitumor effects in some patients with glioblastoma. This compound is being applied in a phase II trial for hepatocellular carcinoma, glioblastoma, and advanced pancreatic carcinoma. In hepatocellular cancer, high serum levels of the tumor marker alpha-fetoprotein (AFP) indicate an advanced stage of the disease. Interestingly, in patients with high AFP concentrations, a significant difference in time to tumor progression and overall survival between responders and nonresponders has been reported (Neuzillet *et al.*, 2015).

An extended list of TGFβ pathway inhibitors has also been published (Neuzillet *et al.*, 2015).

14.9 TGFβ Superfamily Members in Tumor Cachexia

Patients with advanced tumor stages often develop cachexia, which is a wasting syndrome associated with weight loss, muscle atrophy, anemia, fatigue, and weakness. Many cancer-related deaths are, at least in part, attributed to this syndrome. Since members of the BANG subfamily of the TGFβ superfamily regulate skeletal muscle stem cells and myogenesis, it has been speculated that TGFβ superfamily members may be involved in cachexia and particularly in muscle atrophy.

Activins are members of the TGFβ superfamily, which contain the typical cysteine knot motif. Activins are secreted as homodimers or heterodimers of related β-subunits. Homodimers of βA chains form activin A, homodimers of βB chains form activin B, and heterodimers of a βA and βB chain form activin AB. Activin A and activin B signal through the type 2 receptor ACTRIIB. The activin βA and βB unit can heterodimerize with the inhibin α-subunit to form inhibin A (α-βA) and inhibin B (α-βB), respectively. Inhibins oppose many actions of activins (Harrison *et al.*, 2005).

Inhibin-α deficient mice first pointed to an important role played by TGFβ superfamily members in tumor cachexia. Inhibin-α knockout (k.o.) mice develop gonadal tumors, which are followed by a cachexia-like wasting syndrome with anemia and severe weight loss. The concentration of activins was more than 10-fold higher in the serum of the k.o. mice compared to wild-type (WT) mice, indicating that the wasting syndrome might have been induced by activins. In order to prove this hypothesis, a double knockout mouse was generated, which was devoid of both inhibin-α and the ACTRIIB receptor, through which activins

signal. These mice still developed gonadal tumors, which, however, were no longer accompanied by the cachexia-like syndrome (Coerver *et al.*, 1996; Matzuk *et al.*, 1994). In an attempt to develop a drug against tumor cachexia, a soluble recombinant ACTRIIB construct was administered to tumor-bearing mice. Without treatment, body weight and mass of the gastrocnemius muscle were

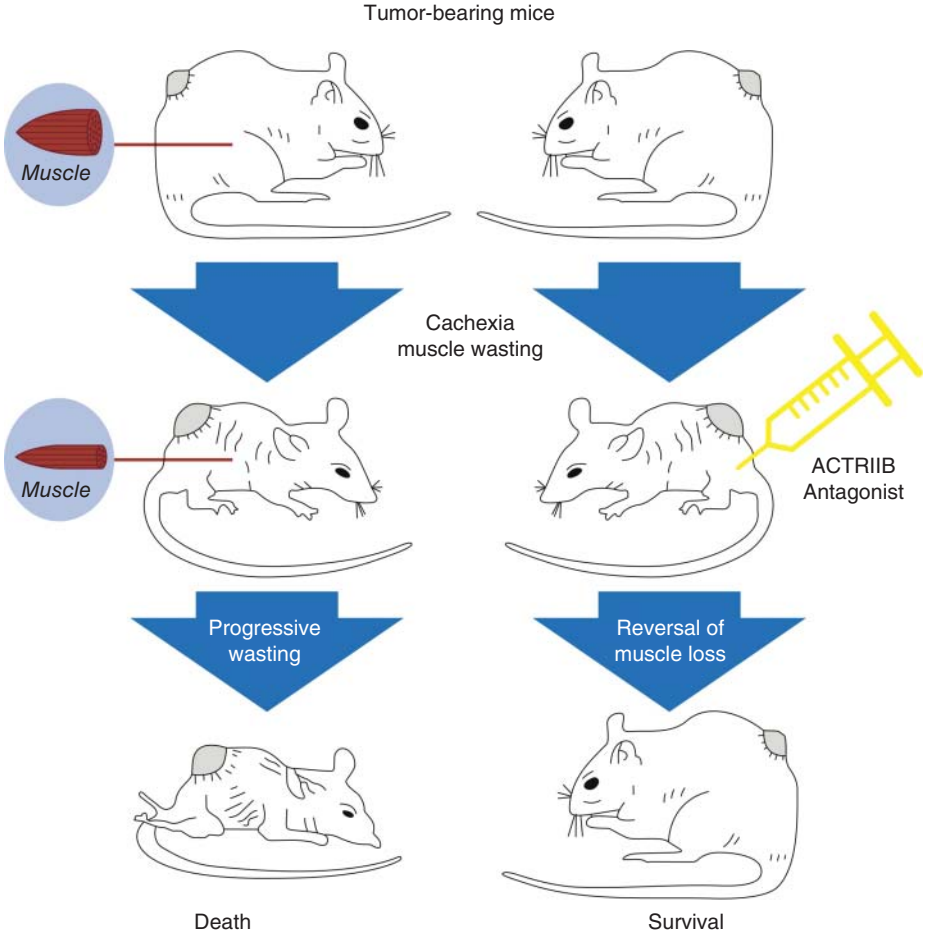

Figure 14.11 Role of TGFβ in cancer-associated cachexia. Mice bearing colonic carcinoma transplants develop cachexia and muscle wasting. Administration of a soluble recombinant ACTRIIB receptor construct reversed cachexia and wasting without affecting tumor growth. (Zhou *et al.*, 2010), (graphical abstract, modified, with permission).

20–45% reduced in the k.o. mice compared to the WT controls. Treatment with soluble ACTRIIB reversed these parameters back to or even beyond the levels in the WT controls. Interestingly, a single dose of soluble ACTRIIB caused the doubling of the gastrocnemius muscle in the k.o. mice within 2 weeks. Treatment with a soluble ACTRIIB receptor was also effective in mice bearing transplants of a colonic carcinoma. The mice were treated when weight loss began. Injections of the soluble receptor not only prevented further weight loss but also caused a rapid gain in body weight without affecting tumor growth (Zhou *et al.*, 2010) (Figure 14.11).

Myostatin (GDF 8) is a member of the TGFβ superfamily specifically associated with the regulation of skeletal muscle mass. The loss of myostatin function is associated with increased muscle mass, both in humans and in animals (McNally, 2004). Conversely, the application of myostatin leads to muscle loss and cachexia. Similarly to activins, myostatin binds to the ACTRIIB receptor. In the aforementioned colonic carcinoma transplant model, myostatin transcription was induced in skeletal muscle. When the soluble ACTRIIB receptor was applied to the tumor-bearing animals, the expression of the myostatin encoding *Mstn* gene in the gastrocnemius muscle was prevented. This finding suggests that ligands recognized by the ACTRIIB receptor can induce the transcription of myostatin in skeletal muscle, thereby inducing the loss of muscle mass in tumor-associated cachexia (Zhou *et al.*, 2010).

14.10 Outlook

In carcinomas associated with defects in the DNA MMR system, such as HNPCC, inactivation of the *TGFBR2* gene is a common event. In carcinomas of the pancreas, colorectum, and other sites, biallelic loss or inactivation of the *SMAD4* gene is often observed, typical of a classical tumor suppressor gene. In a genome-wide DNA sequence analysis of pancreatic cancer, mutations in genes involved in TGFβ signaling were detected in each of the tumors analyzed. These and other findings firmly establish that the TGFβ signaling pathway is frequently inactivated in major human malignancies. The tumor suppressive effects of TGFβ signaling are counteracted by the stimulation of EMT in tumor progression. In addition, the TGFβ signaling pathway is interconnected with other tumorigenic signaling pathways. In order to increase the efficiency of drugs that interfere with TGFβ signaling, the molecular basis of protumorigenic effects of TGFβ signaling must be understood in detail. Based on this knowledge, predictive tests may be developed as basis for an effective, individualized, combinatorial therapy. In patients with advanced cancer stages, interference with activin signaling may assist in fighting tumor cachexia.

Nomenclature

Nomenclature of genes and proteins of the TGFβ signaling pathway.

Recommended name (UniProt)	Alternative name (UniProt)	Short name (UniProt)	Short name used in text	Gene
Transforming growth factor beta-1		TGF-beta-1	TGFβ1	*TGFB1*
Bone morphogenetic protein 1		BMP-1	BMP1	*BMP1*
Inhibin beta A chain	Activin beta-A chain			*INHBA*
Inhibin beta B chain	Activin beta-A chain			*INHBB*
Inhibin alpha chain				*INHA*
TGF-beta receptor type-2		TGFR-2	TGFR2	*TGFBR2*
Activin receptor type-2A (Activin receptor type-IIA)		ACTRIIA	ACTRIIA	*ACVR2A*
Activin receptor type-2B (Activin receptor type-IIB)		ACTRIIB	ACTRIIB	*ACVR2B*
Bone morphogenetic protein receptor type-2 (BMP type-2 receptor)		BMPR-II	BMPR2	*BMPR2*
TGF-beta receptor type-1	Activin receptor-like kinase 5	TGFR-1	TGFR1	*TGFBR1*
		ALK5, ALK-5		
Serine/threonine-protein kinase receptor R3	Activin receptor-like kinase 1	SKR3	ALK1	*ACVRL1*
	TGF-B superfamily receptor type I	ALK-1		
Bone morphogenetic protein receptor type-1A (BMP type-1A receptor)	Activin receptor-like kinase 3	BMPR-1A	ALK3	*BMPR1A*
		ALK-3		
Bone morphogenetic protein receptor type-1B (BMP type-1B receptor)	Activin receptor-like kinase 6 (mouse)	BMPR-1B	BMPR1B	*BMPR1B*
		ALK-6 (mouse)	ALK6	

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