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Heinz Peter Nasheuer
Editor

Genome Stability and Human Diseases

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This review book is dedicated to
Dr. Teresa Wang
the Klaus-Bensch Professor of Pathology
Stanford University, California,
to celebrate her life-long achievements
in the fields of
Genome Stability and Cell Cycle Regulation

Preface

Since the discovery of the DNA structure by Crick, Franklin, Watson and Wilkins in 1953 and even before researchers have been highly interested in the molecular basis of the inheritance of genes and of genetic disorders (Franklin and Gosling, 1953; Watson and Crick, 1953a, b; Wilkins et al., 1953a, b). In addition, these publications were the foundation of a new research stream coined "Molecular Biology". At the same time but remaining nearly unrecognized, cell biologists A. Howard and S. R. Pelc published a nearly as fundamental discovery when they discovered DNA replication as a timely scheduled process between two cell divisions and coined the names G1, S and G2 phase introducing the concept of the eukaryotic cell cycle (Howard and Pelc, 1953). Prior to this, for about half a century, cell biologists had studied mitosis and the remaining was considered as a "black Box" called interphase. Further understanding of the structure, assembly and replication of DNA in the nucleus as well as knowing the mechanisms cell cycle regulation has not only yielded numerous Nobel prizes for scientists but ever since also resulted in better understanding of the molecular bases of diseases to introduce more rational treatments including improved treatments of cancer.

During her career Teresa Wang was one of the scientists, who with great success has enhanced our understanding of cancer cell biology since the early1970s when the American President Richard Nixon proclaimed the "War on Cancer". It was nearly at the same time when Teresa Wang started her career at Stanford University where her main research focus has been in the fields of cell growth, cell division and genome stability. She contributed numerous milestones to the field such as cloning and expressing the first human replicative DNA polymerase, study regulation of replication proteins in the cell cycle, in dependence of proliferation and after DNA damage to name a few. During her research career, she has served as a role model for a huge number of life scientists, who work in her group but also interacted with her at all stages of their research career. A number of them contributed to this book. Teresa Wang to whom this book is dedicated was not only an exceptional scientist but was honored with numerous awards including the Klaus-Bensch Professorship of Pathology at Stanford University. Despite her busy university life she also found time to look after her family including her mother, her husband and raising two children. During my research career at for me an especially sensitive decision-making time I was one of these lucky ones, who spent time in her lab and published some of

my most important discoveries with her. And I can only write in short: “Thank you very much, Teresa, for all your support and help throughout my scientific career.”

Galway, Ireland Heinz Peter Nasheuer
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Coming Full Circle: Cyclin-Dependent Kinases as Anti-cancer Drug Targets

Robert P. Fisher

Abstract Because the normal control of cell proliferation is disturbed in cancer, the cyclin-dependent kinases (CDKs) that trigger DNA synthesis and mitosis have been popular targets for inhibition with small molecules, but the jury is still out on whether this will be an effective anti-tumor strategy. There is debate about which of the multiple CDKs active during the mammalian cell cycle might be good targets, reflecting fundamental confusion about what, precisely, those different CDKs really do. In the classical view, based largely on their activation timing in cycling cells, different CDKs are specialized to perform discrete functions during distinct cell-cycle intervals. A revisionist model has emerged in which all functions essential to cell division can be performed by a single catalytic subunit, based on the ability of cells to proliferate and animals to survive when individual CDKs are removed by gene deletion or depleted by RNA interference. That those situations in no way resemble ones in which CDKs are inhibited pharmacologically is often overlooked or downplayed. A more nuanced – and accurate – picture is now coming into view, thanks to recent studies that reveal kinetically distinct pathways of activation for closely related CDKs and CDK-specific roles in the temporal control of S phase. The basic question of whether CDKs can be effectively targeted in cancer has yet to be answered but can now be addressed in chemical-genetic model systems that approximate the situation – still hypothetical – of truly selective CDK inhibition in vivo.

Keywords Cell cycle · Cancer · Cyclin-dependent kinase (CDK) · Chemical genetics · Checkpoints · DNA replication · DNA damage · Transcription

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Abbreviations

AS	analog-sensitive
APC	anaphase-promoting complex
CAK	CDK-activating kinase
CDK	cyclin-dependent kinase
DN	dominant negative
MEF	mouse embryonic fibroblast
Plk1	Polo-like kinase 1
P-TEFb	positive transcription elongation factor b
rAAV	recombinant adeno-associated virus
Rb	retinoblastoma protein
RNAi	RNA interference
Pol II	RNA Polymerase II
TFIIH	transcription factor IIH

Introduction

Cell proliferation is inherently threatening to genome integrity; errors in duplicating the genetic material during S phase, or in segregating the duplicated chromosomes at mitosis, must be detected and if possible corrected to prevent mutations or chromosomal aberrations from arising during cell division. To ensure fidelity of genetic transmission, the cell-cycle machinery depends on surveillance mechanisms – DNA structure checkpoints – that sense damaged, unreplicated or misreplicated DNA, and send inhibitory signals to arrest cell-cycle progression while repair pathways operate to remove the lesion (reviewed in Melo and Toczyski, 2002; Sancar et al., 2004; Harrison and Haber, 2006; Su, 2006). In fission yeast and mammalian somatic cells, the primary targets of that signaling are the cyclin-dependent kinases (CDKs) that govern entry to both S phase and mitosis (reviewed in Morgan, 2007). CDKs are more than just passive receivers of negative signals in response to DNA damage, however, and play active roles both in the choice and proper functioning of DNA repair pathways, and in the initiation, maintenance and termination of checkpoint signaling (reviewed in Yata and Esashi, 2009; Wohlbold and Fisher, 2009).

Cancer is a disease of inappropriate cell proliferation, which arises when cells evade normal constraints on their growth or division. Oncogenic transformation often entails derangement of the mechanisms that ensure the stable inheritance of genes and chromosomes during mitotic cell division (reviewed in Malumbres and Barbacid, 2009). Because CDKs play critical roles in both the commitment to cell division and the quality control mechanisms that safeguard genome integrity, they represent obvious, but potentially risky, therapeutic targets in human cancers (Shapiro, 2006). One rationale for inhibiting CDKs is simple: block all CDK activity and cell proliferation should cease. The toxicity of such a treatment might be tolerable in the short term, given that most adult tissues do not turn over rapidly and therefore do not depend on ongoing cell division. In the longer run, of course, cell

division is essential for survival, and removing the block to allow regeneration of normal tissues (e.g. hematopoietic or gut epithelial cells) will also allow the tumor to "grow back." Therefore, imposition of a pure cell-cycle arrest, even if it is possible, can never be sufficient to eradicate a cancer. Another problem is that complete CDK blockade might not be achievable in vivo with even the most potent CDK inhibitor, so that before the threshold of CDK activity required to stop division was crossed, off-target effects on other kinases would produce dose-limiting toxicity. In fact, members of the CDK family participate in other essential processes such as gene expression (reviewed in Fisher, 2005; Peterlin and Price, 2006; Zhou and Yik, 2006), which are required even in non-dividing cells. Most of the CDK-selective compounds that have been developed so far inhibit both "cell-cycle" and "transcriptional" CDKs (Shapiro, 2006; Malumbres and Barbacid, 2009), and so might cause unacceptable toxicity in vivo even while staying on target.

The obvious reasons for trying to inhibit CDKs in cancer cells may not be valid or practicable, but less obvious approaches – and some less obvious or even counterintuitive target CDKs – might hold more promise. The commitment to entering a round of cell division has been a focal point for attempts at pharmacologic intervention, but with little success and much uncertainty about which CDK to aim for. The non-canonical roles of CDKs in response to DNA damage may be vulnerable in cancer cells, which divide more rapidly and show more genomic instability than do normal ones, but more insight is needed into what those roles are, and which CDKs are responsible, in mammals. Finally, the transcriptional CDKs should not be considered off-limits a priori, based on the unexamined assumption that any disruption of their function will shut down gene expression globally and therefore be toxic to healthy tissues; recent evidence points to more specialized functions of these CDKs, and some potential advantages over the cell-cycle CDKs as anti-cancer drug targets (Fisher, 2005; Lolli and Johnson, 2005). In this essay, I will argue for re-evaluation (yet again) of interphase CDKs as potential drug targets, for sustained interest in the transcriptional CDKs, and above all for better genetic models in which to investigate the normal functions of CDKs and evaluate their druggability. First, however, I compare the still-evolving model of cell-cycle control by CDKs in mammalian cells with the more settled picture of the same process in yeast.

Overview of Cell Cycle Control in Metazoans and Yeast

CDKs are Ser/Thr protein kinases that consist of an intrinsically inactive or weakly active catalytic subunit, the CDK, and a positive regulatory subunit, the cyclin (reviewed in Morgan, 2007). To be fully activated, CDK/cyclin complexes additionally require phosphorylation by a CDK-activating kinase (CAK) (reviewed in Fisher, 2005). CDKs are the principal triggers of both the DNA synthesis (S) phase and mitosis in all eukaryotes, and the cell cycle can be modeled as a "CDK cycle" in which progress is measured by the quantity and quality of CDK activity present at a given point ("quality" here meaning principally the subunit compositions of active

CDK/ cyclin complexes). The periodic oscillation of total CDK activity – from low as cells exit mitosis, through intermediate levels as they commence and complete S phase, to high as they enter mitosis and back to low again as mitotic cyclin is abruptly destroyed by the anaphase-promoting complex (APC) – is necessary for orderly progression of all eukaryotic cell cycles. For example, the restriction of replication origin-licensing to periods of very low CDK activity, coupled with a requirement for active CDK to fire origins, ensures that a given origin can only fire once per cell cycle (Diffley, 2004). Thus, quantitative regulation of CDK activity may be sufficient to ensure both the faithful duplication of the genome and the strict alternation of S phase and mitosis (Stern and Nurse, 1996).

It is never quite as simple as that in nature, however. Even in the fission yeast *Schizosaccharomyces pombe*, which has one CDK catalytic subunit (Cdk1, encoded by the *cdc2*$^{+}$ gene) needed for both G1/S and G2/M transitions (Stern and Nurse, 1996), and can divide nearly normally with only a single cyclin (Fisher and Nurse, 1996), additional cyclins play important roles in fine-tuning the cell cycle (Mondesert et al., 1996; Martín-Castellanos et al., 2000). There is also unexpected complexity in the number of CAKs and, therefore, the number of different ways CDK/cyclin complexes can become activated (Lee et al., 1999; Saiz and Fisher, 2002).

The budding yeast *Saccharomyces cerevisiae* likewise depends on Cdk1 (encoded by *CDC28*) to promote both S-phase and mitotic entry, but the single catalytic subunit must partner with specific cyclin subtypes during different intervals to ensure normal coordination of the cell cycle: Cln1, -2 and -3 for passage of Start, the point of commitment to cell division during G1; Clb5 and -6 to promote timely and coordinated S phase; and Clb1, -2, -3 and -4 for normal progression into mitosis (Morgan, 2007). There are functional overlaps and redundancies, and no single cyclin is essential for viability, as is the major mitotic cyclin in *S. pombe* (Booher et al., 1989). For example, loss of the cyclins that normally activate Cdk1 to trigger replication delays but does not prevent S-phase onset, which instead occurs upon expression of Clb3 and Clb4 in *clb5Δ clb6Δ* mutants (Piatti et al., 1996). The entire repertoire of cyclins is probably needed to make Cdk1 such a versatile enzyme, however, and to make the yeast cell cycle robust and responsive to internal and environmental cues (Loog and Morgan, 2005; Bloom and Cross, 2007).

In metazoans, there has been more extensive duplication and diversification of the genes encoding CDK catalytic subunits. Individual CDKs have distinctive temporal profiles of activation during the mammalian cell cycle, and bind different cyclins with clear preferences both in vivo and in vitro (Fig. 1). During early G1, D-type cyclins are expressed in response to mitogenic signaling, and bind Cdk4 and Cdk6 to promote G1 progression by phosphorylating the pocket proteins: the retinoblastoma tumor suppressor protein Rb, p107 and p130. This alleviates pocket-mediated repression of E2F target genes needed for G1/S progression, including *cyclin E*, the product of which binds to Cdk2 and triggers S-phase entry. The Cdk2/cyclin E complex phosphorylates Rb on additional sites not efficiently modified by Cdk4/cyclin D complexes (Zarkowska and Mittnacht, 1997), fully activating E2F-1 to drive

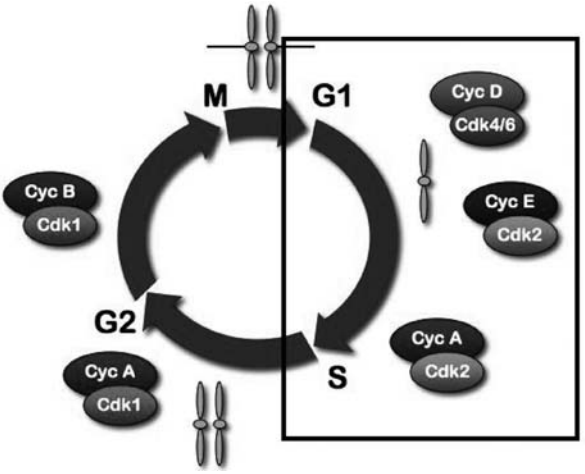

Fig. 1 The classical model of mammalian cycle control. In this model, based on measurements of expression pattern and activation timing, multiple CDK catalytic subunits partner with different cyclins to perform specialized functions at distinct points in the cell cycle. Recent results obtained by knocking out *Cdk* genes in mice have indicated that cells can divide and maintain the normal alternation of S phase and mitosis in the absence of Cdk2, Cdk4 and Cdk6, i.e. when none of the normal "interphase" CDK/cyclin complexes (Boxed) can form (Santamaria et al., 2007)

expression of additional cell-cycle regulated genes, including *cyclin A* and *Cdc2* (which encodes Cdk1) (Harbour et al., 1999; Zhang et al., 2000). Cyclin A2 (the isoform expressed in somatic cells) accumulates during S phase and G2 and binds to both Cdk2 and Cdk1 in mammalian cells (Pagano et al., 1992) before being degraded in APC-dependent manner just before entry to mitosis. Finally, Cdk1/cyclin B complexes, which assemble during S and G2 phases, are activated to trigger entry to mitosis (Morgan, 2007).

Cdk2 Becomes Dispensable

In the classical model, dependence on distinct CDKs at different points in the cell cycle appeared to be a defining feature of metazoan cell division (Sherr and Roberts, 2004), with the multiple catalytic subunits providing essential specialization of functions in much the same way that different types of cyclin confer unique specificities on the budding yeast CDK (Bloom and Cross, 2007). This seemed plausible – or even inevitable – given that a mammalian somatic cell typically expresses *fewer* different cyclins than does a vegetative *S. cerevisiae* cell. Early attempts to define the functions of individual mammalian CDKs in vivo seemed to bear this out; overexpression of dominant-negative (DN) CDKs – catalytically inactive mutant proteins that retain cyclin-binding ability – arrested the cell cycle

at different points depending on which mutant CDK was overexpressed. In human U2OS osteosarcoma cells, transient overexpression of Cdk1-DN led to an arrest in G2, as expected for the major effector kinase in the G2/M transition, whereas Cdk2-DN overexpression resulted in an accumulation of cells with a G1 DNA content (van den Heuvel and Harlow, 1993). Subsequent experiments, with Cdk2-DN expressed from an inducible promoter in stably transfected U2OS cells, suggested additional, essential roles for Cdk2 in S phase and G2 (Hu et al., 2001). Specificity in CDK-cyclin pairing in vitro also supported the idea that different catalytic subunits would be needed to form active complexes with different cyclins; for example, purified cyclin E was unable to form a stable complex with Cdk1 even when the two proteins were incubated at high concentrations in the presence of a CAK, whereas Cdk2/cyclin E complexes formed readily (Desai et al., 1995).

The expression of Cdk2-DN caused delay or arrest at different points in the cell cycle depending on the culture conditions and levels of expression (van den Heuvel and Harlow, 1993; Hu et al., 2001) – variability that could be explained by different degrees of interference with endogenous Cdk2 function, and/or the relatively long cell-cycle interval during which Cdk2 is maximally active (Rosenblatt et al., 1992). That the dependence on Cdk2 *for any essential function* might vary among cell types was suggested by the failure of Cdk2-DN to arrest or even delay cell-cycle progression in a number of human cancer-derived cell lines. Similarly, depletion of Cdk2 by RNA interference (RNAi) and antisense DNA-based strategies was without effect on cell cycle progression in multiple human cell lines (Tetsu and McCormick, 2003).

This study suggested that overexpression of Cdk2-DN might have interfered with cellular functions other than those of wild-type Cdk2, and raised serious questions about the reliability of the dominant-negative approach. (It also prompted the authors to assert, with questionable justification, that Cdk2 was not likely to be a good target for anticancer chemotherapy.) A bigger apparent blow to the classical model came, however, from disrupting the *Cdk2* gene in mice; two independent studies revealed that *Cdk2–/–* mice were viable, and that murine embryonic fibroblasts (MEFs) lacking Cdk2 could divide more-or-less normally in culture (Berthet et al., 2003; Ortega et al., 2003). At around the same time, it was reported that the lethality caused by knocking out both *cyclin E* genes in mice was due to a placental defect and could be rescued by extra-embryonic expression of cyclin E; cells lacking cyclin E could proliferate when maintained as continuously cycling cultures but had a defect in cell-cycle reentry from a quiescent state (Geng et al., 2003). It seemed that previous work assigning unique functions to cyclin E and Cdk2 in the essential task of initiating DNA replication must have been flawed, either in experimental design or in interpretation of the data (Roberts and Sherr, 2003).

Subsequent studies investigated how animals could survive – and cells proliferate – in the absence of Cdk2. The unsurprising answer was that, in the absence of "interphase" CDKs, other CDKs – most prominently, Cdk1 – could substitute in complexes with cyclins that normally bound to Cdk2, Cdk4 or Cdk6 (Aleem et al., 2005; Santamaria et al., 2007). In an extreme example, MEFs lacking Cdk2, -4 and -6 due to gene disruption were able to maintain the strict alternation of

S phase and mitosis and proliferate (Fig. 1), but did so more slowly than did wild-type MEFs (Santamaria et al., 2007). In vivo, the requirements for interphase CDK functions are more stringent; male and female *Cdk2–/–* mice are infertile because of a failure in meiosis (Berthet et al., 2003; Ortega et al., 2003) and, although no single interphase CDK is strictly essential for viability (Rane et al., 1999; Tsutsui et al., 1999; Berthet et al., 2003; Ortega et al., 2003; Malumbres et al., 2004), combined loss of Cdk2 and Cdk4 causes embryonic lethality (Berthet et al., 2006). Nonetheless, the mammalian cell division machinery is able to withstand the loss of all cell-cycle CDKs, save one, and still perform its basic, essential function. This appeared to elevate that one (Cdk1) to the status of über-CDK, master regulator of the entire cell cycle (Bashir and Pagano, 2005), while relegating the others to minor or auxiliary roles, which might perhaps be effectively targeted in cancers arising in specific tissues, but not as a general anti-tumor strategy (Hochegger et al., 2008; Malumbres and Barbacid, 2009).

What's Wrong with This Picture?

Before accepting this, the revisionist view of the mammalian cell cycle, we should first ask what kind of information we glean from gene knockouts in mice (or from knockdown of protein levels in cultured cells). Do we uncover all the functions – or even the principal functions – of that gene or its product in a physiologic setting? In the realm of drug discovery we should decide what information we need about a specific enzyme we are evaluating as a potential anticancer target. Do we learn more from phenotypes that result from the disappearance of that protein or from the consequences of inactivating its catalytic function?

Although homozygous disruption can tell us whether a gene is strictly essential for viability, it does not address whether the product of that gene performs an essential function, perhaps exclusively, when it is present. We should care about this scenario for at least two reasons. First, from the basic scientist's standpoint, if one is interested in how biological processes are regulated, one wants to know who is normally doing the regulating. The mere fact that Cdk1 can take over the functions of Cdk2 when the latter is removed or depleted from the cell does not mean that Cdk1 normally performs those functions, *or that interfering with Cdk1 activity will disrupt processes normally regulated by Cdk2* (see below for an example). This leads to a second reason, of paramount importance in drug discovery, to look beyond data obtained from knockout and knockdown experiments: neither gene disruption nor RNAi mimics the situation in which enzymatic activity is inhibited by a small molecule (Weiss et al., 2007). In the case of CDKs, most such compounds in pre-clinical or clinical development target the enzymes' active sites, not their expression levels or protein–protein interactions (Shapiro, 2006; Malumbres and Barbacid, 2009). Therefore, knockout mice or cells in which CDK levels have been manipulated with RNAi (or antisense DNA oligonucleotides) are poor models for predicting the efficacy of anti-CDK chemotherapy.

Closer to the Mark: Insights into CDK Function from Chemical Genetics

Chemical genetics provides a way to inhibit a single CDK in vivo, and thereby obtain the kind of information not accessible by knockout or knockdown strategies. The most common approach is the analog-sensitive (AS) strategy, in which the ATP-binding site of the kinase is mutated to replace a conserved bulky residue with a less bulky Gly or Ala residue, creating extra space to accommodate bulky ATP derivatives. In most cases, the mutant kinase retains enzymatic and biological activity while becoming susceptible to inhibition by bulky, non-hydrolyzable purine analogs that do not bind tightly to (and therefore do not effectively inhibit) wild-type kinases (Knight and Shokat, 2007). In one successful application of this strategy in vivo, budding yeast Cdk1 was shown to have different inhibitory thresholds for its two distinct execution points in the cell cycle; *cdc28-as* cells arrested in G2 at low doses, and in G1 at high doses, of inhibitor (Bishop et al., 2000). Although this result was obtained in budding yeast, it is perhaps the strongest indication to date that quantitatively different thresholds of CDK activity are needed to enter S phase and mitosis, consistent with the model of CDK-dependent cell-cycle regulation derived from work in fission yeast (Stern and Nurse, 1996).

Because of the ease of gene disruption and replacement, chemical genetics has been used extensively to map signaling pathways involving CDKs in yeast. For example, inhibition of Cdk1-as after introduction of a single double-strand break in an *S. cerevisiae* chromosome revealed a requirement for CDK activity in the DNA strand resection step of homologous recombination repair (Ira et al., 2004). The AS-kinase strategy also allowed the dissection of CDK functions in transcription by RNA polymerase (Pol) II. The relative contributions to Pol II phosphorylation and transcription by Kin28, the CDK associated with transcription initiation factor TFIIH, and Srb10, the CDK component of the Pol II Mediator complex (budding yeast orthologs of mammalian Cdk7 and Cdk8, respectively), were measured by inhibiting AS versions of each kinase individually and together (Liu et al., 2004; Muratani et al., 2005). The chemical-genetic strategy also revealed a requirement for Kin28 activity in normal 5′-end capping of Pol II transcripts in budding yeast (Kanin et al., 2007). More recently, selective inhibition of AS version of two essential CDKs in fission yeast – Mcs6, orthologous to Cdk7/Kin28, and Cdk9, an ortholog of metazoan positive transcription elongation factor b (P-TEFb) – revealed that they act in sequence on select subsets of genes, in order to couple transcript elongation with capping (Viladevall et al., 2009). A similar relationship between Kin28 and the Cdk9 ortholog Bur1 was uncovered in budding yeast, also based partly on results obtained with AS alleles (Qiu et al., 2009).

The limited, but apparently lethal, effect of inhibiting transcriptional CDK function on gene expression raises anew the idea that these kinases might be effectively targeted in cancer cells (Fisher, 2005; Lolli and Johnson, 2005). In fission yeast, the genes dependent on CDKs for full expression are enriched for ones involved in cell division (Lee et al., 2005; Viladevall et al., 2009), and it has recently been

suggested that the most potent anti-proliferative effects of general CDK inhibitors in mammalian cells are mediated primarily through inhibition of Cdk7 and Cdk9, rather than Cdk1 and Cdk2 (Scrace et al., 2008). Perhaps consistent with this notion, human *Cdk7*$^{as/as}$ HCT116 colon carcinoma cells undergo discrete cell-cycle arrests with failure to activate cell-cycle CDKs only when treated with high (micromolar) doses of inhibitory analogs, but lose proliferative capacity and die even when exposed to much lower doses (<100 nM) for extended periods (Larochelle et al., 2007), possibly due to derangements in gene expression.

The mammalian cell-cycle machinery, like the transcription machinery in both higher and lower eukaryotes, depends on multiple CDK catalytic subunits, and is therefore a candidate for dissection by the chemical-genetic strategy. AS alleles of the relevant *Cdk* genes can be introduced into vertebrate cells by a number of methods, although none is as easy (or as rapid) as gene replacement in yeast. In one study, the endogenous Cdk1 was eliminated from chicken DT40 cells by gene disruption, and its essential function was rescued by a transgenic, AS version of *Xenopus* Cdk1. Besides allowing rapid and reversible chemical inhibition of the G2/M transition, the DT40 cell system provided an elegant demonstration that Cdk1 activity is *not* required for S phase when Cdk2 is present and active; inhibitory analogs only blocked G1/S progression of *Cdk1-as* cells if the *Cdk2* gene was also knocked out (Hochegger et al., 2007).

Dissection of the mammalian cell-cycle machinery by chemical genetics has thus far depended on homologous gene-targeting with recombinant adeno-associated virus (rAAV) vectors (Kohli et al., 2004). In one example, the two endogenous loci encoding the essential Polo-like kinase 1 (Plk1) were knocked out in human cells, and the loss of viability upon conditional disruption of the second allele was rescued by transgenic expression of *Plk1-as* (Burkard et al., 2007). To dissect the functions of human Cdk7, which performs dual functions as the catalytic subunit of CAK and the TFIIH-associated kinase, we replaced both wild-type copies of *Cdk7* with AS alleles in HCT116 cells by homologous gene-targeting; inhibition of Cdk7-as in G1 or G2 delayed S-phase onset or blocked entry to mitosis, respectively, revealing the essential function of Cdk7 in activating the cell-cycle CDKs (Larochelle et al., 2007).

Cdk2 – Back in the Saddle?

The introduction of a chemical switch into the CDK activation pathway enabled us to order the steps in the assembly and activation of different CDK/cyclin complexes in human cells (Fig. 2). In vivo, Cdk1 and Cdk2 follow kinetically distinct paths to activation, even though they share an activating kinase, Cdk7, and at least one partner, cyclin A (Larochelle et al., 2007; Merrick et al., 2008). Cdk1 must bind a cyclin to be recognized and phosphorylated on its activation segment (T-loop) by Cdk7, but it must be phosphorylated on the T-loop to form a stable complex

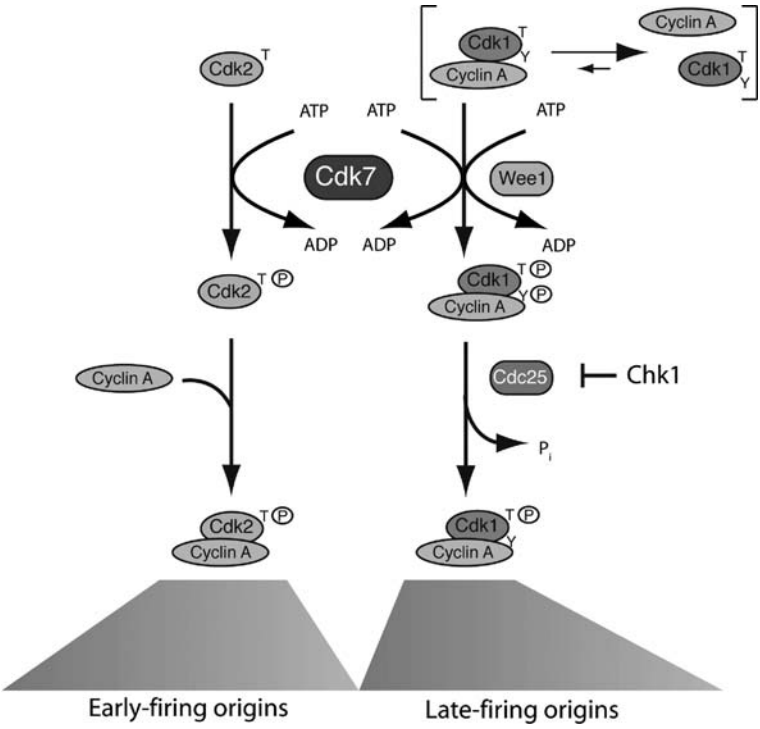

Fig. 2 Kinetically distinct activation pathways and temporally separate functions of Cdk2/cyclin A and Cdk1/cyclin A. Cdk2 can be phosphorylated by Cdk7 as a monomer, before binding cyclin A, whereas Cdk1 must associate with cyclin to be phosphprylated on both its activating (T) and inhibitory (Y) residues. This gives Cdk2 a kinetic advantage in binding cyclin A, and may explain why Cdk1-cyclin A binding does not occur efficiently until mid- to late-S phase (Merrick et al., 2008). This delay may contribute to the temporal coordination of replication origin-firing during S phase; premature activation of Cdk1/cyclin A, by ablation of Chk1, causes normally late-firing origins to fire early in S phase (Katsuno et al., 2009)

with cyclin – a mutual dependence that may contribute to the switch-like nature of Cdk1/cyclin B activation (Larochelle et al., 2007). In contrast, in the predominant pathway of Cdk2 activation in vivo, Cdk7 phosphorylates monomeric Cdk2, which then binds cyclin to become active; preventing Cdk2 T-loop phosphorylation by inhibiting Cdk7 does not impair Cdk2-cyclin binding (Merrick et al., 2008).

The differences in activation kinetics have consequences for the division of labor between Cdk1 and Cdk2 (Fig. 2). Cdk2 is ~10-fold less abundant than Cdk1 in vivo (Arooz et al., 2000; Merrick et al., 2008). Nevertheless, because of its less stringent requirements for both T-loop phosphorylation and cyclin-binding, Cdk2 is able to exclude Cdk1 from cyclin E complexes throughout the cell cycle, and to delay the assembly of Cdk1/cyclin A complexes until mid-S phase (Merrick et al., 2008). Temporal separation between Cdk2/cyclin A and Cdk1/cyclin A activation may be enforced by multiple mechanisms. In addition to the competition with Cdk2 for

cyclin A-binding, Cdk1 activity is held in check until mid-S phase by inhibitory phosphorylation of its Tyr-15 residue, through a pathway dependent on the checkpoint kinase Chk1, which promotes turnover of the Tyr-15 phosphatase Cdc25A (Katsuno et al., 2009). Both Cdk2 in mammalian cells and the S-phase-specific form of Cdk1 in budding yeast may be relatively refractory to this inhibition, because of properties conferred by their specific regulatory subunits (Chow et al., 2003; Keaton et al., 2007).

The normal sequence of CDK activation during S phase – Cdk2/cyclin A followed by Cdk1/cyclin A – appears to establish a temporal program of replication origin-firing; when the Cdk1-inhibitory mechanism dependent on Chk1 is circumvented, origins that normally initiate replication late in S phase fire prematurely at the beginning of S phase (Katsuno et al., 2009). This has an interesting parallel in *S. cerevisiae*, in which the S-phase cyclin Clb5 is specifically required for the activation of origins late in S phase (Donaldson et al., 1998). It remains to be seen what consequences disruptions in the S-phase program will have for genome stability in mammalian cells, or whether precocious activation of Cdk1 has other effects on normal cell-cycle coordination.

Conclusions and Perspectives

Therefore, in a "normal" cell cycle, Cdk1 activity is low or absent during an interval spanning the G1/S transition and early S phase. The delay in Cdk1 activation, moreover, *depends on Cdk2*, specifically, its advantage in binding cyclin E – which does not bind Cdk1 detectably in wild-type human cells – and cyclin A – which binds Cdk1 only after apparently saturating Cdk2 (Merrick et al., 2008). It follows that the normal, CDK-mediated regulation of this cell-cycle interval occurs predominantly through Cdk2, and that Cdk1's ability to substitute for Cdk2 in complexes with cyclins E and A is a case of "pseudoredundancy," in which one member of an enzyme family takes over the function of another, closely related family member, only in the latter's absence (Madhani et al., 1997). Thus, in gene knockout and knockdown experiments, eliminating or reducing expression of Cdk2 allowed other CDKs to usurp its normal functions. Conversely, the levels of catalytically inactive Cdk2-DN needed to displace endogenous, wild-type Cdk2 from active complexes – the rationale underlying the dominant-negative strategy – probably also perturbed complex formation by Cdk1, e.g. with cyclin A. As a result, we still do not know what effect the truly selective inactivation of Cdk2's enzymatic activity might have on cell proliferation, or survival. Therefore, whereas removing or depleting Cdk2 by gene disruption or RNAi may have revealed the robustness of the cell-cycle machinery in mammalian cells, it may have obscured actual regulatory mechanisms controlling G1 progression and S-phase entry. Because these pathways might contain legitimate targets for anticancer agents, future drug discovery efforts should be based on chemical-genetic methods that do not grossly distort the cell-cycle regulatory network by simply eliminating its key players.

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Core and Linker Histone Modifications Involved in the DNA Damage Response

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Abstract The stability of the genome is constantly under attack from both endogenous and exogenous DNA damaging agents. These agents, as well as naturally occurring processes such as DNA replication and recombination can result in DNA double-strand breaks (DSBs). DSBs are potentially lethal and so eukaryotic cells have evolved an elaborate pathway, the DNA damage response, which detects the damage, recruits proteins to the DSBs, activates checkpoints to stall cell cycle progression and ultimately mediates repair of the damaged DNA. As the DSBs occur in the context of chromatin, execution of this response is partly orchestrated through the modification of the DNA-bound histone proteins. These histone modifications include the addition or removal of various chemical groups or small peptides and function to change the chromatin structure or to attract factors involved in the DNA damage response, and as such, are particularly important in the early stages of the DNA damage response. This review will focus on such modifications, the enzymes responsible and also highlights their importance by reporting known roles for these modifications in genome stability and disease.

Keywords Chromatin · Core histones · Linker histones · DNA damage response (DDR) · Double strand breaks (DSBs)

Abbreviations

53BP1	p53 binding protein 1
ABRA1	Abraxas-BRCA1-A complex subunit
ac	acetylated
ADP	adenosine di-phosphate
AF4	ALL1 fused gene from chromosome 4
ASF1A	anti-silencing function 1A

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ATM	ataxia telangiectasia mutated
ATR	ATM and Rad3-related
BRCA1	breast cancer 1 early onset
BRCT	BRCA1 c-terminal
CAF-1	chromatin assembly factor 1
CBP/p300	CREB binding protein/Histone acetyltransferase p300
ChIP	chromatin immunoprecipitation
Chk1	checkpoint kinase 1
Chk2	checkpoint kinase 2
Crb2	crumbs homologue 2 (Rad9 homologue)
DDR	DNA damage response
DNA	deoxy-ribonucleic acid
DNA-PK	DNA dependent protein kinase
DSB	double strand break
DUB	deubiquitinase
E	Glutamic acid
ES	embryonic stem
EYA	eyes absent homologue
FHA	forkhead associated
FRAP	fluorescence recovery after photobleaching
grp	grapes (*Drosophila Chk1*)
H1	Histone 1
H2A	Histone 2A
H2B	Histone 2B
H2AX	Histone 2A.X
H3	Histone 3
H4	Histone 4
H2Av	Histone 2Av
HDAC	histone deacetylase
hMOF	human MOF (a.k.a. KAT8)
HP1	heterochromatin protein 1
HP1-β	heterochromatin protein 1 beta
HR	homologous recombination
IR	ionizing radiation
JNK1	Jun N-terminal kinase
K	Lysine
KAT	lysine acetyltransferase
KDM	lysine demethylase
KMT	lysine methyltransferase
LOH	loss of heterozygosity
lok	long form of nuclear kinase (*Drosophila Chk2*)
MDC1	mediator of DNA checkpoint 1
me	methylated
MEF	mouse embryonic fibroblast
MIU	motif interacting with ubiquitin

MOF	males absent on the first
MRN	Mre11-Rad50-Nbs1
NBS1	Nijmegen breakage syndrome 1
NHEJ	non-homologous end joining
ph	phosphorylated
PIKK	phosphatidylinositol-3 kinase related kinase
PRMT	protein arginine methyltransferase
Q	Glutamine
R	Arginine
Rad51	radiation (sensitive) 51
RAP80	receptor associated protein 80
RNAi	RNA interference
RNF168	ring finger protein 168
RNF8	ring finger protein 8
ROS	reactive oxygen species
S	Serine
SIRT1	silent mating type information regulation homologue 1
SIRT2	silent mating type information regulation homologue 2
SNF2H	sucrose non-fermenting protein 2 homologue
Su(var)3-9	suppressor of variegation 3-9
SUMO	small ubiquitin-like modifier
SUV39H	suppressor of variegation 3-9 homologue (human)
T	Threonine
Tip60	HIV Tat-interacting protein 60 kDa (a.k.a. KAT5)
Trrap	transformation/transcription domain-associated protein
ub	ubiquitin
UBC13	ubiquitin conjugating enzyme E2 13
UIM	ubiquitin interaction motif
WICH	WSTF-ISWI chromatin remodelling
WSTF	William's syndrome transcription factor
Y	Tyrosine

Introduction

In eukaryotic cells the genetic material is packaged into a complex nucleoprotein structure, called chromatin. This packaging is necessary to fit all of the three billion base pairs of DNA in our genome into the relatively small cell nucleus and subsequently, to facilitate management of DNA-templated processes such as gene transcription, DNA replication, chromosome condensation, segregation and DNA repair. Chromatin is composed of repeating units called nucleosomes, which consist of 146 base pairs of DNA wrapped ~1.7 times around a core histone octamer (Luger et al., 1997) that then usually associates with one linker histone (for review see Woodcock et al., 2006). These nucleosome repeats can be further packaged and

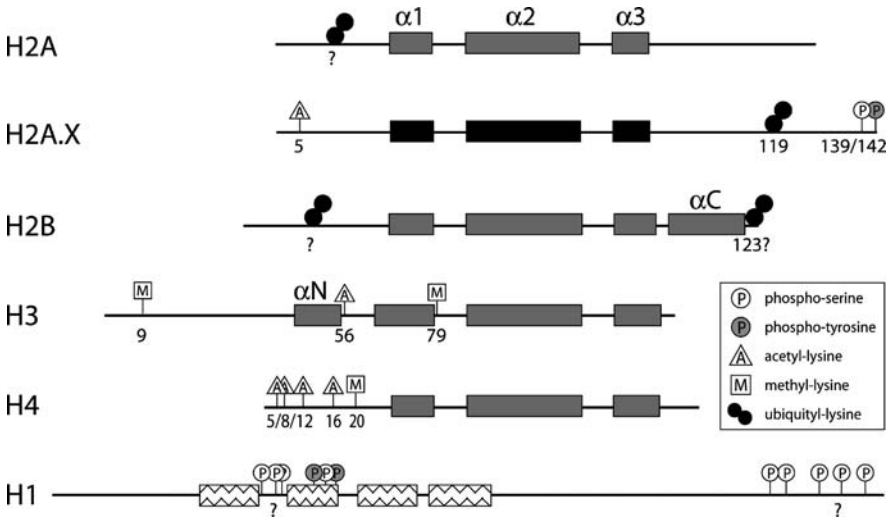

Fig. 1 Location of histone modifications relevant to the DDR. Location of covalent histone modifications involved in the DNA DSB response in the linear histone sequence. *Rectangles* represent structured alpha-helical regions. Unknown modification sites are indicated with a question mark. Figure adapted from (Costelloe et al., 2006)

folded into higher order structures known as heterochromatin. The histone octamer is composed of eight core histone proteins; two each of H2A, H2B, H3 and H4. Histones are small, globular, basic proteins. They each have two domains; a histone fold domain rich in alpha helical repeats, which is required for histone–histone interactions and the formation of the nucleosome, and a flexible amino-terminal tail domain (Fig. 1) that may lie outside the nucleosome and interact with other proteins (Luger et al., 1997). Histones are subject to a number of post-translational covalent chemical modifications. These modifications may directly alter chromatin structure or they may mediate binding of non-histone proteins, either event being instrumental in facilitating the aforementioned processes. Disruption of normal chromatin modifications can have severe consequences for the cell, potentially resulting in genomic instabilities that may manifest as cancer in an organism. This review will focus on chromatin modifications important for maintaining the integrity of the eukaryotic genome by facilitating the process of DNA repair.

Histone Modifications

There are at least eight different types of histone modifications reported to date which together affect over 60 different amino acids on the core histones (Fig. 1; Kouzarides, 2007). The modification of histones is usually mediated by enzymes with a broad specificity that often target more than one histone residue and even non-histone substrates. Most modifications are reversible through the action of

counter-acting enzymes. The known histone modifications reported to date are summarised below.

Acetylation: the addition of an acetyl (ac) group to lysine (K) residues by K-acetyltransferases (KATs; formerly histone acetyltransferases). Acetyl groups can be removed through the action of histone deacetylases (HDACs; for detailed review of enzymes see Smith and Denu, 2009).

Methylation: the addition of methyl (me) groups to lysine or arginine (R) residues by K-methyltransferases (KMTs) or protein arginine methyltransferases (PRMTs), respectively. Methyl-lysine modifications may be removed by K-demethylases (KDMs; (Smith and Denu, 2009). Although, there is no known arginine demethylase, the deiminases (mentioned below) may possibly perform an analogous function to demethylases by converting methylarginine into peptidylcitrulline (Thompson and Fast, 2006).

Phosphorylation: the addition of a phosphate group to serine, threonine or tyrosine residues by various kinases. These modifications may be removed by phosphatases (PPTases; Bradbury, 1992; Johnson and Turner, 1999).

Ubiquitylation: the addition of the protein Ubiquitin (ub) to lysine residues by ubiquitin ligases. This modification may be removed by Deubiquitinases (DUBs; Shilatifard, 2006; Vissers et al., 2008).

ADP-ribosylation: the addition of one or more molecules of ADP-ribose to glutamate by mono-ADP-ribosyltransferases or poly-ADP-ribose polymerases respectively (Faraone-Mennella, 2005). This modification may be removed by poly-ADP-ribose glycohydrases (Hassa and Hottiger, 2008).

Sumoylation: the addition of the protein SUMO (small ubiquitin-like modifier; su) to lysine residues by SUMO ligases. This modification may be removed by SUMO proteases (Mukhopadhyay and Dasso, 2007).

Deimination: the conversion of the amino acid arginine into the amino acid citrulline by peptidylarginine deiminases (Thompson and Fast, 2006).

Isomerisation: the switching between the cis- and trans- conformation of the peptidyl prolyl bonds of the amino acid proline mediated by the peptidyl−prolyl cis/trans isomerases (Andreotti, 2003).

Mechanism of Action

There are two general mechanisms that explain how modification of histones helps to orchestrate the various DNA-templated processes.

The first is based on the charge hypothesis which proposes that the presence of many basic (positively-charged) residues, such as lysine and arginine, in the core histones helps them to interact with the negatively charged DNA (Grunstein, 1997). Modifications affecting the overall charge of the histones may be expected to reduce their affinity for the DNA thus weakening histone–DNA interactions or even interactions between nucleosomes. This would in turn relax the packaging or higher-order folding of the chromatin making it less condensed and euchromatic, allowing access of various factors to the DNA. Acetylation of lysine residues is

the best characterised in this regard; it is known that the addition of an acetyl group results in loss of the net positive charge of this residue. In addition to this, recent evidence from the Peterson laboratory supporting this charge hypothesis has shown that nucleosomes which are artificially acetylated on histone H4 at lysine 16 (H4K16ac) inhibit the formation of the 30 nanometer chromatin fibre and prevent the formation of higher order chromatin structures/heterochromatin (Shogren-Knaak et al., 2006). Other modifications may also affect the net charge of histones in a similar manner. For example, the deimination of arginine to citrulline would result in a loss of positive charge (Smith and Denu, 2009), although there is no evidence to date suggesting that this has any biological effect. The addition of a phosphate group during histone phosphorylation also decreases the net positive charge. In the case of the linker histone, H1, phosphorylation of an amino-terminal region creates a "charge patch" (Dou and Gorovsky, 2000) that affects H1's association with DNA (Dou et al., 2002). However, it is unclear whether phosphorylation of the core histones directly alters the interaction between the DNA and histones.

The second mechanism, based on the "histone code" hypothesis, is applicable to all histone modifications and involves the active recruitment of non-histone "effector" proteins to specifically modified histones (Strahl and Allis, 2000; Turner, 1993; Turner, 2000). The recruited effector protein then functions to bring about a specific process associated with its biochemical activity. Recruitment of the protein to a modified histone is mediated by specific domains within the protein which can recognise and bind to various modified amino acids of the histone. This idea of active recruitment is gaining support as the past 20 years of research have witnessed the identification of many of the enzymes that modify histones in a specific manner and the corresponding effector proteins that selectively bind these modified histones. However, the situation is more complicated; it is rarely the case that one protein will bind to one specific type of modified histone. In fact, many effector proteins possess more than one binding domain and/or form a complex with a number of other proteins, each of which themselves may bind other modified histones. Moreover, there is a degree of interaction between the 60 or more known histone modifications themselves, whereby a modification at one residue may inhibit or promote the modification of another residue. Undoubtedly, it is this complexity that allows these modifications to orchestrate many DNA-templates processes that exist in the cell with such precision. Henceforth this review will focus on histone modifications which are important in the DNA damage response, in particular the early and late stages of double strand break (DSB) repair, and discuss how alterations in certain modifications in these contexts results in genome instability.

Histone Modifications in DNA Repair

The genome is under constant attack by numerous internal (free radicals) and external agents (radiation, chemicals) that frequently cause damage to the DNA (Pandita and Richardson, 2009; Ataian and Krebs, 2006). These genotoxic agents result in

various types of DNA lesions that, if not properly repaired, may lead to genome instabilities proving fatal to the organism. There are a number of repair pathways which the cell may employ to repair damaged DNA including; (i) base excision repair, which can remove and repair specific types of incorrect or damaged DNA bases; (ii) nucleotide excision repair recognises regions of DNA containing aberrant bases due to their abnormal structure and chemistry and orchestrates removal and repair of the DNA in this region; (iii) mismatch repair involves the removal and replacement of incorrect DNA bases that have been introduced during DNA replication or other DNA-templated processes; (iv) DSB repair recognises lesions that cut both DNA strands and repairs the break either by homologous recombination (HR) or non-homologous end-joining (NHEJ).

The specific type of lesion and the circumstances of the cell determine which repair pathway will be engaged (Ataian and Krebs, 2006). However, all the DNA damage response pathways must, (i) detect the damaged DNA, (ii) activate a DNA damage checkpoint, (iii) repair the lesion and (iv) restore the chromatin to its original state. With the important exception of the actual physical repair of the DNA lesion, recent data have shown that histone modifications play an important role in orchestrating these events (Table 1).

Table 1 Role of histone modifications in repair of DSBs

Histone	Modification	Mediated by	Function
H1	Phosphorylation	PIKK kinases	Release of linker histones
H2AX	S139 phosphorylation	PIKK kinases	Recruitment of MDC1, MRN complex, ATM, RNF8, RNF168 and UBC13
	Y142 phosphorylation	WSTF	Recruitment of JNK1 kinase
	K5 acetylation	Tip60	Pre-requisite for K119 ubiquitylation
	K119 ubiquitylation	RNF8 & UBC13 & RNF168	Histone exchange/chromatin remodelling
H2A	Ubiquitylation	RNF8 & UBC13 & RNF168	Recruitment of BRCA1 and 53BP1
H2B	Ubiquitylation	RNF8 & UBC13 & RNF168	Recruitment of 53BP1
H3	H3K9 tri-methylation	SUV39H	Redistribution of phospho-HP1-beta promotes γH2AX foci formation following DNA damage
	K79 methylation	DOT1L	Recruitment of 53BP1
	K56 acetylation	P300/CBP	Chromatin reassembly, checkpoint inactivation
H4	K16 acetylation	MOF	Sensing of DSB ATM activation?
	K20 di-methylation	SET proteins	Recruitment of 53BP1
	K5, 8, 12 acetylation	Tip60	Chromatin relaxation allowing access of repair proteins including BRCA1, 53BP1, Rad51

DSBs are the most dangerous in terms of affecting genome stability, as they can result in chromosomal rearrangements which may lead to cancer. DSBs can be caused by external or internal insults such as; ionizing radiation (IR), chemical agents and reactive oxygen species (ROS), but importantly these are also naturally generated during V(D)J recombination of the immune system, DNA replication and meiosis (Helleday et al., 2007; Povirk, 2006). As DSBs are a normal part of cell physiology, efficient mechanisms for repairing such breaks have evolved. Eukaryotic cells employ one of two mechanisms to deal with this type of damage; NHEJ which involves the direct ligation of the broken DNA ends, and HR which repairs the break using an undamaged homologous chromosome (van Gent et al., 2001). Many of the proteins involved in the response to DSBs have been identified, however, the first event in the DNA damage response (DDR), sensing the lesion, is still unclear. This sensing may simply be recognition of broken DNA ends through a resultant topological change in the DNA, although it may be more complex in nature involving the release of some factor from the chromatin surrounding the site of the break or exposure of a previously hidden signal due to changes in the DNA structure.

Core Histone Modifications in the Repair of DSBs

Phosphorylation of H2AX at S139

Phosphorylation of the histone H2A variant, H2AX at serine 139 (γH2AX), is a well-characterised modification that plays a key early role in the cell's response to DSBs (reviewed in detail in van Attikum and Gasser, 2005 and Srivastava et al., 2009); see also chapter "Structure and Function of Histone H2AX" by Pinto and Flaus in this book). H2AX accounts for 2–25% of the total histone H2A pool in mammalian cells (Goldknopf et al., 1975). It contains a highly conserved and unique carboxy-terminal tail containing the amino acid sequence SQEY which is phosphorylated following the recognition of a DSB. This phosphorylation is carried out by members of the phosphatidylinositol-3 kinase-like family of kinases (PIKK), including ATM (ataxia-telangiectasia mutated) and DNA-PK (DNA-dependent protein kinase) in response to IR-induced breaks (Burma et al., 2001), and ATR (ATM- and Rad3-related) following DNA replication stress (Chanoux et al., 2009; Ward and Chen, 2001). ATM is a key kinase that gets recruited to the damage site and activated by the MRN (Mre11-Rad50-Nbs1) complex (Lee and Paull, 2005; Paull and Lee, 2005). This results in phosphorylation of a number of substrates, including H2AX, which can be seen by immunofluorescence microscopy minutes after induction of DNA damage as large spots/foci of γH2AX that accumulate over 50–1,000 kilobases surrounding the actual break (Rogakou et al., 1998; Srivastava et al., 2009).

This epitope provides a platform that assists the recruitment of several DDR proteins such as MDC1 (mediator of DNA checkpoint 1; Stucki et al., 2005), 53BP1

(p53 binding protein 1; Ward and Chen, 2001; Fernandez-Capetillo et al., 2002), BRCA1 (breast cancer 1, early onset; Manke et al., 2003), Microcephalin (Wood et al., 2007) and NBS1 (Nijmegen breakage syndrome 1; Schultz et al., 2000; Figs. 2a, b). These proteins contain BRCT (BRCA1 C-terminal) domains, which can bind to phospho-serine residues (Manke et al., 2003; Rodriguez and Songyang, 2008) of γH2AX or other proteins in the foci (Botuyan et al., 2004; Chapman and Jackson, 2008) and from here can transduce signals to effector kinases including Chk1 (checkpoint kinase 1) and Chk2 (checkpoint kinase 2) which in turn control a number of downstream targets resulting in a cell cycle delay and expression of DNA repair genes (Motoyama and Naka, 2004; Niida and Nakanishi, 2006). Mice in which the *H2AX* gene is deleted are sensitive to IR and display repair defects, chromosomal instabilities and impaired localisation of NBS1, 53BP1 and BRCA1 to DSBs (Celeste et al., 2002). However, these mice are able to activate a checkpoint response following IR, suggesting that the main function of γH2AX is to recruit and retain proteins involved in the physical repair of the DNA. Nevertheless, it is still possible that γH2AX is involved in checkpoint activation albeit with some degree of redundancy in this pathway.

Phosphorylation of H2AX at Y142

Recently it has been shown that a tyrosine residue of H2AX, (Y142) which is in close proximity to S139 is also phosphorylated. A novel kinase WSTF (William's syndrome transcription factor) that along with SNF2H (Sucrose non-fermenting protein 2 homologue) forms part of the WICH (WSTF-ISWI chromatin remodelling) complex, has been shown to be responsible for this phosphorylation (Xiao et al., 2009). Unlike S139, Y142 is phosphorylated in un-damaged cells and becomes de-phosphorylated by the EYA (Eyes absent homologue) phosphatases following DNA damage (Cook et al., 2009; Krishnan et al., 2009; Xiao et al., 2009). De-phosphorylation of Y142 following DNA damage is proposed to be part of a switch which enables the cells to decide between a DNA repair or an apoptotic response (Cook et al., 2009). The model proposed is that de-phosphorylation of Y142 along with phosphorylation of S139 following IR promotes binding of DNA repair proteins including MDC1, Mre11 and Rad50 (Cook et al., 2009). However, if phosphorylation persists at Y142 in addition to S139 a stress-response kinase, JNK1 (Jun N-terminal Kinase), preferentially binds and is expected to elicit an apoptotic response (Cook et al., 2009). In support of this *H2AX*-deficient MEFs (mouse embryonic fibroblasts) expressing exogenous H2AX mutant for Y142 display a weakened apoptotic response following high-doses of IR in comparison to MEFs expressing the wild-type H2AX (Cook et al., 2009).

However, there are still some grey areas in the interpretation of these data; de-phosphorylation of Y142 following DNA damage would also remove the binding site for JNK1 kinase. Furthermore, inhibition of Y142 phosphorylation using RNAi (RNA interference) for the WSTF kinase or mutation of the Y142 residue results

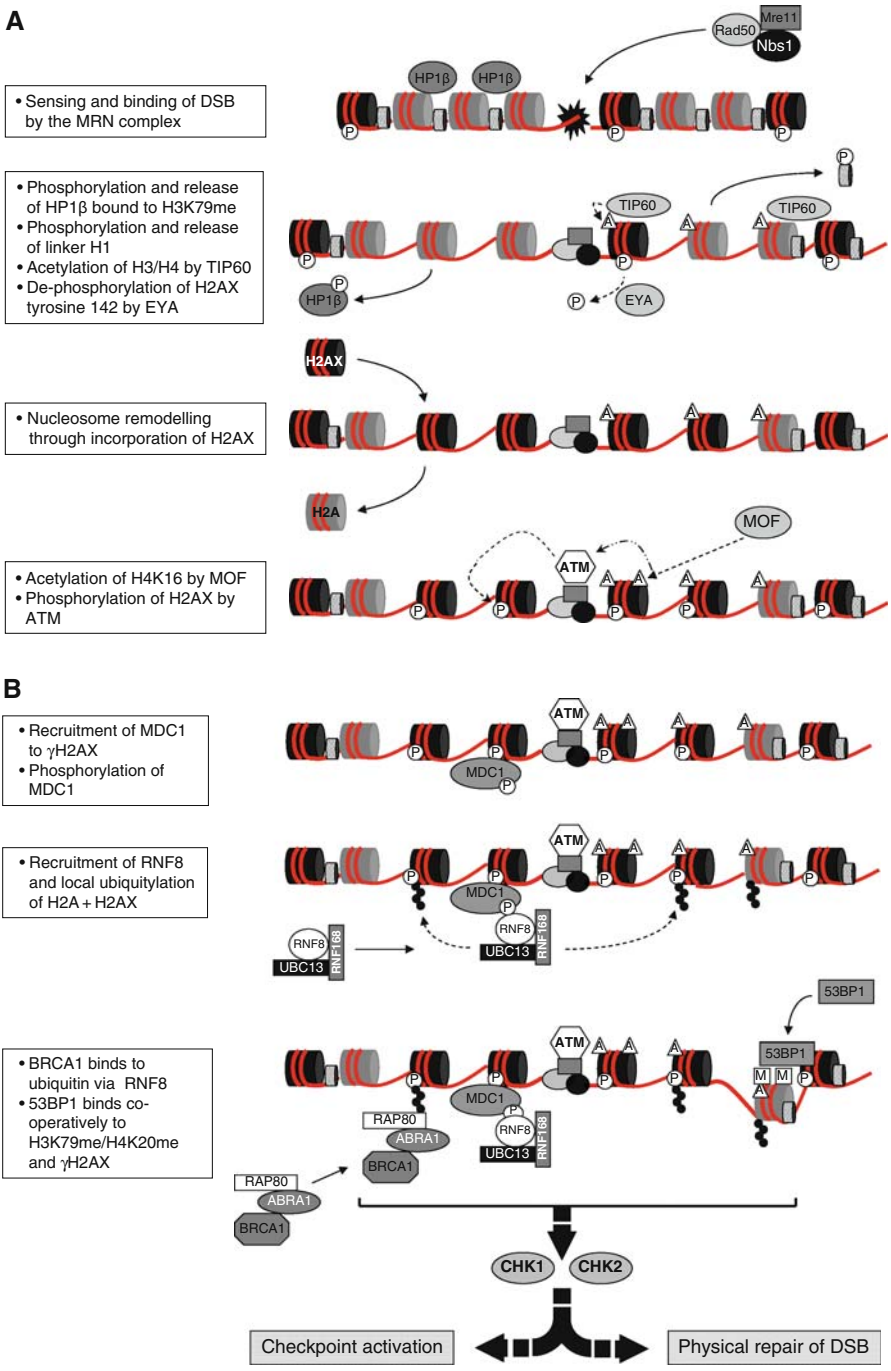

Fig. 2 Model showing stages of the DDR and associated modifications

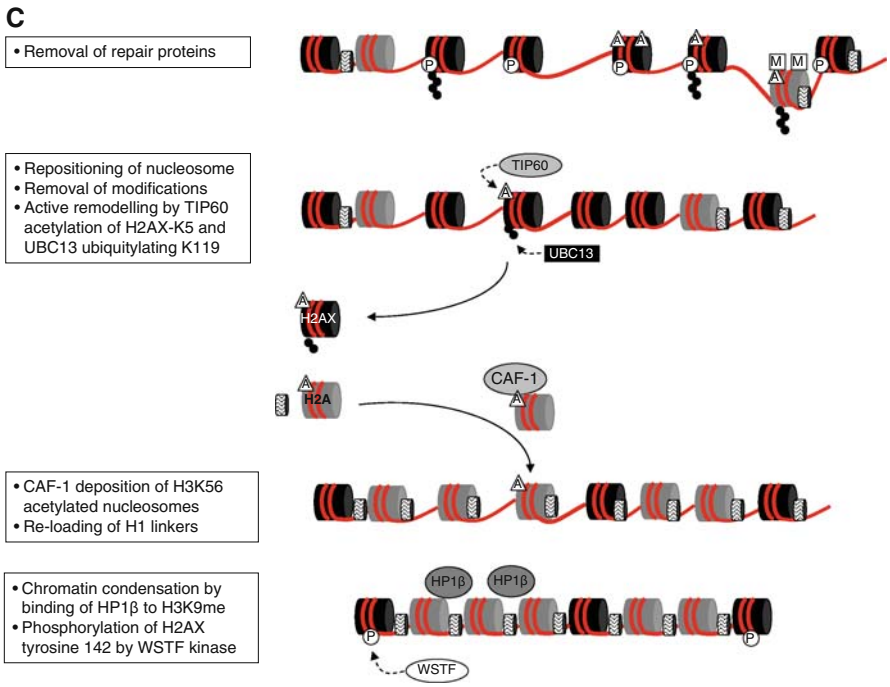

Fig. 2 (continued)

in impaired phosphorylation of S139 of H2AX and impaired recruitment of DNA damage response proteins (Cook et al., 2009; Xiao et al., 2009). This suggests that although Y142 is de-phosphorylated following IR some basal level of phosphorylation may be needed to promote or maintain S139 phosphorylation and the DNA damage repair processes.

Methylation of H3K79

Methylation of various lysine residues on histones H3 and H4 has also been suggested to play a role in the early events of the DNA damage response (Huyen et al., 2004; Kouzarides, 2007). 53BP1 is a conserved DDR protein that rapidly localises to DSBs following DNA damage (Anderson et al., 2001; Schultz et al., 2000) and contributes to the phosphorylation and activation of the Chk2 effector kinase (Wilson and Stern, 2008). 53BP1 possesses a tandem tudor domain that mediates its in vitro binding to histone H3 methylated at lysine 79 (H3K79me) and which is required for its targeting to DSBs (Huyen et al., 2004). Furthermore, depletion of DOT1L, the methyltransferase responsible for H3K79me, inhibits the recruitment of 53BP1 to DSBs, providing further evidence that this modification is important for the recognition of breaks by 53BP1. Curiously, no detectable change has been observed for

overall H3K79 methylation levels in response to damage by irradiation. It has therefore been suggested that 53BP1 can sense DSBs via changes in chromatin structure that expose a previously concealed H3K79me binding platform (Huyen et al., 2004; Fig. 2b).

Methylation of H4K20

Separate structural and functional studies on the mechanistic role of histone methylation in 53BP1 recruitment to DSBs by Botuyan and colleagues, have suggested that methylated histone H4 at lysine 20 (H4K20me), and not H3K79me, is the key binding site (Botuyan et al., 2006). Both 53BP1 and its *Saccharomyces pombe* homologue Crb2 were found to be recruited to DSBs through binding to H4K20me via their tandem tudor domain (Botuyan et al., 2006; Sanders et al., 2004). Binding of both proteins is preferential for di-methylated H4K20 (mono-methylated to a lesser degree) and is blocked by tri-methylated H4K20 (Botuyan et al., 2006) suggesting that the chromatin state may be a factor in 53BP1 recruitment; tri-methylated H4K20 is enriched at pericentric heterochromatin (Schotta et al., 2004). As with H3K79 there was no detectable change in total H4K20 methylation levels in response to DNA damage (Botuyan et al., 2006). However, in light of recent observations implying that nucleosomes are very dynamic structures permitting rapid access to proteins, even at concealed regions of chromatin (Li et al., 2005), the authors suggested that exposure of previously concealed modifications following damage is an unlikely mechanism and proposed cooperation of another histone modification in the recruitment of 53BP1 (Botuyan et al., 2006). The probable candidate for such cooperation is γH2AX, where it has been suggested that 53BP1 is recruited to γH2AX through its BRCT domain and then becomes stabilised on chromatin through binding to methylated histones via its tudor domains (Ward et al., 2003; Fig. 2b).

Methylation of H3K9

The importance of methylation of histone H3 at lysine 9 (H3K9me) in chromatin organisation is well established (Daniel et al., 2005; Jenuwein and Allis, 2001). It is preferentially found in heterochromatic regions of the genome where it acts as a binding site for HP1 (heterochromatin protein 1) which is thought to mediate the formation of higher order chromatin structures. H3K9me is mediated by the SUV39H (suppressor of variegation 3-9 homologue) enzymes in humans (Rea et al., 2000) or by Su(var)3-9 in *Drosophila* (Czermin et al., 2001). Recent studies in *Drosophila* using *Su(var)3-9* mutants have shown that loss of this H3K9me2 modification results in spontaneous DNA damage that occurs preferentially at heterochromatin (Peng and Karpen, 2009). Cells from these flies were found to have increased genomic instability, where 1.1% of the mutant cells displayed

chromosomal deletions, duplications and translocations, with no wild-type cells having these defects. The mutant flies also had a 2.5-fold higher frequency of loss of heterozygosity (LOH) at a particular gene compared to wild type (Peng and Karpen, 2009). These slight increases in genomic instabilities are probably due to recurring DNA damage in these animals. However, the flies are generally healthy and fertile, but this is dependent on an intact DNA damage checkpoint (Peng and Karpen, 2009). Flies that are double mutant for *Su(var)3-9* and either of the checkpoint genes *grp* or *lok* (*Chk1* or *Chk2* homologues) were not viable (Peng and Karpen, 2009).

The authors proposed that the causes for this increased damage to heterochromatin could be that there is a defect in DNA replication; with heterochromatic DNA being incompletely replicated due to a shorter S-phase in these mutants or DNA being replicated too quickly for this type of repetitive heterochromatin (Peng and Karpen, 2009). These scenarios would result in stalled replication forks or collapsed forks leading to DSBs. A second proposal is that H3K9me is required in the early sensing of the DNA damage (Peng and Karpen, 2009). Another explanation for this increase in damage is that HP1 is required for formation of heterochromatin (Daniel et al., 2005), and loss of H3K9me results in loss of HP1 binding. Euchromatin is more susceptible to DNA damage than heterochromatin (Falk et al., 2008) and so these mutants display more genomic instabilities (Peng and Karpen, 2009). This is supported by an interesting finding in mammalian cells where, in response to DNA damage HP1 becomes phosphorylated and subsequently released from H3K9me in chromatin. This promotes H2AX phosphorylation and a proper DNA damage response (Ayoub et al., 2008). This makes sense as a logical early response to damage in heterochromatin would be to unfold these regions of highly condensed chromatin through release of HP1, facilitating access of repair factors (Fig. 2a).

Ubiquitylation of H2A and H2B

A novel pathway in the DDR involving another histone modification, ubiquitylation, has recently gained attention and helps explains the recruitment of some key DDR proteins (for recent reviews see Panier and Durocher, 2009 and van Attikum and Gasser, 2009). This pathway involves enzymes that ubiquitylate the histones H2A, H2B and H2AX at sites of DNA damage, subsequent recruitment of factors that recognize and bind to these modified histones, and co-recruitement of important DDR proteins such as BRCA1.

The first player in this pathway is the E3 ubiquitin ligase, RNF8 (RING finger protein 8) which contains an FHA (fork-head associated) domain at its amino-terminus and a RING finger domain at its carboxy-terminus (Mailand et al., 2007). RNF8 accumulates at sites of damage shortly after recruitment of γH2AX, MDC1 and NBS1. This accumulation is dependent on RNF8 binding to the ATM-phosphorylated amino-terminus of MDC1, via its FHA domain (Huen et al., 2007; Kolas et al., 2007). RNF8 is then expected to target the E2 ubiquitin-conjugating

enzyme UBC13 to H2A, H2B and H2AX to ubiquitylate these histones on unknown lysine residues (Doil et al., 2009; Huen et al., 2007; Mailand et al., 2007). Another E3 ligase, RNF168 (RING finger protein 168), also accumulates at damage sites in an RNF8-dependent manner. In addition to its RING finger domain, RNF168 contains two MIU (motif interacting with ubiquitin) domains, which it uses to bind to histones ubiquitylated by RNF8. RNF168 also associates with UBC13 to ubiquitylate or poly-ubiquitylate H2A and H2AX (Zhao et al., 2007) and it is believed that the RNF8 mediated ubiquitylation is amplified and stabilised by RNF168 (Panier and Durocher, 2009). Currently it is unclear which specific histone residues become ubiquitylated in this pathway (Fig. 2b).

These ubiquitylation events prove important for recruitment of the DDR protein BRCA1. BRCA1 exists in several complexes, one of which contains RAP80 (receptor-associated protein 80) and ABRA1 (Abraxas-BRCA1-A complex subunit). RAP80 contains two ubiquitin interaction motifs (UIMs), which are able to bind poly-ubiquitin chains and mediate localisation of the BRCA1 complex to ubiquitylated histones at the site of DNA damage (Kim et al., 2007; Fig. 2b).

Acetylation of Histone H2AX

Further evidence of interplay and crosstalk between histone modifications is exemplified by a study from Ikura and colleagues examining the acetylation of H2AX by the lysine acetyltransferase Tip60 (HIV Tat-interacting protein, 60 kDa). This enzyme is required for acetylation of H2AX at lysine 5 early in the response to DNA damage (Ikura et al., 2007). It was shown that Tip60 mediated acetylation was necessary for the subsequent ubiquitylation or poly-ubiquitylation of H2AX at lysine 119 by UBC13. This ubiquitylation results in the release of H2AX from chromatin at the site of damage (Ikura et al., 2007; Fig. 2c). Currently the mechanism of release and its exact relevance is unclear. It may facilitate chromosome remodelling by altering the structure of chromatin around the site of damage to facilitate access of repair factors. Interestingly, a similar role for Tip60 has also been reported in *Drosophila*, where Tip60 acetylation of the phosphorylated H2AX orthologue, phospho-H2Av, results in its removal and exchange for a non-phosphorylated H2Av (Kusch et al., 2004). However, in the Ikura study, phosphorylation of H2Av was not a prerequisite for acetylation by Tip60 (Ikura et al., 2007).

Acetylation of Histone H4

These aforementioned acetylations at HA2X may function to remodel the chromatin to a more open relaxed state to allow access for the repair machinery. Recent studies on histone H4 acetylation support this theory. Murr and co-workers have shown that acetylation of H4 mediated by Tip60 in complex with Trrap (Transformation/transcription domain-associated protein) facilitates accumulation

of DDR proteins, including 53BP1, BRCA1 and Rad51, following DNA damage (Murr et al., 2006; Fig. 2a). Importantly, this study also showed that artificially inducing a relaxed chromatin state could partially rescue phenotypes resulting from abrogation of this acetylation. This suggests that an important role of acetylation is remodelling the chromatin environment to facilitate repair, either by relaxing the chromatin to expose pre-existing binding sites for repair factors or by initiating histone exchange for variants needed to attract repair factors (Murr et al., 2006).

Whereas Tip60 is the KAT responsible for the majority of acetylation of lysines, 5, 8 and 12 on histone H4 (Ikura et al., 2000), thus implicating these modified residues in the DNA damage response, acetylation of histone H4 at lysine 16 is mediated by another acetyltransferase, human MOF (hMOF; males absent on the first; Taipale et al., 2005). It is not clear whether this H4K16ac would affect chromatin organization or function to recruit other proteins, but it is important in the DNA damage response. It has been shown that depletion of hMOF results in altered activation of ATM and aberrant repair kinetics of DSBs following IR (Gupta et al., 2005; Taipale et al., 2005). It has been suggested that hMOF functions upstream of ATM activation (Gupta et al., 2005). However, little is known about how hMOF and the resulting H4K16ac affect ATM recruitment and activation. Acetylation occurs in a preferential manner on histone H4, taking place on lysine 16 before lysines 5, 8 and 12 (Turner and Fellows, 1989). It is possible therefore that elimination of H4K16ac could alter subsequent acetylations and in this manner impair chromatin decondensation and the DNA repair process. However, as H4K16ac is the most abundant of these modifications (Munks et al., 1991; Turner and Fellows, 1989) and as H4K16ac levels have been found to be marginally decreased in the vicinity of DSBs during NHEJ in yeast (Downs et al., 2004; Jazayeri et al., 2004) it is likely that this mark also plays an active role in the response to DSBs. In addition, this particular modification is clearly important for maintenance of genome stability as it has been implicated in tumorigenesis in a number of studies which will be discussed later (Fraga et al., 2005; Pfister et al., 2008).

Acetylation of Histone H3

Following a DSB, nucleosomes are removed from the broken ends of the DNA (Tsukuda et al., 2005; van Attikum et al., 2007). This removal is mediated by chromatin remodelling complexes and facilitates either histone exchange or DNA end resection (the 5′–3′ digestion of the DNA end to create single stranded DNA; van Attikum et al., 2007). On completion of DNA repair the chromatin structure needs to be restored. This requires the re-loading of the histones onto the DNA, which is achieved in a similar way to nucleosome assembly on DNA during DNA replication. Recent work in yeast has shown that acetylation of lysine 56 in the globular core of H3 plays an important role in the reassembly of chromatin in both scenarios (Chen et al., 2008; Li et al., 2008). Chen and colleagues found that in wild type cells, reassembly occurs with similar kinetics for both replication and repair;

however, a mutant strain lacking H3K56ac was unable to reassemble chromatin at the break even though repair of the DSB occurred normally. Although this strain can repair DSBs it is sensitive to DNA damage, probably because the cells cannot overcome a DNA damage checkpoint in the absence of H3K56ac, which results in cell death (Chen et al., 2008). This led the authors to suggest that the altered chromatin structure containing reassembled H3K56ac histones is part of a signal (in conjunction with other modifications) to switch off the DNA damage checkpoint and enable cells to continue cycling once damage has been repaired (Chen et al., 2008; Fig. 2c). More recently, the same research group has identified the *Drosophila* and human enzymes involved in regulating H3K56 acetylation (Das et al., 2009).

Again, the authors found that the level of H3K56ac on chromatin increased in response to various types of DNA damage. Interestingly, this modification co-localises in damage-induced foci with γH2AX. The enzymes responsible for this H3K56ac are the CBP (CREB binding protein) and p300 acetyltransferases, and the histone chaperone ASF1A (anti-silencing function 1 homolog A) is also required. Furthermore, the authors showed that CAF-1 (chromatin assembly factor 1) is needed for assembly of the H3K56ac histones onto DNA. RNAi experiments identified SIRT1 and SIRT2 (silent mating type information regulation homolog 1,2) as deacetylase enzymes that can remove H3K56ac (Das et al., 2009). Although there is abundant H3K56 acetylation of chromatin throughout the cell cycle (Das et al., 2009) it clearly plays a role in the DDR, presumably in the reassembly of chromatin after repair and in switching off a checkpoint, as proposed for yeast (Chen et al., 2008). However, loading of acetylated H3K56 may occur earlier and function to hold the chromatin in an open structure to help in the repair process. Determination of the timing of H3K56 acetylation in human cells at the sites of damage will help settle this matter. Similarly, elucidating the kinetics of deacetylation at this residue following repair will clarify whether this H3K56ac needs to be removed to restore chromatin to its original/unmodified state for the full repair process to be completed.

The Modification of Linker Histones During DNA Repair

The linker histone, H1, associates with the inter-nucleosomal linker DNA. Its exact position in the nucleosome is controversial, but it is generally thought that the central globular domain localises to the nucleosome-linker DNA interface at the position where it can interact with the DNA as it enters and leaves the nucleosome (Travers, 1999). The carboxy-terminal domain is able to interact with the linker DNA and aids in the folding of nucleosome arrays to form higher order chromatin (Happel and Doenecke, 2009). Not all nucleosomes contain a linker histone and some may contain more than one as has been deduced from the finding that the average ratio of linker histones per nucleosome varies from 0.45 to 1.3 depending on the type of cell (Woodcock et al., 2006). Histone H1 is also subject to post-translational modifications including phosphorylation, methylation,

acetylation and ubiquitylation (Garcia et al., 2004; Wisniewski et al., 2007). However, in comparison to modification of the core histones, very little is known about the function of H1 modifications.

Studies in *Tetrahymena* suggest a role for the phosphorylation of H1 that may be relevant to the DDR. As mentioned above, phosphorylation of an amino-terminal region of H1 creates a charge patch (Dou and Gorovsky, 2000). Mutational studies mimicking either a non-phosphorylated or hyper-phosphorylated H1 followed by FRAP (Fluorescence recovery after photobleaching) experiments showed that phosphorylation at several proximal residues increases H1 dissociation from chromatin (Dou et al., 2002). In vitro experiments suggest that in addition to a charge effect, phosphorylation may also alter the affinity of H1 for DNA through a change in structure of the molecule (Roque et al., 2008).

In response to DNA damage, chromatin becomes decondensed, presumably to facilitate genome surveillance and access of repair factors. As H1 is involved in chromatin compaction (Fan et al., 2005), Murga and colleagues examined the effect of reduced levels of H1 on the DNA damage response (Murga et al., 2007). They found that triple-knockout mouse embryonic stem (ES) cell lines (with three of the six H1 genes inactivated resulting in a decondensed chromatin phenotype; Fan et al., 2005) had a resistance to DNA damaging agents, an enhanced checkpoint response and an increase in the amount of activated DDR proteins in comparison to wild-type cells (Murga et al., 2007). It is possible that one of the DNA damage response kinases could phosphorylate H1 resulting in its release from chromatin allowing decondensation of the chromatin and access of the DDR proteins (Fig. 2a).

Indeed, there is precedent for release of linker histones in the repair of double strand breaks. Konishi and colleagues found that the linker histone H1.2 gets released from the nucleus into the cytoplasm (along with the other H1 subtypes) following high doses of X-ray irradiation where it induces the release of cytochrome c from the mitochondria leading to the induction of apoptosis (Konishi et al., 2003). The phosphorylation status of H1 could act as an indicator to the cell by relating the degree of damage sustained to the genome. At lower levels of damage, H1 becomes phosphorylated, gets released from the nucleosome, allowing chromatin decondensation and access of repair proteins. If the damage is extensive and beyond repair, then much more H1 would get phosphorylated and released. This could exceed a critical level at which point it may signal to the mitochondria causing release of cytochrome c and instructing the cell to undergo apoptosis. Alternatively, specific linker histone subtypes could preferentially associate with certain types of chromatin and release of that subtype upon damage could result in a particular cell response. Interestingly, H1.2 depletion results in decreased expression of certain cell-cycle genes and a cell cycle arrest (Sancho et al., 2008), suggesting that this linker helps in the transcriptional regulation of the cell cycle. Aberrant regulation of such genes could be detrimental to the organism. Perhaps for this reason cells have evolved the H1.2 mediated apoptotic response.

There also appears to be interplay between modifications of core and linker histones as the ubiquitylation and acetylation state of nucleosomes are able to influence H1 dynamics. The carboxy-terminal tails of H2A and H2AX are able to interact

with linker histones (Luger et al., 1997). These tails are the sites of modifications involved in the early response to DNA DSBs (Ikura et al., 2007; van Attikum and Gasser, 2005), suggesting that the modification state of these tails could influence the binding status of H1, either directly through physical interactions or indirectly through the recruitment of other histone modifying or remodelling factors. In support of this Zhu and colleagues found that deubiquitylation of H2A, at K119, by the enzyme 2A-DUB (H2A-Deubiquitinase) promoted the phosphorylation and subsequent release of H1 from the nucleosome (Zhu et al., 2007).

Model for Integrated Role of Histone Modifications in Repair of DSBs

DSBs induced by genotoxic agents are sensed in a currently unknown manner with one of the first consequences being recruitment of the MRN complex to the site of DNA damage where it binds to the broken ends of the double stranded DNA. Following this the kinase ATM is recruited to the break which initiates a plethora of signalling events that culminate in repair of the DSB (Figs. 2a, b).

One of the key early events in the damage response pathway is phosphorylation of H2AX by ATM in the vicinity of the break. Accumulation of γH2AX surrounding the break may be dependent on redistribution of the HP1-β from its normal chromatin context (Ayoub et al., 2008). Acetylation of H4 by the Trrap-Tip60 complex early in the response also serves to relax chromatin compaction (Murr et al., 2006) as does the removal of the linker histone H1 (Murga et al., 2007). These events contribute to chromatin decondensation surrounding the break which facilitates the binding of repair factors to the site of damage (Fig. 2a).

MDC1 binds to γH2AX via its BRCT domain and then also gets phosphorylated by ATM (Stucki et al., 2005). This chromatin-recruited MDC1 protein acts as a master regulator of the DNA damage response by recruitment of late repair proteins to the damage site, including the RNF8 E3 ubiquitin ligase. At the site of damage RNF8 along with RNF168 and UBC13 induces and maintains ubiquitylation of H2A and H2AX histones. These ubiquitylated histones attract the BRCA1 protein, which binds ubiquitin via its interacting proteins RAP80 and ABRA1 (Huen et al., 2007; Kolas et al., 2007; Mailand et al., 2007). Changes in chromatin structure due to ubiquitylation in the region may also expose histone modifications such as H4K20me2 or H3K79me which helps recruit the late repair protein 53BP1 (Botuyan et al., 2006; Huen et al., 2007). BRCA1 and 53BP1 then serve to activate the downstream effector proteins which activate cell-cycle checkpoints and carry out the physical repair of the damage (Wilson and Stern, 2008; Fig. 2b).

Following repair, the DNA damage checkpoint needs to be switched off, the damaged induced modifications need to be removed and the chromatin state needs to be restored. Chromatin becomes reset through the action of various deubiquitinases, phosphatases, demethylases and histone deacetylases, which remove the modifications. Other mechanisms that restore chromatin structure could be histone exchange

or degradation of ubiquitylated histone proteins followed by new histone deposition. Ubiquitylation of H2AX mediated by UBC13 (Ikura et al., 2007) may serve a role in chromatin remodelling via histone exchange in the later stages of the repair process. Acetylation of H3 by CBP/p300 is required for its loading onto DNA during chromatin reassembly (Das et al., 2009) and this loading is probably part of a signal to switch off the DNA damage checkpoint (Chen et al., 2008; Fig. 2c).

This is a speculative model that has overlooked the complicated process of physically repairing the DNA. The DNA damage response is much more complex than suggested in this model and there are likely to be many unknown players still to be identified. Moreover, there is bound to be a high degree of cross-talk between modifications, feedback loops and compensatory pathways that will make unravelling the DNA repair process even more difficult. High resolution chromatin immunoprecipitation (ChIP) experiments examining specific histone modifications at regulated DSBs in both euchromatic and heterochromatic landscapes will help to identify the timing and interdependence of the various modifications.

Aberrant Histone Modifications Cause Genome Instability and Disease

Considering their important role in repair of damaged DNA and in regulation of many DNA-templated processes, it is not surprising that incorrect modification of histones is implicated in disease. Such defects have been most widely reported in cancer.

The H2AX gene in humans, *H2AFX*, is located on chromosome 11q23.2-23.3, a region which is often lost in human cancers and elevated levels of the phosphorylated form of H2AX, γ-H2AX, are reported in a number of pre-malignancies implicating it as an important tumour suppressor gene (for further review see Srivastava et al., 2009).

Loss of H4K16ac and H4K20me3 has also been reported as a common hallmark of human cancer (Fraga et al., 2005). The acetyltransferase responsible for H4K16ac, hMOF, has also been reportedly down-regulated in a number of human tumours and expression levels of hMOF serve as a prognostic tool for patient outcome, with lower expression correlating with worse overall survival rates in medulloblastoma patients (Pfister et al., 2008).

H3K9 methylation levels can also be prognostic for cancer with H3K9 trimethylation correlating with tumour stage, invasiveness and overall poorer survival in gastric adenocarcinomas (Park et al., 2008). This study also examined H4K16 acetylation and H4K20 trimethylation but these did not correlate with any of the clinicopathological variables examined. This is an interesting finding as it differs from that of Pfister et al. where reduced H4K16 acetylation was associated with poorer prognosis and survival. Gupta and colleagues further reported that H4K16 acetylation, and the HAT responsible (MOF) was increased in tumours, and overexpression of MOF correlated with oncogenic transformation and tumour growth

(Pfister et al., 2008; Gupta et al., 2008). These data demonstrate that cancer is a very epigenetically heterogeneous disease and suggests that different histone modifications may have different prognostic value in different cancer types.

In addition to those mentioned above, a number of other histone modifications have been reportedly implicated in cancer including, acetylation of H2AXK5, H3K9, H3K18, H4K12 and methylation of H3K27 and H4R3. These alterations may not only be important on their own but also in combination with alterations at other sites (for review see Lennartsson and Ekwall, 2009).

Histone Modifying Enzymes and Cancer

Some of the enzymes involved in the DNA damage response have been implicated in human diseases, where again, cancer predominates. This contribution to disease often arises through mutation of the genes encoding the enzymes, but may also be due to translocation of two genes.

The *P300* gene is located on chromosome 22q13 and this region frequently undergoes loss of heterozygosity in a variety of cancer types, which directly affects the *P300* gene locus (Bryan et al., 2002). Missense and truncating mutations of *P300* have also been reported in a number of primary tumour samples and tumour cell lines (Gayther et al., 2000). Both the *CBP* and *P300* gene loci have been shown to undergo translocations resulting in P300/CBP-MLL (mixed-lineage leukemia) fusion proteins in haematological malignancies. The CBP-MLL fusion protein has been reported in haematological malignancies following treatment with topoisomerase II targeting drugs (Rowley et al., 1997; Sobulo et al., 1997). These drugs aim to trigger the cells instrinsic cell death mechanisms by stablizing otherwise transient DNA breaks created by the normal physiological function of the topoisomerase II enzyme. This CBP-MLL fusion protein has been shown to possess the ability to induce oncogenic transformation which is expected to result from combining the capacity of MLL to bind DNA with the ability of CBP to alter chromatin structure (Lavau et al., 2000). In addition to its role in cancer, *CBP* is the most frequently targeted gene in Rubenstein-Taybi syndrome (Petrij et al., 1995), a malformation syndrome whose phenotype includes growth defects, facial abnormalities, skeletal abnormalities and mental retardation (Rubinstein and Taybi, 1963). Mutations in P300 are also causative in a small percentage of Rubenstein-Taybi cases (Roelfsema and Peters, 2007).

The MLL protein itself is a methyltransferase and translocations involving MLL with over 50 fusion partners, which can result in leukaemia have been identified to date (Slany, 2005). One common translocation, t(4;11)(q21;q23), involves fusion of MLL to AF4 (ALL1 fused gene from chromosome 4) and results in acute lymphoblastic leukaemia. MLL is the human homologue of the *Drosophila* trithorax protein and is responsible for methylation of H3K4, which is associated with transcriptional activation and is important for regulating transcription of *Hox* gene clusters (for review see Shilatifard, 2006). AF4 normally associates with the DOT1L methyltransferase to methylate H3K79. It has recently been shown that ectopic H3K79 di-methylation via the MLL-AF4 fusion protein results in aberrant gene

expression in leukaemia as a result of ectopic targeting of DOT1L to incorrect gene promoters by MLL-AF4 (Krivtsov et al., 2008). Aberrant H3K4 tri-methylation, the normal target for MLL, has also been found to be coincident with this mis-targeted H3K79 di-methylation to create large abnormal chromatin domains in MLL-AF4 leukaemia cells which results in mis-regulation of gene expression (Guenther et al., 2008). It is important to note that in addition to altered transcription patterns, changing the normal chromatin structure in this way may render the DNA in these regions more susceptible to damage by genotoxic agents. Furthermore mutations in enzymes responsible for histone modifications that are important for repair of damaged DNA may also result in genomic instability in these cells.

We are now gaining insights into the roles of both novel and well-known histone modifications in the maintenance of genome stability. However, there is still much to be learned and with further research new discoveries will provide us with prognostic and therapeutic tools in the fight against diseases of genomic instability.

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Chromatin Assembly and Signalling the End of DNA Repair Requires Acetylation of Histone H3 on Lysine 56

Thomas Costelloe and Noel F. Lowndes

Abstract The packaging of DNA into chromatin results in a barrier to all DNA transactions. To facilitate transcription, replication and repair histone proteins are frequently post-translational modified. Such covalent additions to histone residues can modulate chromatin folding and/or provide specificity to docking surfaces for non-histone chromatin proteins. In the budding yeast, one such modification, transient acetylation of histone H3 on residue lysine 56 (H3K56ac); occurs on newly synthesized H3 molecules and facilitates their deposition onto newly replicated DNA during S phase. H3K56ac also has a role in chromatin reassembly following DNA damage in S phase. Importantly, the completion of H3K56ac-dependent chromatin reassembly appears to be required for resumption of cell proliferation after DNA repair. Emerging evidence, although not without conflict, suggests that H3K56ac is not only present in human cells, but is similarly regulated and required for chromatin reassembly.

Keywords Chromatin · Histones · Acetylation · H3K56 · DNA repair · Genome stability

Abbreviations

Asf1	Anti-Silencing Function 1
CAF-1	Chromatin Assembly Factor 1
CBP/p300	CREB Binding Protein/ Histone acetyltransferase p300
CPT	Camptothecin
DDR	DNA Damage Response
DSB	Double Strand Break
FACT	Facilitates Chromatin Transcription
HAT	Histone Acetyltransferase
HDAC	Distone Deacetylase

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Hst3/Hst4	Homologue of Sir2 3/4
MMS	Methyl Methane-sulfonate
PH	Pleckstrin Homology
Pob3	Pol1 Binding 3
Rtt109/Rtt106	Regulator of Ty1 Transposition 109/106
Sir2	Silent mating type Information Regulation 2
Top1	Topoisomerase 1

Introduction

The DNA of a single eukaryotic cell is compacted into the cell nucleus as chromatin, a hierarchical scheme of folding. The first level of DNA compaction is the wrapping of 147 bp of DNA into 1.65 left-handed superhelical turns around a histone octamer to create the nucleosome core particle (Davey et al., 2002; Kornberg and Lorch, 1999; Luger et al., 1997). The histone octamer itself is comprised of two molecules each of the histones H2A, H2B, H3 and H4 (Fig. 1). Each histone protein has both

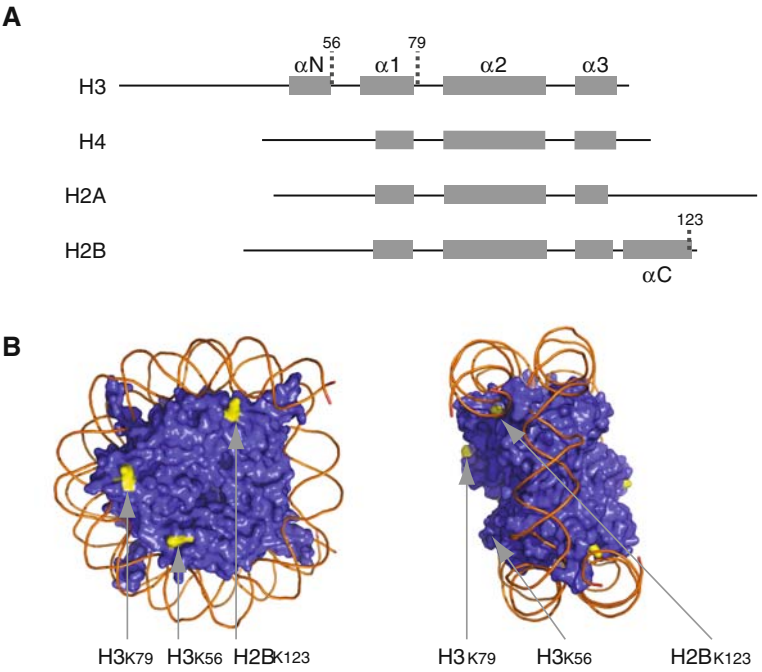

Fig. 1 Histone modifications involved in the DDR. (**a**) Location of H3K56, H3K79 and H2BK123 in the linear histone sequence. (**b**) Location of H3K56, H3K79 and H2BK123 in the nucleosome, indicated in *yellow* and also by *arrows*. Note that the location of H3K79 and H2BK123, two other modifiable lysines in the nucleosome core involved in the DDR, are shown for comparison

an ordered histone fold domain, which mediates histone–histone and histone–DNA interactions that are crucial for the assembly of the nucleosome core particle, and a flexible amino-terminal tail domain, which protrudes from the nucleosome core particle. In higher eukaryotes, nucleosome core particles, measuring approximately 10 nm in diameter, are joined together by variable lengths of linker DNA (roughly between 18 and 65 bp). This linker DNA gives rise to the "beads on a string" appearance upon high salt extraction. Linker histones shield the negative charge of linker DNA to promote folding of chromatin into a higher-order structure known as the 30 nm fiber. Further packaging of nucleosomes into higher-order structures results in the highly condensed state typical of metaphase chromosomes (Schalch et al., 2005).

In response to DNA damage, detection of lesions and repair of DNA must be carried out in this chromatin environment. Therefore, cells have evolved strategies to overcome this chromatin barrier in order to gain access to DNA, including mechanisms to manipulate this chromatin structure. These include covalent histone modifications, ATP-dependent chromatin remodelling and the incorporation of histone variants (Downs et al., 2007; Kouzarides, 2007; Saha et al., 2006).

One of the major means of regulating the chromatin structure is through covalent modification of histone proteins. Histone modifications can increase or decrease the higher order folding of chromatin, and specific histone modifications can alter their chemophysical properties and generate modification-specific docking surfaces for chromatin interacting proteins. A striking feature of histones, and particularly their tails, is the large number and type of modifications that are possible. These include acetylation, phosphorylation, methylation, ubiquitylation, sumoylation and ADP ribosylation (Kouzarides, 2007).

The role of histone modifications in chromatin folding and transcriptional regulation is becoming very well defined. More recently, efforts have begun to elucidate a role for histone marks in the DDR, and this is discussed in detail elsewhere in this volume (see Chubb and Rea, and Pinto and Flaus, and references therein). In this article we focus on the role of acetylation of lysine 56 of histone H3 (termed H3K56ac) in the budding yeast *Saccharomyces cerevisiae*. We also discuss recent evidence consistent with the evolutionary conservation of this modification in human cells and how misregulation of this histone "mark" is implicated in cancer.

Histone Acetylation in the DDR

Along with modifications including phosphorylation, methylation and ubiquitylation, acetylation of histones has been shown to play an important role in the DDR (Table 1). Acetylation of conserved lysine residues on the tails of H3 and H4 is important for normal cell growth and an efficient DDR following treatment with damaging agents such as MMS or breaks induced by the endonuclease *EcoR1* (Bird et al., 2002; Choy and Kron, 2002; Qin and Parthun, 2002). Similarly, mutation of the histone acetyltransferases (HATs) responsible for these acetylation events, NuA4, Gcn5 and Hat1, also confers sensitivity to DSB-inducing agents (Downs

Table 1 Histone modifications involved in the DNA damage response

Histone residue	Modification	Enzyme	Function	References
H2AS129	Phosphorylation	Mec1, Tel1	Stable retention of DDR checkpoint proteins at DSB, DSB repair	Downs et al. (2007)
H3K79	Methylation	Dot1	Rad9 recruitment to DSBs, checkpoint activation. Marks euchromatin	Giannattasio et al. (2005), Grenon et al. (2007), Huyen et al. (2004), van Leeuwen et al. (2002), Wysocki et al. (2005)
H3K4	Methylation	Set1	Checkpoint activation. H3K4me1 localised to silenced chromatin, H3K4me2, H3K4me3 mark 5′ region of active genes	Giannattasio et al. (2005), Wysocki et al. (2005)
H2BK123	Ubiquitylation	Rad6-Bre1	Checkpoint activation	Giannattasio et al. (2005)
H3K56	Acetylation	Rtt109	Modification regulated in cell cycle and DNA damage dependent manner. Required for chromatin assembly following DNA replication and DNA repair. H3K56ac signals checkpoint recovery following DNA damage	Chen et al. (2008), Driscoll et al. (2007), Han et al. (2007), Hyland et al. (2005), Li et al. (2008)
H3K9, K14, K18, K23, K27	Acetylation	Hat1, Gcn5	Acetylation of N-terminal lysines of H3 required for efficient DDR following MMS treatment	Li et al. (2008), Qin and Parthun (2002)
H4K5, K8, K12, K16	Acetylation	NuA4	Acetylation of N-terminal lysines of H4 plays minor roles in DDR following MMS or CPT treatment	Bird et al. (2002), Choy and Kron (2002)

et al., 2004; Qin and Parthun, 2002). In mammalian cells, the Tip60 HAT is also recruited to sites of DSBs, and is required for acetylation of H4 and efficient homologous recombination (HR) (Murr et al., 2006). Similarly, hMOF is required for DNA damage-induced acetylation of H4K16, and defective acetylation of H3 and H4 has been linked to defective cellular responses to DNA damage (see Chubb and Rea, and references therein). Finally, H2A acetylation has also been implicated in the DDR, an event carried out by the HAT NuA4, and shown to be required for efficient repair of DNA damage (Bird et al., 2002; Moore et al., 2007).

H3K56ac in the DDR

More recently, acetylation of H3K56 has been shown to be required for an effective DDR, in particular, after exposure to agents that primarily result in lesions during DNA replication, for example, following treatment with the chemotherapeutic agent, camptothecin (CPT) (Hyland et al., 2005; Masumoto et al., 2005). CPT induces DNA DSBs specifically in S-phase by preventing the transient cleavage and religation of DNA Topoisomerase I (Top1). In a normal S-phase, Top1 acts to prevent the buildup of superhelical strain around the elongating replication fork by transiently cleaving and religating a single strand of duplex DNA via a covalent 3′ phosphotyrosyl enzyme-DNA intermediate. Normally, these cleavage complexes are short-lived intermediates, but CPT stabilizes this complex by slowing the rate of DNA religation (for review see (Pommier et al., 2006)). This CPT-induced stabilized complex can then lead to a DSB if it blocks a replication fork.

In an unperturbed cell cycle, acetylation of histone H3 on lysine 56 is regulated in a cell cycle-dependent manner. H3K56 becomes acetylated on newly synthesised histones during S-phase and is then deposited onto chromatin behind the advancing replication fork. In the absence of DNA damage, this histone modification disappears as cells progress through the G2 phase of the cell cycle (Hyland et al., 2005; Masumoto et al., 2005). However, in the presence of DNA damage in S phase, or particularly after exposure of cells to agents that induce lesions during S phase (e.g. CPT, and MMS), H3K56ac is maintained in a checkpoint-dependent mechanism, i.e. dependent on the Rad9 and Mec1 checkpoint proteins (Masumoto et al., 2005; Thaminy et al., 2007).

Interestingly, the first factor identified as being required for this acetylation event did not exhibit HAT activity. Rather, Asf1 (Anti-Silencing Function 1) is a histone chaperone that binds H3/H4 dimers upon their synthesis and presents these dimers to the H3K56 histone acetyltransferase (HAT) activity (Recht et al., 2006). Subsequently, the specific HAT responsible for H3K56ac was identified in budding yeast as Rtt109, a novel HAT that does not show sequence conservation with other known HATs. Rtt109 was previously identified as a regulator of transposition of the yeast Ty1 retrotransposon (Driscoll et al., 2007; Han et al., 2007). Two homologous histone deacetylases, or HDACs, are responsible for the deacetylation of H3K56ac (Celic et al., 2006; Maas et al., 2006; Miller et al., 2006). Interestingly, these HDACs

are homologues of Sir2 and hence abbreviated, Hst3 and Hst4 (Homologues of Sir Two). Sir2 is a conserved NAD+ dependent histone deacetylase of the Sirtuin family, and is required for silencing at telomeres, the HML and HMR mating type loci, as well as at the rDNA locus. *SIR2* is one of four Silent Information Regulator genes in yeast, but is the only one that is highly conserved from archaea to humans. Sir2 has also been implicated in the DNA damage response, relocalising to sites of DNA damage, and being required for efficient repair of DSBs (Lee et al., 1999).

Hst3 and Hst4 are also cell cycle regulated. In the absence of exogenous DNA damaging agents, Hst3, which is maximally expressed in the late S and G2 phases of the cell cycle, actively deacetylates H3K56ac. Hst4, which is maximally expressed in M and G1 phases of the cell cycle, then maintains the deacetylated state until new histones are synthesised in late G1 and S-phase. However, in the presence of DNA damage, Hst3/Hst4 are phosphorylated in a Mec1-dependent manner, subsequently ubiquitylated and targeted for degradation by the proteosome, thus, in the absence of sufficient histone deacetylase activity, high levels of the normally transient H3K56ac mark are maintained.

Indeed, tight regulation of H3K56 acetylation is required not only for an effective DNA damage response following DNA damage in S phase, but also for normal cell cycle progression in the absence of exogenous damaging agents. Mutations that result in abrogated H3K56ac, such as loss of the modified residue, the HAT or chaperone (*h3K56R, rtt109*Δ and *asf1*Δ, respectively) all result in pleiotrophic cellular phenotypes. These include increased doubling time (at least in part explained by a failure of a subpopulation of cells to undergo mitosis and the resultant loss of these cells from the proliferating population) and sensitivity to genotoxic agents (Celic et al., 2006; Driscoll et al., 2007; Han et al., 2007; Hyland et al., 2005; Masumoto et al., 2005; Recht et al., 2006). Interestingly, mutations that result in hyperacetylation of H3K56, such as loss of the H3K56ac specific HDACs (*hst3*Δ*hst4*Δ), also results in similar cellular phenotypes (Brachmann et al., 1995; Celic et al., 2006; Thaminy et al., 2007). Thus, perturbations that result in either too little or insufficient levels of H3K56ac result in similarly negative consequences for the cells, underlying the importance of exquisite regulation of this important histone mark.

The precise molecular mechanisms underlying the role of H3K56ac in maintaining normal proliferation and genomic stability remained unclear until two recent independent reports began to provide insight into the role of H3K56ac in chromatin assembly. Li and colleagues reported that acetylation of H3K56 is required for chromatin assembly following DNA replication (Li et al., 2008). They present evidence indicating that acetylation of H3K56, dependent on the Rtt109 HAT and Asf1 histone chaperone, facilitates nucleosome assembly by increasing the affinity of CAF-1 and Rtt106 for H3/H4 dimers which facilitates their deposition onto newly synthesised DNA (Li et al., 2008). CAF-1 (Chromatin assembly Factor-1) is a conserved three subunit protein complex (consisting of Cac1, Cac2 and Cac3) that has previously been shown to deposit newly synthesised H3 and H4 onto replicating DNA to

form nucleosomes (Groth et al., 2007). This histone deposition also requires the histone chaperones Asf1 and Rtt106 (Regulator of Ty1 Transposition 106). Evidence from Li and colleagues suggests that following acetylation of H3K56, mediated by Rtt109 and Asf1, H3K56ac-H4 dimers are transferred to CAF-1 and Rtt106, which bind preferentially to H3K56ac, ensuring rapid formation of $(H3/H4)_2$ tetramers and subsequent nucleosome formation (Li et al., 2008).

Importantly, Li and colleagues have identified a specific region of Rtt106 that recognises H3K56ac (Li et al., 2008). This region bears homology to a domain of a protein termed Pob3 (Pol1 Binding) that adopts a structure related to the Pleckstrin Homology domain (an approximately 100–120 residue structure implicated in intracellular signalling by binding phosphatidylinositol lipids). Pob3 is a subunit of FACT (Facilitates Chromatin Transcription), an abundant nuclear complex that can destabilise the interaction between the H2A/H2B dimer and the H3/H4 tetramer of the nucleosome and therefore promotes nucleosome assembly and disassembly during transcription, replication and other processes that traverse chromatin. In fact, Li and colleagues have shown that FACT copurifies with H3K56ac, suggesting that this PH-like domain represents a new family of acetyl-lysine binding motifs (Li et al., 2008).

An independent report from the Tyler lab has also provided evidence for a role of H3K56ac in chromatin assembly following DNA damage (Chen et al., 2008). The authors found that H3K56ac, Asf1 and Rtt109 were required for chromatin reassembly following repair of an HO-induced DSB. Surprisingly, even though the *h3K56R*, *asf1Δ* and *rtt109Δ* mutants all display sensitivity to DNA damaging agents, their data indicated that DNA repair of the HO-induced DSB was successfully completed. Cell death appears to be a consequence of failure of the mutant cells to recover from the DNA damage checkpoint. Interestingly, the defect in chromatin reassembly observed in the *asf1Δ* mutant was rescued by introducing the acetyl-mimic *h3K56Q* mutation in these cells, supporting their observation that the nucleosome reassembly at sites of DNA damage is dependent on the H3K56ac pathway.

A possible explanation for these data is that during chromatin assembly/reassembly histone H3 lysine 56 acetylation following DNA replication or repair signals the completion of chromatin assembly/reassembly (Fig. 2). In the case of DNA replication, newly synthesised free histone H3 molecules (as H3/H4 dimers) are acetylated on residue K56, which increases their affinity for the CAF1 which is required for re-incorporating newly synthesised histones into new nucleosomes behind the replication fork (Fig. 2c). Removal of this acetyl mark is dependent on the HDACS, Hst3 and Hst4 (Fig. 2d). However, following DNA damage, these HDACs are down regulated in a Mec1-dependent manner, resulting in persistence of the H3K56ac histone mark. Upon completion of DNA repair, this mark is required for proper reassembly of chromatin and, intriguingly, required for the signal that repair is complete and the consequent down-regulation (often termed recovery) of the DNA damage checkpoint (Fig. 2d). Thus, in budding yeast at least, it is reformation of normal chromatin, rather than DNA damage per se, that signals the end of the DNA damage response.

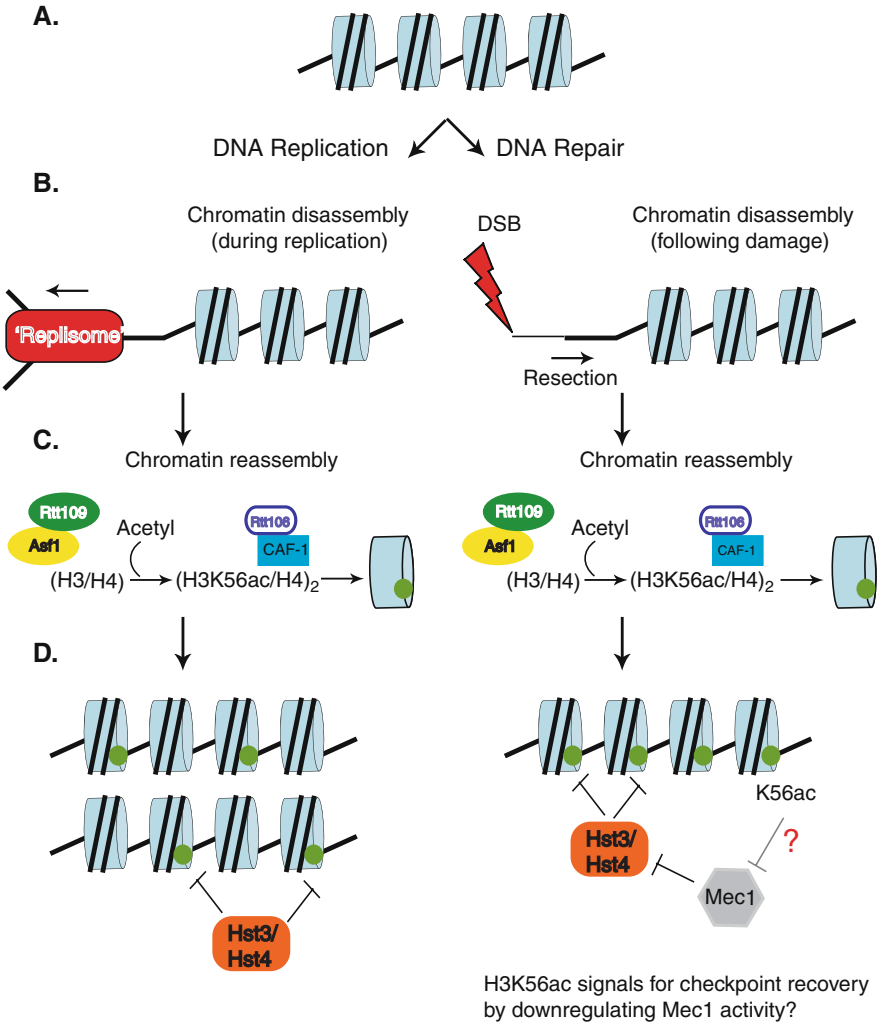

Fig. 2 Model for the role of H3K56ac in chromatin reassembly following DNA replication or DNA damage. (**a**) and (**b**) Following either DNA replication (*left*) or DNA damage (*right*), H3K56ac is required for chromatin reassembly. (**c**) Acetylation of newly synthesised H3 depends on the actions of the HAT Rtt109 and the histone chaperone Asf1. H3/H4 dimers, containing H3K56ac are then passed to CAF-1 and are incorporated into nucleosomes. (**d**) (*Left*) H3K56 acetylation levels reach a maximum of 50% during S-phase, before being deacetylated by the HDACs Hst3/Hst4 as cells progress through G2 phase of the cell cycle. (*Right*) H3K56ac is incorporated into newly reassembled nucleosomes at sites of DNA damage upon completion of repair. This localised increase in H3K56ac around a break site signals checkpoint recovery, possibly by downregulating Mec1 activity. Decreased Mec1 activity in turn leads to increased HDAC activity and subsequent removal of the acetyl mark. *Green circles* indicate H3K56ac

H3K56ac in Human Cells

In addition to the established requirement of H3K56ac in yeast, previous evidence had demonstrated the presence of H3K56ac in *Drosophila* (Xu et al., 2005). However, until recently it had not been known if the functions associated with this modification extended to higher eukaryotes. Three reports demonstrate that not only is H3K56ac preserved in human cells, but that this mark also appears to be regulated in a DNA damage-dependent manner (Das et al., 2009; Tjeertes et al., 2009; Yuan et al., 2009). Two of these studies indicate that, as is the case in yeast cells, H3K56ac increases in response to DNA damaging agents, including MMS and CPT which specifically cause DNA damage in S-phase (Das et al., 2009; Yuan et al., 2009).

The Tyler group have also shown that the HATs responsible for H3K56ac in higher eukaryotic cells are CBP in *Drosophila* and CBP/p300 in human cells (Das et al., 2009). The structurally related CBP and p300 proteins are known to function with numerous transcription factors as transcriptional co-activators. They are only distantly related to yeast Rtt109, contain HAT domains, as well as numerous protein interaction domains connected by long stretches of unstructured regions. Similarly, homologues of yeast Asf1 (termed Asf1 in *Drosophila* and ASF1A (but not ASF1B) in human cells) and the histone chaperone CAF1 (termed Caf1 and CAF-1 in *Drosophila* and human cells, respectively) are also required for formation of H3K56ac and chromatin assembly/reassembly in vivo (Das et al., 2009; Yuan et al., 2009). Moreover, analogously to the deacetylation of H3K56ac by the yeast Sir2-related HDACs, Hst3 and Hst4, deacetylation of H3K56ac is catalysed by homologous Sirtuin proteins; Sir2, in *Drosophila* and SIRT1 and SIRT2 in human cells, (Das et al., 2009; Yuan et al., 2009).

Despite these similarities some conflicting evidence remains to be resolved in these recent reports. The Lou group report that, as is the case in yeast cells, H3K56 is preferentially acetylated during S-phase (Yuan et al., 2009), whereas the Tyler group report that H3K56ac appears to be present throughout the cell cycle; even though the HAT activity of CBP is highest, at the G1/S transition, when newly synthesised histones are present (Das et al., 2009). In addition, Yuan et al. report that the major HDAC in human cells, SIRT1, is expressed throughout the cell cycle. Thus the precise mechanism of histone H3 lysine 56 deacetylation remains to be elucidated.

Interestingly, increased acetylation of histone H3 on lysine 56, as well as upregulated expression of its positive regulator, ASF1A, has been observed in many cancers (Das et al., 2009). It is not yet known whether deacetylation of H3K56ac is defective in some cancer cells displaying elevated levels of H3K56ac. As either too much or too little H3K56ac results in similar phenotypes in yeast, elevated deacetylation of H3K56ac might even occur in any cancers that display abnormally low levels of this histone mark. Regardless of the possibility of abnormally low levels of H3K56 acetylation in some cancers, a direct correlation between tumourigenicity and increased levels of the H3K56ac histone mark has now been defined. Similarly to yeast, increased H3K56ac levels would promote chromatin assembly/reassembly and might reflect underlying high rates of endogenous DNA damage in cancer

cells. However, they could also be causative, as elevated levels of H3K56ac may increase overall genome instability. On the other hand, abnormally low levels of H3K56ac would negatively impact on processes that traverse chromatin such as replication and repair, and thus also increase overall genome instability. Whether H3K56ac also regulates checkpoint recovery in higher cells remains to be determined and deregulated checkpoint recovery could also be a contributing factor in carcinogenesis.

Confoundingly, an independent study has shown that, although H3K56ac appears to be present throughout the cell cycle in human cells, the levels of H3K56ac actually decrease following DNA damage (Tjeertes et al., 2009). The authors also address the differing results found by the Tyler group, but did not detect increased H3K56ac in any of their experiments, and the discrepancies between the findings of these two groups remains unresolved. The authors also provide evidence for a role of human GCN5 in acetylating H3K56. Gcn5 has also previously been shown to be required for acetylating lysine residues in the tail of H3 in yeast, and mutating yeast Gcn5 or the H3 lysine residues caused sensitivity to DNA damaging agents (Choy and Kron, 2002). In the current study, the Jackson laboratory have shown that knocking down hGCN5 using siRNA leads to a reduction, although not a complete loss, of H3K56ac (Tjeertes et al., 2009). The authors also showed a decrease in H3K56ac following knockdown of human p300, although to a lesser extent than knockdown of GCN5. Thus, it appears that although GCN5 has HAT activity towards H3K56, it may have redundant roles with other HATs, including CBP and p300.

Conclusion

From pioneering work in the budding yeast, it is now obvious that acetylation of histone H3 on lysine 56 (H3K56ac) plays an extremely important role in genome stability. It is central to the regulation of chromatin assembly and reassembly that must occur during DNA replication and after DNA repair. Recent studies have provided solid evidence that this modification is conserved in humans, although some ambiguities remain to be resolved. Importantly, abnormally high levels have been reported in some human cancers and the hypothesis that other cancers might have abnormally low levels of H3K56ac remains to be supported by experimental evidence. The fact that cells with over or under regulation of this histone mark are highly sensitive to agents that specifically induce damage during DNA synthesis, such as camptothecin (derivatives of which are commonly used as chemotherapeutic agents), also stresses the importance of this mark. The recent elucidation of the functions of this histone post-translational modification in yeast and higher eukaryotes has been dramatic. No doubt the near future will answer some remaining outstanding questions, including the requirement for H3K56ac in chromatin assembly and chromatin reassembly, as well as a potential role in checkpoint recovery in human cells, analogously to yeast cells. These answers should enhance our understanding

of, as well as bringing further surprises relevant to, the mechanism and function of this modification.

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Structure and Function of Histone H2AX

David Miguel Susano Pinto and Andrew Flaus

Abstract Histone H2AX is a histone variant found in almost all eukaryotes. It makes a central contribution to genome stability through its role in the signaling of DNA damage events and by acting as a foundation for the assembly of repair foci. The H2AX protein sequence is highly similar and in some cases overlapping with replication-dependent canonical H2A, yet the H2AX gene and protein structures exhibit a number of features specific to the role of this histone in DNA repair. The most well known of these is a specific serine at the extreme C-terminus of H2AX which is phosphorylated by Phosphoinositide-3-Kinase-related protein Kinases (PIKKs) to generate the γH2AX mark. However, recent studies have demonstrated that phosphorylation, ubiquitylation and other post-translational modifications are also crucial for function. H2AX transcript properties suggest a capability to respond to damage events. Furthermore, the biochemical properties of H2AX protein within the nucleosome structure and its distribution within chromatin also point to features linked to its role in the DNA damage response. In particular, the theoretical inter-nucleosomal spacing of H2AX and the potential implications of amino acid residues distinguishing H2AX from canonical H2A in structure and dynamics are considered in detail. This review summarises current understanding of H2AX from a structure–function perspective.

Keywords Histone · H2AX · Structure–function · Chromatin structure · DSB · DNA repair

Abbreviations

DSB	double strand break
DDR	DNA damage response
HDE	histone downstream element

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HR	homologous recombination
IR	ionising radiation
NHEJ	non-homologous end joining
PIKK	phosphoinositide-3-kinase-related protein kinase
PTM	post-translation modification
SHL	superhelical location
SLBP	stem–loop binding protein
snRNA	small nuclear RNA
TSS	transcription start site

Introduction

Maintenance of the genome stability is of great importance to all organisms because DNA damage can have serious biological implications including genetic disorders and cancer (McKinnon and Caldecott, 2007). One mechanism for maintaining genome stability is to increase DNA repair (Lengauer et al., 1998) and an important paradigm for DNA repair is the mechanism for identifying and facilitating re-ligation of DNA Double Strand Breaks (DSBs). DSBs are one of the most serious forms of DNA damage because they involve loss of genetic continuity. They arise from a variety of causes including not only the action of DNA damaging agents but also normal functions such as meiosis and antibody class switching. An important player in the DNA Damage Response (DDR) for dealing with DSBs is the histone variant H2AX, which is an integral component of the chromatin packaging of eukaryotic genomes.

Chromatin Structure and Genome Stability

Eukaryotic DNA is not dispersed randomly within the cell nucleus. Instead, it is packaged into the chromatin structure which compacts DNA and organises accessibility to the genome. This chromatin packaging is hierarchical, based on the nucleosome as a fundamental building block. The canonical nucleosome comprises two copies of each core histone (H2A, H2B, H3 and H4) around which 147 bp of DNA is wound in a superhelical spiral (Davey et al., 2002). The nucleosomes are connected by short DNA linkers to form repeating units which subsequently arrange in a number of higher-order structural levels up to condensed metaphase chromosomes.

Despite its modular structure, the arrangement of chromatin is not static and must be “remodeled” during nuclear processes including DNA repair. This remodeling is driven by two general mechanisms: Firstly, molecules such as ATP-dependent remodelers and chromatin-binding proteins can directly modify the structure. Secondly, physiochemical properties of chromatin can be modulated by post-translational modification or insertion of histone variants to alter its stability (Felsenfeld and Groudine, 2003; Ausió, 2006).

H2AX and DNA Repair

H2AX and another histone variant, H2A.Z, were both identified in human cells by their different migration compared to canonical H2A isoforms on SDS and acetic acid–urea gels (West and Bonner, 1980). In this separation, H2AX and H2A.Z were two of four unidentified species arbitrarily labeled T, W, X and Z. Subsequently, it was found that T and W were the ubiquitylated forms of X and Z (West and Bonner, 1980). H2A.Y is an alternative name for macroH2A1. Although originally labeled as H2A.X, the internal period (".") separating the X has fallen into disuse so the H2AX name is almost universally used in the DNA repair field. In contrast, the internal period has historically been retained in H2A.Z, whose major roles have been subsequently associated with transcription (Ausió, 2006).

A distinct function for H2AX was uncovered some 18 years after its initial identification when human and mouse serine 139 was observed to be rapidly phosphorylated in response to treatments that cause DSBs (Rogakou et al., 1998). In structural nomenclature, the phosphorylation occurs on the serine oxygen in the gamma position so the modified form is widely referred to as γH2AX. This γH2AX phosphoprotein is found to be rapidly concentrated around DSBs in centers termed "foci" that can extend for a range of up to 2 Mbp away from the damage site (Rogakou et al., 1999).

The amino acid region surrounding serine 139 matches the consensus recognition sequence for a set of PhosphoInositide-3-Kinase-related protein Kinases (PIKKs) known to be central in the DNA damage response from genetic studies in yeast (Downs et al., 2000). The link between PIKKs and γH2AX formation has been directly demonstrated by biochemical inhibition using mutagenesis in yeast (Downs et al., 2000) and wortmannin in higher cells (Paull et al., 2000).

γH2AX is a widely recognised participant in DSB repair and is one of the earliest markers of damage (Pilch et al., 2003). Other DNA repair-related proteins subsequently congregate at the γH2AX foci during the repair process. Although their recruitment to DSBs is not completely dependent on H2AX phosphorylation, H2AX is an important element in proper damage response foci formation by enhancing the retention of repair factors after their initial recruitment (Celeste et al., 2003b). $H2AX^{-/-}$ mice have moderate defects including radiation sensitivity, growth retardation and immunodeficiency which are consistent with deficiencies in DNA repair (Celeste et al., 2002, 2003a). Importantly, these phenotypes are only moderate and suggest redundancy for the role of H2AX. Nevertheless, karyotypes of H2AX-deleted genomes also reveal a high number of translocations and chromosome rearrangements directly demonstrating increased genomic instability.

Structural Properties of H2AX

Based on the linkage between the early H2AX phosphorylation event and the DDR, a large number of studies have focussed on γH2AX and its subsequent interactions

with the repair mechanism. Less consideration has been given to the biochemical properties of H2AX itself.

H2AX is one of a set of histone H2A proteins encoded in eukaryotic genomes, the human genome holding 21 genes of H2A forms. The canonical human H2A has two biochemically separable isoforms, H2A.1 and H2A.2. No functional difference between those isoforms is known, and the basis of their distinction appears to be dependent on residue 51 encoding, respectively, either a leucine or methionine despite further heterogeneity within each isoform (Bonenfant et al., 2006; Marzluff et al., 2002). Four additional H2A variants with distinct functions are encoded in humans and other higher eukaryotes: H2AX, H2A.Z, macroH2A1, macroH2A2, H2A.F/Z and H2ABbd (Marzluff et al., 2002).

Definition of H2AX

The H2AX variant is principally defined by the capacity to accept phosphorylation on a serine near the C-terminus through the activity of PIKKs such as ATM, ATR and DNA-PK on the consensus motif SQ[E/D]Φ (where Φ is a hydrophobic residue). The number of residues separating this motif from the core histone fold region is variable and has been claimed to correlate with the evolutionary complexity of the organism (Redon et al., 2002). For example, the spacing of 29 residues between the end of the H2AX α3 helix and the phospho serine in *Saccharomyces cerevisiae* and *Giardia lamblia* is 12 residues shorter than in humans and mice.

In higher eukaryotes, H2AX is encoded as a separate histone variant of H2A but in lower organisms such as *S. cerevisiae*, *G. lamblia* and certain protists, the distinguishing H2AX features are merged into the canonical H2A (Malik and Henikoff, 2003; Sullivan et al., 2006) so that the canonical H2A also acts as the H2AX variant. In *Drosophila melanogaster*, the H2AX feature is instead merged with H2A.Z as a single variant, H2AvD, that is distinct from the canonical H2A (Madigan et al., 2002).

Based on phylogenetic analysis, it has been suggested that H2AX appeared multiple times in eukaryotes as an example of parallel evolution (Malik and Henikoff, 2003). However, the differences between metazoan H2AX and canonical H2A sequences are few in number and this could be confounding to phylogenetic algorithms. An alternative hypothesis is that the H2AX function is ancestral and canonical H2A evolved from the H2AX when complete phosphorylation became unnecessary or undesirable as genomes expanded. This would explain the preeminence of the H2AX variant in *G. lamblia* and *S. cerevisiae* compared to the lower abundance in mammals.

It has remained something of a puzzle that no H2AX variant function is identifiable in *Caenorhabditis elegans* (Malik and Henikoff, 2003) or some protists (Sullivan et al., 2006). A search of all predicted *C. elegans* histones protein sequences reveals no PIKK consensus motifs in the coding sequence or in any frame downstream of the annotated stop codons for any of the core histone genes (data not

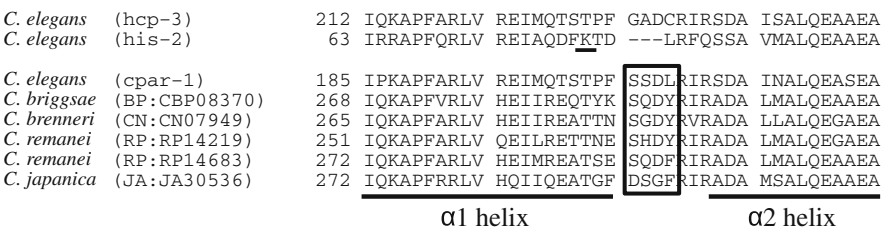

Fig. 1 Alignment of *Caenorhabditis* CENP-A homologues showing conservation of possible PIKK recognition site inserted within H3 structure. The major *C. elegans* CENP-A homologue (hcp-3) and a canonical H3 isoform (his-2) with lysine 79 *underlined* are shown above

shown). However, the related *C. briggsae* genome contains the motif SQDY within the cpar-1 isoform of CENP-A, the centromeric H3-like histone. Alignment of seven known *Caenorhabditis* CENP-A homologues shows that this motif is quite conserved, with the sequence being SSDL in *C. elegans* cpar-1 (Fig. 1). Although these do not strictly conform to the classic PIKK recognition site sequence, non-canonical sites are known to be recognised by them (Sweeney et al., 2005).

This potential merger of H2AX with CENP-A is interesting for several reasons: Firstly, *C. elegans* utilises holocentric chromosomes so cpar-1 is thought to be distributed throughout the genome (Monen et al., 2005). Secondly, cpar-1 appears to be more weakly expressed than the other CENP-A homologue in the *C. elegans* genome, hcp-3 (Monen et al., 2005), recalling the H2AX/H2A ratio in human and mouse chromatin. Finally, the SQDY/SSDL motif is located at small 3-4 residue insertion unique to the core histone fold of CENP-A family proteins. This insertion immediately abuts lysine 79 of canonical H3 which is also implicated in the DDR (Fig. 1). CENP-A family proteins do not have lysine at the equivalent of position 79 of canonical H3.

H2AX Gene

Canonical histone genes in humans are spread over one large and two small clusters named HIST1, HIST2 and HIST3. These are located at 6p21–p22, 1q21 and 1q42 respectively (Table 1). Canonical H2A is encoded by sixteen genes, twelve of which are located in HIST1, three in HIST2 and one in HIST3. H2A variants are located outside these histone clusters in the human genome, with the H2AX-encoding gene H2AFX at 11q23.2–11q23.3 (Ivanova et al., 1994b) (Table 1). Histone variant gene names typically include the letter F for family.

The H2AFX promoter region, 151 bp upstream from the transcription start site, shows higher activity than the typical canonical H2A.1 HIST1H2AE promoter in transcription reporter assays (Ivanova et al., 1994a). There are two CCAAT elements upstream of the TATA box in H2AFX (Fig. 2) compared to a single CCAAT element in the H2A.1. The CCAAT element proximal to the TATA box

Table 1 Localisation of all human canonical and variant H2A genes and proteins (adapted from Marzluff et al., 2002)

Histone cluster	Gene	Protein	Locus
HIST1	H2A A–E, G–M	H2A.1	6p21–22
HIST2	H2A A–C	H2A.1 and H2A.2	1q21
HIST3	H2A	H2A.1	1q42
–	H2AFB3	H2ABbd	Xq28
–	H2AFJ	macroH2A2	12p12
–	H2AFV	H2A.F/Z	7p13
–	H2AFX	H2AX	11q23.2–11q23.3
–	H2AFY	macroH2A1	5q31.3–q32
–	H2AFZ	H2A.Z	4q24

in H2AFX has a significant effect on expression, whereas this element has no apparent effect on promoter activity in the canonical H2A promoter. The transcription factors that bind to the element also bind to the distal CCAAT as well as to three similar elements in H2AFZ but not to the one in the H2A.1 promoter (Ivanova et al., 1994a). This suggests that H2AFX is regulated independently of canonical H2A.

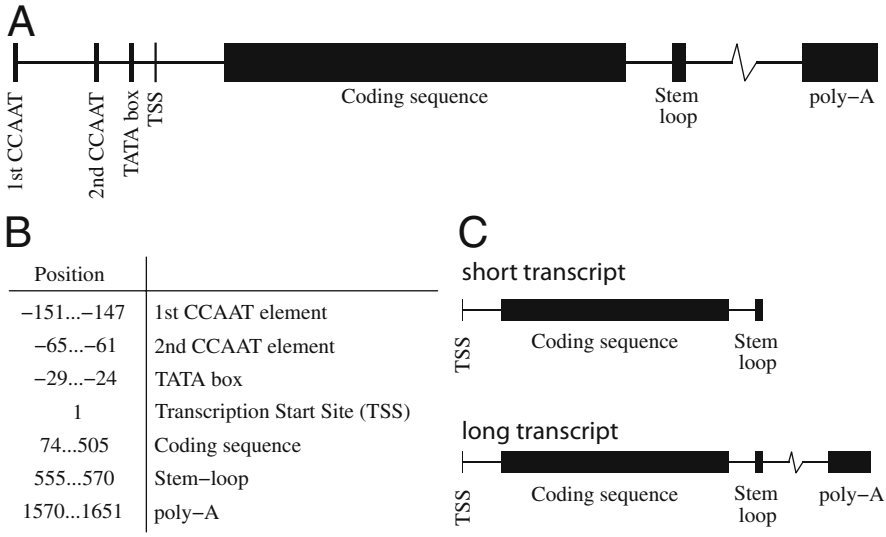

Fig. 2 H2AFX gene and transcripts. **a**. Schematic of H2AFX gene region showing promoter and 3′ mRNA stabilizing elements. **b**. Sequence coordinates of each element in H2AFX relative to transcription start site. **c**. Alternative transcripts of H2AFX. The short transcript (≈600 bp in size) ends in a stem–loop like canonical histones, whereas the long transcript (≈1,600 bp in size) ends in a poly-(A) tail

H2AX Transcripts

A fundamental distinction between histone types is whether their expression is replication-dependent or replication-independent. This difference is a consequence of the requirement for large amounts of canonical histones during S phase to package the newly duplicated genome (i.e. replication-dependence). In contrast, variant or "replacement" histones often appear to be inserted into chromatin to replace canonical histones for functional reasons throughout the cell cycle and are therefore replication-independent (Marzluff et al., 2002).

Canonical histone genes lack introns, probably to circumvent the requirement for primary transcript processing when histones must be rapidly produced at S phase. A number of transcript features appear to enhance the capacity of replication-dependent histone expression by up to 35-fold during S phase. In fact, there is only a 5-fold increase in their transcription rate at S phase, compared to the other phases of cell cycle so regulation acts strongly at the post-transcriptional level (Harris et al., 1991). Replication-dependent histone transcripts lack a poly(A) tail and encode a stem–loop followed by a purine-rich Histone Downstream Element (HDE) downstream of the stop codon. The stem–loop interacts with the Stem–Loop Binding Protein (SLBP) to stabilise the mRNA in S phase (Whitfield et al., 2000) while the HDE interacts with U7 snRNA to direct efficient 3′ end processing (Georgiev and Birnstiel, 1985).

Human H2AX transcripts exhibit characteristics of both replication-dependent and replication-independent histones. The H2AFX gene lacks introns, and has two alternative transcripts: one shorter form contains the characteristic stem–loop, and the other longer form contains a downstream poly(A) tail (Mannironi et al., 1989) (Fig. 2). The combined synthesis of H2AX transcripts has been described as "weakly replication-linked at best" since the H2AFX promoter keeps the levels of both transcripts high through the cell cycle (Ivanova et al., 1994a). However, the cell cycle linkage of the forms is unclear and no study has reported the effect of DNA damage on transcription levels.

H2AX Protein

Despite the large amount of attention paid to the DNA damage-linked serine phosphorylation by PIKKs, the H2AX protein itself has a number of additional unique properties.

The defining feature of H2AX is considered to be the C-terminal region with the SQ[E/D]Φ motif (Fig. 3). As mentioned in section "Definition of H2AX", the number of residues separating this motif from the histone fold is variable and claimed to correlate with the evolutionary complexity of the organism (Redon et al., 2002). The residues responsible for this variable spacing are mainly hydrophilic with a high glycine and proline content suggesting a flexible, unstructured nature so the basis for the correlation could be more directly related to a structural constraint such as the

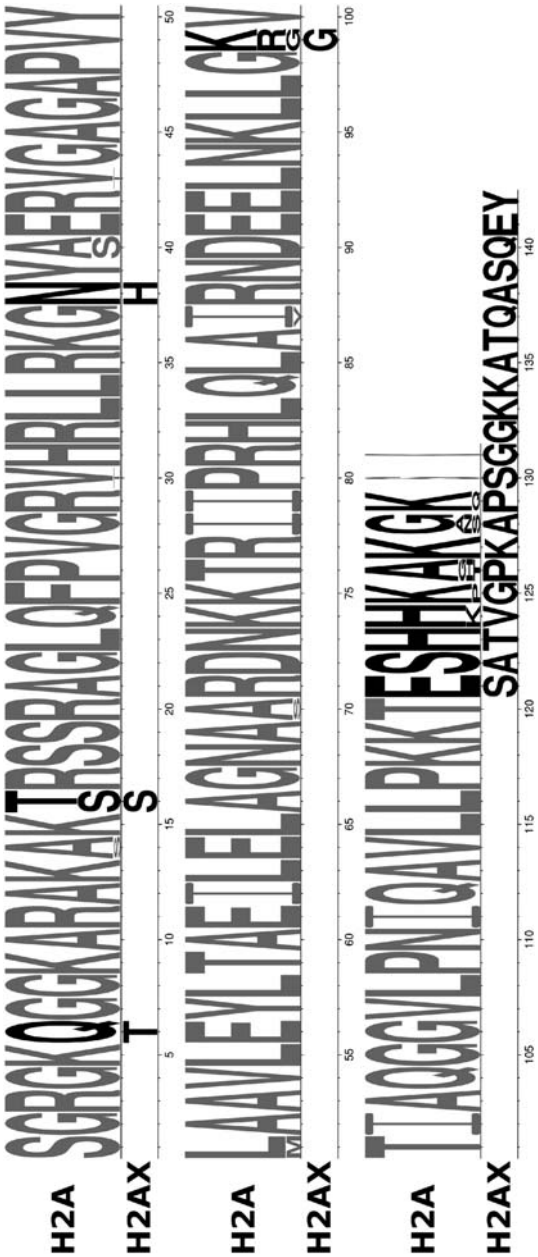

Fig. 3 Sequence logo of all human canonical H2A isoforms showing differences with H2AX below. The 4 residues changes from H2A to H2AX outside the C-terminal region are Gln6Thr, Thr16Ser, Asn38His and Lys99Gly. Alignment of H2A genes was made usign edialign (Morgenstern, 1999) from EMBOSS (Rice et al., 2000) and WebLogo 3 (Crooks et al., 2004).

variation in internucleosomal repeat lengths of organisms which itself shows linkage with evolutionary complexity.

In addition to the C-terminal motif, amino acid residues 6, 16, 38 and 99 of H2AX are different from the human H2A.1 consensus (Figs. 3 and 4). Inspection of the human and *X. laevis* histone based nucleosome structures reveals that H2A

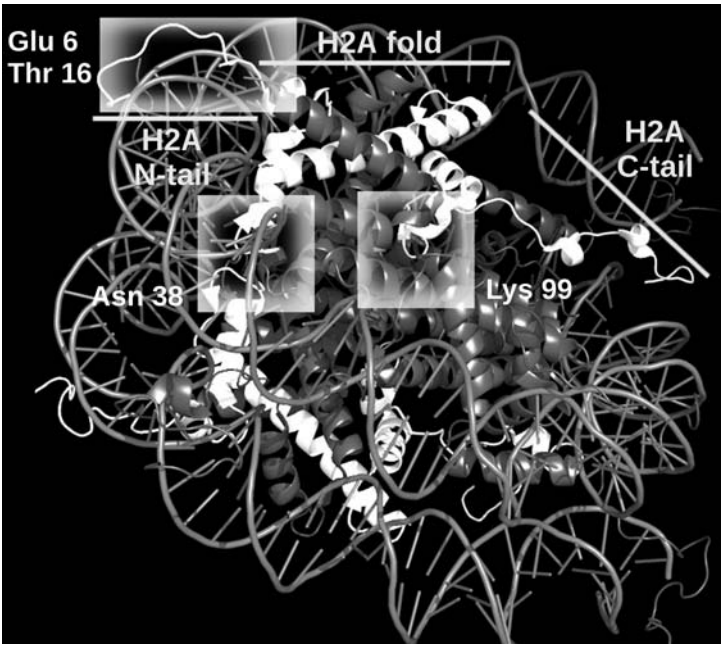

Fig. 4 Nucleosome structure highlighting differences between H2A and H2AX. H2A chain is highlighted and *white frames* indicate the position of the residues that differ between the human canonical H2A and H2AX. Image from PDB structure 1KX5 using PyMOL (DeLano, 2002).

residue 6 is located in the flexible N-terminal tail and residue 16 is located at the very base of the tail (Figs. 4 and 5b) which tracks the minor groove at superhelical location 4.5 (SHL4.5) (Fig. 5a). The substitution of glutamine with threonine at position 6 in H2AX introduces a potential hydroxyl site for post-translational modification that is not present for the glutamine in canonical H2A. In contrast, the threonine to serine substitution conserves the modifiable hydroxyl at position 16.

Asparagine 38 is located in the loop between the α1 and α2 helices of H2A within the nucleosome (Figs. 4 and 5c). Importantly, this residue makes direct contact with the equivalent amino acid in the other H2A–H2B dimer in the nucleosome structure and has been suggested to affect both nucleosome stability and the balance between homotypic and heterotypic combinations (see section "H2AX Distribution in Chromatin") of H2A types within the yeast nucleosome (White et al., 2001). It is possible that the change of residue 38 from asparagine in H2A to histidine in H2AX could also affect nucleosome stability and dynamics. For example, weakening of interactions between the two H2A–H2B histone fold dimers could result in increased nucleosome flexing and impact the ability to condense into stable higher order chromatin structure. Furthermore, the presence of the histidine in H2AX could affect the stabilisation of a second copy of H2AX relative to canonical H2A within the nucleosome. This change of asparagine to histidine at position 38 occurs only in higher organism H2AX and could potentially drive a bias towards either homotypic H2AX-only or heterotypic H2AX–H2A mixed nucleosomes which could

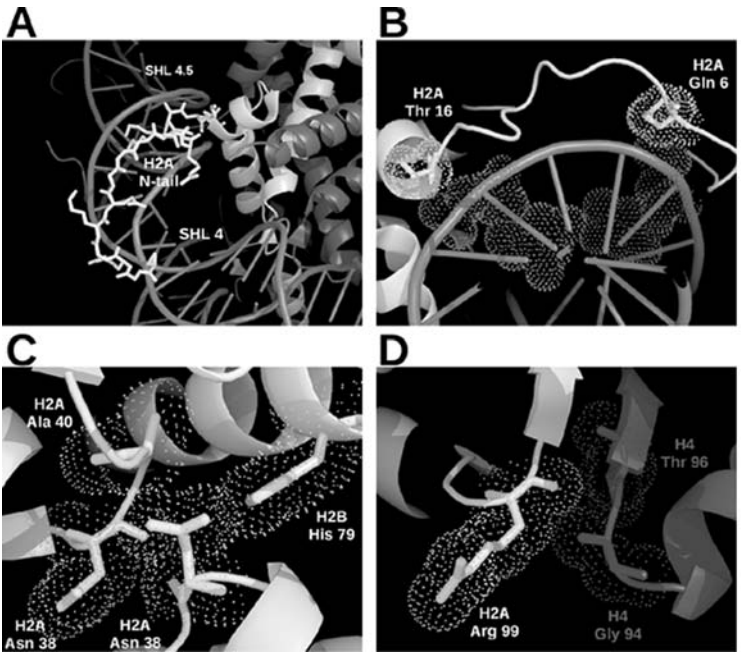

Fig. 5 Structural environment of H2A residues that differ from H2AX. The van der Waals surface of differences and all residues within 5Å are shown as a surface of *dots* over bond sticks. Image from PDB structure 1KX5 using PyMOL (DeLano, 2002). **a**. H2A N-terminal tail encompassing H2AX residues Thr6 and Ser16 passes across minor groove at superhelical location (SHL) 4.5. **b**. Closeup of H2A N-terminal tail minor groove association from A showing canonical H2A Gln6 and Thr16 which become, respectively, Thr6 and Ser16 in H2AX. **c**. Residues around H2A–H2A association in structure showing interaction between paired Asn38 sidechains and adjacent residues. Canonical H2A Asn38 is His38 in mammalian H2AX. **d**. Environment around H2A Arg99 showing unusual absence of close packing. Canonical H2A Arg99 is Gly99 in H2AX

have consequences for the distribution of H2AX in chromatin (see section "H2AX Distribution in Chromatin").

The effect of the final substitution distinguishing canonical H2A and H2AX where lysine becomes glycine at position 99 is less clear. This residue is located in a sharp turn immediately after the α3 helix and points towards the C-terminal ends of H3 and H4 but makes no direct interactions in the nucleosome (Figs. 4 and 5d). Nevertheless, the exchange of the large, positively charged and potentially modifiable lysine for the highly flexible glycine in H2AX could potentially alter stability and flexibility of the nucleosome.

H2AX Post-translational Modifications

Histones typically have a large proportion of amino acid residues which are modified post-translationally for functional reasons so it is significant that three of the four residues distinguishing human H2A and H2AX in the core region are capable of

distinction via post-translational modification (i.e. Thr6 and Ser16 in H2AX vs. Thr16 and Lys99 in canonical H2A).

However, only the phosphorylation of H2AX serine 139 by PIKKs in response to DNA damage has been intensively studied. This modification has been demonstrated to enhance access of restriction enzymes and DNA methylases to the DNA, possibly by reducing nucleosome stability (Heo et al., 2008). In the same study the activity of the FACT complex which can facilitate dissociation of H2A/H2B dimers from nucleosomes was shown to increase after H2AX phosphorylation.

One of the most recently reported post-translational modifications of H2AX related to DSB is the phosphorylation of tyrosine 142 in the PIKK recognition motif of human H2AX (Xiao et al., 2009; Cook et al., 2009). In contrast to the phosphorylation of Ser139, this Tyr142 residue is phosphorylated under normal conditions with DNA damage acting as trigger for its dephosphorylation. The dephosphorylation seems to not only precede the phosphorylation of Ser139, but also to be a prerequisite for the Ser139 phosphorylation. When Tyr142 is phosphorylated, affinity of Ser139 to the DNA damage response factors MDC1, MRE11 and Rad50 is greatly reduced and binding by pro-apoptopic factor JNK1 was found to occur instead. It has therefore been suggested that the phosphorylation status of Tyr142 is a determinant of cell fate after DNA damage.

H2AX can also be subject of acetylation at lysine 5 (Pantazis and Bonner, 1981) and to both mono- and poly-ubiquitylation at lysine 119 dependent on the prior acetylation at Lys5 (Table 2) (Ikura et al., 2007). These modifications are intimately related to DNA repair because their levels increase significantly after exposure to DSB-inducing Ionising Radiation (IR) and appear to drive H2AX eviction from the nucleosome by the action of Tip60 complex and UBC13 (Ikura et al., 2007). However, conflicting data about the interdependence of these effects with phosphorylation has recently been reported (Rios-Doria et al., 2009).

Other modifications unrelated to DNA damage have been reported for H2AX, including a rather unusual biotinylation of Lys9 and Lys13 (Chew et al., 2006) and

Table 2 Reported post-translational modifications (PTMs) for H2AX. Other PTMs present in H2A but not yet related to H2AX include acetylation of lysine 9 and 13 (Zhang et al., 2003), phosphorylation of threonine 120 (Aihara et al., 2004), and the possible methylation of lysine 127 (Zhang et al., 2003)

Residue number	Residue indentity	PTM	Related to DSB	References
1	Serine	Phosphorylation	No	Pantazis and Bonner (1981)
5	Lysine	Acetylation	Yes	Pantazis and Bonner (1981) and Ikura et al. (2007)
9	Lysine	Biotinylation	No	Chew et al. (2006)
13	Lysine	Biotinylation	No	Chew et al. (2006)
119	Lysine	Ubiquitylation	Yes	Ikura et al. (2007)
139	Serine	Phosphorylation	Yes	Rogakou et al. (1998)
142	Tyrosine	Phosphorylation	Yes	Xiao et al. (2009)

the phosphorylation of Ser1 (Pantazis and Bonner, 1981). By homology to canonical H2A, it is probable that Lys9 and Lys13 can also be acetylated (Zhang et al., 2003) and Thr120 phosphorylated (Aihara et al., 2004). Another interesting possible post-translational modification is a methylation at Lys127 (Zhang et al., 2003). Although it was inconclusive whether Lys125 or Lys127 is the target of this methylation, it is tempting to speculate that it occurs at Lys127 since this residue is the only one conserved in the C-terminal of all human H2A sequences (Fig. 3).

H2AX Distribution in Chromatin

The original estimates of H2AX abundance in human cells reported cell line specific values from 2.5 to 25% of total H2A in asynchronous immortalised cell lines (Rogakou et al., 1998). These values were determined by densitometry of Coomassie-stained, acid-extracted histones in two-dimensional gels. A 10% abundance value of H2AX has become accepted despite wide differences in the study and the fact that HeLa cells were reported to contain 2.5% H2AX.

Although it is tempting to interpret 10% abundance as implying every tenth nucleosome will contain H2AX, combinatorial features of nucleosomes make the statistics of spacings between H2AX occurrences in the chromatin fibre more complex. H2AX can be incorporated either as one or as two copies per nucleosome (Fig. 6a), and the H2AX-containing nucleosomes can be either randomly or non-randomly distributed along the chromatin fibre (Fig. 6b, c). Random incorporation would lead not simply to each tenth nucleosome containing H2AX, but to a geometric distribution of spacings between H2AX-containing nucleosomes. This predicts many instances of small spacings and some instances of very large spacings, and has clear implications for the ability of γH2AX to signal local damage events as well as for the spreading of the phosphorylation along the chromatin fibre.

Combinatorial Potential in H2AX Distribution

The combinatorial potential for H2AX inclusion has two separate features which could affect the detailed distribution of H2AX along chromatin.

Firstly, either one or two H2AX polypeptides can in principle be present within a nucleosome: Two H2AX copies would give rise to a “homotypic” H2AX/H2AX (“XX”) nucleosome, whereas a single H2AX copy will give rise to a “heterotypic” H2AX/H2A (“XA”) nucleosome (Fig. 6a). It is currently unknown whether there is a bias for either homotypic or heterotypic nucleosomes (see section “H2AX Protein”) although this affects the statistics of H2AX spacing in chromatin since the XA combination yields twice as many H2AX-containing nucleosomes than XX for a given H2AX abundance.

Secondly, the spacing of nucleosomes containing H2AX should have a major influence on its functional roles in DSB signaling, assembling of repair foci and

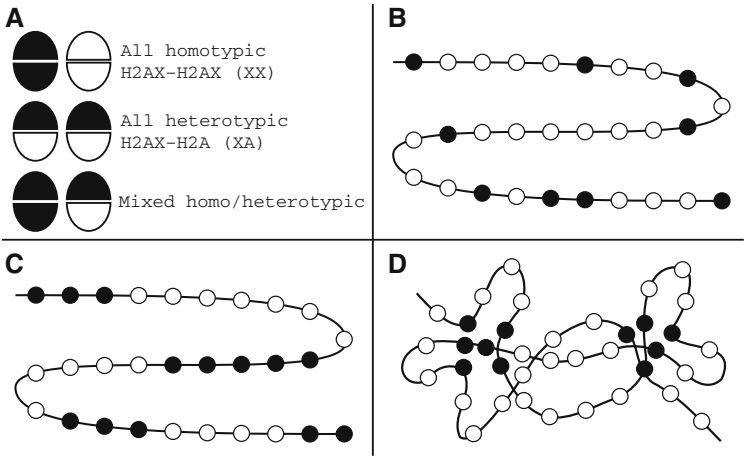

Fig. 6 H2AX distribution in the chromatin. **a**. Schematic of possible H2AX homotypic, heterotypic and mixed nucleosome combinations. *Black semicircle* represents H2AX–H2B dimer and *white semicircle* represents H2A–H2B dimer. **b**. Random incorporation of H2AX into nucleosomes would lead to a random distribution of H2AX-containing nucleosomes. **c**. Selective incorporation of H2AX into nucleosomes would lead to "islands" of H2AX-containing nucleosomes. **d**. Random incorporation of H2AX nucleosomes could also lead to "islands" of H2AX nucleosomes by chromatin reorganization

facilitating the repair machinery. H2AX nucleosomes could be randomly distributed (Fig. 6b) or subject to clustering in one (Fig. 6c) or three dimensions (Fig. 6d). Any mechanism randomly assembling chromatin from pools of XX and/or XA versus canonical H2A–H2A ("AA") nucleosomes will give rise to a geometric distribution of spacings between H2AX (Fig. 7a). This distribution predicts a bias towards small spacings (Fig. 7a).

Simulation of Random H2AX Inclusion

Simple computational simulations reveal interesting features in this H2AX spacing distribution. In the simplest case of H2AX assembling in a mixture of XA and XX nucleosomes, 10% overall H2AX abundance would generate an average of 4.3 nucleosomes between H2AX occurrences along the chromatin fibre (Fig. 7b). The mean spacing is highly sensitive to H2AX abundance (Fig. 7a), so 2.5 and 25% H2AX abundances yields means of 19.3 – 1.3 nucleosomes, respectively (Fig. 7b). Similar results arise for calculations where only heterotypic XA nucleosomes can assemble and homotypic XX nucleosomes are structurally precluded. In contrast, if heterotypic XA nucleosomes are precluded and only XX nucleosome structures can assemble, then 10% H2AX abundance yields a mean spacing of 9 nucleosomes between H2AX occurrences. The mean spacings for 2.5 and 25% H2AX abundance are 39 – 3 nucleosomes respectively (Fig. 7b).

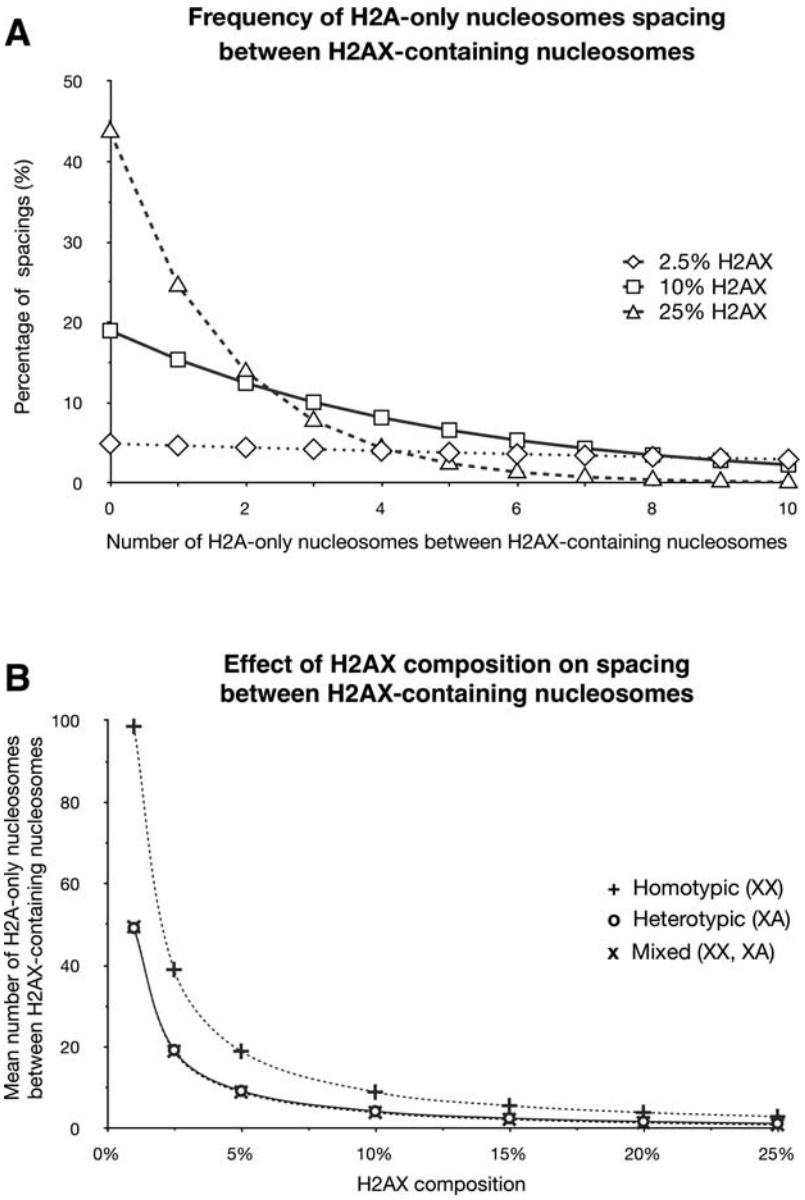

Fig. 7 Simulations of H2AX spacing distributions. **a**. Distribution of spacings between instances of H2AX for mixed population of homotypic and heterotypic nucleosomes at abundances of 2.5% (*dot*), 10% (*solid*) and 25% (*dashed*) H2AX in total H2A pool. **b**. Effect of abundance on mean H2AX spacing for homotypic (H2AX–H2AX) only, heterotypic (H2AX–H2A) only, and mixed homotypic+heterotypic nucleosome combinations

Functional Implications of H2AX Distribution

These simple models of random nucleosome incorporation have interesting implications. The occurrence of occasional large H2AX spacings could limit both processive γH2AX spreading along the chromatin fibre and the proximity of H2AX in solenoidal higher order chromatin packaging. At 10% H2AX abundance, 23% of nucleosomes in mixed XA and XX nucleosomes will be spaced by more than 6 nucleosomes and in the extreme case of 2.5% H2AX abundance, 84% of solely homotypic XX nucleosomes would be spaced by more than 6 nucleosomes.

This sensitivity of H2AX spacing in chromatin to abundance provides a potential opportunity for the cell to regulate responsiveness to damage. For example, if H2AX expression is up-regulated the mean proximity of randomly inserted H2AX will rapidly increase and effects such as processive γH2AX spreading and retention of DDR factors at foci will be significantly enhanced.

It is unknown whether H2AX distribution varies between euchromatin and heterochromatin. However, differences in H2AX response have been reported according to the condensation level of chromatin and phosphorylation of Ser139 has been observed to occur preferentially in euchromatin (Cowell et al., 2007). This preference is overcome during replication of heterochromatin when it is in a less condensed state (Cowell et al., 2007). The distinction between active and inactive chromatin can also be regulated, as demonstrated for phosphorylation of KAP–1 by ATM reducing the access of DNA repair proteins to heterochromatic regions of the genome (Goodarzi et al., 2008).

Possibility of Non-random H2AX Distribution

If H2AX nucleosome incorporation is not a random process (Fig. 6b), inhomogeneity could also exist at a more local level. For example, small "islands" of higher density H2AX nucleosomes could be interspersed within broader regions with lower relative abundance of the variant (Fig. 6c). A recent study using a novel high-resolution microscopy observed several thousand small spatial clusterings of H2AX and pointed to a mutual exclusivity of H2AX and the phosphorylated form (Bewersdorf et al., 2006). This would be consistent with a clustering model (Fig. 6d) that enhanced the kinetics of the damage signaling at foci, perhaps by making use of a chromatin structural feature such as the chromosomal scaffold (Bewersdorf et al., 2006). The inherent clustering and active insertion of H2AX in the DDR could also drive larger scale chromosomal rearrangements through chromatin stability (Fig. 6d) (Heo et al., 2008).

Functional Roles of H2AX

Phosphorylation of H2AX serine 139 by PIKKs to generate "γH2AX foci" is an early and characteristic feature of DSB events. This modification is thought to be the primary identifier of the location of DNA damage and would therefore be central to the function of H2AX.

The γH2AX foci extend for 2–30 Mbp along the chromatin fibre (Rogakou et al., 1999), implying the involvement of a span of $10^4 - 10^5$ nucleosomes per individual DSB repair event. At 10% H2AX abundance, this would involve up to $10^2 - 10^4$H2AX molecules and hence a $10^2 - 10^4$ fold amplification of DSB event signal. A direct link between the site of a lesion and a single focus has been observed (Rothkamm, et al., 2003), suggesting that there is a linkage between γH2AX and the repair mechanism. Many protein factors have been identified, which depend directly or indirectly on the phosphorylation of H2AX at serine 139. Thereafter, it appears to act as a foundation for recruitment of DDR factors at DSB sites (Paull et al., 2000). As a consequence, H2AX performs a role in both localisation and structuring of the repair focus.

Initiation of H2AX Phosphorylation as a Reporter of DSB Events

The process of establishing H2AX phosphorylation at the characteristic terminal motif can be performed by any of the three PIKKs ATM, ATR and DNA–PK. Their induction and binding characteristics suggest that H2AX phosphorylation for focus generation can be distinguished by an initiation phase when a small number of phosphorylations are made at nucleosomes adjacent to the break, and a spreading phase in which a larger region of phosphorylation extends one-dimensionally from either side of the break. The structural exposure of the serine 139 site through chromatin flexibility will be crucial determinant of the modification event (see section "H2AX Post-translational Modifications").

ATM has been considered a strong candidate as the principal kinase responsible for the initiation phase of general damage events because it responds to changes in chromatin conformation expected when a spontaneous DSB event releases local superhelical tension (Bakkenist and Kastan, 2003). ATR appears to be linked to replication stress or UV damage events which lead to breaks as indirect consequences, so ATR is recruited by ATRIP which detects single-stranded DNA. DNA–PK is localised to DSBs in complex with the end-binding protein Ku, so such an association will act to limit the distance from the damaged end on which DNA–PK can act (Walker et al., 2001) and such an end-dependent mechanism would be sensitive to H2AX abundance and distribution.

Spreading of H2AX Phosphorylation as a Damage Signal Amplifier

The conventional model for γH2AX focus formation suggests that after initiation in the immediate vicinity of the break by ATM and/or DNA–PK, amplification occurs by spreading through the action of MDC1 binding to γH2AX (Stucki et al., 2005). MDC1 in turn recruits the MRN complex (Mre11–Rad50–Nbs1) via direct interaction with Nbs1 (Lukas et al., 2004) and the MRN complex further activates ATM (Uziel et al., 2003). This generates a positive feedback loop to drive spreading of

the phosphorylation modification away from the break. Hence H2AX acts both as signal and target of phosphorylation in the spreading phase. Each focus acts independently even when several foci are formed in the immediate vicinity of each other (Kruhlak et al., 2006), suggesting a one dimensional diffusion along the chromatin fibre.

How the signal spreads over megabase but non-infinite distances is unknown. It is possible that non-homogeneous H2AX distribution could contribute to the localisation of γH2AX stochastically through random occurrence of large spacings between H2AX that the spreading mechanism could not bridge (see section "H2AX Distribution in Chromatin"). Consistent with this, high resolution microscopy reveals that H2AX is not randomly distributed but organized into discrete clusters which would control the expansion of the signal (Bewersdorf et al., 2006).

Since levels of phosphorylated H2AX rise rapidly in response to damage and then reduce over time (Rogakou et al., 1998) it is necessary to remove either the phosphate or the entire γH2AX. The timing of this process is unclear but must depend on the presence of γH2AX binding factors such as MDC1 which could stabilise γH2AX or obscure the phosphate group (Stucki et al., 2005). In *S. cerevisiae*, dephosphorylation is achieved by removal of phosphorylated H2AX from nucleosomes and subsequent dephosphorylation by the HTP–C complex (Keogh et al., 2006). In higher eukaryotes the mechanisms remain unclear since several phosphatases have been implicated in the process and these can variously dephosphorylate H2AX within nucleosomes or after removal (Chowdhury et al., 2005; Kimura et al., 2006; Chowdhury et al., 2008). In addition, the FACT complex which facilitates nucleosome exchange has enhanced activity on phosphorylated H2AX (Heo et al., 2008) suggesting at least one pathway involving displacement for extra-nucleosomal dephosphorylation. A background level of H2AX remains phosphorylated even in the apparent absence of DNA damage, but the reason for this is unknown (Rogakou et al., 1998).

γH2AX and Chromatin Structural Remodelling

Intrinsically, H2AX phosphorylation must take place within the context of chromatin structure so both the Non-Homologous End Joining (NHEJ) and Homologous Recombination (HR) pathways can efficiently undertake DSB repair. To facilitate this, chromatin decondenses near the DSB (Kruhlak et al., 2006) but the mechanism for this remodeling is unclear.

The modified serine 139 of H2AX is located near the DNA entry/exit point on the nucleosome (Fig. 4) so one putative mechanism for the chromatin structural change is to be driven directly by the chemical properties of the added phosphate group. *S. cerevisiae* mutants with the serine 139 equivalent mutated to glutamate to mimic the phosphate charge show increased micrococcal nuclease sensitivity consistent with such a destabilisation (Downs et al., 2000) and phosphorylated human H2AX renders chromatin more susceptible to restriction enzymes and DNA methylase

(Heo et al., 2008). However, a separate analysis of chromatin structure, also in yeast, harboring the glutamate mutation did not find evidence of direct chromatin structural effects (Fink et al., 2007).

An alternative indirect mechanism for linking H2AX phosphorylation with chromatin disruption is by recruitment of proteins to drive remodeling. A number of different ATP-dependent chromatin remodeling activities have been implicated in this process, including RSC, SWI/SNF, INO80 and SWR (reviewed in Downs et al., 2007), as well as other nucleosome modifying enzymes such as the NuA4 histone acetyltransferase. There is also evidence that chromatin chaperones and binding proteins contribute to the process of chromatin dynamics at DSBs. For example, the FACT complex, which participates in exchange between H2A and H2AX, has greater ability to mobilise γH2AX than unphosphorylated H2AX (Heo et al., 2008). In addition, HP1β, which binds to H3 K9me, has recently been shown to be released by phosphorylation immediately after DSB events and that this contributes to H2AX phosphorylation by PIKKs (Ayoub et al., 2008).

Both direct and indirect mechanisms for chromatin remodeling depend on H2AX phosphorylation, and hence require an independent initiation step. The PIKKs ATM and DNA–PK can achieve this by detecting changes in chromatin structure or appearance of DNA ends, respectively (Bakkenist and Kastan, 2003; Burma and Chen, 2004). However, the impact of chromatin on PIKK initiation is difficult to probe because H2AX phosphorylation occurs very rapidly after DSBs, making it difficult to temporally distinguish factors which remodel chromatin to enable initial PIKK access from downstream events which undertake remodeling to amplify γH2AX around the site.

Furthermore, despite the intimate link between H2AX phosphorylation and chromatin remodeling at the DSB site, local decondensation of chromatin occurs at similar levels on both wild type and $H2AX^{-/-}$ cell lines when ATP is not depleted (Kruhlak et al., 2006). This suggests that the role of H2AX phosphorylation in driving the chromatin remodeling is redundant with other pathways.

γH2AX and Localisation of DSB Repair Proteins

Since H2AX phosphorylation is one of the earliest events after a DSB, this suggests it may play a role in subsequent recruitment of the active repair proteins. This is supported by the absence of RAD51 and BRCA1 at DSB foci when γH2AX phosphorylation is prevented (Paull et al., 2000). However, NBS1, BRCA1 and 53BP1 are recruited to the sites of damage in $H2AX^{-/-}$ cell lines which display only moderate sensitivity to ionising radiation but fail to maintain focal localisation (Celeste et al., 2003b). It has therefore been suggested that the crucial role of H2AX phosphorylation is not as a direct agent of repair factor recruitment, but of retention of these factors in the vicinity of the DSB (Celeste et al., 2003b). This role in defining a "damage neighborhood" does not necessarily imply a direct role in repair at the break site itself. For example, stimulation of the G2/M checkpoint may result from the accumulation of checkpoint signalling factors at the focus (Fernandez-Capetillo,

et al., 2002). In fact, Chromatin ImmunoPrecipitation (ChIP) revealed that γH2AX is evicted from the region close to the DSB early in the DDR in *S. cerevisiae* and that γH2AX does not strictly co-localise with the active repair complexes (Shroff et al., 2004).

This accumulated retention of DDR factors in the vicinity of a DSB appears to be a complex process where the initiating damage signal is integrated by factors recognising the H2AX phosphorylation and presumably additional chromatin features. For example, human 53BP1 and its putative homologues, *S. cerevisiae* Rad9 and *S. pombe* Crb2, all contain Tudor domains which bind specific methylated histones in chromatin, and BRCT domains which can both mediate dimerisation and bind γH2AX. Despite the similarity in domain structure of Rad9, Crb2 and 53BP1, individual investigations have indicated that they have different binding capabilities. The Rad9 Tudor domain binds H3 K79me (Grenon et al., 2007; Huyen et al., 2004) whereas Crb2 and 53BP1 Tudor domains bind H4 K20me2 (Sanders et al., 2004; Botuyan et al., 2006). Rad9 and Crb2 BRCT domains bind directly to γH2AX (Hammet et al., 2007; Kilkenny et al., 2008) whereas 53BP1 does not, instead relying on an indirect interaction mediated by the BRCT domain of MDC1 which directly binds γH2AX (Lee et al., 2005; Stucki et al., 2005). Some direct interaction between 53BP1 BRCT domain and γH2AX has also been reported by co-precipitation studies, but in a much smaller proportion than Rad9 and Crb2 (Kilkenny et al., 2008). Rad9 and Crb2 can all also dimerise or oligomerise through their BRCT domains (Soulier and Lowndes, 1999; Du et al., 2004) although this domain is not necessary for the oligomerisation of 53BP1 (Adams et al., 2005). The latter instead requires a sequence upstream of its Tudor domain (Ward et al., 2006).

This complex interplay between the combinatorial interactions made by 53BP1, Rad9 and Crb2 with themselves and with γH2AX builds up to generate another level of the structural environment for the repair process. γH2AX therefore acts as a foundation to define the extent of the repair focus through the H2AX distribution and the extent of its phosphorylation.

γH2AX and Maintenance of Proximity of Break Ends

Linked to this role in retaining repair factors in the repair focus, phosphorylated H2AX also appears to function in the bringing together of damaged ends. It has been suggested that by recruiting repair factors which directly associate with the damaged ends, H2AX could prevent diffusion of these ends away from each other (Bassing and Alt, 2004). For example, linkage has been observed in the distribution of cohesin and γH2AX near DSBs (Unal et al., 2004) so γH2AX-dependent cohesin association would promote the stabilisation of sister chromatids to facilitate HR. Furthermore, localisation of self-interacting factors by their association with γH2AX nucleosomes could bring together distant break ends. For example, 53BP1 is suggested to localise to break ends by direct interaction with nucleosomes and indirect interaction through MDC1 (Huyen et al., 2004; Botuyan et al., 2006; Eliezer et al., 2009). Oligomerisation of 53BP1 has also been reported to enhance

association of distant ends, thereby facilitating long range recombination and NHEJ (Difilippantonio et al., 2008; Dimitrova et al., 2008).

γH2AX and Complementary Damage Signalling via Ubiquitylation

A secondary pathway of signaling by ubiquitylation of both canonical H2A and H2AX has recently been uncovered which appears to derive directly from γH2AX, and therefore act as a complementary amplification of the damage signal (Panier and Durocher, 2009). Recognition of H2AX phosphorylation by MDC1 leads to recruitment of an initiating ubiquitylation by RNF8 and UBC13 which is subsequently amplified with the involvement of RNF158, and possibly maintained by Rap80 and BRCT1. The direct role of the ubiquitylation remains to be clarified because it can act in factor recruitment as well as affecting the structure, stability or turnover of histones including H2AX itself.

Conclusion

Despite H2AX having a highly similar primary sequence or even overlapping identity with canonical H2A, it is clear that the DNA damage-linked function of this histone variant is highly specific. Its functional role is as an amplifier of the damage event signal, a foundation for marshaling repair factors, and a promoter of the chromatin dynamics required to complete the repair process. It is clear that the phosphorylation of serine 139 by PIKKs generates an epitope which is crucial to these functions. Nevertheless, it is important to note that the DNA damage response is only moderately defective in $H2AX^{-/-}$ cells, suggesting that complementary mechanisms must operate redundantly with H2AX functions. Much remains to be appreciated about γH2AX structure and function, but this must ultimately be based on the unique distinguishing features of the H2AX gene and protein.

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The Initiation Step of Eukaryotic DNA Replication

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Abstract Eukaryotic initiation of DNA replication is a tightly regulated process. In the yeasts, S-phase-specific cyclin Cdk1 complex as well as Dfb4-Cdc7 kinase phosphorylate the initiation factors Sld2 and Sld3. These factors form a ternary complex with another initiation factor Dbp11 in their phosphorylated state, and associate with the origin of replication. This complex mediates the loading of Cdc45. A second complex called GINS and consisting of Sld5 and Psf1, 2 and 3 is also loaded onto the origin during the initiation process, in an interdependent manner with the Sld2/Sld3/Dpb11 complex. Both complexes cooperate in the recruitment of the replicative DNA polymerases, thus executing the initiation and subsequent establishment of the replication fork. Cdc45 and GINS are essential, well-conserved factors that are retained at the elongating replication fork. They form a stable helicase complex with MCM2-7 and mediate its contact to the replicative DNA polymerases. In contrast, the Sld2/Sld3/Dpb11 complex critical for the initiation is not retained by the elongating replication fork. Sld2 displays limited homology to the amino-terminal region of RecQL4 helicase, which may represent its metazoan orthologue, whereas Sld3 homologues have been identified only in fungi. Dbp11 and its fission yeast homologue Cut5 are members of a large family of BRCT-containing proteins including human TopBP1 and fruit fly Mus101. Similar principles of regulation apply also to human initiation of DNA replication, despite obvious differences in the detailed mechanisms. The regulatory initiation cascade is intimately intertwined with the cell cycle apparatus as well as the checkpoint control.

Keywords Cell cycle · Cdc45 · Checkpoint · Cut5 · DNA replication · Dpb11 · GINS · Initiation · Sld2 · Sld3 · TopBP1

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Abbreviations

Cdc	cell division cycle
Cdt	Cdc10-dependent transcription
CDK	cyclin-dependent kinase
Csm3	chromosome segregation in meiosis protein 3
Ctf4	chromosome transmission fidelity protein 4
DDK	Dbf4-dependent kinase
Dpb	DNA polymerase B subunit
Drc1	DNA replication and checkpoint protein 1
dsDNA	double-stranded DNA
GINS	Go, Ichi, Nii, and San
MCM	mini-chromosome maintenance
Mrc1	mediator of replication checkpoint protein 1
Mus	nitrogen mustard-sensitive
ORC	origin recognition complex
pre-RC	pre-replicative complex
pre-IC	pre-initition complex
Pob3	DNA polymerase α binding protein 3
Pol	DNA polymerase
Psf	Partner of Sld five
RPA	replication protein A
ssDNA	single-stranded DNA
Sld	synthetically lethal with Dpb11-1
Spt16	suppressor of protein Ty 16
TopBP1	DNA topoisomerase II binding protein
Tof1	topoisomerase 1-associated factor 1

Introduction

DNA replication is a highly controlled process. In eukaryotes, the "once per cell cycle" rule is enforced by global regulation of the formation of the pre-replication complex during the M and G1 phases of the cell cycle, also called replication licensing (Blow and Dutta, 2005; DePamphilis et al., 2006; Nasheuer et al., 2002). Since recent reviews give an excellent account of pre-replication complex formation and its regulation, this issue and the factors involved, including the MCM2-7 complex and Mcm10, will not be discussed here.

The entry into S phase is marked by the initiation of DNA synthesis. This process requires of S phase-specific cyclin-dependent kinase (S-CDK) and Dbf4-dependent kinase Cdc7 (DDK) activity (Sherr and Roberts, 2004; Masai et al., 2005; Oehlmann et al., 2007). S-phase-specific cyclins A and E CDK proteins are controlled at the expression level, and furthermore, S-CDK and DDK activities are tightly regulated by inhibitory as well as activating post-translational modifications. These control mechanisms prevent on the one hand a premature onset of the S phase in general,

and govern the timely firing of each origin on the other hand. During recent years, it became apparent that unwinding of the DNA at the replication origin, and probably even more important, loading of the replicative DNA polymerases (Pols), onto the origin are the critical steps of regulation of the individual origins. This review will concentrate on the factors and mechanisms involved in this process.

The Regulators of DNA Replication Initiation

Dpb11, Cut5 and TopBP1

Albeit many disciplines in biosciences have contributed to our current understanding of DNA replication and its initiation, yeast genetics has been instrumental for the identification of novel factors involved. Among these, the *DPB11* gene was identified as a multi-copy suppressor of temperature-sensitive mutations of the Pol ε genes *POL2* and *DPB2* in the search for novel factors that interact genetically with Pol ε in the budding yeast (Araki et al., 1995). Dpb11 protein also interacts physically with Pol ε, but this interaction appears to be transient and probably restricted to the initiation of replication (Masumoto et al., 2000). Dpb11 associates with early firing origins of replication at the same time as Pol ε at the onset of S phase. But in contrast to Pol ε and Cdc45, Dpb11 does not associate with chromosomal DNA distal to the origins when DNA replication progresses (Aparicio et al., 1997; Masumoto et al., 2000) indicating that Dpb11 does not migrate with the progressing DNA replication fork. This also argues for a function specific for the initiation, but not elongation, of Dpb11.

Dpb11 was found to be homologous to the fission yeast Cut5/Rad4 protein. As Dpb11, Cut5 is essential for cell viability and is required for DNA replication as well as cell cycle control (Araki et al., 1995; Saka and Yanagida, 1993; Saka et al., 1994; Reid et al., 1999). Both proteins share a repetitive structure containing two pairs of BRCT domains. BRCT domains constitute a phospho-peptide binding region that has been found in a variety of proteins from bacteria to men (reviewed in Rodriguez and Songyang, 2008). BRCT domains have been first identified in BRCA1 and are common among DNA damage response and repair proteins (Bork et al., 1997; Callebaut and Mornon, 1997). In addition to binding to phospho-proteins, BRCT domains have also been implicated in binding of unphosphorylated target proteins as well as various DNA structures (Yamane and Tsuruo, 1999; Glover et al., 2004). Despite the low conservation of Dpb11/Cut5 at the sequences level, these proteins represent probably a ubiquitous replication and DNA damage response factor in eukaryotes. Orthologues have also been identified in plants and several metazoans, including human TopBP1 and fruit fly Mus101 (see “Function of TopBP1 in Genome Stability” by Sokka et al., this book for a detailed review on metazoan TopBP1 homologues), reviewed in (Garcia et al., 2005). These homologues have a more complex structure with additional BRCT domains, which may be associated with additional functions. As their yeast counterparts, the metazoan proteins have

been implicated in DNA replication. The Dpb11 homologue in *Xenopus*, XCut5 is required for the transition from the pre-replication to the pre-initiation complex. In particular, the loading of Cdc45 and of Pol ε onto the origin depends on Dpb11/XCut5 (Masumoto et al., 2000; van Hatten et al., 2002; Hashimoto and Takisawa, 2003).

Hiroyuki Araki and his co-worker continued their search for new factors involved in the initiation of DNA replication by seeking for genetic interaction partners of *DPB11*. In a screen for genes synthetically lethal with *DPB11*, the group of *SLD* mutants was discovered (Kamimura et al., 1998). The mutants fell into six complementation groups, thus representing six different genes. *SLD1* encodes Dpb3, which is the third largest subunit of Pol ε. *SLD4* is identical to *CDC45*, and *SLD6* is the same as the checkpoint kinase $RAD53^{CHK2}$. The three other *SLD* genes were found to be novel and not yet characterized.

Sld2 – A New Player in the Initiation of DNA Replication

SLD2 has been the first of the new *SLD* genes to be characterized (Kamimura et al., 1998 reviewed in Nasheuer et al., 2007). The same gene has been independently identified as a dosage suppressor of the *dpb11-1* mutation named *DRC1* (DNA replication and checkpoint 1) (Wang and Elledge, 1999). Sld2 is a protein of 453 amino acids in budding yeast and 337 amino acids in fission yeast without similarity to any characterized structural domain. Sld2 is a substrate of S-CDK activity (Masumoto et al., 2002; Noguchi et al., 2002). During S phase, the Sld2 protein becomes phosphorylated at least at six different serine and threonine residues both in fission and in budding yeast, and this modification facilitates an interaction with Dpb11/Cut5. Phosphorylation and apparently the Sld2-Dpb11 interaction is required for DNA replication, since a mutant deficient for all six phosphorylation residues is defective in chromosomal DNA replication, as are *SLD2* gene deletions (Masumoto et al., 2002; Noguchi et al., 2002). Surprisingly, these multiple phosphorylations at canonical motifs do not play a direct role in complex formation, but are barely the prerequisite for the phosphorylation of a further, non-canonical site within a 28 amino acid sequence that is responsible for binding of the carboxy-terminal BRCT pair of Dpb11 (Tak et al., 2006).

Recent reports suggest that RecQL4 protein may be the functional orthologue of Sld2 in animals. RecQL4 is one of five RecQ-like helicases identified in humans (reviewed in Bachrati and Hickson, 2008). Mutations in the *RecQL4* gene have been associated with Rothmund-Thompson, RAPADILINO and Baller-Gerold syndromes. Patients with these syndromes exhibit various physical and mental developmental abnormalities, increased risk of osteosarcoma, and features of premature aging. Disease-causing mutations lead to truncations or amino acid substitutions in the RecQ helicase domain. Sequences amino-proximal to the RecQ domain that are unique to metazoan RecQL4 are generally not affected in the patients. It is this amino-terminal region that shows limited homology to yeast

Sld2 (Sangrithi et al., 2005; Matsuno et al., 2006). Mice with a disruption in the helicase domain are viable, but exhibit growth retardation and genomic instability (Hoki et al., 2003; Mann et al., 2005). In contrast, mice with a homozygous disruption of exons 5–8 coding for part of the amino-terminal region die very early during embryogenesis (Ichikawa et al., 2002), emphasizing the importance of these sequences. RecQL4 appears essential for DNA replication, since depletion of the protein from *Xenopus* egg extracts inhibited initiation of DNA replication (Sangrithi et al., 2005; Matsuno et al., 2006). The amino-terminal fragment of RecQL4 lacking the helicase domain is sufficient to rescue the replication activity (Matsuno et al., 2006). This fragment included the Sld2 homology region and is able to bind XCut5. In RecQL4 depleted extracts, the pre-replication complex assembled normally, and Cut5, GINS and Cdc45 were loaded onto the chromatin. Instead, RecQL4 depletion suppresses the loading of DNA polymerases and RPA (Sangrithi et al., 2005; Matsuno et al., 2006). Recently results suggest that the amino-terminal, Sld2-like domain of RecQL4 promotes ATP-dependent DNA unwinding independently of the RecQ domain (Xu and Liu, 2009), despite the absence of conserved helicase motifs. Therefore, Sld2 and its metazoan homologue may have a direct role in the DNA unwinding at the origin of DNA replication.

Sld3– The Initiator of Initiation

Sld3 is another essential *SLD* gene identified in yeast by genetic screening (Kamimura et al., 2001; Nakajima and Masukata, 2002). Sld3 is a protein of 668 amino acids in fission and budding yeast that forms a complex with Cdc45. As for Sld2, no functional information can be extracted from the primary structure of Sld3. It is required for the association of yeast Cdc45 with the MCM2-7 complex, recruitment of Cdc45 to origins of DNA replication, and subsequent loading of RPA to the origin (Kamimura et al., 2001; Nakajima and Masukata, 2002; Pollok et al., 2003). In the budding yeast, Sld3 and Cdc45 associate simultaneously to origins at the time of origin firing, and their origin association is mutually dependent. Instead, in the fission yeast, Sld3 associates to origins without Cdc45 in the *nda4-108/mcm5* mutant (Yamada et al., 2004; Kanemaki and Labib, 2006). These studies indicate that loading of Sld3 and formation of an unstable complex with MCM2-7 is the initial step in the assembly of the initiation complex (Fig. 1). This is followed by the loading of Dpb11/Cut5, GINS and finally Cdc45 followed by unwinding of DNA at the origins (Nakajima and Masukata, 2002; Yabuuchi et al., 2006). However, differences in the loading order can be observed between early and late firing origins in fission yeast. In fact, association of *S. pombe* Sld3 with Cut5 and Cdc45 may occur before their loading at late firing origins (Yabuuchi et al., 2006). Sld3 loading requires DDK, but not S-CDK activity, whereas the later steps during initiation depend on S-CDK phosphorylation of Sld3 (Nakajima and Masukata, 2002; Yabuuchi et al., 2006; Tanaka et al., 2007b). Sld3 phosphorylated by S-CDK binds to the amino-terminal BRCT repeats of Dpb11 (Zegerman and Diffley, 2007).

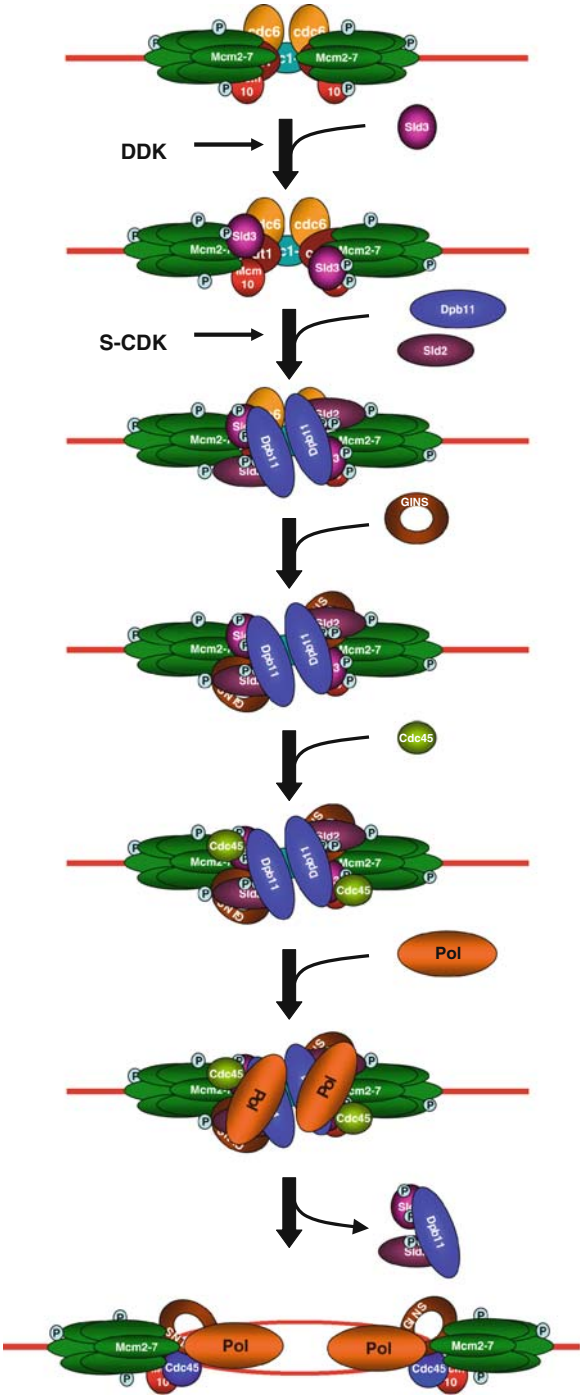

Fig. 1 A model for the ordered assembly of the pre-initiation complex. The names of the proteins follows the nomenclature of the budding yeast *Saccharomyces cerevisiae*, where this process has been studied in most detail. A similar order of events is likely to take place also in other eukaryotes despite some variation in the factors involved. See text for details

After establishment of DNA replication forks, Sld3 is no longer required for the completion of DNA replication. This is in line with Sld2 and Dpb11, but in contrast to Cdc45 and GINS that are components of the elongation machinery, too. And in difference to the other factors involved, no structural or functional homologue of Sld3 could be identified thus far in organisms other than fungi, suggesting some diversity in the regulation of initiation complex formation in different groups of eukaryotes.

The Replication Factor Cdc45

Discovery and Characterization of Cdc45

CDC45 was first described as a cold-sensitive cell division cycle mutant in *Saccharomyces cerevisiae* (Moir et al., 1982 for review Nasheuer et al., 2007). Subsequently it has been shown to be essential for the initiation (Aparicio et al., 1997; Hardy, 1997; Owens et al., 1997; Uchiyama et al., 2001a) and elongation of DNA replication (Bauerschmidt et al., 2007; Pacek and Walter, 2004; Tercero et al., 2000) in a variety of eukaryotic organisms. The importance as replication factor is underlined by the fact that Cdc45 is highly conserved from yeast to man and that conservation between men and mice amounts to 92% identical amino acids. On the other hand, Cdc45, which is called Sna41 in *Schizosaccharomyces pombe*, and Tsd2 in *Ustilago maydis*, is apparently not present in archeabacteria (Grabowski and Kelman, 2003; Matsunaga et al., 2001), the third kingdom of life that shares many replication and transcription factors with the eukaryotes (Sclafani and Holzen, 2007). Sequence comparisons revealed only a conserved domain with acidic amino acids and a bipartite nuclear localization sequence (NLS) (Hopwood and Dalton, 1996; Loebel et al., 2000; Miyake and Yamashita, 1998; Shaikh et al., 1999; Zou et al., 1997). In yeast, Cdc45 is transported into the nucleus through the classical NLS transporter importin α (Pulliam et al., 2009).

Expression of Cdc45 and Its Control

Like many other genes involved in DNA replication, Cdc45 expression is regulated by the E2F family of transcription factors (Arata et al., 2000; Loebel et al., 2000; Stevens et al., 2004). There are several splice variants of its mRNA described. The main form, known as Cdc45L, lacks both the complete exon 7 as well as 36 base pairs from exon 18 (Kukimoto et al., 1999; Saha et al., 1998). Obviously, there exist some other forms of Cdc45 in various human tissues as detected by Northern blot analyses (Shaikh et al., 1999). Cdc45 mRNA expression in *S. cerevisiae* is maximal at the G1/S transition, whereas the protein level remains nearly constant over the cell cycle (Hardy, 1997; Owens et al., 1997). Similar results were also observed for Cdc45 mRNA and protein from fission yeast and humans (Saha et al., 1998;

Uchiyama et al., 2001b), the latter being in accordance with regulation by E2F/Rb. Cell cycle dependent degradation of Cdc45 is most likely achieved by an anaphase promoting complex (APC/C)-mediated ubiquitylation and subsequent degradation by the 26S proteasome. This was inferred from the presence of various APC/C-specific destruction boxes in the primary sequence and the enrichment of Cdc45 in the presence of proteasome inhibitors (Pollok and Grosse, 2007). This argues for a dynamic equilibrium in the protein levels supported by a half life of approximately 10 h (Pollok et al., 2007). In accordance with these regulatory circuits, differentiated or quiescent cells do not express Cdc45, but may turn on transcription and translation after the addition of growth factors or heavy metal ions (Arata et al., 2000). Since Cdc45 expression is tightly associated with proliferation this protein may be a promising candidate for a novel proliferation marker in cancer cell biology (Pollok et al., 2007). Highest protein levels were found in 0–4 h old embryos of the fruit fly *Drosophila melanogaster* (Loebel et al., 2000), which apparently stockpile this protein to allow several rounds of rapid cell proliferation without ongoing transcription. The highest amounts of human Cdc45 mRNA were found by Northern blots in proliferating tissues such as testis, placenta, thymus, thyroid glands, and colon epithelia, whereas non- or slowly proliferating tissue such as liver, brain and kidney were practically devoid of this mRNA (Shaikh et al., 1999).

As already mentioned thermo-sensitive yeast mutants of Cdc45 displayed a growth-arrest phenotype at the G1/S transition of the cell cycle at the restrictive temperature (Hennessy et al., 1991; Hopwood and Dalton, 1996; Miyake and Yamashita, 1998; Uchiyama et al., 2001a; Zou et al., 1997). Comparably, depletion of Cdc45 from *Xenopus* oocyte extract resulted in the inhibition of replication initiation and elongation (Mimura and Takisawa, 1998; Pacek and Walter, 2004). Interestingly, an RNA interference (RNAi) mediated Cdc45 knockdown caused chromosome-condensation in *D. melanogaster* (Christensen and Tye, 2003) and fragmented chromosomes in *Arabidopsis thaliana* (Stevens et al., 2004), whereas RNAi knockdown of Cdc45 in human tumor cells induced apoptosis (Feng et al., 2003). Heterozygous CDC45$^{+/-}$ mice did not display any anatomical abnormalities whereas homozygous CDC45$^{-/-}$ mice died during the peri-implantation state of the blastocyste about 7 days after conception (Yoshida et al., 2001). In humans the CDC45 gene is localized to chromosomal region 22q11, a region where micro-deletions are associated with the DiGeorge syndrome, a developmental defect that is thought to be due to a haploinsufficient expression of this part of the human chromosome. DiGeorge patients typically develop heart failures, insufficient aortas, craniofacial abnormalities, and underdeveloped thymus and parathyroid glands, which in turn give rise to immunodeficiencies and hypocalcemiae, respectively (Shaikh et al., 1999). Fluorescence in situ hybridizations indicate that at least 90% of all patients with DiGeorge syndrome display mono-allelic micro-deletions of the chromosomal region 22q11 including the coding region of CDC45. The resulting hypomorphic expression of Cdc45 together with its essential role in replication and proliferation may be one reason for the underdevelopment of the thymus and the parathyroid glands. On the other hand, since heterozygous mutant mice develop into adulthood without any apparent abnormalities, it is unlikely that hemizygosity

of CDC45 alone is responsible for the cardiac and craniofacial defects of this congenital syndrome (Yoshida et al., 2001).

Dynamics of Cdc45 in the Cell

In the cell, Cdc45 is mainly localized within the nucleus of yeast cells, as detected by live cell imaging with fusions between Cdc45 and the green fluorescent protein (Hopwood and Dalton, 1996). Confirmatory results were obtained with hemagglutinin-tagged Cdc45 (Owens et al., 1997). Similarly, immunofluorescence studies with *Drospophila* embryos revealed chromatin-bound Cdc45 during interphase and dissociation from chromatin and a corresponding cytoplasmic localization during mitosis, when the cell nucleus is dissolved. Biochemical fractionations with human U2OS cells displayed a cytoplasmic and a nuclear distribution of Cdc45 during S phase (Saha et al., 1998), whereas immunoprecipitation and immunoflourescence experiments with HeLa S3 cells revealed a nuclear localization during interphase and chromatin-bound Cdc45 during S and G2 phase (Bauerschmidt et al., 2007).

Interaction Partners of Cdc45

Cdc45 interacts with a variety of other proteins, as determined by genetic interactions, two-hybrid screens and pull-down techniques. There is plentiful data describing the binding of Cdc45 to the MCM2-7 helicase of eukaryotic cells (Bauerschmidt et al., 2007; Dalton and Hopwood, 1997; Dolan et al., 2004; Gambus et al., 2006; Hennessy et al., 1991; Hopwood and Dalton, 1996; Kneissl et al., 2003; Kubota et al., 2003; Kukimoto et al., 1999; Loebel et al., 2000; Masai et al., 2006; Masuda et al., 2003; Mimura and Takisawa, 1998; Moyer et al., 2006; Pacek et al., 2006; Pacek and Walter, 2004; Uchiyama et al., 2001b; Zou and Stillman, 2000) as well as to the GINS complex (Bauerschmidt et al., 2007; Gambus et al., 2006; Kubota et al., 2003; Moyer et al., 2006; Pacek et al., 2006). Similar to Cdc45, the GINS complex is also recruited to the activated origins of replication, where it assembles with Cdc45 and the MCM2-7 proteins to form the Cdc45/MCM2-7/GINS (CMG) complex (see below), the presumed replicative helicase (Moyer et al., 2006; Boskovic et al., 2007). Cdc45 has also been shown to associate with Dpb11/Cut5/Mus101/TopBP1 either directly (Hashimoto and Takisawa, 2003; Wollmann et al., 2007) or indirectly via Sld3 (Kamimura et al., 1998; Nakajima and Masukata, 2002; Yamada et al., 2004). Cdc45 has been described to interact with all three replicative DNA polymerases of eukaryotes, i.e. Pol α (Aparicio et al., 1999; Hashimoto and Takisawa, 2003; Kubota et al., 2003; Kukimoto et al., 1999; Mimura et al., 2000; Mimura and Takisawa, 1998; Uchiyama et al., 2001a, b), Pol δ (Bauerschmidt et al., 2007) and Pol ε (Bauerschmidt et al., 2007; Kubota et al., 2003; Zou et al., 1997). In this respect it came as no surprise that Cdc45 also interacted

with the presumptive Pol α chromatin loader Mcm10 (Christensen and Tye, 2003; Gregan et al., 2003; Homesley et al., 2000; Loebel et al., 2000; Ramachandran et al., 2004; Sawyer et al., 2004) and the single-strand DNA binding protein RPA (Bauerschmidt et al., 2007; Dalton and Hopwood, 1997; Zou and Stillman, 2000). Thus, Cdc45 may act as a tether that bridges the MCM2-7-GINS helicase complex (see below) with the elongating DNA polymerases (Bauerschmidt et al., 2007; Kim et al., 1996).

The Role of Cdc45 During DNA Replication

DNA replication starts during S phase of the cell cycle at distinct origins of replication. Bidirectional DNA synthesis from each of the about 25,000 origins (in humans) follows a still ill-defined program with many origins firing early in S phase, fewer origins starting later and only a couple of origins starting very late the DNA synthesis phase of the cell cycle (Vogelauer et al., 2002). Apparently, at least in yeast Cdc45 is loaded into the early firing origins rather early and in the late firing origins late (Aparicio et al., 1999; Zou and Stillman, 2000), which taken together with its relative low abundance observed in the human cell (Pollok and Grosse, 2007) suggests a regulatory function, mainly since origin recruitment of Cdc45 is tightly associated with an activation of these origins. Until now there is only limited evidence for a phosphorylation of Cdc45 by any of the S phase kinases, though Cdc45 from *S. cerevisiae* is phosphorylated at least in vitro by Dbf4-Cdc7 (Weinreich and Stillman, 1999). Since in higher eukaryotes and particularly in mammals there is no known homologue of the yeast Sld3 protein, the question arose of how mammalian Cdc45 may become recruited to the initiation complex. One suggestion is that Cdc45 binds directly to the Dpb11 homologue TopBP1 (Schmidt et al., 2008). This might require phosphorylation of human Cdc45 by S phase kinases. Interestingly, a proteomic approach revealed a cluster of phosphorylation sites at position 130–151 of human Cdc45 (Dephoure et al., 2008), which however has not yet been shown to become phosphorylated by S phase kinases or being a binding module for TopBP1's BRCT domains. Another possibility is that TopBP1 binds unphosphorylated Cdc45 but may nevertheless be regulated by internal phosphorylation events. In this regard it is noteworthy to mention that depletion of protein phosphatase 2A from *Xenopus* egg extracts abolish loading of Cdc45 into the pre-replicative complex and that the initiation of eukaryotic DNA replication is regulated at the level of Cdc45 loading by a combination of stimulatory and inhibitory phosphorylation events (Chou et al., 2002).

A Phosphorylation Switch for the Initiation of DNA Replication

Recent experiments have shown that S-CDK-dependent phosphorylation of Sld2 and Sld3 initiates DNA replication in budding yeast (Zegerman and Diffley, 2007; Tanaka et al., 2007b). Dpb11 forms a ternary complex with the replication initiation

factors Sld2 and Sld3 when these become phosphorylated (Fig. 1). This complex then controls the association of Cdc45 and the replicative DNA polymerases with the origins of DNA replication (Masumoto et al., 2000). Tanaka and co-workers (Tanaka et al., 2007b) were able to demonstrate that a phospho-mimetic form of Sld2 (Sld2-11D) confers S-CDK-independent DNA replication when combined with either the *JET1* mutation of Cdc45, or overexpression of Dpb11. Both *JET1* and Dpb11 over-expression overcomes the requirement for Sld3 phosphorylation for initiation of DNA replication. Zegerman and Diffley (2007) fused an *Sld3* mutant that cannot become phosphorylated by S-CDK and that is deficient for DNA replication with the sequence for the amino-terminal BRCT domain pair of Dpb11. In a strain where S-CDK activity was inhibited at the same time, almost no DNA replication occurs. But when wild-type *SLD2* is in addition replaced by the phospho-mimetic *SLD2-T84D* variant, extensive DNA replication occurs, bypassing the requirement for S-CDK activity. Therefore, the phosphorylation of Sld2 and Sld3, and their subsequent binding by Dpb11 represents the minimal requirement for CDK-dependent activation of replication initiation in yeast (Tanaka et al., 2007a). The subsequent recruitment of Cdc45 into this complex (via Sld3) might be the rate-limiting step for the formation of an active replicative DNA helicase, i.e. the Cdc45-MCM2-7-GINS complex (Moyer et al., 2006; Boskovic et al., 2007; Aparicio et al., 2009).

As discussed above, the corresponding regulatory mechanisms in higher eukaryotes are poorly understood. Considering the roles of TopBP1/XCut5, RecQL4 and Cdc45 for the loading of the replicative DNA polymerases and establishment of the replication fork both in *Xenopus* and in human cells, a similar regulatory network as in yeast can be assumed for vertebrates. The targets for S-CDK and DDK among these proteins have not yet been identified. What is more, it remains unclear, which vertebrate factor takes over the role of yeast Sld3. Since human Cdc45 interacts directly with TopBP1, it is conceivable that human TopBP1 abrogates a requirement of Sld3. But in yeast, DDK-dependent loading of Sld3 appears to be the most upstream event in the initiation cascade, and a comparable regulatory step is not yet in sight in higher eukaryotes.

GINS: An Evolutionarily Conserved Key Player in DNA Replication

Identification of the GINS Complex

The GINS complex consists of four paralogous proteins (Sld5, Psf1, Psf2 and Psf3), whose encoding genes are present in all sequenced eukaryotic genomes. The name GINS is the acronym of the Japanese words “Go-Ichi-Ni-San” which mean “five-one-two-three”. The genes coding for the subunits of the GINS complex were identified by independent research groups in 2003. In their genetic analyses in *S. cerevisiae* aimed at discovering novel interaction partners of *DPB11* (Kamimura et al., 1998) Araki and co-workers identified mutations in the *SLD5* gene, which

were lethal in a *DPB11* temperature-sensitive mutant yeast strain (Takayama et al., 2003). In the same study, *PSF1*, *PSF22* and *PSF3* were identified as Partners of Sld Five by a combination of multi-copy suppression analysis and two-hybrid screens. Cell-cycle studies on yeast strains bearing thermo-sensitive mutants of the *SLD5* and *PSF1* genes revealed a defect of DNA replication under non-permissive conditions suggesting that GINS could be involved in DNA replication. Consistently, Ch-IP (Chromatin-Immuno-Precipitation) assays indicated that budding yeast GINS first associates with replication origins and then with proximal sequences during S phase (Takayama et al., 2003).

In a contemporaneous study Kubota and colleagues reported the identification and biochemical characterization of the GINS complex from *Xenopus laevis* (Kubota et al., 2003). Antibodies raised against each subunit of the *Xenopus* GINS complex were used to demonstrate that the four proteins form stable complexes in frog egg extracts. Furthermore, the four proteins were co-expressed in insect cells and found to co-purify. Sedimentation through glycerol gradients of the recombinant as well as endogenous complex indicated an apparent molecular weight of about 100 kDa consistent with an equimolar stoichiometric ratio of the four subunits. *Xenopus* GINS was found to be required for DNA replication, because immunodepletion of GINS abolished the incorporation of dNTPs into sperm chromatin. This effect was rescued by addition to the depleted egg extract of the full complex but not by an Sld5-Psf1-Psf2 sub-complex (Kubota et al., 2003). In *S. cerevisiae* as well as in *Xenopus* egg extracts, the GINS complex was found to be tightly associated with replicating chromatin fractions. Here it interacted with Cdc45 and the MCM2-7 complex and was loaded onto replicating chromatin together with Cdc45 in a mutually dependent fashion (Kubota et al., 2003; Takayama et al., 2003).

In a subsequent study in budding yeast, the GINS subunits were shown to be essential for the establishment of the DNA replication fork at firing origins as well as for normal progression of the replisome away from these origins (Kanemaki et al., 2003). *S. cerevisiae* strains were produced in which the gene of interest was fused to a "heat-inducible degron" cassette. The latter targeted the protein for rapid proteolysis at 37°C so that the immediate effects of bulk protein depletion could be evaluated. Using this strategy, three genes were identified (named *CDC101*, *CDC102* and *CDC105*) and found to be essential for the initiation and elongation phases of DNA replication (Kanemaki and Labib, 2006; Kanemaki et al., 2003). By immunoprecipitation experiments and mass spectrometry the corresponding proteins were found to be part of a complex together with a fourth component referred to as Yol146w. These four budding yeast genes corresponded to *SLD5* (*CDC105*), *PSF1* (*CDC101*), *PSF2* (*CDC102*) and *PSF3* (Yol146w).

The essential physiological role of GINS was also demonstrated in higher eukaryotes. Homozygous *PSF1* knockout mice died in uterus at around the time of implantation due to a defect in proliferation of the inner cell mass (Ueno et al., 2005). A subsequent two-hybrid screen confirmed that murine Sld5 was an interaction partner of murine Psf1 (Kong et al., 2006). Interestingly, both alleles of *PSF1* are required for the maintenance of the pool size of immature hematopoietic cells and acute bone marrow regeneration (Ueno et al., 2009).

A bioinformatic analysis revealed that the subunits of the GINS complex belong to a family of paralogous proteins, despite the low level of primary structure conservation (Makarova et al., 2005). In this report proteins orthologous to the eukaryotic GINS subunits were also identified in Archaea, as described below.

The Archaeal GINS Complex

The four subunits of the GINS complex display similar peptide chain lengths (around 220 amino acid residues) but very limited sequence identity. A bioinformatics analysis indicated particular similarities between Sld5 and Psf1 on the one hand and between Psf2 and Psf3 on the other hand (Makarova et al., 2005). This study also revealed that Archaea possess GINS homologues. Some archaeal species (such as *Sulfolobus solfataricus* and *Pyrococcus furiosus*) contain two members of the GINS family, one similar to Sld5/Psf1 and the other more close to Psf2/Psf3 (Marinsek et al., 2006; Yoshimochi et al., 2008). Other archaeal species (such as *Methanothermobacter thermoautotrophicus* and *Archeoglobus fulgidus*) apparently possess a single GINS protein which is homologous to Sld5/Psf1 (Makarova et al., 2005; Yoshimochi et al., 2008). Biochemical studies showed that the Sld5/Psf1 (GINS15) and the Psf2/Psf3 (GINS23) homologues of *Sulfolobus solfataricus* form a 2:2 tetrameric complex (Marinsek et al., 2006). The *Sulfolobus* GINS complex co-immunoprecipitated from cell extracts together with the MCM-like protein and the heterodimeric eukaryotic-like DNA primase. Two-hybrid analyses confirmed that the GINS23 subunit interacts with MCM and DNA primase (Marinsek et al., 2006). However, the *Sulfolobus* GINS complex did not exert any effect on the catalytic functions of these interaction partners in vitro.

More recently, Ishino and colleagues reported the biochemical characterization of the GINS complex of *Pyrococcus furiosus* (Yoshimochi et al., 2008). Similarly to the *S. solfataricus* GINS complex, it consists of two subunits (GINS15 and GINS23) forming a 2:2 tetramer. Nonetheless, differently from the *Sulfolobus* GINS, the ATPase and strand displacement activities of the *Pyrococcus* MCM complex were stimulated by GINS in vitro (Yoshimochi et al., 2008). However, the molecular mechanism for this activation is not clear because the *Pyrococcus* GINS was unable to bind nucleic acids in band shift assays and did not form a stable complex with MCM under gel filtration conditions. In addition, Ch-IP assays revealed that the *Pyrococcus* GINS preferentially associated with the chromosomal replication origin during the exponential growth phase but not in non-replicating cells. Moreover, a two-hybrid analysis showed that the *Pyrococcus* GINS interacts with the Cdc6/Orc1 homolog (Yoshimochi et al., 2008). These results suggest that, as observed for the eukaryotic GINS complex, the archaeal GINS may play a role in the initiation and/or the elongation phase of DNA replication.

The archaeal species, whose genome does not contain a Psf2/Psf3 homologue, might possess a very simplified version of the GINS complex consisting of only one Sld5/Psf1-like subunit. Alternatively, it is plausible that for these species the

in silico analyses failed because of the high sequence divergence of the Psf2/Psf3 homologue. The characterization of the archaeal GINS complex suggests that the four eukaryotic GINS subunits may have evolved from a common evolutionary origin by subsequent events of gene duplications and permutations (Makarova et al., 2005). This hypothesis has been found to be consistent with the recent structural analyses of the human GINS complex (Chang et al., 2007; Choi et al., 2007; Kamada et al., 2007).

Structural Studies on the GINS Complex

The first structural observations of the GINS complex were carried out by transmission Electron Microscopy (EM) (Kubota et al., 2003). This analysis revealed for the first time that the *Xenopus* GINS adopts a ring-like (or C-shaped) structure with an average diameter of 95 Å and a central pore of about 40 Å. This molecular shape was considered reminiscent of PCNA (Proliferating Cell Nuclear Antigen), the homotrimeric sliding clamp of Pol δ. Accordingly, it was proposed that GINS might act as a processivity factor for Pol ε (Kubota et al., 2003). Based on a gene multi-copy suppression analysis and two-hybrid assays, the four subunits of the GINS complex were proposed to be arranged in the order Psf2:Sld5:Psf1:Psf3 (Takayama et al., 2003). This subunit arrangement was recently confirmed for the human GINS complex by a combination of mass spectrometry and monoclonal antibody mapping using EM (Boskovic et al., 2007). In this study a three-dimensional reconstruction of the GINS complex has revealed a horseshoe-like shape with a central hole of 30–35 Å in diameter, large enough to encircle dsDNA (double-stranded DNA) and ssDNA (single-stranded DNA). According to this study, the central hole has the shape of a funnel because its diameter on one side is 70 Å wide, whereas it is narrower (about 25 Å) on the opposite site indicating the possibility of different functions for each side of the complex. In this report, the human GINS complex was found to bind nucleic acids with a clear preference for ssDNA. Based on the report that the *Drosophila* GINS complex is stably associated with MCM2-7 and Cdc45, the authors propose a model where GINS acts as a co-factor for the MCM2-7 replicative helicase by encircling ssDNA in its central hole (Boskovic et al., 2007).

However, the crystal structure of the human GINS complex as solved by three groups seems not to be consistent with the proposal that the central hole encircles ssDNA. The overall structure as well as the fold of the individual subunits and their interactions were essentially the same in all three publications (Chang et al., 2007; Choi et al., 2007; Kamada et al., 2007), although two groups used crystals of the complex with deletion of the last 50 amino acid residues of Psf1 (Choi et al., 2007; Kamada et al., 2007). The resulting Psf1-truncated form of GINS was as stable as the complex containing full-sized Psf1, indicating that the missing Psf1 fragment is not essential for tetramer formation and complex stability. This agrees well with the full-length structure where the C-terminal 51 residues of Psf1 were not visible suggesting that this portion of the polypeptide chain is intrinsically disordered (Chang

et al., 2007). The hetero-tetrameric GINS complex resembles a trapezoid with Sld5 and Psf1 forming the top layer and Psf2 and Psf3 associated at the bottom. Few contacts are observed between Sld5 and Psf3 and between Psf1 and Psf2. This subunit arrangement is fully consistent with the results of the genetic analyses and two-hybrid assays carried out in *S. cerevisiae* (Takayama et al., 2003). One important finding was that the fold of Sld5 and Psf2 is similar to that of Psf1 and Psf3, respectively, despite the limited sequence identity within each couple of subunits. In addition, each subunit is composed of two structural domains: an α-helix-rich (A) domain and a β-strand-rich (B) domain. These two domains are found in the order A-B in Sld5 and Psf1, whereas they are inverted (B-A) in Psf2 and Psf3. The A domain consists of four α-helices (with the exception of Psf2 whose A domain contains 2 α-helices and one β-strand) forming an arc; the B domain consists of two small anti-parallel β-sheets forming a jelly-roll structure. The linker region connecting the A and B domains is only 6 residues long in Psf2 and Psf3 but 21 residues in Sld5 and possibly also in Psf1. The B domain of Sld5, Psf2 and Psf3 is stably anchored to the respective A domain whereas in Psf1 the B domain is loosely associated to the A domain. The unstructured B domain of Psf1 is likely involved in physical interaction with other DNA replication factors. To test this hypothesis, the *Xenopus* DNA replication system was used (Kamada et al., 2007). Frog egg extracts, immuno-depleted of the GINS complex, were unable to replicate DNA while DNA replication activity was restored by adding the recombinant full-length human GINS complex. In contrast, addition of a complex containing a deletion of the Psf1 B domain or addition of the Psf1 B domain alone ($Psf1_{140–196}$) or a combination of these two were not sufficient to support DNA replication in depleted extracts. Analysis of the chromatin-bound fraction from frog egg extracts by western blots revealed that the ORC and MCM2-7 complexes were associated with chromatin in the immuno-depleted extracts supplemented with either the intact or the mutant human GINS complex. Conversely, chromatin-association of Cdc45 and Pol ε varied according to the replication activity of each GINS mutant used to complement the egg extracts.

By modelling of the Psf1 B domain on the structure of the corresponding Sld5 B domain Kamada et al. identified the linker region and residues in Psf1 that could be exposed and form the binding interface for other proteins (Kamada et al., 2007). The corresponding residues in Sld5 are involved in binding Psf2. Substitution of these residues of Psf1 with alanine reduced (although did not completely abolish) the ability of the GINS complex to support the DNA replication activity in immunodepleted *Xenopus* egg extracts. Similar effects were observed by mutating specific residues that are likely to be located in the Psf1 linker region. Therefore, the Psf1 B domain located on the surface of the GINS complex plays a critical role in the initiation process where it mediates chromatin-association of other replication factors, such as Cdc45 and Pol ε. In addition to the Psf1 B domain, other unstructured regions were observed in both Psf3 and Sld5 in the X-ray structure. In particular, the Psf1 B domain is close to the unstructured C-terminal tail of Psf3 (residues 194–216) and to the disordered fragment of Sld5 (residues 65–71). On the same side, but on the other end of the hetero-tetramer, lies an unstructured region within the Psf3 B

domain, which may also serve as a protein-binding site. This putative wide distribution of the protein interaction surfaces may allow simultaneous interactions of more than one binding partner to the GINS complex.

An important issue regarding the function of the GINS complex is whether its central pore exists and is accessible. One high-resolution crystallographic study showed that the central cleft that is almost closed at the bottom (Kamada et al., 2007). Choi and co-workers reported that the human GINS complex contains a central channel, but only with an internal diameter of about 5 Å that is not large enough to accommodate nucleic acids (Choi et al., 2007). In contrast, in the X-ray structure of human GINS by the Chen group the diameter of the central pore is 10 Å and a mechanism was suggested by which the opening of the central pore may be regulated (Chang et al., 2007). In fact, a careful inspection of the crystal structure revealed that a 16-residues loop from the N-terminus of Psf3 is not tightly bonded to the pore surface and therefore may regulate its accessibility by moving outside and inside this central cavity. Upon the removal of this N-terminal 16-residues loop of Psf3 the diameter of the central pore is increased from 10 to 18 Å. Multiple sequence alignments indicate that the first 16 N-terminal residues of Psf3 are only present in human and higher eukaryotes. In addition, the human GINS complex, in which Psf3 bears a truncation of the first 10 or 18 residues from the N-terminus, was found to be as stable as the hetero-tetramer containing full-sized Psf3 (Chang et al., 2007). The latter proposed that the central pore may be involved in holding a domain of MCM2-7 complex, Cdc45 or a DNA polymerase at the replication fork or, alternatively, that it can bind DNA in its open state.

GINS in the Initiation and Elongation Phases of DNA Replication

The abundance and composition of GINS were found to be constant during the cell cycle in *S. cerevisiae* (Takayama et al., 2003). Association of GINS to chromatin at the replication origins takes place at the onset of S phase and requires the activity of both S-CDK and DDK (Kanemaki and Labib, 2006; Yabuuchi et al., 2006). The ordered assembly of various initiation factors to the pre-RC and their regulation by the cell-cycle kinases has already been discussed above in detail. Importantly, both in *S. cerevisiae* and *S. pombe* Sld3 is required for the association of GINS to replication origins and two-hybrid assays revealed that fission yeast Sld3 directly interacts with GINS (Yabuuchi et al., 2006). Also Dpb11-Sld2 are loaded in an interdependent way with GINS in both budding and fission yeast and a direct association among these factors was suggested on the basis of two-hybrid studies (Takayama et al., 2003; Yabuuchi et al., 2006).

It has become clear that GINS is required not only for the establishment but also for the progression of the DNA replication fork in budding yeast (Takayama et al., 2003; Kanemaki et al., 2003). Several studies revealed that GINS is a stable component of the eukaryotic replisome. Calzada and co-workers described a method for inducing pausing of the replisome at natural Replication Fork Barriers

(RFBs) in specially-engineered *S. cerevisiae* strains where a RFB is located in the vicinity of specific early replication origins (Calzada et al., 2005). Ch-IP assays revealed that paused replisomes contain MCM2-7, Cdc45, GINS, the proteins forming the fork protection complex (Mrc1, Tof1 and Csm3) and Pols α and ε. A similar approach was employed to dissect the molecular anatomy of the replisome in *Xenopus* egg extracts where sequence-specific replication fork pausing was induced with biotin-streptavidin-modified plasmids. Here, the replicative Pols α, ε and δ, GINS, MCM2-7, Cdc45 and MCM10 were identified as components of the vertebrate replisome (Pacek et al., 2006). In the presence of aphidicolin, a DNA polymerase inhibitor, MCM2-7, Cdc45 and GINS were still found to be associated to the pause sites, whereas the replicative DNA polymerases showed a more dispersed distribution along the plasmid DNA. This result suggested that MCM2-7, Cdc45 and GINS form a stable heterologous complex with DNA unwinding activity (the so-called "unwindosome") that could be "uncoupled" from the machinery responsible for DNA synthesis. Association of GINS and MCM2-7 to the replication fork was also demonstrated by a proteomic study carried out in *S. cerevisiae* (Gambus et al., 2006). A yeast strain, which expressed differently tagged versions of Sld5 and MCM4, was created to purify complexes containing both MCM2-7 and GINS from cell extracts. After digesting the chromosomal DNA, the so-called Replisome Progression Complexes (RPCs), large protein assemblies (>1,400 kDa), were purified. Mass spectrometry identified the components of the RPCs, which included (in addition to GINS and MCM2-7) Cdc45, MCM10, DNA topoisomerase I, factors involved in the stabilization of stalled forks (Mrc1, Tof1 and Csm3), in sister chromatid cohesion (Ctf4), and in chromatin-remodelling (Spt16 and Pob3). The RPCs are formed only during S phase and disappear at the end of chromosomal replication, but are not disassembled when forks from early-firing origins are stalled by inhibitors of DNA synthesis (Gambus et al., 2006).

A complex containing Cdc45, MCM2-7 and GINS was also isolated by Moyer and colleagues from extracts of *Drosophila* embryos (Moyer et al., 2006). Biochemical and immunological analyses identified the six proteins of the MCM2-7 complex and four subunits of GINS as components of these purified samples. This protein assembly was called CMG (Cdc45/MCM2-7/GINS). Enzymatic assays revealed that the CMG complex possessed an ATPase-dependent DNA helicase activity with 3′–5′ directionality (Moyer et al., 2006).

The structural analyses of the human GINS suggest that this complex mediates the interaction with other replication factors. Two-hybrid analyses revealed that *S. cerevisiae* Psf1 and Dpb2 (the 60-kDa subunit of Pol ε) interact physically and, thus, a direct contact between GINS and Pol ε at the replication fork in budding yeast can be postulated (Takayama et al., 2003). In addition, a direct interaction between fission yeast Psf3 and subunits of the MCM2-7 complex has been detected by the two-hybrid system (unpublished data in Yabuuchi et al., 2006). A direct physical interaction between the N-terminal portion of the *Sulfolobus* MCM complex and the Psf2/Psf3 homolog has also been detected in a two-hybrid screening (Marinsek et al., 2006). Therefore, it is plausible that Psf3 is responsible for directly contacting the MCM complex within the replisome. However, also Psf1 could be

involved in the formation of a complex with Cdc45 and the MCM2-7 proteins, according to the analysis performed using the *Xenopus* egg extracts (Kamada et al., 2007). Furthermore, the *Sulfolobus* GINS complex was found to stably interact with the eukaryotic-like heterodimeric DNA primase in vitro and in vivo through the Psf2/Psf3 (GINS23) subunit (Marinsek et al., 2006). This finding is consistent with a recent report that the recombinant human GINS complex physically interacts in vitro with the Pol α-primase complex and stimulates its DNA synthesis activity (De Falco et al., 2007). This interaction is likely to take place through the Psf2 subunit, as recently proposed (Chang et al., 2007) and to involve other replication factors, such as MCM10 and Ctf4 (Zhu et al., 2007).

Initiation and Checkpoint

A Role for Initiation Factors During Checkpoint Response

When an ongoing replication fork encounters an obstacle or experiences a diminution of nucleotide building blocks it stops and triggers an intra S phase checkpoint mechanism that in turn prevents loading of initiation factors onto distal origins and subsequent firing of the later ones (Machida et al., 2005). This mechanism is conserved from yeast to man (Aparicio et al., 1999; Costanzo et al., 2003; Falck et al., 2002; Liu et al., 2006; Petersen et al., 2006). Signalling is typically initiated by the phosphatidyl inositol kinase-like kinases (PIKK) ataxia telangiectasia mutated (ATM) or ataxia telangiectasia and Rad-3 related (ATR) that phosphorylate the effector kinases Chk2 and Chk1, respectively. Inhibition of ATM or ATR by caffeine leads to an accumulation of Cdc45 on chromatin (Costanzo et al., 2003; Falck et al., 2002; Shechter et al., 2004). There is an intimate interplay between the checkpoint apparatus and DNA initiation factors that regulate initiation in response to DNA damage or physiological stress, but also during normal DNA replication. Most critical for the activation of replication are the S phase kinases Cdk2 (S-CDK) and Cdc7 (DDK). These proteins are regarded as being the most important targets for checkpoint control during S phase. ATM initiated signalling targets Cdk2 (Costanzo et al., 2000), while ATR mediated signalling targets also Cdc7 (Costanzo et al., 2003; Shechter et al., 2004). Several replication and initiation factors, including Sld2 have been implicated in checkpoint control (Wang and Elledge, 1999; Tourrière and Pasero, 2007). But without doubt, the initiation factor Dpb11/Cut5 and its metazoan TopBP1 homologues have emerged as the key player for the checkpoint regulation of initiation of replication. Budding yeast Dpb11 is required for S phase checkpoint control, as has Cut5 in fission yeast (Saka et al., 1994; Araki et al., 1995), reviewed in (Garcia et al., 2005). This role appears to be universal in eukaryotes (reviewed in "Function of TopBP1 in Genome Stability" by Sokka et al., this issue). It has been best studied in vertebrates, where TopBP1 and its homologues have been shown to represent a general activator of ATR (see "Function of TopBP1 in Genome Stability" by Sokka et al., this issue for a detailed review on this topic).

This function has also been confirmed for the yeast orthologues (Navadgi-Patil and Burgers, 2008; Mordes et al., 2008). There exists apparently a negative feedback loop, where TopBP1 is recruited by the checkpoint apparatus involved in ATR activation. This in turn leads to inhibition of the S phase kinases S-CDK and DDK, preventing initiation-activating phosphorylation of Sld2 and Sld3. It is therefore not surprising that ATM and ATR and their mediator kinase Chk1 affect also initiation of DNA replication in undamaged cells. They are believed to inhibit the firing of distal origins by inactivating phosphorylation of the S phase kinases and the MCM2-7 complex (Fisher and Méchali, 2004; Shechter et al., 2004; Shechter and Gautier, 2005).

DNA Initiation Factors and Stalled DNA Replication Forks

The DNA initiation factors discussed in the review also appear to have a role in the stabilization and re-activation of stalled replication forks. Cdc45 interacts with the mediator of the replication checkpoint 1 (Mrc1, known as claspin in mammals) and the topoisomerase 1-associated factor 1 (Tof1, known as Tim1 or *timeless* in mammals) (Katou et al., 2003). Mrc1/claspin is necessary for the activation of the intra S phase checkpoint whenever a replication fork stalls (Kumagai and Dunphy, 2000; Alcasabas et al., 2001). After the loading of Cdc45 both Mrc1/claspin and Tof1/Tim1 are recruited into the replication origin and all three proteins co-migrate with the ongoing replication fork (Katou et al., 2003; Osborn and Elledge, 2003). When such a fork hits a replication blockade Mrc1, Tof1 and other factors prevent disassembly of the replication machinery (Calzada et al., 2005; Katou et al., 2003; Nitani et al., 2006). Moreover, both proteins are important for the recovery of DNA synthesis at stalled forks after the block has been removed (Tourrière et al., 2005). The resulting stabilization of stalled replication forks is fundamental for preventing genomic instability in eukaryotes (Branzei and Foiani, 2006). This function primarily depends on the ATR pathway, including Mrc1/claspin. Stalled replication forks are also stabilized in a checkpoint-independent manner where the key players are Tof1/Tim1 plus Mrc1 (Katou et al., 2003; Tourrière et al., 2005). Although TopBP1 and its orthologues are not part of the progressing DNA replication fork, they have been shown to re-localize to the sites of stalled replication and they are involved in the restart of the stalled replication fork (Mäkiniemi et al., 2001). It is conceivable that a regulatory switch comparable to the replication initiation reaction is also required for the replication restart.

Conclusions

Eukaryotes have restricted replication to a specific phase of the cell cycle dedicated to extensive DNA synthesis. This has led to the invention of a sophisticated regulatory mechanism that ensures correct timing of origin firing. It is probably for the

sake of an efficient regulation that the critical targets of S-phase kinase-dependent promotion of replication, Sld2 and Sld3, are proteins that are dedicated to the assembly of the replication fork, but are not required for fork progression. Dpb11 (and its metazoan TopBP1 orthologues) likely represents a master controller that integrates mitogenic cell cycle and checkpoint signalling to regulate the initiation at the level of the individual origin of replication.

The role of the Sld2-Sld3-Dpb11 phosphorylation switch lies in the modification of the pre-replication complex that leads to the loading of Cdc45, GINS and eventually the replicative DNA polymerases.

Since no enzymatic activities have been assigned to Cdc45 and GINS, it is likely that these factors play rather structural roles at the replication fork. Cdc45 and GINS have been on the one hand proposed as MCM2-7 helicase cofactors that form together the CMG unwindosome, and on the other hand as bridging factors between the replicative helicase and DNA polymerases. They not only seem to coordinate helicase and DNA polymerases action but may also retrieve and integrate signals from several pathways to uncouple the CMG helicase from the Pols δ and ε.

Despite the picture emerging for the initiation and its regulation, major challenges are left for the future: the mechanisms of the regulatory switch in metazoan initiation are still to be elucidated, the structural understanding of the initiation complex is still rudimentary, and the interplay between initiation and checkpoint control awaits to be defined in detail. These and many other unresolved issues will provide topics for many years of DNA replication research.

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Non-coding RNAs: New Players in the Field of Eukaryotic DNA Replication

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Abstract The machinery required for the replication of eukaryotic chromosomal DNA is made up of proteins whose function, structure and main interaction partners are evolutionarily conserved. Several new cases have been reported recently, however, in which non-coding RNAs play additional and specialised roles in the initiation of eukaryotic DNA replication in different classes of organisms. These non-coding RNAs include Y RNAs in vertebrate somatic cells, 26T RNA in somatic macronuclei of the ciliate *Tetrahymena,* and G-rich RNA in the Epstein-Barr DNA tumour virus and its human host cells. Here, I will give an overview of the experimental evidence in favour of roles for these non-coding RNAs in the regulation of eukaryotic DNA replication, and compare and contrast their biosynthesis and mechanisms of action.

Keywords DNA replication · Initiation · Non-coding RNA · ORC recruitment · Origin of replication

Abbreviations

ARS	autonomously replicating sequence
CDK	cyclin-dependent kinase
DS	dyad symmetry element
EBER1	EBV-encoded small nuclear RNA 1
EBNA1	Epstein Barr virus nuclear antigen 1
EBV	Epstein Barr virus
FMRP	fragile X mental retardation protein
GINS	go-ichi-ni-san (five-one-two-three in Japanese)
HMG	high mobility group
hY RNA	human Y RNA
LR	linking region

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MCM	mini-chromosome maintenance
ORC	origin recognition complex
OriP	origin of plasmid replication
PCNA	proliferating cell nuclear antigen
rDNA	ribosomal DNA
RFC	replication factor C
RGG	arginine/glycine-rich repeats
RNP	ribonucleoprotein
RPA	replication protein A
rRNA	ribosomal RNA
TIF	type I element binding factor

Introduction

The protein machinery required for the replication of chromosomal DNA is evolutionarily conserved in eukaryotes with regards to function, structure and main interaction partners. It includes proteins of the pre-replication complex (e.g. ORC, Cdc6, Cdt1 and MCM2-7), the pre-initiation and initiation complexes (e.g. Cdc45, GINS, Mcm10, RPA, DNA polymerase α/primase), protein kinases controlling initiation (e.g. cyclin/CDK complexes and Dbf4/Cdc7), and proteins of the replication fork elongation complex (DNA polymerases α, δ, ε, RFC, PCNA, Fen1, DNA ligase and many others). These protein complexes have been extensively reviewed, in this volume and elsewhere (Bell and Dutta, 2002; Takeda and Dutta, 2005; Machida et al., 2005; Nasheuer et al., 2007; DePamphilis et al., 2006; Arias and Walter, 2007).

Additional levels of control have evolved on top of this core machinery (Arias and Walter, 2007), mainly around the initiation step. For instance, in metazoa the activity of Cdt1 protein is controlled by two proteins that have no counterparts in unicellular organisms, geminin and MCM9 (Lutzmann et al., 2006; Lutzmann and Mechali, 2008). Geminin is required at low concentrations to load the MCM2-7 complex onto chromatin in concert with Cdt1 and MCM9, whereas higher geminin concentrations as found in S and G2 phases inhibit the Cdt1/Mcm9-dependent loading of new MCM2-7, thus preventing re-licensing and unwanted re-replication (Lutzmann et al., 2006; Lutzmann and Mechali, 2008).

A new class of factors, which play essential roles in the regulation of DNA replication in different organisms have recently been identified. These factors are RNAs that do not code for proteins. Such “non-coding RNAs” are already recognised as cellular regulators of key events including RNA splicing, editing, translation and degradation, heterochromatin formation and development. This field has been extensively reviewed, for recent examples see: (Hogg and Collins, 2008; Ponting et al., 2009; Carthew and Sontheimer, 2009; Ghildiyal and Zamore, 2009; Amaral and Mattick, 2008). Non-coding RNAs function through very different pathways, but key mechanistic principles include mediating base-specific interactions with other

nucleic acids (e.g. snoRNA in RNA editing, miRNA in RNA translation, siRNA in RNA degradation), providing protein binding sites for the assembly of functional ribonucleoprotein complexes (RNPs) (e.g. XIST RNA in heterochromatin formation, roX RNA in dosage compensation, 7SK RNA in transcription factor assembly) or direct catalysis (e.g. ribozymes and riboswitches).

In the following discussion, I will give an overview of experimental evidence in favour of non-coding RNAs playing a role in the regulation of eukaryotic DNA replication. I will not consider RNA molecules that serve as templates for DNA synthesis, such as the RNA component of telomerase, or short complementary RNAs synthesised by DNA primase to prime DNA strand synthesis. The objective of this review is to provide an initial conceptual framework for non-coding RNAs as new players controlling eukaryotic DNA replication.

Non-coding RNAs in Eukaryotic DNA Replication

Over the last three years, and in three different experimental systems, three independent cases for non-coding RNAs regulating aspects of eukaryotic DNA replication have been published. Below, I will discuss them in order of appearance.

Y RNA

The first example of a non-coding RNA involved in eukaryotic DNA replication is Y RNA, which is required for the functional reconstitution of chromosomal DNA replication in a mammalian cell-free experimental system (Christov et al., 2006). The initiation and elongation stages of mammalian chromosomal DNA replication can be reconstituted biochemically in cell-free systems derived from cultured somatic cells (Krude, 2006). Templates for chromosomal DNA replication are nuclei isolated from cells synchronised in late G1 phase of the cell cycle. Chromosomal DNA replication is initiated in these nuclei upon incubation in extracts from human S phase cells (Krude et al., 1997; Stoeber et al., 1998), or from asynchronously proliferating human cells (Krude, 2000). These cell extracts thus contain essential factors that interact with late G1 phase template nuclei, leading to the establishment of new replication forks in these nuclei.

Fractionation experiments in this system led to the isolation of RNA from the activating cytosolic extract by anion-exchange and arginine-affinity chromatography (Christov et al., 2006). Chromosomal DNA replication is reconstituted by this RNA fraction in the presence of two additional protein fractions (Christov et al., 2006), which contribute the single stranded DNA binding protein RPA (Szüts et al., 2003), the sliding clamp PCNA (Szüts et al., 2005), and other proteins. The active component of the essential RNA fraction comprises the small non-coding human Y RNAs (hY1, hY3, hY4 and hY5 RNA). Testing of individual recombinant hY RNAs confirmed that they are essential to reconstitute chromosomal DNA replication in vitro, and that they are functionally redundant with each other (Christov

et al., 2006). Loss-of-function experiments have further confirmed a requirement of Y RNAs for the reconstitution of human chromosomal DNA replication. Specific ribonucleolytic degradation of Y RNAs in the human cell extract by targeting RNAseH activity with anti-sense DNA oligonucleotides to individual hY RNAs inhibits the activation of DNA replication in the template nuclei, and addition of an excess of a non-targeted hY RNA overcomes this inhibition again (Christov et al., 2006).

These biochemical experiments did not distinguish between a function of Y RNAs in the either initiation or elongation stages of chromosomal DNA replication. To differentiate between these possibilities, a single molecule analysis of DNA replication fork dynamics was performed, showing that degradation of Y3 RNA in a human cell extract drastically reduces the number of active replication forks initiated in late G1 or S phase template nuclei in vitro (Krude et al., 2009). In contrast, Y3 RNA degradation has no significant effect on individual DNA replication fork progression speeds (Krude et al., 2009). These findings were confirmed by bulk nascent strand analysis (Krude et al., 2009). Therefore, the execution point for Y RNA function is the initiation stage, and not the chain elongation stage of mammalian chromosomal DNA replication.

A functional requirement of hY RNAs for mammalian chromosomal DNA replication has also been found in cell-based systems. Degradation of hY1 or hY3 RNAs in proliferating human cells by RNA interference reduces the proportion of replicating cells in the treated cell population and leads to a cytostatic inhibition of cell proliferation (Christov et al., 2006, 2008). Consistent with a functional role in cell proliferation, Y RNAs are over-expressed in human solid tumours (e.g. carcinomas and adenocarcinomas of the lung, kidney, bladder, prostate, colon and cervix), when compared to normal reference tissues of the same origin (Christov et al., 2008).

Taken together, these observations provide the first precedent for a non-coding RNA as an essential factor in eukaryotic DNA replication. Y RNAs have been known for 25 years, but their function had remained elusive. Therefore, these observations also demonstrate for the first time a direct essential function for Y RNAs in a metabolic process in eukaryotes.

Y RNAs were originally discovered in the early 1980 s as an RNA component of Ro RNPs (Lerner et al., 1981), which also contain Ro60 protein (Chen and Wolin, 2004) and other potentially associated proteins. The four human Y RNAs are encoded in single copy genes, clustered into a single locus on chromosome 7 (Hendrick et al., 1981; Matera et al., 1995; Maraia et al., 1994, 1996). They are transcribed by RNA polymerase III, the primary Y RNA transcripts are not modified, and they fold into characteristic stem-loop structures (Farris et al., 1999; Teunissen et al., 2000). The partially complementary 5′ and 3′ terminal domains form a double-stranded stem structure, which is linked by a single-stranded loop domain containing secondary stem-loops. The terminal stem contains conserved binding sites for Ro60 and La proteins (Green et al., 1998; Stein et al., 2005; Wolin and Cedervall, 2002), which have been implicated in protecting Y RNAs from exonucleolytic degradation, amongst other functions (Chen and Wolin, 2004; Wolin and Cedervall, 2002).

Inactivation or deletion of these binding sites does not inhibit the ability of the mutant Y RNA to support initiation of chromosomal DNA replication (Christov et al., 2006; Gardiner et al., 2009), indicating that neither Ro60, La, nor their binding sites on Y RNAs fulfil an essential role in DNA replication. Consistently, genetic knockouts of Ro60 orthologues in different organisms show no effect on DNA replication (Chen et al., 2003, 2000; Labbe et al., 1999; Xue et al., 2003).

Y RNAs have been conserved during evolution (Pruijn et al., 1993; Farris et al., 1995; Mosig et al., 2007; Perreault et al., 2007). Homologues of the four human Y RNAs have been found in all major classes of vertebrates investigated. Individual gene-loss events have occurred during evolution in most classes, resulting in an uneven number of expressed Y RNAs in extant vertebrates (Pruijn et al., 1993; Farris et al., 1995; Mosig et al., 2007; Perreault et al., 2007). For instance, zebrafish only express Y1, chicken express Y3 and Y4, and mice express Y1 and Y3 RNAs. Small RNAs with structural features similar to Y RNAs, or genes coding for candidate Y RNAs have also been identified in a few isolated non-vertebrates, including the prokaryote *Deinococcus radiodurans*, the nematode *Caenorhabditis elegans* (van Horn et al., 1995; Chen et al., 2000), the lancelet *Branchiostoma floridae* (Mosig et al., 2007) and the insect *Anopheles gambiae* (Perreault et al., 2007). These few non-vertebrate Y RNAs are evolutionarily distant from vertebrate Y RNAs as they cannot be assigned to any of the four vertebrate Y RNA clades (Perreault et al., 2007). However, no Y RNA genes have been found in the genomes of fungi, plants, echinoderms and other invertebrates.

The function of Y RNAs in chromosomal DNA replication is conserved in vertebrates (Gardiner et al., 2009). Y RNAs from fish, amphibians, reptiles, birds and mammals can fully substitute for human Y RNAs to permit the initiation of DNA replication in late G1 phase human nuclei, whereas non-vertebrate Y RNAs cannot (Gardiner et al., 2009). These functional data show that a feature of vertebrate Y RNAs required for the initiation of chromosomal DNA replication is evolutionarily conserved. The most conserved structural domain of vertebrate Y RNAs is their double-stranded terminal stem, whereas the central loop domain is diverse in nucleotide sequence and structure (Pruijn et al., 1993; Farris et al., 1995; Mosig et al., 2007; Perreault et al., 2007). Systematic mutagenesis experiments have shown that the central portion of the evolutionarily conserved double-stranded stem is essential for reconstituting chromosomal DNA replication, whereas the central loop and the terminal stem are dispensable for this function (Gardiner et al., 2009). A short 9 bp double-stranded RNA helix representing the central stem of hY1 RNA is actually sufficient to substitute for any full-length vertebrate Y RNA in reconstituting chromosomal DNA replication in vitro, and its insertion into a folded inert RNA backbone generates a functional Y RNA in vitro (Gardiner et al., 2009).

A potential direct molecular interaction between Y RNA and the core protein machinery required for chromosomal DNA replication in vertebrate somatic cells has not yet been reported. This is a focus of ongoing work in my laboratory at the time of writing. However, several key conclusions regarding Y RNA function in chromosomal DNA replication can be drawn. Firstly, Y RNAs are not required for actual DNA strand synthesis in eukaryotes, because this synthesis can be

faithfully reconstituted from purified proteins in the absence of Y RNA (Challberg and Kelly, 1989; Waga and Stillman, 1998), and because degradation of Y RNA does not significantly influence replication fork speeds in mammalian cell nuclei (Krude et al., 2009). Secondly, the execution point of vertebrate Y RNA function is the initiation of DNA replication, i.e. the activation of somatic replication origins and the establishment of replication forks. However, this requirement does not apply to all eukaryotes because DNA replication takes place in non-vertebrate organisms such as yeast, plants and invertebrates that do not have Y RNA genes. It seems likely, therefore, that Y RNAs are not a core component of the conserved eukaryotic DNA synthesis machinery, but probably act as an additional but essential switch, providing another layer of control over the initiation step in vertebrate somatic cells. This postulated role would be broadly analogous to the role of vertebrate-specific MCM9 and geminin proteins in the control of the conserved DNA replication licensing protein Cdt1 (Lutzmann et al., 2006; Lutzmann and Mechali, 2008). It is also conceivable, and not mutually exclusive with this working model, that Y RNAs are involved in checkpoint control or developmental regulation of the initiation step of chromosomal DNA replication.

Future work on the identification of the Y RNA interacting proteins will replace this tentative working model with a more sophisticated description of the protein-RNA network in control of the initiation of chromosomal DNA replication in vertebrates.

26T RNA

The unicellular ciliate *Tetrahymena thermophila* contains two types of nuclei in a single cell: a small, diploid, silent, germline micronucleus, and a large, polyploid, transcriptionally active, somatic macronucleus. The macronuclei develop from micronuclei through a pathway involving chromosome fragmentation and amplification. After conjugation and fusion of two haploid pronuclei, the resulting diploid micronucleus divides mitotically and half the progeny differentiates into macronuclei. During this macronuclear development, the five monocentric chromosomes become fragmented into about 250–300 small chromosomes and each fragment chromosome amplifies to copy numbers of about 45. An exceptional case here is the ribosomal DNA (rDNA) chromosome, of which about 9,000 copies are generated. The 21 kbp rDNA chromosome is an inverted repeat with a central non-transcribed spacer and two divergently transcribed 35S rRNA genes. The non-transcribed spacer contains a defined origin of replication, the rDNA origin, which is essential both for the amplification of the chromosome during development and for subsequent once-per-cell cycle vegetative replication.

Initiation of DNA replication at the rDNA origin during amplification and vegetative growth is under the control of *cis*-acting A/T-rich "type I elements" (Reischmann et al., 1999). They are recognised by four sequence-specific *trans*-acting protein complexes, the "type I element binding factors" TIF1 to TIF4 (Umthun et al., 1994; Saha and Kapler, 2000; Mohammad et al., 2000, 2003). These

TIFs bind to single-stranded DNA target sequences, implying that DNA exists in an unwound state at the rDNA origin. Biochemical, immunological and bioinformatical analyses have established that TIF4 is the *Tetrahymena thermophila* origin recognition complex, ORC (Mohammad et al., 2003, 2007).

It was recently shown that the binding of *Tetrahymena* ORC to the rDNA origin of replication is regulated by a non-coding RNA (Mohammad et al., 2007). ORC (TIF4) binds to the T-rich DNA strand of the type I element in an ATP-dependent manner, but not to the A-rich strand, or the corresponding DNA duplex (Mohammad et al., 2003, 2007). Ribonuclease treatment eliminates this binding (Mohammad et al., 2007). Therefore, *Tetrahymena* ORC is a ribonucleoprotein (RNP), which binds to an rDNA origin-specific single-stranded DNA in an RNA-dependent manner. The ORC-associated RNA was isolated and sequenced, and its nucleotide sequence corresponds to 282 nucleotides of the 3′ terminus of the mature 26S rRNA (Mohammad et al., 2007). It was therefore designated 26T RNA. In pull-down assays using recombinant 26T RNA, the authors have found a specific association of the ORC RNP complex with the rDNA origin, but not with the fully complementary rRNA coding sequence or rDNA promoter sequences which also contain type I elements. Complementary mutations of 26T RNA and the target origin DNA confirm that their interaction is nucleotide sequence-specific, mediated by specific base pairing between the 26T RNA and the single-stranded origin DNA. Finally, mutations of 26T RNA, which disrupt recognition of the rDNA origin sequences, result in an inhibition of the activation of the rDNA origin in vivo.

Taken together, these experiments provide experimental evidence for a role of a non-coding RNA in the specification of ORC binding to a particularly active origin of DNA replication, the rDNA origin. The molecular mechanism responsible for this specification involves the specific base pairing between the non-coding RNA with a single-stranded target DNA sequence, thereby recruiting ORC complex to the target origin DNA sequence.

A question arising from this work is whether the RNA-dependent mechanism of ORC recruitment to DNA replication origins is the rule in *Tetrahymena*, or whether it serves a specialised role dedicated to the over-amplified rDNA origin. Chromatin immunoprecipitation (ChIP) experiments showed that ORC is bound specifically to the rDNA origin sequence, but not to other sites on the rDNA macronuclear chromosome. This binding was detected throughout the cell cycle and the ORC complex does not dissociate from chromatin after the initiation of DNA replication and the degradation of the regulatory subunit Orc1p (Mohammad et al., 2007; Donti et al., 2009). Recently, another replication origin termed ARS1 was isolated from *Tetrahymena,* using a plasmid maintenance assay (Donti et al., 2009). ARS1 lacks type I elements, and therefore 26T RNA-binding sites, which suggests that this origin may recruit ORC independently of 26T RNA (Donti et al., 2009). In contrast to the rDNA chromosome, ORC binds indiscriminately to random sites on the ARS1-containing macronuclear chromosome in G2 phase following DNA replication, but relocalises to the ARS1 origin in G1 phase, prior to initiation of DNA replication. Following initiation and Orc1p degradation, the ORC complex dissociates in S phase until it re-associates again with the replicated daughter chromosomes

at random positions in G2 phase (Donti et al., 2009). These data show that the binding specificity of ORC to the ARS1 origin is regulated during the cell cycle and does not depend on specific base-pairing between the 26T RNA and origin DNA, whereas the binding of ORC to the rDNA origin does not change during the cell cycle and depends on the interaction between 26T RNA and the origin DNA.

In conclusion, the employment in *Tetrahymena* of a sequence-specific, non-coding RNA for targeting ORC to the rDNA origin represents a specific case of regulating the activity of this particular origin. ORC recruitment to non-rDNA origins and initiation of DNA replication can proceed without the requirement for complementary base-pairing between unwound origin sequences and the ORC-specific 26T RNA. One could argue here that a key function for the non-coding 26T RNA in the control of rDNA replication is linked to the efficient and site-specific recruitment of ORC complex to this particular replicon, which is highly amplified during development. The employment of a non-coding RNA, which increases specificity or affinity of ORC for this origin would thus provide a selective advantage under competitive demand for limited replication initiation factors. However, it cannot be excluded at present that 26T RNA may also play a general structural role in the ORC complex, which is independent from the function of targeting ORC to the rDNA origin.

Structured G-Rich RNA

Epstein-Barr virus (EBV) is an oncogenic herpesvirus, which infects human cells and becomes established as a latent extrachromosomal minichromosome replicating under once-per-cell cycle control in the host cell. Latent EBV DNA replication initiates at the origin of plasmid replication (oriP) and is under the control of the virus-encoded protein, EBNA1 (Lindner and Sugden, 2007). EBNA1 binds to specific DNA elements of oriP and recruits the host cell origin recognition complex (ORC) to a particular element of oriP, the dyad symmetry element (DS) (Schepers et al., 2001; Dhar et al., 2001). The EBNA1:ORC interaction integrates latent viral replication into the cellular replication cycle (Chaudhuri et al., 2001; Ritzi et al., 2003). This recruitment of human ORC, which has no specific DNA binding activity of its own, to a specific DNA sequences through association with EBNA1 is an attractive model and precedent for the establishment of replication origins in human cells.

A recent molecular analysis of ORC recruitment to oriP has provided clear evidence for a structured non-coding RNA as a key factor mediating the interaction between ORC and EBNA1 (Norseen et al., 2008). Deletion analysis has identified two domains in the amino terminal half of EBNA1, termed linking regions 1 and 2 (LR1 and LR2), which are required and sufficient for interaction with ORC, as measured by immunoprecipitation from human cell extracts. These LR domains consist of arginine/glycine-rich repeats (RGG motifs), and substitution of these motifs with alanine prevented interaction with ORC. When fused to the DNA binding domain of EBNA1, LR1 or LR2 promoted plasmid replication from oriP whereas the alanine

substitution mutants did not, indicating that ORC recruitment via this domain is required to establish a functional origin of DNA replication. EBNA1 binds RNA via the RGG motifs (Snudden et al., 1994; Lu et al., 2004), suggesting that ORC recruitment via these motifs might be influenced by RNA. Indeed, treatment of EBNA1 immunoprecipitates with ribonucleases disrupts the interaction between EBNA1 and ORC, thus establishing that it is RNA-dependent (Norseen et al., 2008).

The EBNA1-associated RNA is very heterogeneous in size and includes the EBV-encoded small nuclear RNA1 (EBER1), its own mRNA and other transcripts (Snudden et al., 1994; Lu et al., 2004; Norseen et al., 2008). EBNA1 displays a preference for G-rich RNA with particular secondary structures (Lu et al., 2004; Norseen et al., 2008). ORC1 protein also binds to G-rich RNA, but not to G-poor RNA, and tertiary [EBNA1/G-rich RNA/ORC] complexes can be formed in vitro provided the RNA exceeds a critical length (Norseen et al., 2008). These experiments have therefore established a role for structured G-rich RNA in the EBNA1-dependent recruitment of ORC to replication origins. G-rich RNA has the potential to form a complex four-stranded G-quadruplex structure (Huppert, 2008), so it becomes an interesting and testable hypothesis that G-quadrupex RNA mediates the interaction between EBNA1 and ORC.

RGG motifs are not unique to LR1 or LR2 of EBNA1. The RGG motif of the RNA-binding protein FMRP (fragile X mental retardation protein) interacts specifically with G quadruplex RNA (Darnell et al., 2001, 2004). This example supports the notion that G quadruplexes may be the relevant RNA structure that mediates the interaction between the RGG motifs of EBNA1 and ORC. The small cellular non-histone chromatin protein HMGA1a can substitute for the amino terminal half of EBNA1 to support oriP-dependent plasmid replication, plasmid maintenance and binding to ORC (Sears et al., 2003; Thomae et al., 2008). HMGA1a and ORC also interact in discrete domains in human cell nuclei in vivo, which consist of A/T-rich heterochromatin (Thomae et al., 2008). HMGA1a contains an RGR motif, which like the RGG motifs of EBNA1, is able to recruit ORC in a manner dependent on structured G-rich RNA (Norseen et al., 2008). This observation opens up the possibility that structured G-rich (possibly quadruplex) RNA may also mediate the recruitment of ORC to cellular chromatin via HMGA1a or other RGG motif containing cellular proteins. This hypothesis is supported by the observation that treatment of cellular chromatin with ribonucleases releases a fraction of ORC (Norseen et al., 2008). It is therefore a possibility that the RNA-dependent recruitment of ORC to cellular chromatin may contribute to the establishment of cellular origins of replication.

Conclusions

Over the last three years, clear evidence in favour of regulatory roles for non-coding RNAs in eukaryotic DNA replication has emerged from model systems as distant as mammalian chromosomes (Christov et al., 2006, 2008; Norseen et al., 2008;

Gardiner et al., 2009; Krude et al., 2009), ciliated protozoa (Mohammad et al., 2007) and DNA tumour viruses (Norseen et al., 2008). The underlying RNAs range from defined primary transcripts of single-copy genes (Y RNAs), discrete processing products of larger transcripts (26T RNA), to quite heterogeneous populations (structured G-rich RNA).

One common feature of the cases reported so far is that these non-coding RNAs are involved in the initiation step of DNA replication. The underlying molecular mechanisms are either still unclear, or involve the recruitment of the initiator protein ORC to specific DNA sequence elements in order to establish active DNA replication origins. RNA-dependent recruitment of ORC to DNA replication origins can be mediated through various mechanisms, either by hybridisation of the ORC-associated non-coding RNA with single-stranded origin DNA (Mohammad et al., 2007), or by providing interfaces for high-affinity protein-protein interactions (Norseen et al., 2008).

A second common principle is the requirement of particular nucleotide sequence elements for non-coding RNA function in DNA replication. However, the identity of the involved sequence elements differs drastically in the three examples discussed. For Y RNAs, all that is necessary and sufficient for their function is a single turn of double-stranded RNA helix containing a patch of defined nucleotide sequence (Gardiner et al., 2009). 26T RNA requires a partially complementary nucleotide sequence to hybridise with single-stranded origin DNA in order to stimulate site specific initiation at the rDNA origin (Mohammad et al., 2007). G-rich nucleotide elements, possibly in quadruplex configuration, are required on larger RNAs to bind the RGG Boxes of EBNA1 or HMGA1a to mediate the recruitment of ORC to oriP and, possibly, some cellular origins (Norseen et al., 2008).

A further common feature emerging from these cases is that non-coding RNAs are not part of the evolutionarily conserved core machinery for eukaryotic DNA synthesis, but seem to have evolved as additional layers of control. Without Y RNA, initiation of DNA replication is inhibited in vertebrates, but not in non-vertebrates (Gardiner et al., 2009). Without 26T RNA, non-rDNA chromosomes do replicate efficiently in *Tetrahymena* and even the rDNA minichromosomes can be replicated passively from different origins to some extent (Mohammad et al., 2007). Without the structured G-rich RNA, replication of oriP-dependent episomes would be compromised in human host cells, but only a fraction of cellular origins may be affected, if any (Norseen et al., 2008). Therefore, it seems reasonable to conclude that non-coding RNAs provide additional levels of control for the core protein machinery for eukaryotic DNA replication. This would be in keeping with the well-established but diverse regulatory roles of a plethora of non-coding RNAs in the field of gene expression.

This may only be the initial wave of non-coding RNAs as new players in the field of DNA replication. Exciting discoveries of novel participants, and detailed analyses to unravel the molecular mechanisms by which these non-coding RNAs perform their regulatory roles lie ahead.

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Function of TopBP1 in Genome Stability

Miiko Sokka, Sinikka Parkkinen, Helmut Pospiech, and Juhani E. Syväoja

Abstract Human DNA topoisomerase IIβ-binding protein 1 (TopBP1) and its orthologues in other organisms are proteins consisting of multiple BRCT modules that have acquired several functions during evolution. These proteins execute their tasks by interacting with a great variety of proteins involved in nuclear processes. TopBP1 is an essential protein that has numerous roles in the maintenance of the genomic integrity. In particular, it is required for the activation of ATM and Rad3-related (ATR), a vital regulator of DNA replication and replication stress response. The orthologues from yeast to human are involved in DNA replication and DNA damage response, while only proteins from higher eukaryotes are also involved in complex regulation of transcription, which is related to cell proliferation, damage response and apoptosis. We review here the recent progress in research aimed at elucidating the multiple cellular functions of TopBP1, focusing on metazoan systems.

Keywords BRCT · Checkpoint · DNA damage response · Replication · TopBP1

Introduction

Human DNA topoisomerase IIβ-binding protein 1 (TopBP1) was originally isolated in a two-hybrid screen as a protein binding to the C-terminal domain of topoisomerase IIβ (Yamane et al., 1997). The 180 kDa protein contains eight Brca1 C-terminus (BRCT) repeats which are common in proteins involved in DNA damage repair or cell cycle control. The diversity of the sequence and function of the BRCT superfamily suggests that BRCT domains are interaction modules forming homo/hetero BRCT multimers, BRCT-non-BRCT interactions, and interactions

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with DNA strand breaks (Huyton et al., 2000; Saka et al., 1994a). Binding of BRCT domains may be dependent on phosphorylation of the target domain (Manke et al., 2003; Yu et al., 2003).

Orthologues of mammalian TopBP1 include Xcut5/Xmus101 in *Xenopus,* Mus101 in *Drosophila*, MUS-101 in *Caenorhabditis elegans*, Mei1 in *Arabidopsis thaliana*, Dpb11 in *Saccharomyces cerevisiae*, and Cut5/Rad4 in *Schizosaccharomyces pombe* (Makiniemi et al., 2001; Garcia et al., 2005; Nasheuer et al., 2007). When compared to vertebrate TopBP1, lower metazoan and yeast counterparts are smaller. Consequently, proteins from *Drosophila*, *C. elegans* and yeasts contain seven, six and four BRCT repeats, respectively (Fig. 1). It appears that both gains as well as loss of additional BRCT repeats, and obviously also adaptation of the functions, have occurred during evolution.

After early observations on the requirement of yeast Dpb11 and Cut5 for onset of DNA replication and for cell cycle control (Araki et al., 1995; Saka et al., 1994b; see also Pospiech et al. in this issue for a more detailed Overview on eukaryotic initiation of replication) the research reports on the function of vertebrate TopBP1 and lower metazoan orthologues started to accumulate at the beginning of this decade. The *Drosophila Mus101* gene was cloned and found to be involved in DNA repair, replication and mitosis in 2000 (Yamamoto et al., 2000). The human protein was associated with DNA replication and DNA damage response (Makiniemi et al., 2001) and *S. cerevisiae* Dpb11 with the initiation of replication (Masumoto et al., 2000). The finding that human and *Xenopus* TopBP1 activates the ATR-ATRIP complex (Kumagai et al., 2006), a regulator of normal DNA replication and replication stress response has been crucial for recent progress. However, the mechanism how TopBP1 integrates the different aspects of cell cycle machinery and DNA metabolism awaits further explanation. An excellent review on TopBP1 and its homologues was published some years ago, focusing mainly on the yeast

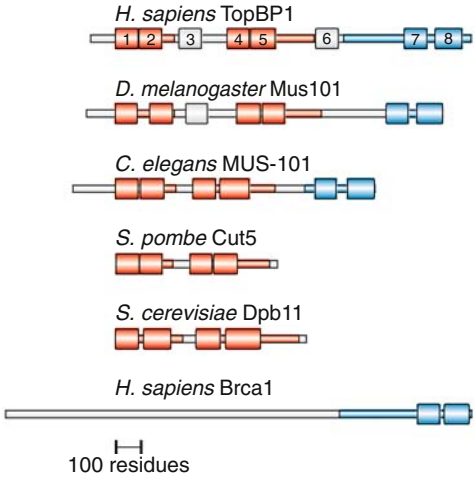

Fig. 1 Structural conservation of TopBP1 orthologues and Brca1. BRCT domains are Boxed. N-terminal regions similar to the Cut5 are in *red* and C-terminal regions similar to the Brca1 are in *blue*

homologues (Garcia et al., 2005). In the current review we wish to concentrate on the extensive progress made thereafter. The focus of this review is on the metazoan orthologues, which are structurally more complex than the corresponding yeast proteins and hence execute, at least partially, different functions.

Role of TopBP1 in DNA Damage Signaling

Identification of TopBP1 as a Damage Response Protein

TopBP1 has been initially implicated in the DNA damage response by cell biological experiments. During S phase TopBP1 and Brca1 are found to co-localize in foci that are distinct from replication foci (Makiniemi et al., 2001). When replication forks are stalled by hydroxyurea or 4-NQO, TopBP1 together with Brca1 are re-localized to replication foci, suggesting that TopBP1 plays a role in rescue of stalled replication forks (Makiniemi et al., 2001; Yamane et al., 2002). This behavior has been already previously reported for Brca1 (Scully et al., 1997). UV-irradiation also induced TopBP1 foci that co-localized with Brca1, but only a fraction of these foci represented sites of ongoing DNA replication (Makiniemi et al., 2001). Induction of DNA double strand breaks by γ-irradiation or treatment with the radiomimetic chemical zeocin resulted in foci that again co-localized with Brca1 but not with ongoing DNA replication detected by PCNA (Makiniemi et al., 2001; Yamane et al., 2002). This DNA damage dependent targeting of TopBP1 to Brca1 foci suggests a role for TopBP1 in the DNA damage response. Additional evidence for this notion has been provided by showing that TopBP1 interacts with Rad9 (Makiniemi et al., 2001; Greer et al., 2003). Rad9 forms a repair clamp with Rad1 and Hus1 (9-1-1) that is structurally related to PCNA (Thelen et al., 1999; Parrilla-Castellar et al., 2004). The interaction is mediated by the C-terminus of Rad9 (Makiniemi et al., 2001; Greer et al., 2003) and BRCT domains four and five (Makiniemi et al., 2001), and by domains one and two (Delacroix et al., 2007) of TopBP1 (Fig. 2). Interestingly, the focus formation in response to γ-irradiation was found to be dependent on the fifth BRCT domain of TopBP1 (Yamane et al., 2002), suggesting that interaction with Rad9 may be required for the targeting of TopBP1 to the sites of DNA damage. The interaction is regulated by phosphorylation of Rad9 (Greer et al., 2003; St. Onge et al., 2003). The interaction, including its dependence on the phosphorylation of Rad9, seems to be conserved in evolution from yeasts to human (Garcia et al., 2005).

Involvement of ATM/ATR in TopBP1 Mediated Damage Response

ATM (ataxia-telangiectasia mutated) and ATR (ataxia-telangiectasia mutated and RAD3-related) are the two major players in the initiation and regulation of DNA damage response. ATM and ATR are large phosphoinositide 3-kinase-related

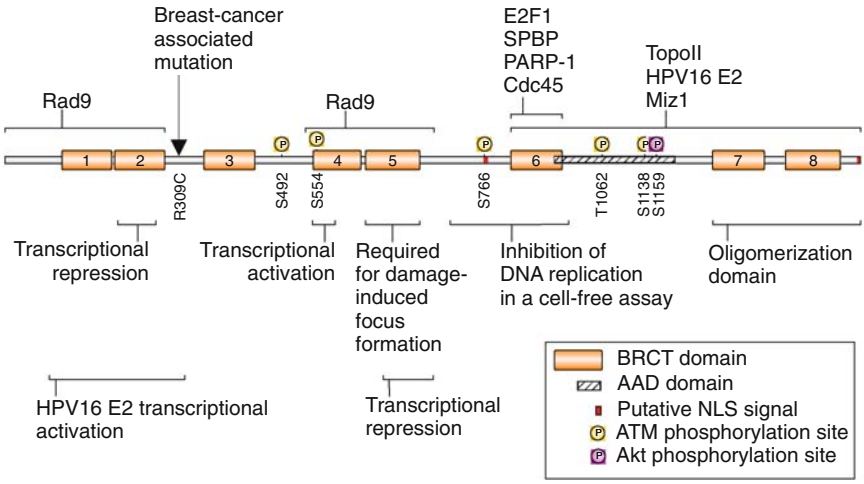

Fig. 2 Assignment of functions to the TopBP1 protein. BRCT domains are Boxed and the ATR activating domain (AAD) is hatched. Putative nuclear localization signals (NLS) are marked in *red*. ATM phosphorylation sites are depicted with *yellow circles* and the Akt phosphorylation site with a *purple circle*. The corresponding amino acid residues are indicated below the diagram. Functional domains and interaction sites are marked below and above the diagram, respectively. A breast-cancer-associated mutation Arg309Cys is marked with an *arrow*. See text for details

serine-threonine kinases (PIKK) that share, together with DNA-dependent protein kinase, significant sequence homology and a related overall structure (Abraham, 2001; Nasheuer et al., 2002). Their biochemical properties and functions are similar, and they share many substrates. ATM is activated mainly by DNA double-strand breaks and ATR by other DNA lesions that block DNA replication. However, ATR is also activated by double-strand breaks, and it seems to be more important than ATM for genomic integrity. Once activated, both signaling molecules control cell cycle transitions, DNA repair, apoptosis and senescence, by phosphorylating a number of target proteins.

Phosphorylation of histone H2AX (γH2AX) is considered to indicate DNA strand breaks (Rogakou et al., 1998). Phosphorylation of H2AX by DNA-PKcs, ATR and ATM is followed by recruitment of other DNA damage response proteins including NBS1, 53BP1 (Celeste et al., 2002; Yamane et al., 2002) and TopBP1 (Yamane et al., 2002; Greer et al., 2003). Coimmunoprecipitation of TopBP1 and 53BP1 indicate a physical interaction between the two proteins (Yamane et al., 2002). Promyelocytic leukaemia protein (PML), a multifunctional tumor suppressor, was also found to colocalize with TopBP1 in response to ionizing radiation (Xu et al., 2003). Overexpression of PML seemed to stabilize TopBP1. Honda and co-workers (Honda et al., 2002) found that γ-irradiation diminish ubiquitination of TopBP1 by hHYD ubiquitin ligase and subsequent proteosome-degradation. This Results in the up-regulation and stable co-localization of TopBP1 with γH2AX.

TopBP1 is a phosphoprotein (Makiniemi et al., 2001) and it is phosphorylated in response to hydroxyurea treatment as well as DNA damage (Yamane et al., 2003). In

response to ionizing radiation, TopBP1 is phosphorylated at several sites by ATM in vitro (Fig. 2). However, formation of TopBP1 foci in response to ionizing radiation is not dependent on ATM (Yamane et al., 2003). Formation of UV induced TopBP1 foci was inhibited by the PIKK inhibitor caffeine (Herold et al., 2002), indicating that ATM/ATR was needed in this case. These Results suggest that TopBP1 is in a first step recruited to the sites of DNA damage after phosphorylation of another ATM/ATR target, and only in a second step phosphorylated by ATM/ATR.

Activation of ATR by TopBP1

During the past 3 or 4 years, TopBP1 has been established as an essential activator of ATR, which is discussed in more detail below. ATR is activated by numerous DNA lesions, such as replication stress, double-strand breaks (DSBs), base adducts, and DNA strand crosslinks (for review see Cimprich and Cortez, 2008). The recognition of these various substrates can be challenging for a sensor protein. Current evidence suggests that the single-stranded DNA (ssDNA) generated during processing of the DNA damage and replication block serves as a general signal for ATR activation, integrating a plethora of signals into a single marker of damaged DNA. Stretches of ssDNA can be detrimental to cells and are rapidly coated with replication protein A (RPA). ATR is localized to ssDNA-bound RPA through its essential binding partner ATRIP (ATR interacting protein). In addition to RPA, ATR activation needs the stimulation of the 9-1-1 checkpoint clamp. In response to DNA damage the 9-1-1 clamp is loaded onto DNA and binds the junction between single-stranded and double-stranded DNA. In analogy to PCNA, the clamp loader Rad17-RFC opens the ring structure facilitating loading of the 9-1-1 clamp onto chromatin.

Kumagai and co-workers (Kumagai et al., 2006) found that TopBP1 protein greatly increases ATR kinase activity in an in vitro assay. TopBP1 associates with ATR in an ATRIP-dependent manner. They mapped the ATR-activating domain (AAD) of TopBP1 to reside between BRCT domains six and seven (Fig. 2). They further showed that this domain itself is sufficient for ectopically activating ATR-dependent signaling in both human cells and *Xenopus* egg extracts, and that a single point mutation in the AAD domain abrogates this activity. The data from another group confirm that re-localization of ectopically expressed AAD domain from cytoplasm to nucleus is enough to activate ATR (Toledo et al., 2008). The down-regulation of TopBP1 prevents the phosphorylation of ATR kinase targets Chk1, Nbs1, Smc1 and H2AX (Liu et al., 2006). On the other hand, activating ATR in the absence of DNA damage by over-expressing AAD promotes the phosphorylation of Chk1, Smc1, H2AX and Rad17 (Toledo et al., 2008). Thus, the activation of ATR by TopBP1 seems to be general in nature. TopBP1 may be required for activation of ATR towards numerous substrates.

The exact mechanism how TopBP1 activates ATR-ATRIP is currently not known, and would need structural insight into the individual proteins. One possibility is that TopBP1 functions as a scaffold or landing pad for the proteins to be phosphorylated

by ATR. However, it is more likely that the activation is promoted by conformation changes in ATR-ATRIP upon TopBP1 binding. This is suggested by the fact that TopBP1 stimulates ATR-ATRIP kinase activity towards different substrates. It appears that the interaction between TopBP1 and ATR-ATRIP is loose and transient, and ATR-ATRIP is active only as long as TopBP1 is bound. Indeed, TopBP1 separates from the complex upon gel filtration and ATR-ATRIP kinase activity reverts to its initial low level (Kumagai et al., 2006).

Further mechanistic insight into activation is provided by a study where the TopBP1 binding sites in ATR and ATRIP are described (Mordes et al., 2008). Association of TopBP1 with ATRIP facilitates the interaction between ATR and TopBP1. Mutations in the TopBP1 interaction region of ATRIP impair the ability of the cells to recover from replication stress and to induce cell cycle arrest, and decrease viability of the cells. TopBP1 associates with a specific PIKK regulatory domain in the ATR C-terminus (next to the kinase domain), which is similar to the regulatory domains of other PIKK. Mutations in the regulatory domain of ATR do not affect the basal kinase activity, but prevents full activation.

As the activation of ATR takes place upon contact with TopBP1, the correct timing of association of these two proteins must be tightly controlled in cells. The checkpoint clamp 9-1-1 could fulfil this regulatory function. Rad9 phosphorylated at Ser387 binds TopBP1 and directs it to the vicinity of ATR-ATRIP (Delacroix et al., 2007; Lee et al., 2007). Although the interaction site in TopBP1 was originally mapped to a region corresponding BRCT domains four and five, recent data suggest that the binding occurs via BRCT domains one and two, both in human cells and *Xenopus* egg extracts (Delacroix et al., 2007; Lee et al., 2007). Interestingly, tethering the AAD domain of TopBP1 to PCNA or histone H2B bypasses the need for the loading of 9-1-1 complex onto DNA (Delacroix et al., 2007).

Although it appears that the 9-1-1 complex is highly important for localizing TopBP1 to ATR-ATRIP, this may not always be the case. This is suggested by the fact that loss of ATR phenotype is more severe than the phenotype resulting from the loss of Rad1 or Hus1 (Cimprich and Cortez, 2008; and references therein). There are several implications that TopBP1 does not necessarily need the 9-1-1 clamp for ATR activation. In *Xenopus* egg extracts, Rad1 was phosphorylated in an ATR and TopBP1-dependent manner, without the need for Rad9 C-terminus (Lupardus and Cimprich, 2006). Minimally, ATR activation needs only ATRIP and TopBP1 proteins (Kumagai et al., 2006). In a more physiologically relevant in vitro assay containing DNA, ATR-ATRIP, TopBP1 and Chk1 as a substrate, TopBP1 stimulated ATR kinase activity three to four fold in the presence of bulky DNA lesions compared to undamaged DNA (Choi et al., 2007). The stimulation was shown to be dependent on TopBP1 binding to the damaged DNA template. A recent work from the same group further shows that TopBP1 binds DNA through its C-terminus, containing AAD and BRCT domains seven and eight (Choi et al., 2009). Interestingly, antibodies against the XMus101 C-terminus containing BRCT domains seven and eight, but not the AAD domain, abolished the Chk1 phosphorylation in a *Xenopus* egg extract assay, where oligonucleotide A_{70}-T_{70} duplexes were used to mimic DNA damage (Yan et al., 2006).

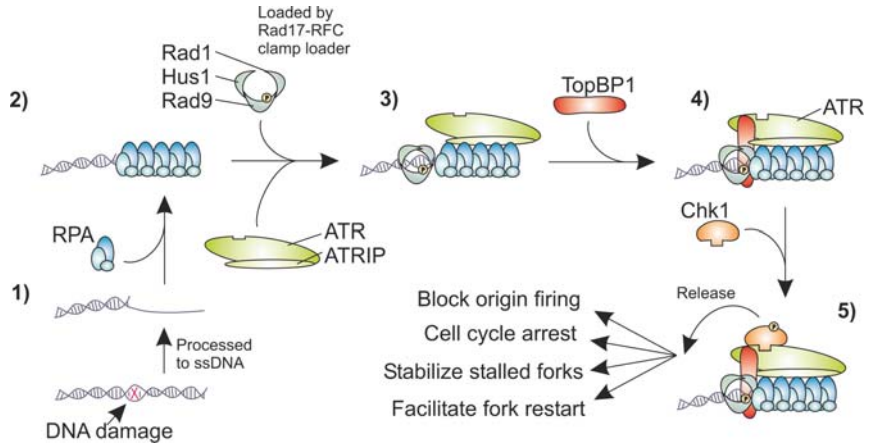

Fig. 3 A model on the function of TopBP1 in ATR activation. DNA damage that blocks replication fork (e.g. bulky base adduct) leads to the formation of single-stranded DNA (ssDNA), which is rapidly bound by replication protein A (RPA). ATR-ATRIP and Rad9-Rad1-Hus1 (9-1-1) complexes are then independently recruited to DNA. ATRIP binds to RPA and 9-1-1 complex is loaded by Rad17-RFC. TopBP1 is recruited to the damage sites via binding to constitutively phosphorylated Rad9. Once recruited, TopBP1 greatly enhances kinase activity of ATR towards Chk1, which mediates many of the responses to DNA damage. These include blockage of firing new origins, and stabilization and restart of stalled replication forks. For clarity, several additional factors and phosphorylation events involved have been omitted

The data published to date support an ATR activation model presented in Fig. 3. After formation of single-stranded DNA regions in response to genomic insult, the ATRIP and 9-1-1 clamp recruit the ATR and TopBP1 proteins, respectively, to the sites of DNA damage. The re-localization of the proteins facilitate the association of TopBP1 with ATRIP, which brings the AAD domain of TopBP1 into contact with ATR. The association of TopBP1 then induces a conformational change in the ATR kinase domain such that substrates can be readily phosphorylated by ATR.

In the absence of TopBP1, ATR has a basal kinase activity (Kumagai et al., 2006; Mordes et al., 2008). Efficient activation of ATR by TopBP1 may be required to achieve sufficient signal amplification for the proper execution of cellular responses to DNA damage. Such an amplification is also observed during the activation of ATM. A BRCT-domain containing mediator of DNA damage checkpoint protein 1 (MDC1) mediates the interaction between phosphorylated H2AX and ATM, providing a positive feedback loop between the three proteins (Lou et al., 2006). In the absence of MDC1, many ATM signaling events are defective.

Implications of TopBP1 in Response to DNA Double-Strand Breaks

TopBP1 can also activate ATR when phosphorylated at a specific residue in the AAD domain. Phosphorylation of *Xenopus* TopBP1 at Ser1131 (corresponds to human

Ser1138) by ATM strongly enhanced association with ATR (Yoo et al., 2007). When *Xenopus egg* extract was supplemented with mutant TopBP1 that cannot be phosphorylated at Ser1131, phosphorylation of Chk1 in response to DNA double strand breaks was abolished, but not in response to DNA replication stress caused by UV or aphidicolin treatment.

TopBP1 has been shown to physically interact with Nbs1 by co-immunoprecipitation (Morishima et al., 2007). Localization of TopBP1 to foci after ionizing radiation seemed to be dependent on functional Nbs1, since such foci were not formed in Nijmegen breakage syndrome cells. The authors also analysed sister chromatid exchange (SCE) levels in cells where TopBP1 was downregulated by siRNA. Both spontaneous and DNA damage-induced SCE levels were reduced in TopBP1 silenced cells. However, this is not surprising considering that ATR-activated Chk1 plays a role in homologous recombination (Sorensen et al., 2005) and TopBP1 is essential for activation of ATR.

ADP-Ribosylation of TopBP1

One of the post-translational protein modifications is poly(ADP-ribosyl)ation, where polymers of ADP-ribose are formed from donor NAD^+ molecules and covalently attached to glutamic acid, aspartic acid or lysine residues of a target protein. The process is catalysed by the poly(ADP-ribose) polymerase (PARP) family of proteins (reviewed in Woodhouse and Dianov, 2008). The best known of these proteins is PARP-1, which is implicated in transcription, chromatin remodeling, apoptosis and DNA repair. Since TopBP1 contains a sequence homologous to the auto-(ADP-ribosyl)ation site of PARP-1 (Yamane et al., 1997), Wollmann and coworkers (Wollmann et al., 2007) investigated the possible interaction of the two proteins. Indeed, pull-down and co-immunoprecipitation experiments showed that TopBP1 and PARP-1 interact both in vitro and in vivo. The authors further demonstrated that the interaction is dependent on BRCT domain six of TopBP1, and that this domain is ADP-ribosylated by PARP-1. Their Results also indicate that binding of the transcription factor Miz-1 by TopBP1 is regulated by ADP-ribosylation. Binding to TopBP1 is lost after UV irradiation, but when the cells were treated with a PARP inhibitor prior to UV treatment, the Miz1-TopBP1 interaction was retained in a dose-dependent manner. However, the study did not demonstrate that TopBP1 is directly ADP-ribosylated in response to UV, and thus leaves a possibility that ADP-ribosylation of some other protein or proteins is needed for the interaction between Miz-1 and TopBP1.

Regulation of TopBP1 Activity

TopBP1 seems to be a critical component in activation of ATR-dependent DNA damage response. It is recruited to damaged DNA sites very early, where it activates ATR kinase towards numerous, perhaps all of the ATR targets. Very little is

known about the processes that regulate this activity of TopBP1. It seems to be clear that no post-translational modifications of TopBP1 are required for the activation of ATR, since this activation can be achieved with recombinant TopBP1, or with the AAD domain alone. However, TopBP1 modifications that regulate its localization and/or protein concentration are more likely to occur. It has been shown that the ectopically expressed AAD domain, lacking any DNA binding activity (Choi et al., 2009), is able to activate ATR when relocalized from cytoplasm to nucleus (Toledo et al., 2008). The activation can then last even for several days, without any apparent DNA damage, ultimately leading to cellular senescence. This shows that the ATR activation can occur simply by increasing the likelihood of a contact between the activator and the kinase. Current research suggests that, in the normal cellular environment, control over ATR activation is achieved by regulating the localization of TopBP1, and possibly also the concentration of the soluble protein. Several layers of control mechanisms may exist in the recruitment of TopBP1 to sites of DNA lesion. On the one hand, TopBP1 can be recruited to the sites of action by signal recognition proteins such as Rad9. Also other interaction partners of TopBP1, like Nbs1, 53BP1 and PARP-1 are all known to bind DNA lesions early in damage response (Table 1). The binding of TopBP1 to these proteins may provide additional means to

Table 1 Interaction partners of TopBP1 and proposed functions of these interactions

Interacting protein	Function of the interaction	Reference
53BP1	–	Yamane et al. (2002)
ATR	Activation of ATR	Kumagai et al. (2006)
Brg1/Brm	Recruitment of Brg1/Brm	Liu et al. (2004)
c-Abl	Regulation of c-Abl protein levels	Zeng et al. (2005)
Cdc45	Recruitment of Cdc45 to origins of replication	Schmidt et al. (2008)
E2F1	Inhibition of E2F1 transcription activity	Liu et al. (2003)
Geminin[a]	–	Yoshida (2007)
HDAC1	Recruitment of HDAC1	Zeng et al. (2005)
HPV16 E2	Co-activation/recruitment of E2	Boner et al. (2002)
Miz1	Inhibition of Miz1 transcription activity	Herold et al. (2002)
Nbs1	–	Morishima et al. (2007)
PARP-1	–	Wollmann et al. (2007)
PML	–	Xu et al. (2003)
DNA Polymerase ε	–	Makiniemi et al. (2001)
Rad9	Re-localization of TopBP1 after DNA damage	Delacroix et al. (2007)
SPBP	Co-activation of Ets1 transcription activity	Sjottem et al. (2007)
DNA Topoisomerase IIβ[a]	–	Yamane et al. (1997)

[a] The interaction is based only on yeast two-hybrid data.

regulate activation of ATR. On the other hand, it has been reported that TopBP1 can directly bind to DNA lesions such as DNA breaks, single-stranded DNA and bulky DNA adducts in vitro (Yamane and Tsuruo, 1999; Choi et al., 2009). Redundancy in the recruitment of TopBP1 might have evolved to make sure that the pathways preserving genetic integrity are efficiently activated during genomic distress.

TopBP1 in DNA Replication

A role of TopBP1 during DNA replication has already been suggested by analysis of the genes of the yeast orthologues *Dpb11* and *Cut5*. They are both essential for cell viability and required for the onset of S phase DNA replication as well as cell cycle control (Araki et al., 1995; Nasheuer et al., 2007; Saka et al., 1994b). Dpb11 protein is required for the transition from the pre-replication to the initiation complex. Recent experiments have shown that Dpb11 binds the replication initiation factors Sld2 and Sld3 (Synthetically lethal with Dpb11), when being phosphorylated by S-phase CDK (Zegerman and Diffley, 2007; Tanaka et al., 2007a). This complex controls the association of Cdc45 and DNA polymerase ε with the origins of DNA replication (Masumoto et al., 2000). The phosphorylation of the Sld proteins and their subsequent binding by Dpb11 represents the minimal requirement for CDK-dependent activation of DNA replication initiation (Tanaka et al., 2007b). A comparable role emerges also for TopBP1 and its metazoan orthologues. Mutations in the *Mus101* gene coding for the *Drosophila* TopBP1 orthologue exhibit DNA replication defects. Moreover, siRNA-mediated knock-down of TopBP1 demonstrates its requirement for the G1-to-S phase transition in human cells (Jeon et al., 2007). Depletion of the TopBP1 orthologue XCut5 from *Xenopus* egg extracts prevents chromatin binding of Cdc45 and DNA polymerases (Hashimoto and Takisawa, 2003; van Hatten et al., 2002), and XCut5 appears to be required for an S-phase CDK-dependent process in the initiation of DNA replication. Hashimoto and coworkers (Hashimoto et al., 2006) subsequently showed that a fragment comprising the first five BRCT domains conserved from the yeast was sufficient to support the replication function of the TopBP1 orthologue in the clawed frog. *Xenopus* Cut5 also interacts with RecQ4, a replication protein exhibiting a limited homology with yeast Sld2 (Matsuno et al., 2006; Sangrithi et al., 2005), suggesting again a similar course of events in metazoans compared to the yeasts, despite the absence of an orthologue of the Sld3 protein. XCut5 was furthermore shown to be required for the chromatin association of GINS (Kubota et al., 2003), a ring-like protein complex required both for initiation and elongation of DNA replication (see “The Initiation Step of Eukaryotic DNA Replication” by Pospiech et al., this issue). Utilising a cell-free replication system in isolated HeLa cell nuclei, a role of TopBP1 in human DNA replication has also been shown (Makiniemi et al., 2001). But in contrast to the work with *Xenopus*, the sixth BRCT domain of human TopBP1 was particularly important for the replication function of the human protein. Both an antibody against this BRCT domain as well as a recombinant fragment of TopBP1 comprising

BRCT6 inhibited DNA replication in isolated nuclei. A similar BRCT6 fragment also diminished chromatin binding of Cdc45 when overexpressed in human cells (Schmidt et al., 2008), and it was shown that the sixth BRCT domain, and, to a lesser extend, the first and second BRCT domains of human TopBP1 are able to bind directly to Cdc45, an interaction that is mediated by Sld3 in yeast. TopBP1 has also been found to bind DNA polymerase ε (Makiniemi et al., 2001).

Taken the Results presented above together, TopBP1 and its vertebrate homologues apparently have an important function during the initiation of DNA replication leading to the loading of Cdc45 and DNA polymerases comparable to the yeast orthologues. Still, there appear to be considerable differences between the regulatory mechanisms operating in metazoans compared to lower eukaryotes, and between different groups of metazoans. Without further experimental evidence, it remains unclear if the separate regions implicated in DNA replication in *Xenopus* and humans, respectively, reflect barely discrepancies between the experimental systems, differences between embryonic compared to normal DNA replication, or a very recent gain of function of the evolutionarily lastly acquired BRCT domain.

Based on the analysis of TopBP1 knock-down in human cells, a dual role of TopBP1 for the G1/S transition has been suggested (Jeon et al., 2007). Apart from its function for loading replication components onto chromatin for initiation of DNA synthesis, TopBP1 is also necessary for the activation of cyclin E-Cdk2 kinase, probably due to its transcriptional activity (see below).

Although the involvement of TopBP1 and its orthologues in the elongation reaction of DNA replication has been discussed in the yeasts and metazoans, no experiment has confirmed that the protein moves with the replication fork in unperturbed cells. However, it has an important role in the stabilization and reinitiation of stalled DNA replication forks (Makiniemi et al., 2001; Parrilla-Castellar and Karnitz, 2003). Here, one could envision TopBP1 to be involved in the reloading or reactivation of replication factors similar to the initiation reaction.

A Role of TopBP1 During Mitosis and Meiosis

Reini and co-workers (Reini et al., 2004) studied the localisation of TopBP1 during mitosis. During prophase, TopBP1 formed numerous clear foci that did not colocalise with centromeres or centrosomes. The nature of these foci remained obscure since the authors were not able to relate them to known nuclear structures. However, from metaphase to late stages of mitosis, TopBP1 concentrated mainly into centrosomes. In anaphase, the TopBP1 signal was additionally detected at the midbody area, which is the region of cell division. TopBP1 is one in a rapidly growing list of centrosome-associated proteins. These include also cyclin B-Cdk1, p53, Brca1, Chk1, Chk2, Cdc25B and Aurora-A (Kramer et al., 2004). Centrosomes play an important role during the initiation of mitosis, as activation of cyclin B-Cdk1, the critical step in onset of mitosis, initiates at the centrosomes (Jackman et al., 2003). Activation of cyclin B-Cdk1 depends on the dephosphorylation by

Cdc25 phosphatase isoforms, in particular Cdc25B, whose activity is in turn tightly regulated. Cdc25B is activated and localises to the centrosomes after phosphorylation by Aurora A kinase (Dutertre et al., 2004). On the other hand, Cdc25B is negatively regulated by Chk1, counterbalancing the effect of Aurora A. Importantly, Chk1 controls Cdk1 activity during unperturbed cell cycle progression (Hu et al., 2001), and it has recently been shown that this regulatory role of Chk1 is essential for cell proliferation and independent of its DNA damage response function (Wilsker et al., 2008). Cdc25 activity is also inhibited after G2/M checkpoint activation in a Chk1- and Chk2-dependent manner (reviewed by Kramer et al., 2004). The current data suggest that the centrosomes present a platform where various signals from the cell cycle apparatus and checkpoint control are integrated to regulate progression of mitosis. Although the exact function of TopBP1 at the centrosomes has not been defined yet, it is likely that TopBP1 plays here a similar role as integrator, adaptor and activator, as it does in other cell cycle stages. In line with this, Yamane and co-workers (Yamane et al., 2003) found that downregulation of TopBP1 partially abrogates the G2/M checkpoint by destroying the regulation of Chk1 kinase.

TopBP1 resides at chromosomes in meiotic prophase cells in mice (Reini et al., 2004). TopBP1 foci are abundant during early prophase I and localize mainly to histone γ-H2AX positive domains, where DNA double strand breaks initiate recombination (Reini et al., 2004; Perera et al., 2004). At this stage, TopBP1 exhibits an almost identical pattern to that of ATR. At later meiotic prophase stages, TopBP1 localises to the X and Y chromosome cores, whereas the female XX pair does not accumulate TopBP1, indicating that staining in the male is specific for the asynapsed region of the XY chromosome pair. The Results suggest that ATR and TopBP1 monitor meiotic recombination and may be required for activation of the meiotic recombination checkpoint (Perera et al., 2004).

TopBP1 and Regulation of Transcription

TopBP1 has been reported to regulate transcription by interacting or modulating activities of transcription factor E2F1, Miz1, Ets1 and human papillomavirus E2 protein. Based on the present case studies a picture takes shape suggesting that TopBP1 may have a role as a global modifier of transcription in response to cellular stress by selective binding and regulation of transcription factors. It can therefore be expected that interaction with several other transcription factors will emerge in future.

Regulation of E2F1

The E2F family of transcription factors (E2F1-8) regulates the expression of genes involved in a wide range of pathways, including cell proliferation, development, DNA damage response and apoptosis. E2F is known to regulate in particular genes required for DNA synthesis through the retinoblastoma tumor suppressor pathway.

The first member of the E2F family, E2F1, regulates genes inducing both cell proliferation and apoptosis. It is needed for G1/S transition and is selectively induced when DNA is damaged. The control of these contradictory pathways leading either to life or death seems to come from signal-transduction pathways. Mitogenic signals promote proliferation and inhibit E2F1 apoptosis activity, whereas signals from DNA damage response pathway promote apoptosis (for reviews see Stevens and La Thangue, 2004; Polager and Ginsberg, 2008).

During DNA damage, TopBP1 regulates E2F1 by binding to its N-terminus through the sixth BRCT domain (Liu et al., 2003). Crucial for this interaction is phosphorylation of TopBP1 at Ser1159 by the Akt/protein kinase B (PKB) and subsequent oligomerization of TopBP1 through its seventh and eighth BRCT domains (Liu et al., 2006), as well as phosphorylation of E2F1 by ATM or ATR. The control of E2F1 apoptotic potential is known to require the action of the Ras-phosphoinositide 3-kinase-Akt signaling pathway (Hallstrom and Nevins, 2003). The interaction between TopBP1 and E2F1 represses transcriptional and apoptotic activity of E2F1, and its activity to induce entry into S phase. Regulation of E2F1 transcriptional activity is mediated by Brg1/Brm, a component of chromatin-remodeling complex SWI/SNF (Liu et al., 2004). This regulation prevents E2F1-dependent apoptosis during DNA damage and possibly during normal cell growth, since the interaction between TopBP1 and E2F1 was also found at the G1/S transition. An involvement of Brg1/Brm suggests that TopBP1 recruits chromatin modifiers to promoters of apoptotic genes to repress E2F1-mediated transcription. Work on the *Drosophila Mus101* gene has provided further evidence on the role of TopBP1 in condensation of heterochromatic regions (Yamamoto et al., 2000). It has also been proposed that the fourth BRCT domain is a transcriptional activator domain and that the second and fifth domains are repressor domains (Wright et al., 2006). Modification of chromatin could be part of this regulation.

In conclusion, binding of oligomeric TopBP1 to E2F1 seems to suppress E2F1-induced apoptosis when it is not desirable. Interaction of TopBP1 and Miz1 or HPV16 E2, and repression of Miz1 transcriptional activity, are also dependent on Akt phosphorylation of TopBP1 (Liu et al., 2006). This suggests that oligomerization of TopBP1 is a general mechanism in the control of transcription factors (for model see Fig. 4). Interestingly, a similar oligomerization mechanism has been recently reported for the binding of E2F1 to microcephalin (MCPH1) (Yang et al., 2008). MCPH1 is a DNA damage response protein that is involved in checkpoint activation and apoptosis (for review see Chaplet et al., 2006). Like TopBP1, oligomerization of MCPH1 is mediated by BRCT domains, but contrary to TopBP1, binding of MCPH1 to E2F1 promotes its apoptotic activity.

SPBP and Ets1 Activation

Nuclear factor SPBP (Stromelysin-1 PDGF responsive element binding protein) is a transcriptional co-regulator that enhances or inhibits the activity of several transcription factors. Sjottem and co-workers (Sjottem et al., 2007) have shown that TopBP1 interacts with SPBP. SPBP and TopBP1 both enhanced the transcriptional

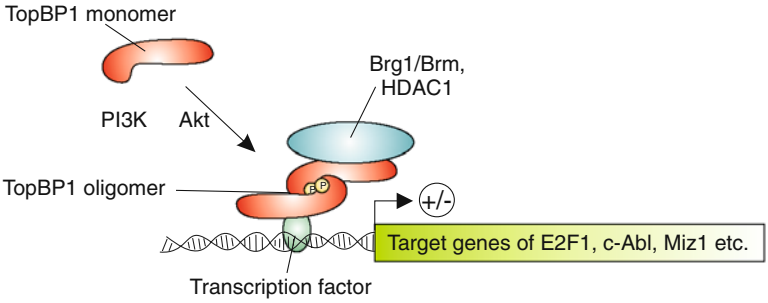

Fig. 4 A model of the function of TopBP1 as a transcriptional regulator. Oligomerization of TopBP1 in response to PI3K signaling and Akt phosphorylation is required for binding to transcription factors E2F1, c-Abl, Miz1 and possibly others. Recruitment of chromatin remodeling proteins Brg1/Brm or histone deacetylase 1 (HDAC1) by TopBP1 induces chromatin changes that may either repress or induce transcription

activity of Ets1 on *MMP3* (matrix metalloproteinase 3) and the *c-Myc* P1/P2 promoters, but the effect was more than additive when the two proteins were included together.

Ets1 is known to contribute to the regulation of cellular differentiation in haematopoietic cells. In a variety of other cells it promotes invasive behavior by regulating metalloproteinase and certain growth factor receptor genes (For review see Dittmer, 2003). As in the case of E2F1, the interaction was mediated by the sixth BRCT domain of TopBP1, and Akt phosphorylation of TopBP1 at Ser1159 was needed for transcriptional activation of Ets1. This suggests that also in this case, oligomerization of TopBP1 is required for the interaction with the transcription factor. It has been shown that E2F1 and Ets1 compete for the same binding site in the *c-Myc* promoter (Albert et al., 2001). Ets1 activates the *c-Myc* expression (Roussel et al., 1994; de Nigris et al., 2001) while E2F1 functions as a repressor (Albert et al., 2001). Thus, TopBP1 may positively affect *c-Myc* expression by stimulating Ets1 and repressing E2F1 transcriptional activities. As a transcription factor, c-Myc is known to activate genes involved in growth and protein synthesis, and to repress growth arrest and anti-proliferative genes (Adhikary and Eilers, 2005)

Miz1 and UV Damage Response

Miz1 is a Myc-associated protein that, depending on whether bound to c-Myc or not, either represses or activates, respectively, transcription of genes encoding the cell cycle inhibitors p15Ink4b and p21Cip1 (for review see Adhikary and Eilers, 2005). When not bound to c-Myc or other transcription factors, Miz1 activates genes involved in cell adhesion, metabolism, cell cycle arrest and apoptosis.

Herold and co-workers (Herold et al., 2002; Herold et al., 2008) have shown that TopBP1 associates with Miz1 in unstressed cells and early after UV irradiation. Yeast two-hybrid experiments suggest that the POZ domain of Miz1 and a region

within BRCT domains six to eight of TopBP1 are required for the interaction. Like in the case of E2F1 and SPBP, also the Miz1-TopBP1 interaction requires Akt phosphorylation and subsequent oligomerization of TopBP1.

The authors suggest that in the absence of DNA damage the interaction is required for the inhibition of Miz1 transcription activity, and for preventing TopBP1 degradation. When Miz1 is downregulated by RNAi, TopBP1 is ubiquitinated by the HectH9 ubiquitin ligase and subsequently degraded. On the other hand, overexpression of TopBP1 inhibited transactivation of *p15Ink4b* by Miz1 in a reporter assay. When cells were treated with a low UV dose, the interaction was lost with a few hours delay and concomitant downregulation of TopBP1 mRNA and protein levels were observed. In addition, the expression of c-Myc led to dissociation of TopBP1 from Miz1, suggesting that c-Myc antagonizes the binding of TopBP1 to Miz1. The authors propose that recovery after UV damage requires c-Myc expression with subsequent release and degradation of TopBP1 from the protective complex with Miz1.

Interaction with c-Abl

Proto-oncogene *c-Abl* that is frequently altered in chronic myelogenous leukaemia (CML), encodes a non-receptor tyrosine kinase. In most CML cases, chromosome translocation leads to expression of a fusion protein BCR-ABL, which is constitutively active as a tyrosine kinase. The c-Abl protein is involved in DNA damage response, actin dynamics, and mitogenesis. Overexpression of c-Abl leads to cell cycle arrest and apoptosis (for a review see Hantschel and Superti-Furga, 2004).

Zeng and co-workers (Zeng et al., 2005) have found that TopBP1 constitutively interacts with c-Abl. TopBP1 is also a substrate of c-Abl and is phosphorylated at a tyrosine residue in its N-terminus containing the first four BRCT domains. Apart from direct binding to c-Abl, TopBP1 represses the expression of c-Abl, when overexpressed in cells. The repression required histone deacetylation, but not Brg1/Brm, and HDAC1 co-immunoprecipitated with TopBP1, suggesting that TopBP1 recruits HDAC1 to the *c-Abl*. Repression of *c-Abl* expression was also dependent on DNA methylation, since the ectopic expression of TopBP1 failed to repress expression in the presence of DNA methylation inhibitor. The repression was inhibited by c-Abl in a kinase dependent manner, suggesting that tyrosine phosphorylation of TopBP1 by c-Abl interferes with its repressor activity. The negative feed-back loop mechanism of c-Abl expression suggests that TopBP1 may be required for fine-tuning the levels of c-Abl.

Hpv16 E2

Human papillomavirus type 16 (HPV16) is of particular interest of research, since infection with this virus is the cause of 50% or more of cervical cancers in women

(reviewed in Stanley et al., 2007). HPV16 E2 regulates transcription from the viral genome. It binds to specific target sequences and recruits viral and cellular co-activators (for review see (Ham et al., 1991)). The same protein also recruits viral replication factor E1 to the origin of replication. The C-terminus of the E2 protein is responsible for the binding to target sequences, and the N-terminus for the binding of co-activators. This activator domain was found to bind to TopBP1 both in vivo and in vitro (Boner and Morgan, 2002). Yeast two-hybrid system assigned also this interaction with a transcription factor to the C-terminal region of TopBP1 containing BRCT domains six to eight. Although TopBP1 was not essential for transcriptional activation by E2 (Donaldson et al., 2007) activation was enhanced when TopBP1 was overexpressed, and this enhancement was dependent on the amino-terminal region of TopBP1 containing BRCT domains one and two (Boner et al., 2002). Depletion of TopBP1 by anti-TopBP1 siRNA resulted in altered subcellular localization of E2, and led to increased chromatin binding by E2 (Donaldson et al., 2007).

Regulation of TopBP1 *Gene Expression*

In early studies TopBP1 protein levels were found to be upregulated concomitantly with S phase entry after quiescent cells were re-stimulated to enter the cell cycle (Makiniemi et al., 2001). A corresponding increase in mRNA levels suggested that *TopBP1* expression is dependent on proliferation rate. Later it was shown that E2F1 activates *TopBP1* expression in G1/S phase (Yoshida and Inoue, 2004). On the other hand, E2F4 was found to be responsible for the *TopBP1* repression in quiescent cells and after UV damage. Both proteins were also shown to directly bind *TopBP1* gene promoter. Given that TopBP1 binds to E2F1 and represses its transactivation activity (Liu et al., 2003), it is tempting to speculate on the existence of a negative feed-back loop in regulation of TopBP1 protein levels. In addition, *TopBP1* has been shown to be induced by the early growth response 1 protein (Egr-1), which is transiently induced by many environmental signals like growth factors, hormones and stress (Usskilat et al., 2006). Egr-1 bound to the *TopBP1* promoter in vivo, and both the E2F1 and Egr-1 binding sites were necessary for the full stimulation of *TopBP1* expression. This suggests the cooperative action of E2F1 and Egr-1 at the *TopBP1* promoter.

TopBP1 and Cancer

Among all breast cancer cases 10–15% are considered familial, since there is a history of breast cancer in close relatives. Familial breast cancer is characterized by onset at early age and an increased risk of developing breast cancer, while the lifetime risk of developing sporadic breast cancer is about 10% of female population. Mutations in known susceptibility genes, the most prominent being *BRCA1* and

BRCA2, can explain about 50% of familial breast and ovarian cancers. Polygenic susceptibility is likely to explain significant part of the remaining cases (Pharoah et al., 2002).

Germline mutation screening of the *TopBP1* gene in familial breast and ovarian cancer material from 125 Finnish cancer families revealed a heterozygous Arg309Cys (Fig. 2) variant at elevated frequency when compared to healthy controls (Karppinen et al., 2006). The Results suggest that this alteration may be associated with an approximately twofold increased breast and/or ovarian cancer risk. This alteration is located at an evolutionarily conserved consensus splicing site sequence adjacent to the second BRCT domain in the N-terminus of the protein. The physiological consequences of this TopBP1 alteration and its potential role in ovarian/breast cancer predisposition remains to be further studied.

A potential role of TopBP1 in the suppression of breast cancer is also supported by histological studies. Aberrant expression of TopBP1 was found in unselected breast carcinoma tissues (Going et al., 2007). In ordinary breast tissues TopBP1 was predominantly located in the nucleus, while in breast cancers the expression patterns were highly diverse. The dominant staining pattern resembled that seen in normal breast tissue (37/61), but in 2/61 cases TopBP1 staining was not observed at all, and in 22/61 cases the subcellular distribution of TopBP1 differed from that seen in normal breast tissues. These carcinoma tissues showed heterogenous staining in nuclei, nuclear and cytoplasmic staining, or purely cytoplasmic staining. Similar Results were obtained with feline (Morris et al., 2008) and canine (Morris et al., 2009) mammary neoplasia.

Conclusions

Taken the research published on TopBP1 so far together, a picture of a protein with versatile pro-survival functions emerges. In addition to being a central player in the activation of ATR, TopBP1 certainly has a broader role in the regulation of proliferation. Like ATR and Chk1, it is essential for normal S phase progression (Kim et al., 2005; Cimprich and Cortez, 2008) for reasons that are not fully understood. It is known that Chk1 signaling is required for the regulation of initiation of DNA replication in the unperturbed cell (Syljuasen et al., 2005), and it is likely that TopBP1 has a part in it. It may also be needed to stabilize and promote restart of stalled replication forks caused by encountering aberrant DNA structures, which are believed to be quite common in replicating DNA even in the absence of exogenous genomic insult (Zou and Rothstein, 1997; Benard et al., 2001). Therefore, TopBP1-dependent ATR signaling may well be required for efficient completion of DNA replication. Given the importance of TopBP1 in initiation of DNA replication and activation of ATR, the dialogue between the two proteins is likely to be more complex than known so far.

The dependence of the interaction between TopBP1 and transcription factors on the Akt signaling pathway suggests a profound role for TopBP1 in fine-tuning of

signaling events that control cell proliferation. When positively stimulated by Akt, TopBP1 suppresses the expression of cell cycle checkpoint genes (e.g. by binding to Miz1), and the expression of genes that inhibit entry into S phase and promote apoptosis (e.g. by binding to E2F1). Furthermore, binding of TopBP1 to SPBP co-activates the expression of *c-Myc*, which promotes proliferation. TopBP1 seems to regulate transcription in a manner that ensures suppression of checkpoint activation when positive growth signals are present. TopBP1 may well prove to be a central player in global growth control.

Current research on TopBP1 has been mainly focused on the roles of TopBP1 after induced DNA damage or cellular stress. Future research should concentrate more on the roles of TopBP1 in unperturbed cell growth, in order to gain a better understanding on the regulatory functions that TopBP1 performs during the normal cell cycle, especially during DNA replication and mitosis. It will be exciting to define the function(s) that make TopBP1 an essential player in the genome stability orchestra.

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Eukaryotic Single-Stranded DNA Binding Proteins: Central Factors in Genome Stability

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Abstract The single-stranded DNA binding proteins (SSBs) are required to maintain the integrity of the genome in all organisms. Replication protein A (RPA) is a nuclear SSB protein found in all eukaryotes and is required for multiple processes in DNA metabolism such as DNA replication, DNA repair, DNA recombination, telomere maintenance and DNA damage signalling. RPA is a heterotrimeric complex, binds ssDNA with high affinity, and interacts specifically with multiple proteins to fulfil its function in eukaryotes. RPA is phosphorylated in a cell cycle and DNA damage-dependent manner with evidence suggesting that phosphorylation has an important function in modulating the cellular DNA damage response. Considering the DNA-binding properties of RPA a mechanism of "molecular counting" to initiate DNA damage-dependent signalling is discussed. Recently a human homologue to the RPA2 subunit, called RPA4, was discovered and RPA4 can substitute for RPA2 in the RPA complex resulting in an "alternative" RPA (aRPA), which can bind to ssDNA with similar affinity as canonical RPA. Additional human SSBs, hSSB1 and hSSB2, were recently identified, with hSSB1 being localized in the nucleus and having implications in DNA repair. Mitochondrial SSBs (mtSSBs) have been found in all eukaryotes studied. mtSSBs are related to prokaryotic SSBs and essential to main the genome stability in eukaryotic mitochondria. Recently human mtSSB was identified as a novel binding partner of p53 and that it is able to stimulate the intrinsic exonuclease activity of p53. These findings and recent results associated with mutations in RPA suggest a link of SSBs to cancer.

Keywords DNA replication · DNA repair · DNA damage response · Single-stranded DNA · Signalling

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Abbreviations

9-1-1 complex	Rad9-Hus1-Rad1 complex (fission yeast and human) equivalent to the Rad17-Mec3-Ddc1 complex in budding yeast
aRPA	alternative replication protein A
A-T	Ataxia telangiectasia
ATM	ataxia telangiectasia-mutated
ATR	ATM-Rad 3-related protein
ATRIP	ATR-interacting protein
BER	base excision repair
BRCA1/2	breast cancer-associated protein 1/2
CDK	cyclin-dependent kinase
DBD	DNA-binding domain
DNA-PK	DNA-dependent protein kinase
DDR	DNA damage response
DSB	double-strand break
dsDNA	double-stranded DNA
E. coli	*Escherichia coli*
HR	homologous recombination
mt	mitochondrial
MBP	maltose binding protein
MMR	mismatch repair
NER	nucleotide excision repair
OB-fold	oligosaccharide/oligonucleotide-binding fold
PCNA	proliferating cell nuclear antigen
PIKK	phosphoinositol-3 kinase-like protein kinase
Pol α	DNA polymerase α–primase
Pol δ	DNA polymerase δ
Pol ε	DNA polymerase ε
Pot1	protector of telomeres 1
RAD	Radiation-induced mutation
RPA	replication protein A
ROS	reactive oxygen species
SSB	single-stranded DNA-binding protein
ssDNA	single-stranded DNA
S. cerevisiae	*Saccheromyces cerevisiae*
SV40	simian virus 40
TopBP1	topoisomerase II-binding protein 1

General Overview

Single-stranded DNA binding proteins (SSBs) are involved in almost every aspect of eukaryotic DNA metabolism including DNA replication, DNA recombination and all major types of DNA repair such as nucleotide excision, base excision,

double-strand break, and DNA mismatch repair (NER, BER, DSBR, and MMR, respectively) (Binz et al., 2004; Fanning et al., 2006; Wold, 1997). However, RPA is also a major player in DNA damage signalling. SSBs are not only essential to maintain the integrity of the genome in the nucleus of eukaryotic cells but they have also been found in eukaryotic mitochondria as being essential for the DNA metabolic activities in this cellular compartment. Recently the long established view, that the heterotrimeric protein complex replication protein A (RPA) is the only nuclear SSB whereas the mitochondrial SSB (mtSSB) is the only other eukaryotic SSB and has exclusively functions in mitochondria, has been challenged: an "alternative" form of heterotrimeric RPA (aRPA) was found and biochemically analysed, in which the second largest canonical RPA subunit RPA2 is substituted by a homologue RPA4 (Keshav et al., 1995; Mason et al., 2009). The heterotrimeric complex aRPA binds to ssDNA but seems not to be associated with any DNA repair pathway tested whereas aRPA rather inhibits the activity of canonical RPA in simian virus 40 (SV40) DNA replication *in vitro* (Keshav et al., 1995; Mason et al., 2009). These differential activities of RPA and aRPA might open new avenues in the regulation of DNA metabolic pathways. Moreover, Richard et al. (2008) reported two additional human genes coding for SSBs, hSSB1 and hSSB2. They are located on chromosomes 12q13.3 and 2q32.3, respectively. One of them, hSSB1, has been characterized in some detail and its domain organization is more closely related to archael SSB than to eukaryotic RPA (Richard et al., 2008). Detailed biochemical and cell biological analyses of hSSB1 suggested that the protein may play a role in DNA repair (Richard et al., 2008). In addition, human mtSSB may have additional functions to its well-known role in the mitochondrial DNA replication since human mtSSB was determined as a protein-binding partner of p53 tumour suppressor and able to stimulate the exonuclease activity of p53 (Mummenbrauer et al., 1996; Wong et al., 2009). This review provides a summary of the present knowledge of the structures, functions and activities of eukaryotic SSBs as well as their impact on human diseases. Taking the properties of RPA-DNA complexes into consideration the review discusses a "molecular counting" mechanism involved in the initiation of DNA damage signalling.

Replication Protein A

RPA is a heterotrimeric complex composed of three subunits with a size of 70, 32 and 14 kDa, which are known as RPA70, RPA32 and RPA14 or alternatively RPA1, RPA2 and RPA3, respectively, with all three subunits being essential in yeast (Binz et al., 2004; Fanning et al., 2006; Wold, 1997). RPA was first identified in human cell extracts as an essential factor in SV40 DNA replication but RPA complexes have been since determined in all eukaryotes with conserved subunit structure and amino acid sequences (Binz et al., 2004; Fanning et al., 2006; Wold, 1997). Prior to the detection of RPA, several proteins binding to ssDNA cellulose were identified in biochemical analyses but RPA was the first found to be directly associated with DNA metabolism (Fairman and Stillman, 1988; Grosse et al., 1986; Jong et al.,

1985; Wobbe et al., 1987; Wold and Kelly, 1988). RPA preferentially interacts with single-stranded DNA (ssDNA) in a high affinity mode whereas it binds with a much lower binding affinity to double-stranded DNA (dsDNA) and RNA (Nasheuer et al., 1992; Wobbe et al., 1987; Wold and Kelly, 1988). RPA is known to be a crucial component in DNA replication, DNA recombination and DNA repair (Braet et al., 2007; Wold, 1997; Iftode et al., 1999).

During chromosomal DNA replication RPA is associated with the initiation and elongation process (Wold, 1997; Iftode et al., 1999). In the eukaryotic cell cycle RPA is necessary for activation of the pre-replication to form the initiation complex and for the ordered loading of essential initiator functions, e.g. the DNA polymerase α–primase (Pol α) complex, to origins of replication (Nasheuer et al., 2002, 2007; Oehlmann et al., 2007). Studies of the SV40 DNA replication system revealed that SV40 TAg, RPA, topoisomerase I and Pol α closely interact to allow initiation of the leading strand synthesis at the viral origin of replication (Hurwitz et al., 1990; Khopde et al., 2008; Murakami et al., 1992; Nesper et al., 1997; Oehlmann et al., 2007; Ott et al., 2002; Smith et al., 2002; Taneja et al., 2007a, 2007b, Trowbridge et al., 1999; Voitenleitner et al., 1997; Weisshart et al., 2000, 2004a). First, RPA is required very early during the unwinding of the origin DNA in cooperation with the replicative helicase SV40 TAg and, secondly, RPA supports Pol α to synthesize the first Okazaki fragment (Oehlmann et al., 2007; Schub et al., 2001; Smith and Nasheuer, 2002; Taneja et al., 2007a, 2007b). In addition, RPA serves as "fidelity clamp" for Pol α (Maga et al., 2001). The latter does not have an intrinsic proof-reading exonuclease but can interact with the tumour suppressor protein p53 with p53 having 3′ to 5′ exonuclease activity able to remove misincorporated nucleotides and damaged DNA (Melle and Nasheuer, 2002; Mummenbrauer et al., 1996, Wong et al., 2009). At the transition from the initiation to the elongation reaction in eukaryotic DNA replication RPA is involved in the DNA polymerase switch from Pol α, which has a DNA polymerase with intermediate processivity, to DNA polymerase δ (Pol δ), which in association with proliferating cell nuclear antigen (PCNA) has high processivity (Hübscher et al., 2000, 2002; Nasheuer et al., 2007; Yuzhakov et al., 1999). Multiple RPA-DNA and RPA-protein interactions are necessary for the elongation reaction of DNA replication including those to the clamp loader replication factor C (RF-C) and PCNA, the actual replication clamp that is the processivity factor of Pol δ. Here, the polarity of the RPA-DNA complex enables the RPA2 subunit to monitor the length of the RNA-DNA primers during lagging strand DNA synthesis in mammalian cells (Mass et al., 1998). Interestingly the early initiation steps have been found to be host-specific in various polyomaviral DNA replication systems (Brückner et al., 1995; Mahon et al., 2009; Schneider et al., 1994; Smith et al., 2002; Stadlbauer et al., 1996).

In cellular DNA replication with linear chromosomes as the template, RPA is also involved in the control of telomerase activity to replicate chromosomal ends, the telomeres. Human HeLa cells deficient in RPA show a decrease in the ability of telomerase to elongate DNA primer (Rubtsova et al., 2009). Addition of small amounts of purified RPA to RPA-depleted cell extracts restores the telomerase activity but adding an excess of RPA to these extracts inhibits the enzyme

activity. In contrast, prokaryotic SSB does not stimulate telomerase activity but actually inhibits the enzyme (Rubtsova et al., 2009). Moreover, RPA interacts with Pot1 and Werner helicase to prevent instability of telomeres (Ahn et al., 2009). However, RPA together with Blooms or Werner helicase may also be involved in the fusion of chromosome ends after telomere loss (Wang and Baumann, 2008).

During the initiation of DNA repair processes RPA binds to ssDNA of the partially unwound DNA in a polar fashion (de Laat et al., 1998). RPA2 is positioned by RPA1 on partial duplex DNA and contacts the 3′-terminus of the primer (Kolpashchikov et al., 1999). This polarity allows RPA together with XPA to direct the two endonucleases XPF and XPG for their precise DNA cleavage 3′ and 5′ of a lesion on the damaged strand (de Laat et al., 1998). The gap filling process will then occur by additional interaction between RF-C, PCNA and Pol δ or ε. In addition to these NER processes, RPA also participates in various steps of homologous recombination (HR) (Daboussi et al., 2002; Song and Sung, 2000; West, 2003). Recent studies revealed that the protein CtIP performs the resection of DSB and the production of ssDNA region in S and G2 phase to allow the activation of ATR-dependent DNA damage signalling and the binding of RPA to these DSBs (Sartori et al., 2007). In budding yeast, the coordinated interactions of RPA, Rad51 and Rad52 modulate the formation of Rad51 nucleoprotein filaments, which mediate DNA strand exchange, a key step in HR (Song and Sung, 2000; Sugiyama and Kowalczykowski, 2002). In mammals, Rad51 colocalizes with the tumour suppressor proteins BRCA1 and BRCA2 in DNA damage-induced nuclear foci and forms a tight complex with BRCA2, which in turn influences the activities of their partner recombination factors and the efficiency of recombination (Pellegrini et al., 2002). Moreover, BRCA1, the BRCA2-Rad51 complex, and Rad54 cooperate with RPA during the strand invasion reaction of HR (Shiloh, 2003; van Komen et al., 2002). Recent findings suggest that RPA controls DNA recombination and genome stability by associating with BRCA2, which might be involved in tumour suppression (Wong et al., 2003). RPA is also involved in additional flavors of DNA repair. RPA has been implicated in BER due to its interaction with human uracil DNA glycosylase and XRCC1 stressing its central role in eukaryotic DNA metabolism (Levy et al., 2009; Mer et al., 2000).

Physical Interactions of RPA with DNA

The RPA binds to ssDNA with high specificity and affinity but it also interacts with template primer systems. Especially RPA2 interacts with the 3′- and 5′-ends of a primer that is hybridized to a template whereas RPA binds with significantly lower affinity to dsDNA (Kolpashchikov et al., 1999; Nasheuer et al., 1992; Wold and Kelly, 1988). The heterotrimeric RPA complex associates with DNA in two different confirmations; in an elongated conformation to long stretches larger than 30 nucleotides of ssDNA whereas it is also known to bind ssDNA fragments of 8–10 residues in a globular conformation (Wold, 1997; Iftode et al., 1999).

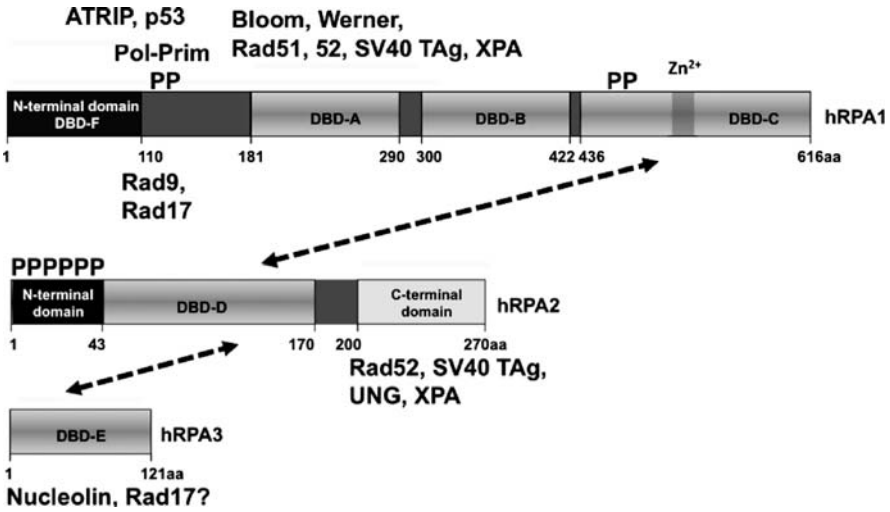

Fig. 1 Physical interactions of RPA. A schematic diagram of the structural and functional domains of the RPA subunits presents the DNA-binding domains (DBDs). Regions of RPA interacting with other proteins and the Zn finger domain are illustrated. Phosphorylation sites in RPA1 and the N-terminus of RPA2 are marked with P. The *arrows* highlight the physical interactions within the RPA complex itself

The RPA complex contains six OB-folds (Oligosaccharide/oligonucleotide-binding fold) with at least one found in the core domain of each subunit (Fig. 1). The OB-fold consists of a five-stranded beta-sheet coiled to form a barrel-helix and an alpha-helix that connects the third and fourth strands (Murzin, 1993). The high affinity ssDNA activities are located in the largest subunit RPA1 (Kenny et al., 1990; Nasheuer et al., 1992; Wold and Kelly, 1988). RPA1 contains four OB-folds, referred to as DNA-binding domains (DBDs) F, A, B and C following their arrangement from the N- to the C-terminus whereas the RPA2 subunit contains DBD-D and RPA3 has DBD-E (Fig. 1). RPA1 has not only DNA-binding activity (Kenny et al., 1990) but is also involved in protein-protein interactions (Dornreiter et al., 1992; He et al., 1995; Weisshart et al., 2000, 2004b). The C-terminus of RPA1 is required for stable interactions with the smaller two RPA subunits (Gomes et al., 1996) whereas the N-terminus is involved in multiple physical interactions with other DNA metabolic factors (reviewed by (Fanning et al., 2006; Wold, 1997) and summarized in Fig. 1). The redox status of RPA seems to significantly affect initial interactions of RPA with ssDNA but has no effect after RPA formed a stable complex with DNA suggesting that redox regulation of the zinc-finger may be involved in mediating the initial RPA-ssDNA interaction to form a stable RPA-ssDNA complex (Bochkareva et al., 2000; You et al., 2000).

The role of RPA1 in human cancer and maintaining cell survival and chromosomal stability in mammalian cells has been highlighted since a mutation changing the amino acid L230P in DBD-A (Wang et al., 2005). Heterozygous mice having the mutation in only one of the coding genes were viable but develop lymphoid tumours

whereas mice, which are homozygous for this mutation (carrying the mutation in both genes coding for RPA1), died during embryonic development. The primary mouse embryonic fibroblasts from heterozygous mice had defects in DSB repair showing chromosomal breaks and aneuploidy (Wang et al., 2005). These findings suggests that role of RPA1 in metabolism is vital for chromosome stability and tumour suppression (Wang et al., 2005).

In contrast to RPA1, the specific role of the two smaller subunits especially RPA3 is still poorly understood. In RPA2, DBD-D has low binding affinity for ssDNA (Dickson et al., 2009). DBD-E in RPA3 provides a structural role and is required for the stable heterotrimeric formation of RPA (Wold, 1997; Iftode et al., 1999). RPA undergoes a conformational change upon binding to ssDNA, which can be analysed by partial proteolytical digestion. RPA3 appears to have a protease resistant structure since even after complete digestion of the two larger subunits RPA3 was shown to be resistant to proteases. RPA2 has a partial resistance to protease digestion resulting in a rapid removal of the N-terminus (Gomes et al., 1996). RPA2 consists of three structural domains; an N-terminal domain, which functions in RPA phosphorylation (Fig. 1), a central DNA binding domain (DBD-D) responsible for subunit interactions and a C-terminal domain with protein-protein interaction activity (Arunkumar et al., 2005; Mer et al., 2000; Nuss et al., 2005; Oakley et al., 2001; Ott et al., 2002 reviewed in Fanning et al., 2006). Upon binding to ssDNA RPA1 becomes more resistant to proteolytic cleavage whereas RPA2 becomes more sensitive (Gomes et al., 1996; Pestryakov et al., 2003). In contrast, in prokaryotic *E. coli* SSB protease sensitivity only increases if the ssDNA oligonucleotide is long enough to allow cooperative binding (Gomes et al., 1996). In yeast it was recently shown that the DNA-binding activity of RPA2 is not essential whereas protein interaction activities of DBD-D of RPA2 are required for viability (Dickson et al., 2009).

Photocrosslinking using nucleotide analogues has been shown to be a valuable tool to analyse structure and functions of DNA binding proteins in DNA metabolic pathways (Hartmann et al., 1988; Lavrik et al., 1998 and "Nucleotide Excision Repair in Higher Eukaryotes: Mechanism of Primary Damage Recognition in Global Genome Repair" by Rechkunova and Lavrik, this book). Studies with RPA and a variety of nucleotide analogues that can be specifically activated by UV revealed that RPA binds in an ordered fashion to ssDNA and that in partial double-stranded DNA the DBD-C and DBD-D are involved in the specific interactions of RPA with 3′-end of a primer annealed to a longer oligonucleotide (Pestryakov et al., 2003, 2004). Recently photocrosslinking techniques revealed that RPA3, which contains an OB-fold, also interacts with DNA (Salas et al., 2009).

The RPA Complex and Its Binding to Proteins

The RPA Complex and DNA Replication

RPA forms a heterotrimeric complex in eukaryotic cells which can also be produced by co-expression of RPA subunits in *E. coli* (Henricksen et al., 1994; Weisshart

et al., 2004b). However, the assembly of the RPA complex is only partially understood. Recent studies using the expression of RPA subunits in *E.coli* suggest the formation of the RPA complex requires a specific assembly of its subunits with the RPA2 and RPA3 interact to form a stable subcomplex, (Henricksen et al., 1994). The subunit RPA1 is difficult to express in *E. coli* but the RPA1 fusion protein with maltose binding protein (MBP) is soluble and functionally active (Weisshart et al., 2004b). MBP-RPA1 forms a soluble and stable complex with RPA2 whereas MBP-RPA1 only weakly binds the smallest subunit RPA3. However, all these purified RPA sub-complexes do not support DNA replication whereas when all three subunits were co-expressed a stable, heterotrimeric RPA complex is formed, which has similar properties to human RPA and is indistinguishable in DNA replication assays from RPA purified from human cells (Henricksen et al., 1994; Weisshart et al., 2004b).

RPA physically interacts with various proteins required for DNA replication and DNA repair and DNA damage signalling (summarized in Fig. 1 and Fanning et al., 2006; Wold, 1997). Recent investigations revealed that protein interaction functions of DBD-D (domain of RPA2) are essential in yeast whereas DNA-binding activities of RPA2 are dispensable for viability of yeast cells (Dickson et al., 2009) underlining the importance of physical RPA-protein interactions for cell functions. RPA physically binds to replication factors such as Pol α and Cdc45 (Bauerschmidt et al., 2007; Braun et al., 1997; Weisshart et al., 2000, 2004b). Polyomaviral DNA replication systems such as SV40, mouse polyomavirus, and the human polyomaviruses BKV and JCV have served as model systems to study eukaryotic DNA replication with the SV40 DNA replication being the best understood. Only a single viral protein the large TAg is necessary for polyomaviral DNA replication and RPA is specifically required for TAg-mediated unwinding of DNA templates containing the viral origin of replication (Dodson et al., 1987; Wold et al., 1988). Studies of the polyomavirus DNA replication systems revealed that SV40 TAg, RPA, topoisomerase I and Pol α closely interact to allow initiation of the leading strand synthesis (Oehlmann et al., 2007). SV40 TAg binds to two regions of RPA one in the N-terminus of RPA1 and the other in the C-terminus of RPA2 (Ott et al., 2002; Fanning et al., 2006). In addition, these interactions may enhance the maturation of Okazaki fragments during DNA replication (Bartos et al., 2006). In addition to the association with TAg, RPA physically interacts with Pol α to support its initiation functions, to serve as "fidelity clamp" for Pol α and to support the polymerase switch from Pol α to Pol δ (Maga et al., 2001). These physical interactions of RPA and Pol α have been localized to the N-terminus of RPA1 and to RPA2 whereas p180 and both primase subunits of are physically involved on the Pol α side (Braun et al., 1997; Weisshart et al., 2000, 2004b).

As mentioned above, RPA interacts with proteins involved in DNA repair such as the nucleotide excision repair proteins XPA and XPG, the tumour suppressor protein p53, and transcriptional activators such as VP16 (He et al., 1993). In addition, the association with p53, which is stimulated by DNA damage, inhibits functions of RPA in DNA replication (Binz et al., 2004; Dutta et al., 1993). RPA physically interacts with Blooms and Werner helicase (Doherty et al., 2005). RPA physically binds

to human uracil DNA glycosylase and XRCC1 linking RPA to BER and stressing its central role in multiple pathways of eukaryotic DNA metabolism (Levy et al., 2009; Mer et al., 2000). The binding to Pot1 and Werner helicase may prevent instability of telomeres whereas together with Blooms or Werner helicase RPA supports the fusion of chromosome ends but only after telomere loss (Wang and Baumann, 2008; Ahn et al., 2009). In summary, the physical RPA-protein interactions serve various important functions to prevent genome instability and cancer in eukaryotic organisms.

The RPA Complex in DNA Repair Processes – Molecular Counting Capabilities

After binding to ssDNA either during DNA replication or in response to DNA damage, RPA is phosphorylated, and this is thought to be an important event in DNA damage response (DDR) (Binz et al., 2004). Recent observations have indicated an involvement of ATR (ATM and Rad3-related) in the RPA2 phosphorylation in response to stalled replication fork in S-phase generated by genotoxic agents such as UV (Olson et al., 2006). A regulatory network has emerged, in which the collaboration of the proteins ATRIP, TopBP1, and the 9-1-1 complex (ATR Interacting Protein, Topoisomerase II-Binding Protein 1, and Rad9-Hus1-Rad1, respectively) together with RPA and putatively other factors activates the phosphoinositol-3 kinase-like protein kinase (PIKK) ATR after DNA damage (Mordes et al., 2008; Xu et al., 2008; see also "Function of TopBP1 in Genome Stability" by Miiko et al., this book). The regulation seems to require multiple protein complexes or "keys". During checkpoint signalling, RPA binds to a region of ssDNA established after DNA damage. To activate ATR RPA must recruit the kinase via ATRIP to the DNA. However, to initiate a DNA damage-dependent signal transduction, at least, TopBP1 must then associated with the RPA-ATRIP-ATR complex. This occurs via an interaction of RPA with the 9-1-1 complex and here especially Rad9. The latter recruits TopBP1, which in turn will activate ATR (Mordes et al., 2008; Xu et al., 2008). Note that 9-1-1 binds to the same N-terminal region of RPA1 and one RPA complex can only bind either to 9-1-1 or ATRIP (Mordes et al., 2008; Xu et al., 2008):

These findings reveal a multi-component network, which is summarized here as a "multi-key" principle, to activate ATR (similar to the requirement of multiple passwords or "keys" to perform a bank transfer on the internet requires). After DNA damage, the initiation of a DDR signal transduction cascade starts with RPA binding to extended stretches of ssDNA and probably short primers synthesized by Pol α followed by the activation of ATR by protein interactions (Byun et al., 2005; Michael et al., 2000). As described before, RPA has high specificity to transitions from ssDNA to dsDNA, to both to 5′ and 3′ ends (Lavrik et al., 1998; Pestryakov et al., 2004). That way multiple RPA molecules would bind to long DNA stretches, which might also have associated scattered RNA-DNA primers, to fully activate ATR. The coordinated activation mechanism would require a minimum of two RPA molecules, but most likely more, to activate ATR (Mordes et al., 2008; Xu et al.,

2008). Depending on the RPA-ssDNA interaction mode, such as with the extended ssDNA-binding mode of RPA being about 50 nucleotides (Blackwell et al., 1996; Lavrik et al., 1998; Pestryakov et al., 2004), the ssDNA region would be at least 100 or multiples of 50 nucleotides to initiate a sustainable DNA damage signal transduction cascade. This multi-factor interaction requirement for initiation of DNA damage-based signalling pathway and checkpoint activation could be seen as a sign of a "molecular counting mechanism". The hypothesis of a multi-key concept or a molecular counting mechanism is supported by the findings that the initial binding of ATR-ATRIP-RPA complex is followed by the activation of the ATR kinase by TopBP1, which is recruited by the 9-1-1 complex binding to RPA (Mordes et al., 2008; Xu et al., 2008; Zou and Elledge, 2003). However, complete activation of ATR may require additional not yet identified protein complexes, which may also associate with RPA. Hypothesizing that all these activation steps of ATR are based on physical interactions with the RPA-ssDNA complex, the activation of a checkpoint by ATR necessarily requires a threshold level of protein-RPA-ssDNA complexes associating with the damaged DNA and its vicinity – "multiple keys" – and introduce a counting reminiscent to primase with accurate counting capabilities with less fidelity in RNA primer synthesis (Arezi and Kuchta, 2000). In this "molecular counting" hypothesis "one unit" would be about 50 nucleotides of ssDNA. The ability of RPA to perform, or more precisely, to initiate and being the base for a molecular counting process would explain the difference between relatively short ssDNA sequences occurring during DNA replication and initiating no checkpoint contra extended ssDNA stretches being induced after DNA damage such as stalled replication forks, which would initiate a DDR-dependent checkpoint. The former might still activate a basal ATR activity involved in coordinating basic cell functions such as feedback process and explain the essential function of ATR during the cell cycle. If RAD9-RPA interaction would be disrupted the ATR signalling to CHK1 would be impaired and the cell would become hypersensitive to replication stress as well as DNA damage (Cimprich and Cortez, 2008; Cortez et al., 2001).

In contrast, various researchers observed that both ATR recruitment to sites of IR-induced DNA damage and its activation require components of the MRN complex as well as ATM (Adams et al., 2006; Falck et al., 2005; Myers and Cortez, 2006). In HR, interactions of RPA, Rad51 and Rad52 modulate the formation of Rad51 nucleoprotein filament and DNA strand exchange (Sugiyama and Kowalczykowski, 2002). These protein-protein interactions are required for genome stability including meiotic recombination, mating-type switching, and survival after DNA damage (Kantake et al., 2003). In mammals, members of the *RAD52* group interact with the tumour suppressor proteins BRCA1 and BRCA2, which in turn influence the activities of recombination factors and the efficiency of HR (Shiloh, 2003). Moreover, recent findings also suggest that RPA controls HR and genome stability by associating with BRCA2, which might be involved in tumour suppression (Wong et al., 2003). BRAC1, the BRAC2-Rad51 complex and Rad54 cooperate with RPA during the strand invasion reaction of HR (Shiloh, 2003; van Komen et al., 2002).

RPA Phosphorylation

RPA is phosphorylated in a cell cycle-dependent manner in S phase and in M phase of a normal cell cycle and dephosphorylated at the end of M phase (Din et al., 1990; Dutta and Stillman, 1992). Cyclin-dependent kinases (CDKs) phosphorylate human RPA2 at one site S23 in S phase and at two sites S23 and S29, which are both canonical CDK recognition sites, in M phase (Stephan et al., 2009). The phosphorylation of RPA influences the binding to dsDNA and to replication factors since in biochemical characterisations the purified mitotic form of human RPA has a weaker binding to dsDNA, DNA-dependent kinase (DNA-PK) and Pol α (Oakley et al., 2003). Recent studies revealed that the N-termini of RPA1 and RPA2 interact, which is diminished in phosphorylated RPA forms (Binz and Wold, 2008).

In eukaryotic cells RPA is also phosphorylated in response to DNA damaging agents suggesting that DDR pathways regulate RPA (Carty et al., 1994; Pan et al., 1995; Zernik-Kobak et al., 1997; Zhou and Elledge, 2000; Zou and Elledge, 2003). Recently a model for the regulation of RPA function by phosphorylation started to emerge: After DNA damage, RPA associates with the site of damage through direct binding with DNA and repair/recombination factors (Binz et al., 2004). In complex with damaged DNA RPA is involved in the initiation of cellular DDRs. In addition, as a response of the signal transduction pathway RPA is then a target of DNA damage-dependent PIKKs, and ATM, ATR and DNA-PK phosphorylated RPA with the N-terminus of RPA2 being a well known substrate and a marker for DNA damage (Binz et al., 2004; Kaufmann, 2007). These kinases and RPA co-localize at the DNA damage site and also physically interact with RPA. Phosphorylation of the RPA2 subunit in response to UV or ionizing radiation causing a conformational change in the RPA complex, which in turn promotes decreased interactions of RPA with protein involved in DNA replication and PIKKs whereas interactions with the p53 tumour suppressor are increased. Associations with proteins involved in DNA repair remain unchanged (Binz et al., 2004). Importantly it is to remember that the UV damage only induces RPA phosphorylation at DNA damage-dependent sites in S phase whereas ionizing radiation or other DSB-causing agents also lead to RPA phosphorylation at these sites independently of cell cycle phases (Anantha et al., 2008; Rodrigo et al., 2000; Stephan et al., 2009).

After phosphorylation, the mobility of RPA2 during gel electrophoresis is significantly reduced, which suggests a conformational change in the subunit in both human and yeast (*S. cerevisiae*) RPA (Din et al., 1990; Dutta and Stillman, 1992). Up to nine potential phosphorylation sites have been suggested within human RPA2 but phosphorylation of RPA is not restricted to the N-terminus of RPA2 (Zernik-Kobak et al., 1997; Binz et al., 2004). Recent data has identified additional phosphorylation sites in response to DNA damage including multiple sites within the RPA1 both *in vitro* and *in vivo* (Nuss et al., 2005). These sites located in the OB-fold of the C-terminus of RPA1 (DBD-C) are likely to play a significant role in the duplex destabilisation activity of RPA (Nuss et al., 2005). Nuss et al. have suggested that these sites contribute to the decrease in affinity of phosphorylated RPA, in comparison to unphosphorylated RPA, for duplex DNA. A recent study has shown the

level of UV-induced RPA phosphorylation increases in the absence of Pol η and DNA-PK is responsible for this phosphorylation of RPA2 during UV-induced DNA damage response pathway (Cruet-Hennequart et al., 2008; for review see "DNA Polymerase η, a Key Protein in Translesion Synthesis in Human Cells" by Cruet-Hennequart et al., this book). The level of phosphorylation necessary to trigger the DNA damage response has yet to be determined but it is possible that the amount of RPA phosphorylated is proportional to the extent of DNA damage. In contrast, there may be a threshold of RPA phosphorylation required to promote down-regulation of DNA replication. It is apparent that the mechanism by which RPA is regulated differs between yeast and mammals, which underlines the importance of investigating RPA regulation in various organisms (Binz et al., 2004).

An Alternative Form of Replication Protein A

Recently a homolog to the RPA2 subunit was identified in human and was called RPA4 (Keshav et al., 1995; Mason et al., 2009). RPA4 shares 47% identity with RPA2 and it has been suggested that selective expression of RPA2 and RPA4 family may affect DNA repair, DNA replication and DNA recombination through regulation of both protein-protein interactions and post-translational modifications (Keshav et al., 1995). Recently RPA4 was expressed together with RPA1 and RPA3 subunit. RPA4 formed a heterotrimeric protein complex with RPA1 and RPA3 and it substituted for RPA2 in the purified complex. The purified complex composed of RPA1, RPA3 and RPA4 is capable of binding ssDNA in a way that is indistinguishable from canonical RPA since this newly established protein complex binds ssDNA with high affinity and low cooperativity (Keshav et al., 1995; Mason et al., 2009). Therefore, it was named as an alternative form of replication protein A, in short aRPA.

However, the RPA4-containing aRPA does not support SV40 DNA replication in vitro but actually inhibits the activity of canonical RPA during replication suggesting that this aRPA has a role in the regulation of human cell proliferation (Mason et al., 2009). Canonical RPA is essential in genome stability. Since the aRPA complex has similar properties in DNA-binding activity as the canonical RPA but seems to interfere with DNA metabolism there is a possibility that RPA4 has a function in maintaining the integrity of the cell or the regulation of pathway in the cellular DNA metabolism. Moreover, these studies revealed the potential for RPA4 as a therapeutic tool or target in preventing cell proliferation in cancer and also as antiviral replication agent through the prevention of viral replication (Mason et al., 2009).

Replication Protein A – The Cancer Link

RPA associates with the tumour suppressor protein p53 and it has been shown that this RPA-p53 interaction inhibits the functions of RPA in DNA replication (Dutta et al., 1993). Recent findings also suggest that RPA controls DNA recombination

and genome stability by associating with BRCA2, which might be involved in tumour suppression (Wong et al., 2003). RPA is essential to the viability of the cell (Wold, 1997). However, mutations in the RPA subunits, which do not interfere with the viability of an organism, can interfere with DNA damage pathways or increase chromosome instability (Mason et al., 2009; Wang et al., 2005). Mutations in RPA1 causes defective DSB repair (Wang et al., 2005; Wold, 1997). To study the role of RPA in human cancer and whether RPA1 is an essential factor in preventing tumour formation and maintaining cell survival and chromosomal stability in mammalian cells a mutation was introduced at nucleotide position 689 of RPA1 changing T to C, which yields a change of the amino acid L230P in one of the DNA binding domains, DBD-A, of RPA1. Heterozygous mice were viable but develop lymphoid tumours whereas mice, which are homozygous for this mutation, died during embryonic development. Analysis of the RPA1 mutation showed that primary heterozygous mutant mouse embryonic fibroblasts carrying it had defects in DSB repair showing chromosomal breaks and aneuploidy (Wang et al., 2005). These findings suggests that role of RPA1 in metabolism is vital for chromosome stability and tumour suppression and that RPA could be used to target tumour formation in humans and that RPA1 is a potential therapeutic tool in the treatment of cancer (Wang et al., 2005).

The Human ssDNA-Binding Protein hSSB1

Recently new human SSBs known as hSSB1 and hSSB2 were discovered with hSSB1 binding characterised in more detail (Richard et al., 2008). Similarly to RPA, hSSB1 binds specifically to ssDNA substrates, particularly to polypyrimidines. hSSB1 is highly conserved in metazoa and contains an OB-fold domain, which is followed by a carboxy-terminal region. The binding affinity of hSSB1 is enhanced with increasing length of its DNA substrate. Upon activation of ATM activity in response to DSBs, hSSB1 is phosphorylated along with several other proteins. The failure to activate ATM activity in A-T (Ataxia telangiectasia) cells results in the inability to stabilise hSSB1 after ionizing radiation in these cells. These findings suggest that ATM activity, which is crucial for cellular signalling in response to DSBs, regulate the function of hSSB1. One result of these analyses is that in an ATM-dependent manner DNA damage triggers the accumulation of hSSB1 in the nucleus, which yields distinct foci that co-localize with several other repair proteins (Richard et al., 2008). hSSB1 is found to form foci at sites of DNA replication and DNA damage. In cells containing both RPA and hSSB1 foci, Richard et al. observed low instances of co-localisation between these human SSBs (<5%). However, RPA foci were shown to be in close proximity hSSB1 suggesting a dual function in DNA repair (Richard et al., 2008). hSSB1 acts as a substrate for ATM and influences diverse endpoints in the DNA damage response including cell cycle checkpoint activation, recombination-based repair and in maintaining genomic stability. Like RPA, it also contributes to HR by promoting Rad51-mediated strand repair. hSSB1 may be

associated with the prevention of tumourigenesis, altering the response of tumours to radiotherapy and DNA-damaging chemotherapies. Although hSSB1 is not essential cells lacking in hSSB1 show signs of increased genome instability, defects in checkpoint activation and are more sensitive to radioactivity (Richard et al., 2008).

Mitochondrial SSBs

Nuclear SSBs (RPA and hSSB1) do not resemble eubacterial SSBs in sequence or structure. However, mtSSBs share a number of conserved regions with *E.coli* but differ otherwise (Ghrir et al., 1991; Wong et al., 2009). mtSSBs are evolutionarily conserved proteins found in all eukaryotes from yeast to humans (Maier et al., 2001). mtSSB binds to ssDNA and its main function is to stabilise the single-stranded regions of mtDNA in the displacement loops (D-loop) (van Tuyle and Pavco, 1985). Depletion of mtDNA has been linked to a number of inherited human diseases. In the absence of mtSSB in *Drosophila*, the majority of mitochondria loose respiratory function due to a loss of mtDNA (Maier et al., 2001). Human mtSSB is a tetramer consisting of two dimers that interact head-to-head and is in D_2 symmetry (Yang et al., 1997). Yang et al. proposed that during binding, ssDNA wraps around mtSSB through four electropositive channels, which is conserved between *Ecoli* SSB and human mtSSB, guided by flexible loops.

Human mtSSB and p53

Mutations in mtDNA are commonly observed in cancer patients. p53 is a key player in maintaining mitochondrial genomic stability through its ability to translocate to mitochondria and physically interact with Pol γ, mitochondrial DNA polymerase (for more details see "The Mitochondrial DNA Polymerase in Health and Disease" by Copeland, this book), in response to mtDNA damage caused by endogenous and exogenous insults such as ROS (reactive oxygen species) (Achanta et al., 2005). Recently Wong et al. (2009) identified human mtSSB as a novel binding partner of tumour suppressor p53 and a component of DNA mitochondrial replisome *in vitro*. p53 interacts with human mtSSB physically through its transactivation domain. Depletion of p53 results in an increase of mtDNA mutation. Therefore, p53 has been implicated in DNA repair in mitochondria during oxidative stress (Wong et al., 2009). Moreover, human mtSSB modestly stimulates $3'$ to $5'$ exonuclease activity of p53, which is an intrinsic to the protein and is able to excise 8-oxodG, suggesting a role for p53 and the mtSSB-p53 complex in genome stability including in mitochondria (Wong et al., 2009).

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DNA Polymerases and Mutagenesis in Human Cancers

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Abstract DNA polymerases (Pols) act as key players in DNA metabolism. These enzymes are the only biological macromolecules able to duplicate the genetic information stored in the DNA and are absolutely required every time this information has to be copied, as during DNA replication or during DNA repair, when lost or damaged DNA sequences have to be replaced with "original" or "correct" copies. In each DNA repair pathway one or more specific Pols are required. A feature of mammalian DNA repair pathways is their redundancy. The failure of one of these pathways can be compensated by another one. However, several DNA lesions require a specific repair pathway for error free repair. In many tumors one or more DNA repair pathways are affected, leading to error prone repair of some kind of lesions by alternatives routes, thus leading to accumulation of mutations and contributing to genomic instability, a common feature of cancer cell. In this chapter, we present the role of each Pol in genome maintenance and highlight the connections between the malfunctioning of these enzymes and cancer progress.

Keywords Cancer · DNA polymerases · DNA damages · DNA repair · Genome instability

Genome Stability Control Mechanisms and the Replication Fork

The understanding of reproduction of normal and cancerous cells has been the objective of intense studies. The cell cycle, the process by which cells progress and divide, lies at the heart of cancer. In normal cells, the cell cycle is controlled by a complex series of signaling pathways by which a cell grows, replicates its DNA and divides (Nasheuer et al., 2002). This process also includes mechanisms to ensure

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that errors are corrected, and if not, that the cells commits suicide (apoptosis) (for review see "Apoptosis: A Way to Maintain Healthy Individuals" by Mondello and Scovassi, this book). In cancer, as a result of genetic mutations, this regulatory process malfunctions, resulting in uncontrolled cell proliferation (Evan and Vousden, 2001). During the early embryonic development, cells may come in a continuous series of cell cycles during which DNA replication and mitotic division occur at high speed. But as embryogenesis unfolds and the demands of cell life in a complex environment set in, a bureaucracy arises, resulting in a cell cycle involving a complex series of molecular and biochemical signaling pathways. The cell cycle has four phases (Fig. 1): the G1, or gap phase, in which the cell grows and prepares to synthesize DNA; the S, or synthesis phase, in which the cell synthesizes DNA; the G2, or second gap phase, in which the cell prepares to divide; and the M, or mitosis phase, in which cell division occurs. The exact execution and interpretation of these steps is a delicate point and, not surprisingly, mistakes of only one phase can lead to cancer. When, due to mutations, the cells remain in a proliferative state, they lose their capacity for terminal differentiation and are ready to grow into a degenerate background. After this, cells can acquire additional mutations for invasion of surrounding tissues and metastatic re-creation of the tumor at distant organs. Many oncogenes and tumor suppressor genes, as well as the therapies that target them, can be linked to cell cycle control.

The key components that make up the checkpoints are grouped in different categories with different functional activities: the effector proteins, the mediators, the sensors and the signal-transducers (Liu et al., 2006). These proteins are acting

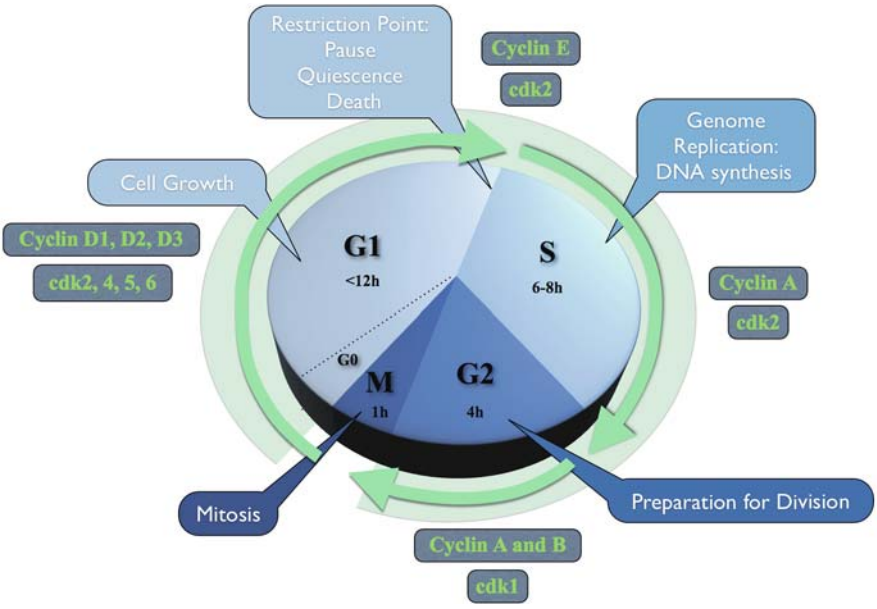

Fig. 1 Schematic representation of the eukaryotic cell cycle phases

through an ordered sequential mechanism or cascade, which leads to either the delay or the arrest of cell-cycle progression, the induction of DNA repair genes or the initiation of apoptosis. Among the signal transducers, an essential role is played by a complex set of enzymes called Cyclin-Dependent Kinases (CDKs) that control the various phases of the cell cycle (Nasheuer et al., 2002). CDKs form a catalytically competent complex together with their regulatory subunits, cyclins, whose level fluctuates during the cell cycle, hence their name. Genetic mutations causing the malfunction or absence of one or more of the regulatory proteins at cell cycle checkpoints can result in the "molecular switch" being turned permanently on, permitting uncontrolled multiplication of the cell, leading to carcinogenesis.

The first step of control during the cell cycle is at the so called G1/S checkpoint, which checks whether the cell is ready or not to replicate the DNA. Cells with intact DNA continue to S phase; cells with damaged DNA that cannot be repaired are arrested and undergo apoptosis. A second checkpoint is in G2 phase, which verifies that DNA replication was successful before it enters the M phase. A third checkpoint operates during mitosis at the anaphase, in order to ensure mitotic spindle formation and chromosome. After the cell has split into its two daughter cells, it enters G_1. In addition, an intra-S phase checkpoint is activated by the presence of a stalled DNA replication fork. The result of this checkpoint activation leads to the block of the cell cycle in S phase and the induction of reparative gene transcription. If the damage couldn't be repaired, apoptosis is promoted.

Compared to DNA replication and mitosis, which follow canonical steps that vary little from cell to cell, the steps controlling entry and progression through G1 are largely dependent on cell type and context. A stem cell that is constantly replenishing the intestinal lining, a lymphocyte suddenly stimulated by antigen, or an angioblast responding to vascular injury, all proceed through G1 phase under different circumstances, different signals, different developmental programs and with different risks of malignant transformation. Ultimately, however, to complete the cycle all cells must fulfil the same essential requirement: they must activate CDKs. The prototypic CDK, Cdk1, associates with cyclins A and B, and acts at the G2/M interface. The progressive accumulation of A and B cyclins during the cell cycle and their abrupt degradation at the onset of anaphase, mediates entry and exit from mitosis, respectively. The drop in Cdk1 activity in M phase allows DNA chromosomal sites known as replication origins to be loaded with a pre-replicative complex (PRC) (Kelly and Brown, 2000; Nasheuer et al., 2007; Oehlmann et al., 2007; Prasanth et al., 2004). This complex contains ORC (origin recognition complex) proteins, Cdc6/18 and Ctd1 (Cdc10-dependent transcript 1), and loads MCM (minichromosome maintenance) complex consisting of the Mcm2 to Mcm7 proteins onto the DNA, licensing these sites for the initiation of replication (for more details see "The Initiation Step of Eukaryotic DNA Replication" by Pospiech et al., this book). The assembly of replication complexes is under strict cell cycle and checkpoint control. Cyclin D is the first cyclin produced in the cell cycle, in response to extracellular signals (e.g. growth factors) during the G1 phase. Different type of cyclin D called D1, D2 and D3 together with CDK2, CDK4, CDK5 and CDK6 can form active cyclin-CDKs complexes. These, in turn, phosphorylate the retinoblastoma

susceptibility protein (pRB). The hyperphosphorylated pRB dissociates from the E2F/DP1/pRB complex and activates the transcription factor E2F. Activation of E2F results in transcription of various genes like cyclin E, cyclin A, DNA polymerases (Pols), thymidine kinase, etc. During the G1/S transition cyclin E thus produced binds to Cdk2, forming the cyclin E-Cdk2 complex. Only when cyclin A binds Cdk2 forming the cyclin A-Cdk2 complex the S phase starts. During the G2 phase cyclin A and cyclinB bind Cdk1. The cyclin B-Cdk1 complex activation causes breakdown of the nuclear envelope and initiation of prophase, and subsequently, its deactivation causes the cell to exit mitosis.

During the replication single stranded DNA (ssDNA) is stabilized and protected by the ssDNA-binding protein replication protein A (RPA) (Shevelev and Hubscher, 2002) (for additional information see "Eukaryotic Single-Stranded DNA Binding Proteins: Central Factors in Genome Stability" by Broderick et al., this book). Human RPA is a heterotrimer with 3 subunits of, respectively, 70, 32 and 14 kDa, called hRPA1, hRPA2 and hRPA3 (Wold, 1997). During S phase, after DNA damage, the DNA-dependent protein kinase (DNA-PK) phosphorylates the 32 kDa RPA subunit. Phosphorylation occurs in response to DNA damage induced by UV light or ionizing radiation (Burns et al., 1996; Carty et al., 1994; Liu and Weaver, 1993). In addition, replication fork arrest due to DNA damage results in uncoupling of helicase from the replisome with accumulation of long stretches of ssDNA. This, in turns, causes the increase in the local concentration of RPA at the stalled fork, inducing the activation of the S-phase checkpoint mediated by the protein kinase ATR (Shechter et al., 2004). Thus RPA can act as a molecular sensor of DNA damage. RPA also interacts with several proteins required for damage recognition and excision in nucleotide excision repair, e.g., XPA, XPG, and ERCC-1/XPF, RAD 52 protein, UDG and FEN1.

To couple DNA replication to the cell cycle machinery, PCNA (proliferating cell nuclear antigen) fulfils essential functions. PCNA is a protein composed of three 29 kDa subunits (258 amino acids each) and performs the essential function of providing replicative Pols with the high processivity required to duplicate an entire genome. The crystal structure shows a closed circular ring formed by association between three monomers (Kolodner, 1996). Each PCNA monomer is composed of two domains that fold to form a "6-fold symmetry" in the PCNA trimer. The presence of PCNA increases processivity of a Pol (as much as 100-fold) by engaging in protein–protein interactions with its outer surface and tethering it to the DNA (Tsurimoto, 1998). PCNA interacts with a multiplicity of DNA associated proteins including but not limited to Pols δ and ε, RFC, FEN1 (Li et al., 1995), DNA ligase I (Levin et al., 1997), p21Cip1/Waf1 (Waga et al., 1994), cyclin CDKs (Koundrioukoff et al., 2000). PCNA is loaded onto DNA in a fixed orientation relative to the direction of DNA, which permits discrimination of newly synthesized DNA strands from parental strands during replication. It may also have a role in orienting the repair process, at least in mismatch repair. PCNA can promote misincorporation catalyzed by Pol δ (Mozzherin et al., 1996) by decreasing the off rate of the Pol δ template-primer complex, e.g., by increasing stability. In fact, it stimulates bypass synthesis by Pol δ 53-fold past an abasic site (Mozzherin et al., 1997) but

in a error-prone fashion leading to incorporation of A in accordance with "A rule". PCNA is also involved in nucleotide excision repair and long-patch base-excision repair pathways (Gary et al., 1999). Due to its ability to bind numerous proteins, PCNA can recruit different partners in response to the activation of either cellular checkpoints and/or DNA repair pathways.

During DNA synthesis the two holoenzymes Pols δ and ε coordinate the synthesis on leading and lagging strands. To ensure that the replicative process has been correctly performed and that the DNA contains all the genetic correct information, the eukaryotic cell has developed the following three mechanisms to control the fidelity of DNA synthesis: (1) the intrinsic mechanisms of steric and energetic exclusion of non-complementary base pairing (base selection) of the Pols that operate at the fork, (2) the proofreading activity of the 3′->5′ exonucleases (exos) of Pols δ and ε or possibly by autonomous exos and (3) the postreplication DNA mismatch repair (Kunkel, 2004). However, certain kinds of DNA damages can often evade these mechanisms, leading to the accumulation of mutations.

DNA Repair and Mutagenesis

DNA itself is highly reactive and is constantly attacked by a broad spectrum of physical and chemical agents, which can alter its biochemical properties. DNA damage, due to environmental factors and normal metabolic processes inside the cell, occurs at a rate of 1,000–1,000,000 molecular lesions per cell per day (Tsurimoto, 1998). While this constitutes only a fraction of the human genome's approximately 6 billion bases (3 billion base pairs), unrepaired lesions in critical genes (such as oncogene or tumor suppressor genes) can lead to the deregulation of cell metabolism, resulting in increase the likelihood of tumor formation.

The main damages to the DNA can be classified in two broad categories (Friedberg et al., 2004; Nouspikel, 2009): exogenous and endogenous. The first group is caused by external agents such as cancer chemotherapy and radiotherapy, virus infection, different type of ultraviolet (UV 200–300 nm) radiation and also X-rays and gamma rays, plant toxins, human-made mutagenic chemicals (especially aromatic compounds that act as DNA intercalating agents). In particular, damages caused by exogenous agents have different origins as industrial and environmental chemicals found in smoke, soot and tar that create a huge diversity of DNA adducts-ethenobases, oxidized bases, alkylated phosphotriesters and crosslinking of DNA. Ionizing radiation such as that created by radioactive decay causes breaks in DNA strands. Thermal destruction at elevated temperature increases the rate of depurination (loss of purine bases from the DNA) and single strand breaks. UV-A light creates mostly free radicals, whereas UV-B light causes crosslinking between adjacent cytosine and thymine bases creating pyrimidine dimers.

There are four main types of damage to DNA due to endogenous cellular processes: (a) hydrolysis of bases, such as deamination, depurination and depyrimidination; (b) alkylation of bases (usually methylation), leading to formation of

7-methylguanine, 1-methyladenine, O6 methylguanine, bulky adduct formation (i.e.: benzo(a)pyrene diol epoxide-dG adduct); (c) mismatch of bases, due to errors in DNA replication, in which a DNA base is skipped over or mistakenly inserted; (d) oxidation of bases [i.e.: 8-oxo-7,8-dihydroguanine (8-oxoG)] and generation of DNA strand interruptions from reactive oxygen species (ROS).

Cells can eliminate some types of DNA damage by chemically reversing it without requiring the synthesis of a new DNA strand. For example, pyrimidine dimers (covalent binding of two adjacent pyrimidine usually formed upon irradiation with UV light) are removed by the enzyme photolyases, which reversibly bind and convert these lesions back to the original bases. Althought photolyases are present and found in many species, from the bacteria to the fungi (Kao et al., 2007), they have not been found in human cells. The guanine bases methylation is directly reversed by the protein methyl guanine methyl transferase (MGMT), that transfers methyl groups from O(6)-methylguanine, and other methylated bases of DNA, to a cysteine residue in the protein itself, thus repairing alkylated DNA in a single-step reaction. Since the methyl group is covalently linked to the enzyme, a MGMT molecule can perform the reaction only once, rendering the reaction stoichiometric rather than catalytic (suicide enzyme) (Kolodner, 1996).

However, the majority of DNA lesions in mammals are removed by six different DNA repair pathways (Fig. 2): mismatch repair (MMR), base excision repair (BER), nucleotide excision repair (NER), translesion DNA synthesis (TLS), homologous recombination (HR), non-homologous end joining (NHEJ). The activity of each of these pathways is essential for ensuring DNA integrity. Thus, malfunctions and alterations in these pathways are often prerequisites to tumor progression. In fact, cancer cells exhibit a high degree of genomic instability, allowing breakage and fusions of chromosomes, inactivating tumor suppressor genes, amplifying drug resistance genes, and consequently becoming more malignant and drug resistant over time.

In the hypothesis that only one strand of the DNA double helix has been injured, the complementary one can be employed as template to drive repair of the defective strand. A number of repair mechanisms indeed repair damages to one of the two DNA daughter strands, by removing damaged nucleotides/bases and replacing them with undamaged ones using the sequence information of the intact complementary strand.

Nucleotide excision repair (NER). This process recognizes bulky, helix-distorting changes such as thymine dimers as well as single-strand breaks (for additional details see “Nucleotide Excision Repair in Higher Eukaryotes: Mechanism of Primary Damage Recognition in Global Genome Repair” by Rechkunova and Lavrik, this book). The enzymes involved in this pathway recognize bulky distortions in the shape of the DNA double helix; in particular, the major lesions recognized and removed by NER are pyrimidine dimers induced by UV. NER is also important in conferring resistance to adduct-forming chemotherapeutic agents such as during platinum-based chemotherapy (Tornaletti and Hanawalt, 1999; Hanawalt, 2002). In mammalian cells several proteins involved in NER has been identified for their association to different genetic complementation groups of severe diseases.

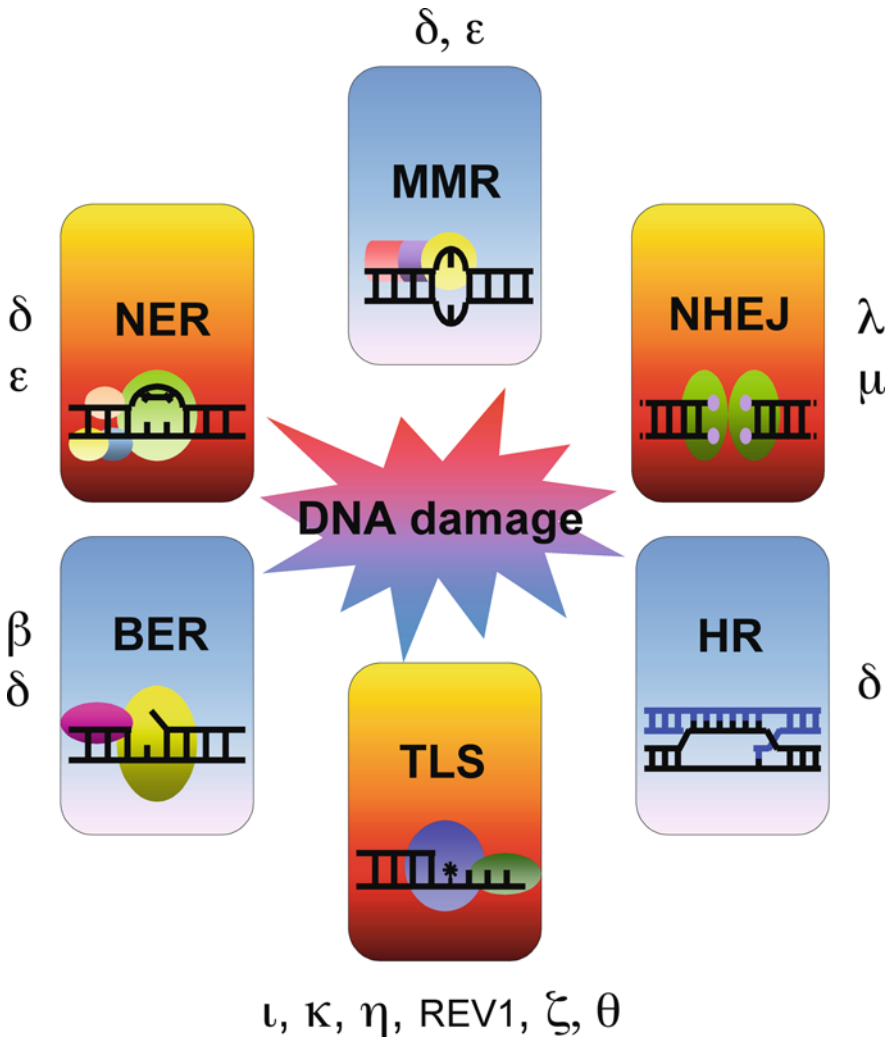

Fig. 2 The major DNA repair pathways operating in eukaryotic cells along with the associated DNA polymerases

The XPA, XPB, XPC, XPD, XPE, XPF, XPG proteins are associated, when defective, to the Xeroderma Pigmentosum (XP) disease, while the CSA and CSB proteins are linked to the Cockayne Syndrome (CS). XP patient exhibit acute sun sensitivity, marked skin changes in exposed areas, susceptibility to skin cancer and frequently progressive neurological degeneration. CS is also a rare human photosensitive disease with a recessive inheritance pattern. CS patients suffer of both neurological and developmental abnormalities. A distinctive feature of XP is the high prevalence of skin cancer, as a result of inefficient repair of UV-induced

lesions, thus directly demonstrating the essential role of NER can be categorized into two classes, global genome NER (GG-NER) and Transcription Coupled NER (TC-NER). GG NER repairs damage in both transcribed and untranscribed DNA strands in active and inactive genes throughout the genome during different phases of the cell cycle. TC-NER occurs when RNA polymerase II encounter a damage that blocks its progression. This results in recruiting NER proteins to correct the damage. In this way the RNA polymerase itself becomes a tool for recognizing DNA damage. Two different sets of proteins are involved in the distortion and recognition of the DNA damage in the two types of NER both needed to lesion recognition. The subsequent steps in GG-NER and TC-NER are similar to each other. XPB and XPD, which have helicase activity unwind the DNA at the sites of damages whereas XPG makes an incision 3′ to the damaged DNA. Than XPF protein in association with ERCC1 introduces the 5′ incision. The dual-incision leads to the removal of a ssDNA with a single strand gap of 25~30 nucleotides. The resulting DNA gap is then filled by Pols δ or $\tilde{\varepsilon}$. Finally, DNA ligase seals the nicks to finish NER.

Base excision repair (BER). BER is the pathway involved in the repair of endogenous lesions such as those arising from oxidation and alkylation of DNA bases (Friedberg et al., 2004; Nouspikel, 2009). For instance, the conversion of a normal guanine to the damaged 7,8-dihydro-8-oxoguanine (8-oxo-G) occurs 10^3–10^4 per cell/per day. In BER specific DNA glycosylases remove the damaged base leading to abasic site formation. Subsequentially AP endonuclease 1 (APE1) incises the strand at the 5′-phosphate, leaving a 5′-deoxyribosephosphate (5′-dRP) group and 3′-hydroxyl (3′-OH) group, resulting in a single strand break (SSB) formation. At this point, dependent of the damage's nature, BER reaction can take two different way namely short patch BER and long patch BER.

In short patch BER, Pol β or Pol λ will perform repair synthesis to replace the excised damaged base, and cleave the deoxyribosephosphate moiety with its lyase activity (Dianova et al., 2004; Tomkinson et al., 2001). Finally the XRCC1/DNA ligase III complex seals the DNA. Some kind of lesions, such as a reduced AP site, a C1 oxidized AP site (Fortini and Dogliotti, 2007) or an adenine opposite 8-oxo-G (Fortini et al., 2003; Hashimoto et al., 2004), can undergo long-patch repair. In this pathway, a 5′-blocking lesion results in Pol β release after which PCNA facilitates excision by the nuclease FEN1, which stimulates strand displacement DNA synthesis (Gary et al., 1999; Liu et al., 2005) by Pols δ or ε (Dianov et al., 2003). Finally, DNA ligase I catalyzes strand ligation. No syndromes have been directly linked to BER defects so far, however, the main enzymes acting in this pathway (Pols β and λ) are often found deregulated in cancer cells. The possible roles of Pols β and λ in spontaneous mutagenesis will be discussed below.

Mismatch repair (MMR). Mispaired nucleotides resulting from replication errors are removed by MMR (Harfe and Jinks-Robertson, 2000a), which is also involved in DNA adducts detection and repair, such as those resulting from platinum-based chemotherapeutic agents (Fink et al., 1998). In the initial step, the heterodimeric MSH complex recognizes the nucleotide mismatch and subsequentially interacts with MLH1/PMS2 and MLH1/MLH3 complexes. Several proteins participate in the process of nucleotide excision and re-synthesis of the DNA. Comparing to

normal cells, tumor cells deficient in MMR have much higher mutation frequencies, exhibiting microsatellite instability (Parsons et al., 1993). Several genes including *MSH2, MSH3, MSH6, MLH1, MLH3, PMS1,* and *PMS2* are involved in MMR. The overwhelming majority of hereditary nonpolyposis colorectal cancers (HNPCC) are attributed to mutations in the MMR genes encoding the MutS and MutL homologues MSH2 and MLH1. HNPCC is characterized by high risk of colorectal cancer and other cancers of the endometrium, ovary, stomach, small intestine, hepatobiliary tract, upper urinary tract, brain, and skin, while a subtype of HNPCC, known as Muir-Torre Syndrome (MTS), is associated with skin tumors. Contrary as would be expected, an intact MMR pathway is required for cisplatin sensitivity rather than resistance (Aebi et al., 1996). This observation suggests that MMR may promote cell death due to DNA breaks after excision of cisplatin adducts or, alternatively it may have also a role as apoptotic signaling.

Under certain circumstances, both DNA strands are damaged. Double-strand breaks (DSBs) are common lesions in all living cells. DSBs are repaired by two alternative mechanisms, dependent on the cell cycle phase: Homologous recombination (HR) act during late S or G2 while Non-homologous end joining (NHEJ) can occur during all the cell cycle and is the major repair pathway of such lesions in multicellular organism (Sancar et al., 2004; Wilson and Lieber, 1999, for additional information see also "Nonhomologous DNA End Joining (NHEJ) and Chromosomal Translocations in Humans" by Lieber et al., and "Fluorescence-Based Quantification of Pathway-Specific DNA Double-Strand Break Repair Activities: A Powerful Method for the Analysis of Genome Destabilizing Mechanisms" by Böhringer and Wiesmüller, this book).

Homologous recombination (HR). In the HR pathway, DSBs are repaired through the alignment of homologous sequences of DNA. The enzymatic system responsible for this action is quite similar to the machinery responsible for chromosomal crossover during meiosis. This pathway allows a damaged chromosome to be repaired using a sister chromatid (available in G2 after DNA replication) or a homologous chromosome as a template. Initially, the complex formed by RAD50, MRE11, and NBS1, which posses a 3′->5′ exonuclease activity, exposes the 3′ ends on both DSBs ends, in a process that may require the protein BRCA1 too (Zhong et al., 1999). The 3′ advancing strand from the damaged chromosome then invades the complementary sequence of the homologous one. For this process both breast cancer susceptibility protein (BRCA1 and 2) and the single strand DNA binding protein RAD51 are required (Moynahan et al., 2001; Davies et al., 2001). The 3′ end of this strand is thought to be extended by a DNA polymerase that reads off of the complementary sequence. After DNA synthesis is extended past the region of the DSB, the 3′ end of the advancing strand returns to the original chromosome and DNA synthesis continues (Khanna and Jackson, 2001; Hoeijmakers, 2001). HR is essentially an error-free pathway, but when DSBs occur during DNA replication, if left unrepaired they can induce the collapse of the replication fork itself. When DSBs arise at the replication fork, the activation of ATM (ataxia telangiectasica mutated)-Chk2 (checkpoint kinase 2) and the ATR (ataxia telangiectasica mutated and Rad3-related)-Chk1 signalling cascades, arrest in S-and G2-phases of the cell

cycle and apoptosis. Chk1 inhibits CDC25 phosphatases by causing their nuclear exclusion or degradation, which in turn prompts the accumulation of inhibitory phosphorylation of CDKs. Chk2 and ATM signal collaborated with p53 to induce different pro-.apoptotic factors (like Puma, Bax, Noxa), oxidative-stress response genes and DNA repair, the expression of p21Cip1/WAF1 and the feedback regulator HDM2 (the human orthologue of the mouse double minute 2, MDM2). In cancer cells, which are often defective in ATR and ATM pathways, unscheduled HR might lead to high rates of chromosome breakage and illegitimate fusions, contribution to genomic instability.

Non-homologous end joining (NHEJ). For efficient rejoining of broken chromosome ends by NHEJ, a number of specific factors are needed, including the complex formed by of XRCC4 and DNA ligase IV, a DNA end binding heterodimer, Ku70 and Ku80, DNA-PK and the recently described accessory factor XLF/Cernunnos. All these factors are essential for the NHEJ reaction and the absence of only one of them can induce sensitivity to double-strand break inducing agents and premature cellular senescence (Mills et al., 2003; Sekiguchi and Ferguson, 2006). NHEJ relies on short homologous sequences called microhomologies present on the single-stranded tails of the DNA ends to be joined. If the structure of broken ends do not show microhomologies, NHEJ employs different factors to process the ends, including the family X DNA polymerases Terminal deoxynucleotidyl transferase (TdT), Pol μ and Pol λ (Nick McElhinny and Ramsden, 2004), to extend the terminus of the broken ends to create such regions. Another important role played by NHEJ is joining hairpin-capped double-strand breaks induced during V(D)J recombination. Such a process generates diversity in B-cell and T-cell receptors in the vertebrate immune system (Budman and Chu, 2005) (see also "Nonhomologous DNA End Joining (NHEJ) and Chromosomal Translocations in Humans" by Lieber et al., this book). However, NHEJ is intrinsically an error-prone process. Thus, high frequencies of DSBs when repaired by NHEJ, can substantially increase the spontaneous mutagenesis rate, accelerating tumor progression.

Translesion synthesis (TLS). In mammalian cells, during DNA replication, there are situations where a lesion in DNA can result in replication fork stalling due to inability of replicative Pols to bypass lesions like abasic (AP) sites or bulky DNA template adducts, thymine-thymine or cyclobutane pyrimidine dimers and cis-platinum adducts (Prakash et al., 2005; Waters et al., 2009). In recent years a variety of so-called translesion Pols have been identified. These enzymes exhibit a distributive DNA synthesis over the lesions, lack the proofreading activity and are usually error-prone in copying canonical DNA templates. Their ability to incorporate nucleotide opposite bulky modified bases is due to a wider nucleotide-binding pocket with respect to the replicative enzymes. Since TLS Pols perform DNA synthesis often in an error-prone manner, this can lead to accumulation of mutations in the newly synthesized DNA. According to the so-called "mutagenesis hypothesis" of carcinogenesis, mutations are a prerequisite for most, if not all, cancers. Since error-prone TLS constitutes a major mechanism of mutagenesis, its deregulated activity can substantially contribute to the mutated phenotype of cancer cells. Indeed, many if not all the TLS enzymes are often found overexpressed in cancer

cells. At least 6 novel putative Pols which belong to the B, X and Y families have been identified as components of the TLS pathway (Hubscher et al., 2002). Their roles in mutagenesis will be discussed below.

DNA Polymerases and Mutagenesis

DNA polymerases act as key players in DNA metabolism (Hubscher et al., 2002). These enzymes are the only biological macromolecules able to duplicate the genetic information stored in the DNA. They are absolutely required every time this information has to be copied, as during DNA replication. However they are also necessary during DNA repair, when lost or damaged DNA sequences have to be replaced with "original" or "correct" copies. As outlined above, in each DNA repair pathways one or more specifics Pols are required depending on damage kind, cellular cycle phase, DNA repair reaction and tissue specific Pols availability. A feature of mammalian DNA repair pathways is their redundancy. For example, DNA double-strand breaks (DSBs) can be repaired by either NHEJ or by HR, while BER, NER, MMR and HR pathways are implicated in repairing single-base damage. Errors made by Pols during both DNA synthesis or postreplicational DNA repair, including translesion synthesis over DNA lesions, can lead to mutation in the genome of both normal and tumor cells and contribute to the overall mutational burden of cancer cells (Hanahan and Weinberg, 2000; Venkatesan et al., 2006). Below, we summarize the known involvement of replicative and repair Pols in mutagenesis.

Replicative Pols

Pol δ. Pol δ plays a major role in genome duplication. This Pol exhibit low misincorporation rates since it possesses an intrinsic 3′->5′ exo activity (proofreading activity) which increase the fidelity. Mutants of Pol δ with inactivating mutations or deletions of the exo domain, exhibits base substitium rates of 10^{-4} while the wild type enzyme shows misincorporation rates of 1–0, 1×10^{-5}. Moreover, defects in the 3′->5′ exo activity in Pol δ, causes cancer susceptibility in mice (Goldsby et al., 2001; Goldsby et al., 2002) indicating that Pol δ proofreading suppresses spontaneous cancer development and suggesting that unrepaired Pol δ errors contribute to carcinogenesis. Pol δ is also implicated in several DNA repair events such long patch BER, NER, MMR (Aboussekhra et al., 1995; Longley et al., 1997), and in translesion synthesis, in combination with the translesion Pol ζ Pol η and Rev 1 (Giot et al., 1997) and its possibly implicated in cell cycle control and in meiotic recombination (Francesconi et al., 1993; Maloisel et al., 2004). Furthermore, reduction in the expression level of wild-type Pol δ results in a mutator phenotype and increased sensitivity to the DNA-damaging agent methyl methane sulfonate (MMS), suggesting that both the quantity and the quality of Pol δ is important in ensuring genome stability (Kokoska et al., 2000).

DNA Repair Pols

Pol β. The major DNA polymerase implicated in BER is Pol β. This enzyme belongs, together with Pols μ and λ, to the Pol family X. It possesses a DNA synthesis specificity for short gaps, an associated dRPlyase activity and is able to associate with other BER enzymes such DNA ligase I, AP endonuclease and XRCC1-DNA ligase III (Idriss et al., 2002). Moreover, Pol β efficiently bypasses cisplatin and oxaliplatin adducts (Vaisman and Chaney, 2000).

Pol β predominantly bypasses the lesion by insertion of a complementary nucleotide to an adjacent downstream template site. This kind of DNA synthesis by "skipping over" the lesion results in both deletion and base substitution errors (Efrati et al., 1997). Many studies show that transcriptional and protein levels of Pol β are higher in cancer tissues, especially solid tumors (e.g. prostate, breast, colon, ovarian) as well as in chronic myeloid leukaemia (Louat et al., 2001). These studies suggest that regulation of Pol β expression may be essential *in vivo* since its up-regulation could contribute to enhancing chromosome instability and tumorigenesis when overexpressed just by 2-fold in cells (Bergoglio et al., 2004; Albertella et al., 2005). These evidences led to the idea that, probably by competing either with replicative Pols or by translesion syntesis over DNA lesions, Pol β causes chromosomal instability. Moreover, IR treatments in cells overexpressing Pol β result in increasing apoptosis, and hypermutator phenotype in surviving cells. According to its proposed role as a mutator enzyme, Pol β exhibit a very low fidelity in DNA synthesis reaction opposite both 8-oxo-G and 1,2-dihydro-2-oxoadenine (2-OH-A) lesions and its overexpression enhances the mutagenicity of oxidative damages and increases apoptosis (Frechet et al., 2002). These data indicate that Pol β act as a determinant factor in both cell death and genetic changes associated with cancer (Frechet et al., 2001).

Pol μ. Pol μ is predominantly expressed in peripheral lymphoid tissues in abundant association with follicular areas of secondary lymphoid tissues (Aoufouchi et al., 2000; Dominguez et al., 2000; Ruiz et al., 2001). Pol μ possesses both DNA template-dependent, template-independent (terminal transferase) activities and, like Pol λ, can perform DNA de novo synthesis. Pol μ is an error-prone DNA-template-dependent polymerase, able to perform DNA translesion synthesis over several damages, and can efficiently extend DNA primers paired with a lesion (Duvauchelle et al., 2002; Zhang et al., 2002). In mammalian cells treated with DNA damaging agents such UV, γ-irradiation and H_2O_2, down regulation of Pol μ mRNA was observed (Aoufouchi et al., 2000). Experimental studies in mice showed that knocking out Pol μ led to a marked depletion of B cells in peripheral lymphoid organs (Bertocci et al., 2002). Moreover, Immunoglobulin light chain gene rearrangement resulted impaired at Vκ–Jκ and Vλ–Jλ junctions levels, causing 40% reduction in the spleen B cell fractions (Bertocci et al., 2003). Pol μ was implicated in malignancy progression since a close correlation was established between its up-regulation in B cells and B-cell non-Hodgkin's lymphomas (B-NHLs) (Chiu et al., 2002).

Pol λ. Pol λ is expressed at the highest level in testis, ovary (Garcia-Diaz et al., 2000) and fetal tissues (Aoufouchi et al., 2000), but it can be detected at lower levels in all proliferating tissues. Pol λ might participate in NHEJ together with Pol μ and TdT, since it possesses a terminal deoxynucleotidyl transferase activity, interacts with the NHEJ factors XRCC4/Ligase IV (Fan and Wu, 2004) and its immunodepletion from a human cell extract led to the inability to perform the NHEJ reaction (Lee et al., 2004). Like Pol β, Pol λ posses a dRP-lyase activity and can efficiently repair uracil-containing DNA in an in vitro reconstituted BER reaction showing 25% efficiency with respect to Pol β (Garcia-Diaz et al., 2002). Recently our research group reported that, in the presence of PCNA and RPA, Pol λ exhibits the highest fidelity in the synthesis reaction opposite of 8-oxo-G and 2-OH-A lesions, suggesting that this enzyme is responsible of the error free bypass of oxidative DNA damage (Maga et al., 2007; Crespan et al., 2007). In addition, we showed that, in a cancer model cell line derived from human fibroblast, cen3tel, Pol λ expression levels, contrary to Pol β, increase during the initial phases of cancer development (Maga et al., 2008). Furthermore, Pol λ is strongly misregulated in many solid tumors leading to the hypothesis that Pol λ expression in cancer cell can compensate the malfunctioning of different repair pathways (Albertella et al., 2005).

Terminal deoxynucleotidyl transferase (TdT). TdT belongs to the Pol X family (Holm and Sander, 1995) and has striking structural homology with Pol μ. It can elongate DNA strands in a template-independent manner (Bollum et al., 1974), a fundamental activity for V(D)J recombination. TdT is overexpressed in several acute leukaemia cells, and this correlates with poor prognosis and low response to chemotherapy (Benedetto et al., 1986). Recently, it has been shown that the nucleoside analogue cordycepine in combination with the antitumor drug coformycine, inhibits TdT and displays selective toxicity against TdT+ leukemic cells, suggesting an important functional role of TdT in the cancerous phenotype. Indeed, a specific inhibitor of TdT showed selective toxicity against the leukemic TdT+ cell line MOLT-4 (Locatelli et al., 2005).

TLS Pols

Pol ζ. Pol ζ belongs to DNA polymerase B family; is composed by Rev3, the catalytic subunit, and the accessory subunit Rev7 (Murakumo et al., 2000). Pol ζ interacts through Rev7 with the protein MAD2, suggesting a function in cell cycle control by affecting the activation of the anaphase promoting complex (APC). Pol ζ is involved in most spontaneous mutations, lesion-targeted mutation via TLS and somatic hypermutation of immunoglobulin genes (Murakumo, 2002; Poltoratsky et al., 2001; Harfe and Jinks-Robertson, 2000b); in combination with other Pols, such as Pol η, Pol ζ acts as a "mismatch extender" in TLS; and is essential in mouse embryonic development (Esposito et al., 2000). *S. cerevisiae* REV3 –/– cells are sensitive to different DNA-damaging agents, such as ionizing radiation (IR), UV light, MMS and cisplatin adducts. These cells, after IR in G2, show a

marked increase of chromosomal breaks in M phase leading to the suggestion of a Rev3 involvement in HR-mediated repair of DSBs. Furthermore, REV3 –/– cells display significant sister chromatid exchange and chromosomal breaks increase also in the absence of exogenous genotoxic stress (Sonoda et al., 2003). Recently, it was found that Pol ζ might be involved in the mutagenesis induced by *N*-methyl-*N*'-nitro-*N*-nitrosoguanidine (MNNG), a carcinogen which can induce gastric cancer. In fact, under low concentration MNNG treatment, the transcriptional level of REV3 gene is upregulated (Zhu et al., 2003); moreover, it was found that the MNNG response element might locate at the REV3 gene promoter region (Yu et al., 2004).

Pol η. Pol η is encoded in *S. cerevisiae* by the RAD30 gene and belong to Y family. In humans, Pol η is encoded by the PolH (xeroderma pigmentosum variant, XPV) gene (for additional information see also "DNA Polymerase η, a Key Protein in Translesion Synthesis in Human Cells" by Cruet-Hennequart et al., this book). It was discovered that a fraction of patients suffering of XP disease did not carry any mutation in NER XP genes (see above), but they showed mutations in the PolH gene, (Masutani et al., 1999; Johnson et al., 1999a), underscoring an essential role of Pol η in preventing spontaneous mutagenesis. One of the major lesions generated by exposure to sunlight is cis–syn cyclobutane dimer (CPDs); the loss of Pol η results in a reduced efficiency to copy DNA containing these lesions (Johnson et al., 1999b, Masutani et al., 2000). Pol η can perform DNA synthesis opposite thymine-thymine (TT) dimers and the bases flanking the lesion with high processivity, switching to less processive and low fidelity synthesis on undamaged DNA. This led to the hypothesis that the ability of Pol η to sense the TT dimer lesion during DNA synthesis progression may facilitate polymerase switching before and after lesion bypass.

Pol η exhibit different fidelity in bypass of 3′ or 5′ thymidine in the TT dimer: the error rate for T to C substitutions at the 5′ T is 32×10^{-4}, a value that is 12-fold lower than at the 3′ T of the dimer (McCulloch et al., 2004). Nevertheless, since Pol η copy undamaged DNA with lower fidelity (Matsuda et al., 2001; Washington et al., 2001) and no other human Pol exhibits comparable efficiency in dimer bypass, it is the most likely enzyme involved in the bypass of CPD lesions. The presence of TT dimers in individuals affected by NER-defective syndromes, such as XP, might lead to the accumulation of mutations by Pol η action, that could contribute to skin cancer spread.

Pol κ. Pol κ is a family Y enzyme encoded by the POLK gene on human chromosome 5. Like Pol η, it can perform DNA synthesis using as substrate DNA containing lesions that substantially distort the DNA helix geometry. Pol κ cannot bypass a TT dimer but it does bypass other kind of lesions such benzo(a)pyrene diol epoxide (BPDE) adducts on the N2 of guanine (Suzuki et al., 2002; Rechkoblit et al., 2002). Pol κ bypass these lesions by insertion of dCMP, thus preventing benzo(a)pyrene-induced mutations. The hypothesis that Pol κ may participate in the error-free bypass of lesions generated by polycyclic aromatic hydrocarbons (PAH) is supported by the observation that expression of the mouse Pol κ gene is under the control of the arylhydrocarbon receptor, a crucial factor for the activation of benzo(a)pyrene into BPDE in mammalian cells (Ogi et al., 2001). Pol κ

expression was diminished in rat mammary carcinoma cell lines and primary mammary carcinomas in comparison to that of the normal tissues (Pan et al., 2005). On the other hand, some evidences exist about a role in tumorigenesis of Pol κ. In fact, ectopic expression of Pol κ promoted DNA strand breaks, aneuploidy as well as tumorigenesis in nude mice (Bavoux et al., 2005). Moreover, non-squamous lung carcinoma showing Pol κ overexpression, displayed loss of heterozygosity compared with adjacent non-tumoral tissues (Bavoux et al., 2005). Taken together, these data suggested that misregulation of Pol κ can promote the emergence of a broad spectrum of genetic disorders associated with a malignant phenotype. In addition, p53-dependent regulation of Pol κ was shown in human cells, as well as in murine cells, and the functional loss of p53 by mutation results in the up-regulation of Pol κ in human lung cancer tissues (Wang et al., 2004b). However, other investigators indicated that Pol κ was primarily regulated by p53 in mouse, but not human cells, and was up-regulated in response to exposure to various DNA-damaging agents in a p53 dependent manner (Velasco-Miguel et al., 2003).

Pol ι. The gene POLI encodes for Pol ι. This gene has only been identified in higher eukaryotes. Pol ι is able to insert nucleotides opposite many lesions present in DNA, like oxidative damages and polycyclic aromatic hydrocarbons adducts (PAH) ones (Vaisman and Woodgate, 2001; Frank et al., 2002), whereas it cannot extend the resulting template/primers efficiently. In this way, for the complete TLS extension a second Pol may by required, e.g. Pol ζ (Guo et al., 2001; Johnson et al., 2000). The fidelity of Pol ι is high opposite template A, showing error frequencies of 10^{-4} to 10^{-5}. Opposite templates G and C, it exhibits a very low fidelity, with error frequencies ranging from 10^{-1} to 10^{-2}. Pol ι has an even lower fidelity opposite template T, with error frequencies ranging from 10^{+1} to 10^{-1} (Haracska et al., 2001a, Tissier et al., 2000; Zhang et al., 2000). A growing body of evidences support the hypothesis of a direct involvement of Pol ι in lung cancer development. By performing systematic candidate gene analyses of the pulmonary adenoma resistance 2 locus, POLI showed 25 nucleotide polymorphisms in its coding region resulting in a total of ten amino acid changes. Such purified Pol ι variants differ in in vitro substrate discrimination (Wang et al., 2004a). Moreover, altered expression of Pol ι and an amino acid changing nucleotide polymorphism were observed in human lung cancer cells, suggesting a possible role of Pol ι in the development of this type of tumor (Wang et al., 2004b). Furthermore a single nucleotide polymorphism in Pol ι (Thr706Ala), which correlated with a significantly higher risk of lung adenocarcinoma and squamous cell carcinoma, was identified (Lee et al., 2003), whereas Pol ι expression resulted elevated in breast cancer cells and correlate with a significant decrease in DNA replication fidelity (Yang et al., 2004).

REV1. REV1 belongs to Y family, its gene is localized on human chromosome 2 and it is ubiquitously expressed in various human tissues (Lin et al., 1999). Yeast and human Rev1 proteins are G template-specific DNA terminal nucleotidyltransferases able to insert only C opposite template G, A and AP sites (Haracska et al., 2001b, Haracska et al., 2002; Lawrence et al., 2000; Masuda et al., 2001). Rev1 interacts with Rev7 and with other Y-family Pols such Pols η, κ and ι, suggesting that this enzyme acts like a scaffold that recruits Pols involved in TLS (Tissier et al., 2004).

Knock down of REV1 mRNA in human cells results in a hypomutable phenotype after UV irradiation (Clark et al., 2003). In addition to its catalytic domain, Rev1 possesses a so-called BRCA1 C-terminal (BRCT) domain. Mice Rev1/BRCT1 double null cells display an elevated spontaneous frequency of intragenic deletions. In addition, these cells were sensitized to exogenous DNA damages. UV-C light induced a delayed progression through late S and G2 phases of the cell cycle and many chromatid aberrations, specifically in a subset of mutant cells, but not enhanced sister chromatid exchanges. UV-C-induced mutagenesis was reduced. All together these data seem to underline a regulatory role of Rev 1 BRCT domain in TLS on a specific nucleotide damage subset (Jansen et al., 2005). Moreover, in Rev1/BRCT1 –/– cells, mutations at TT dimers were absent: the opposite phenotype of UV-C-exposed cells from XPV patients, lacking Pol η. This suggested that the enhanced UV-induced mutagenesis in XPV patients may depend on error-prone Rev1-dependent TLS.

Pol θ. Pol θ is encoded by POLQ gene in human and belongs to DNA polymerase A family (Sharief et al., 1999). Human Pol θ is able to catalyze efficiently DNA synthesis past an AP site and a thymine glycol and displayed high fidelity using canonical DNA substrate (Maga et al., 2002). Unusually, Pol θ both inserts a base opposite an AP site and efficiently extends the misincorporated nucleotide, making it the most efficient known DNA polymerase for AP-site bypass. Like others Pols, Pol θ preferentially incorporated A base opposite an AP site and the best primer extended ending with an A residue opposite an AP. Interestingly, Pol θ inefficiently incorporates dCTP opposite an AP site showing a completely opposite behavior to REV1 preferred reaction at an AP site (Seki et al., 2004). Mutation in Pol θ gene named chaos1 (chromosome aberration occurring spontaneously 1) (Shima et al., 2003) was identified during genetic screening for chromosome instability in the mouse genome, suggesting that Pol θ is potentially involved in cancer susceptibility. Moreover, mutations in this gene in flies were linked to sensitivity to DNA crosslinking agents, elevated frequency of chromosomal aberration and altered DNA metabolism (Boyd et al., 1990). Recently, it was found that vertebrate Pol θ and Pol β cooperate in base excision repair of oxidative DNA damage (Yoshimura et al., 2006).

Telomerase. Telomerase is a specialized Pol responsible for synthesis of telomeres (chromosomal ends) in eukaryotic cells. Terminally differentiated cells loose the telomerase activity, a feature, which has been linked to the ageing process. Because telomeres are essential for genome stability of proliferating cells, changes in telomere's functions are linked to chromosomal abnormalities involved in cancer. Telomerase reactivation, and the consequent telomeres maintenance, is the most common tumor marker, since more than 90% tumor cells express this enzyme (Chang et al., 2004). In addition, in human embryonic kidney cells and in endothelial cells, it was show that telomerase activity had an anti-apoptotic effect, which was post-transcriptionally modulated by phosphorylation and by the interaction with binding proteins such as HSP90 and Akt (Haendeler et al., 2003). Finally, a mouse model showed a direct involvement of telomerase in aging: genetic data indicated that the delayed manifestation of the complex pleiotropic of Werner deficiency relates to telomere shortening (Chang et al., 2004).

Concluding Remarks

A cancer cell feature is genome instability resulting in chromosome breaks and generation of fusions between oncogenes and cellular genes, inactivation of tumor suppressor genes and drug resistance genes amplification. A state of genomic instability can be achieved through the loss of DNA damage signaling and checkpoint pathways or by defects in one or more of the six major DNA repair pathways. For these reasons, understanding how the enzymes implicated in the different DNA repair pathways are regulated in normal and in tumor cells will provide significant novel information on cancer outset. The identification of key proteins involved in these pathways might also provide novel markers for cancer diagnosis and new avenues for the development of antiproliferative (anticancer) drugs. As mentioned above, cancer cell are usually defective for at least one DNA repair pathway. The specific pathway affected is predictive of the kinds of mutations so, under this respect, the mechanisms through which tumor cells respond to and deal with damaged DNA have profound implications in the development of the cancer phenotype. Moreover, the specific DNA repair pathways altered in cancer cells can cause specific tumor drugs sensitivity and could help in the choice of optimal treatment.

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DNA Polymerase η, a Key Protein in Translesion Synthesis in Human Cells

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Abstract Genomic DNA is constantly damaged by exposure to exogenous and endogenous agents. Bulky adducts such as UV-induced cyclobutane pyrimidine dimers (CPDs) in the template DNA present a barrier to DNA synthesis by the major eukaryotic replicative polymerases including DNA polymerase δ. Translesion synthesis (TLS) carried out by specialized DNA polymerases is an evolutionarily conserved mechanism of DNA damage tolerance. The Y family of DNA polymerases, including DNA polymerase η (Pol η), the subject of this chapter, play a key role in TLS. Mutations in the human *POLH* gene encoding Pol η underlie the genetic disease xeroderma pigmentosum variant (XPV), characterized by sun sensitivity, elevated incidence of skin cancer, and at the cellular level, by delayed replication and hypermutability after UV-irradiation. Pol η is a low fidelity enzyme when copying undamaged DNA, but can carry out error-free TLS at sites of UV-induced dithymine CPDs. The active site of Pol η has an open conformation that can accommodate CPDs, as well as cisplatin-induced intrastrand DNA crosslinks. Pol η is recruited to sites of replication arrest in a tightly regulated process through interaction with PCNA. Pol η-deficient cells show strong activation of downstream DNA damage responses including ATR signaling, and accumulate strand breaks as a result of replication fork collapse. Thus, Pol η plays an important role in preventing genome instability after UV- and cisplatin-induced DNA damage. Inhibition of DNA damage tolerance pathways in tumors might also represent an approach to potentiate the effects of DNA damaging agents such as cisplatin.

Keywords DNA polymerase eta · Translesion synthesis · XPV · UV · Cisplatin · DDR

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Abbreviations

ATM	ataxia-telangiectasia mutated
ATR	ATM and Rad3-related
ATRIP	ATR-interacting protein
CPD	cyclobutane pyrimidine dimer
DDR	DNA damage response
DNA-PK	DNA-dependent protein kinase
Pol η	DNA polymerase eta
RPA	replication protein A
XPV	xeroderma pigmentosum variant

Introduction

Environmental and metabolic insults such as radiation, chemical agents and oxidative stress can generate DNA lesions, leading to mutation fixation, DNA strand breaks and genomic instability. DNA damage from exposure to UV radiation can lead to cancer, while DNA damaging agents such as platinum-based drugs are routinely used to kill tumor cells in chemotherapy. Cells that are actively carrying out DNA replication are particularly vulnerable to DNA damage, as endogenous and exogenous events challenge genome integrity by interfering with the progression, stability and restart of the replication fork. A number of DNA repair and DNA damage tolerance pathways counteract the deleterious consequences of DNA damage (Branzei and Foiani, 2008; Chang and Cimprich, 2009; Branzei and Foiani, 2007, see review "DNA Polymerases and Mutagenesis in Human Cancers" by Crespan et al., this book). Despite the presence of dedicated DNA repair pathways, not all damage is removed from the genome before DNA synthesis proceeds. A better understanding of the proteins involved in replication of damaged DNA is of relevance both to cancer initiation, and to cancer treatment using DNA damaging agents to induce replication-blocking lesions (Bartek et al., 2007).

Many DNA damaging agents induce lesions that block DNA synthesis by the replicative DNA polymerases α, δ and ε that normally carry out replication of genomic DNA (see review "DNA Polymerases and Mutagenesis in Human Cancers" by Crespan et al., this book). However, the fact that cells can complete replication in the presence of unrepaired damage indicates the existence of pathways that facilitate replication of damaged DNA without prior lesion removal. These are often referred to as post-replication repair (PRR) pathways, and in recent years have been more clearly defined at the molecular level. The main PRR pathways are translesion synthesis (TLS) involving low fidelity DNA polymerases such as DNA polymerase η (Pol η) (the subject of this review), and error-free PRR, a process that involves fork reversal and template strand switching (Chang and Cimprich, 2009; Hishida et al., 2009).

TLS has been extensively studied over the past decade, and involves the recruitment of specialized DNA polymerases to carry out replication past the lesion site

in the template strand (Branzei and Foiani, 2007; Branzei and Foiani, 2008; Chang and Cimprich, 2009). TLS represents a major mechanism for DNA damage tolerance in all species, being conserved through evolution from bacteria to humans. In *E. coli*, DNA polymerase V, the *umuC* gene product is required for TLS, and the main TLS polymerases, from yeast to humans, show homology to *E. coli* umuC gene. The *RAD30* gene encoding DNA polymerase η was first identified in *S. cerevisiae* (McDonald et al., 1997). The cloning of the human *RAD30* gene in 1999, and the demonstration that mutations in this gene leading to elimination of the full length active Pol η protein were found in all patients with the skin cancer-prone, sun sensitive genetic disease xeroderma pigmentosum variant (XPV) (Cleaver, 1972), provided a major impetus for further research into the process of TLS (Johnson et al., 1999a; Masutani et al., 1999b).

The human *RAD30* (*POLH*) gene is located on chromosome 6p21.1-6p12, and consists of 11 exons, of which exon 1 is untranslated (Yuasa et al., 2000). It was shown that purified Pol η, a 713 amino acid, 78 kDa protein could restore the ability of cell extracts from XPV cell lines to carry out complete replication of plasmid DNA containing a thymine-thymine cyclobutane pyrimidine dimer (CPD) in vitro (Masutani et al., 1999a). DNA polymerase ι (Pol ι), the product of the human *RAD30B* gene (McDonald et al., 1999), is highly homologous to Pol η, but can not substitute for it in TLS at sites of UV-induced damage. Most human TLS DNA polymerases, including Pol η, Pol ι, and Pol κ (Gerlach et al., 1999; Ogi et al., 1999), belong to the Y-family of DNA polymerases (Burgers et al., 2001; Lehmann et al., 2007; McCulloch and Kunkel, 2008). The dCMP transferase Rev1 (Nelson et al., 1996) also belongs to the Y family. However, the Rev3/Rev7 gene product, Pol ζ, which plays an important role in TLS for a number of lesions, belongs to the B family of DNA polymerases (Burgers et al., 2001; Lawrence and Maher, 2001). While TLS is often considered error-prone, several TLS polymerases can carry out error-free bypass of specific lesions (McCulloch and Kunkel, 2008; Prakash et al., 2005). This chapter will focus on Pol η, one of the Y family of DNA polymerases.

DNA Polymerase η, a Member of the Y Family of Polymerases

Unlike the replicative polymerases δ and ε, TLS polymerases, including Pol η, lack a 3′-5′ proofreading exonuclease activity and have high error rates, on the order 10^{-2}–10^{-4}, for base substitutions during DNA synthesis on undamaged DNA (McCulloch and Kunkel, 2008). However, compared to other DNA polymerases, TLS polymerases generally contain a more open active site which can accommodate bulky damaged bases (Friedberg, 2001; Ling et al., 2001; Trincao et al., 2001). This structural feature facilitates nucleotide incorporation opposite lesions in the DNA template; incorporation may be error-free or error-prone depending on the specific lesion to be bypassed.

Sequence alignment of the N-termini of Pol η proteins from lower to higher eukaryotes, revealed five conserved motifs, motifs I–V, and nine highly conserved

acidic residues (Kondratick et al., 2001). Three highly conserved acidic amino acids, present within the motifs I and III, are essential for polymerase activity, by coordinating the two metal ions in the active site necessary for catalytic activity (Kondratick et al., 2001). In human Pol η, an invariant tyrosine (Y52) and arginine (R55) in motif II, and an invariant lysine (K231) residue in motif IV, contribute to nucleotide binding and incorporation during DNA synthesis (Glick et al., 2003; Glick et al., 2001; Johnson et al., 2003). Motif V is a structural feature unique to Y family polymerases, termed the little finger (LF) domain or the polymerase-associated (PAD) domain (Yang and Woodgate, 2007). While all five motifs are essential for DNA synthesis, motif V is much less conserved between the Y-family polymerases than are the other motifs (Prakash et al., 2005). The C-terminus of Pol η contains the nuclear localization signal, as well as a number of structural features that play key roles in the recruitment of the DNA polymerase to sites of DNA damage. These include the PCNA-interacting peptide (PIP) domain (Haracska et al., 2001a), the ubiquitin-binding zinc finger (UBZ) domain (Bienko et al., 2005), and the Rev1-interacting region (Yang and Woodgate, 2007).

Y-family DNA polymerases, including Pol η, differ strikingly from high-fidelity DNA polymerases in that the active site is much more open. Crystallization of the catalytic core of yeast Pol η (Trincao et al., 2001), and of the related Dpo4 DNA polymerase from *Sulfolobus solfataricus* (Ling et al., 2001), with an oligonucleotide containing a thymine-thymine CPD demonstrated that the active site can accommodate both residues of the CPD. Human Pol η is specialized to preferentially insert two adenines opposite thymine-thymine CPDs, to carry out largely error-free bypass of this lesion (Carty et al., 2003; Cleaver et al., 2002a; Cordeiro-Stone and Nikolaishvili-Feinberg, 2002; Johnson et al., 1999a; Masutani et al., 2000; Stary et al., 2003; Thakur et al., 2001; Yao et al., 2001). The importance of Pol η in preventing UV-induced mutations and skin carcinogenesis is underlined by the demonstration that the human genetic disease xeroderma pigmentosum variant (XPV) results from loss of functional Pol η due to mutations in *POLH* (Johnson et al., 1999a; Masutani et al., 1999b). XPV patients account for almost 20% of XP cases and show UV-sensitivity, and strong predisposition to skin cancer (Cleaver, 1972). XPV cells have normal rates of nucleotide excision repair, but have a defect in DNA synthesis, and are hypermutable, after UV-irradiation (Lehmann, 1975; Tung et al., 1996). In the absence of Pol η, mutations in XPV cells accumulate at CPD sites, as a result of error-prone bypass by another TLS polymerase, possibly Pol ι (Gueranger et al., 2008; Stary et al., 2003; Wang et al., 2007). Thus, error-free TLS by Pol η plays a key role in preventing mutation fixation at CPD sites, the most common UV-induced lesion, and reduces the incidence of UV-induced skin cancer in the population (McCulloch et al., 2004; Johnson et al., 1999b). Knockout of the *POLH* gene in mice recapitulates the skin cancer susceptibility observed in Pol η-deficient XPV patients (Lin et al., 2006).

However, while Pol η carries out efficient and accurate replication at sites of thymine-thymine CPDs, for other lesions, Pol η has reduced affinity, poor incorporation rates or low fidelity (Vaisman et al., 2004; Shachar et al., 2009; McCulloch and Kunkel, 2008). UV light induces both CPDs and [6-4] pyrimidine-pyrimidone

photoproducts ([6-4PP]). CPDs are more abundant than [6-4]PPs in UV-irradiated DNA; however [6-4]PPs induce greater structural distortion in the DNA double helix, and probably for this reason are more mutagenic than CPDs, and are repaired faster by the nucleotide excision repair pathway (Vreeswijk et al., 1994). Purified Pol η can not bypass a thymine-thymine [6-4]PP in an oligonucleotide template in vitro (Masutani et al., 2000), but can insert a nucleotide opposite the 3′ base of the [6-4]PP. Combination of Pol η with purified Pol ζ allowed efficient bypass of a [6-4]PP in vitro (Johnson et al., 2001), leading to the proposal that bypass of certain lesions, such as the [6-4]PP, could be accomplished by a two-polymerase mechanism, wherein Pol η (or a related Y family DNA polymerase) inserts a nucleotide opposite the 3′ base of the lesion, with subsequent insertion of a nucleotide opposite the 5′ base, and extension of the primer terminus being carried out by Pol ζ, the *REV3L* gene product (Johnson et al., 2001; Shachar et al., 2009). Consistent with such a role for Pol η, it was found that, compared to extracts of normal cells, Pol η-deficient lymphoblast cell extracts were defective in replication of plasmid DNA containing a single thymine-thymine [6-4]PP in vitro (Yao et al., 2001). Other studies indicate that Pol η plays a role in bypass of [6-4]PP in some, but not all human cell lines, examined (Hendel et al., 2008). An important role for Pol ζ in bypass of [6-4]PPs was reported in the recent study of Shachar et al. (2009) in which gapped templates containing a unique [6-4]PP were transfected into U2OS cells and the level of individual DNA polymerases was down-regulated using siRNA. An investigation of the requirements for replication of modified plasmids containing a site-specific [6-4]PP in chicken DT40 cells also found a key role for Pol ζ in TLS at this lesion (Szuts et al., 2008). The relative contribution of TLS DNA polymerases to bypass of the [6-4]PP in different cell types requires further investigation.

Role of Pol η in Bypass of Lesions Induced by Platinum-Based Chemotherapeutic Drugs

In addition to its main biological role in bypass of UV-induced CPDs, recent interest has focused on the role of Pol η in bypass of lesions induced by platinum-based chemotherapeutic drugs, since damage tolerance by TLS may contribute to the resistance of tumor cells to chemotherapy. Since its accidental discovery 40 years ago, cisplatin (cis-diamminedichloroplatinum (II)) has been successfully used in the treatment of a number of human cancers, including testicular cancer, small cell lung cancer and lymphoma (Kelland, 2007b). Studies on cancer resistance and improvement of drug selectivity towards cancer cells led the development of cisplatin analogues, including carboplatin [*cis*-diammine(1,1-cyclobutanedicarboxylate)-platinum(II)], and oxaliplatin [(*trans*-*R*,*R*)1,2 diaminocyclohexaneoxalatoplatinum(II) (Aabo et al., 1998; Kelland, 2007a). Platinum-based chemotherapeutic agents damage DNA by covalent binding primarily to guanine residues, leading to the formation of monoadducts, intrastrand

and interstrand cross-links (Chaney et al., 2005; Kartalou and Essigmann, 2001). Intrastrand adducts between two adjacent guanines are the most common lesion. Pt-DNA adducts are removed by a combination of DNA repair pathways, including nucleotide excision repair (Chaney et al., 2005; Kelland, 2007b). However, not all platinum lesions are repaired prior to DNA replication, and the role of TLS in preventing DNA replication arrest at unrepaired lesions has received considerable attention in recent years.

Two lines of evidence support a role for Pol η in the response of human cells to replication-blocking platinum-DNA lesions. Firstly, in vitro experiments using purified Pol η and oligonucleotide templates containing a single guanine-guanine cisPt-DNA intrastrand adduct demonstrated that Pol η can bypass the platinum-guanine-guanine adduct more efficiently than other eukaryotic DNA polymerases (Masutani et al., 2000; Vaisman et al., 2000). The accuracy of lesion bypass is influenced by the sequence context of the lesion, but appears to be relatively error-free (Shachar et al., 2009). Secondly, cell lines lacking Pol η are more sensitive to cell killing by platinum-based drugs (Yamada et al., 2003; Bassett et al., 2004; Chaney et al., 2005; Chen et al., 2006; Albertella et al., 2005a). The demonstration that Pol η-deficient cell lines are sensitive to platinum-based chemotherapeutic agents has led to increased interest in the role of this enzyme in determining the outcome of exposure to these agents.

Structural and biochemical analysis of cisplatin-DNA lesion bypass by Pol η has revealed the set of structural features that enable Pol η to carry out replication across these strongly distorting DNA lesions (Alt et al., 2007). The large fragment of yeast Pol η was co-crystallized in a complex with incoming dNTPs and a template containing a site-specific Pt-GG adduct. When Pol η encounters a Pt-DNA lesion, the adduct is situated outside the active site. Two steps are required for lesion bypass. First, there is an efficient and error-free elongation step at the 3′dG, in which the incoming dCTP forms a Watson-Crick base pair with the 3′dG of the Pt-GG adduct in the enzyme active site. Second, there is a slower, less-efficient step which involves incorporation of either dCTP or dATP opposite the 5′dG of the adduct (Alt et al., 2007). This allows for incorporation of either dATP and dCTP at the lesion site, consistent with in vitro studies using purified Pol η in primer extension assays on oligomeric templates containing Pt-GG lesions (Masutani et al., 2000). Site-directed mutagenesis of yeast Pol η demonstrated that arginine 73, located in the enzyme active site plays a key role in bypass of the Pt-GG lesion (Alt et al., 2007). Understanding the structural requirements for lesion bypass by Pol η could aid in the development of modified platinum-based drugs which generate DNA adducts that are bypassed less efficiently in vivo (Alt et al., 2007).

The efficiency of bypass of Pt-GG adducts by purified Pol η in vitro is comparable to the bypass of CPDs; bypass of oxaliplatin lesions is more efficient than bypass of cisplatin lesions (Chaney et al., 2005; Vaisman et al., 2000; Bassett et al., 2003). The greater efficiency of Pol η in bypass of oxaliplatin adducts could account for the lower mutagenicity of oxaliplatin when compared to cisplatin (Chaney et al., 2005). Pol ζ may also play a role in bypass of Pt-GG adducts in vivo, as part of a two-polymerase mechanism for TLS, by extending the primer terminus following

insertion of nucleotides opposite the lesion by Y-family DNA polymerases (Shachar et al., 2009).

Role of Pol η in Bypass of Other Lesions in DNA

As described above, it is clear that Pol η is proficient in bypassing both UV and cisplatin-induced lesions in DNA. In addition to these lesions, Pol η can carry out efficient and accurate replication past an 8-oxoguanine (8-oxoG) lesion in vitro (Haracska et al., 2000b). 8-oxoG results from exposure of mammalian cells to oxidative stress; by promoting error-free replication through the 8-oxoG lesion, Pol η may contribute to reducing mutagenesis and carcinogenesis that could result from mutagenic bypass of this lesion by replicative DNA polymerases (Haracska et al., 2000b). Pol η has also been shown to bypass O^6-methylguanine (me^6G) (Haracska et al., 2000a) in vitro as well as lesions induced by the chemotherapeutic nucleoside analogues AraC and gemcitabine (Chen et al., 2006).

Purified Pol η bypasses N^2-deoxyguanosine DNA adducts formed by benzo[a]pyrene 7,8-diol 9,10-epoxide (BPDE) (Haracska et al., 2001d), butadiene epoxide (Minko et al., 2001), and the acrolein-derived adduct γ-hydroxy-1,N^2-propano-deoxyguanosine (γ-HOPdG) (Minko et al., 2003), but with a lower efficiency at both the nucleotide incorporation and extension steps. In the case of lesions induced by BPDE, DNA polymerase kappa (Pol κ) may play a more important role in TLS (Avkin et al., 2004; Shachar et al., 2009; Zhang et al., 2002).

In addition to its role in TLS during replication of DNA damaged by exogenous agents such as UV light, Pol η has also been implicated in other DNA transactions, including somatic hypermutation (Masuda et al., 2007; Diaz and Lawrence, 2005; Casali et al., 2006), strand invasion during homologous recombination (McIlwraith et al., 2005; Kawamoto et al., 2005), and DNA replication under conditions of nucleotide depletion (de Feraudy et al., 2007). A recent report indicates that naturally occurring DNA structures (such as G4-DNA, H-DNA, or Z-DNA) are also physiological substrates of Pol η, suggesting that Pol η may play a role in preventing genomic instability at certain DNA sequences that are capable of forming unusual secondary structures in human cells (Bétous et al., 2009).

Regulation of Pol η Recruitment

TLS DNA polymerases carry out DNA synthesis on undamaged templates with low fidelity. Thus, access of Y family DNA polymerases to primer termini during replication of genomic DNA in human cells must be tightly regulated, to prevent accumulation of mutations in the genome. Because the active sites of replicative DNA polymerases, including Pol α and Pol δ are unable to accommodate bulky lesions caused by UV or cisplatin, following replication arrest at sites of DNA damage, processive DNA polymerases are temporarily replaced by TLS DNA

polymerases to allow TLS to be carried out at the lesion site. During replication on undamaged DNA, Pol δ forms a stable holoenzyme with proliferating cell nuclear antigen (PCNA), and carries out processive and error-free DNA replication (Garg and Burgers, 2005; Nasheuer et al., 2002, 2007). PCNA plays an important role in DNA replication by modifying the function of the replicative Pols δ and ε, as well as by interaction with a number of proteins involved in cell cycle progression and DNA repair. PCNA is loaded onto DNA by the clamp loader, replication factor C (RFC), forming a homotrimeric ring on the DNA strand. Following loading of PCNA, RFC remains bound to DNA through its interaction with the single-stranded DNA binding protein, replication protein A (RPA) (Bambara et al., 1997; Kelman and Hurwitz, 1998). When Pol δ encounters a damaged base in the template, and is unable to continue replication, a switch to a specialized TLS DNA polymerase such as Pol η is required to allow lesion bypass to occur. The mechanism of the switch between the replicative DNA polymerase and the TLS DNA polymerase at the damage site has been the subject of much investigation. In *E. coli*, the β clamp can bind to both the replicative DNA polymerase III and the TLS Pol IV at the same time, and this interaction is necessary for the polymerase switch (Indiani et al., 2005). In eukaryotic cells, post-translational modification of accessory proteins regulates the recruitment and activity of Pol η. In *S. cerevisiae,* the major modification is ubiquitination of the trimeric sliding clamp, PCNA on lysine 164 by the ubiquitin-conjugating enzyme complex, Rad6/Rad18 (Hoege et al., 2002). Rad6, an E2 ubiquitin-conjugating enzyme acts in concert with Rad18, an E3 ubiquitin ligase, to monoubiquitinate PCNA. Rad6/Rad18-dependent monoubiquitination of PCNA on lysine 164 promotes translesion DNA synthesis involving Pol η (Stelter and Ulrich, 2003; Haracska et al., 2004). Davies et al. (2008) reported that RPA interacts directly with Rad18 in both yeast and mammalian cells, indicating that RPA is also required for DNA damage-induced PCNA ubiquitination (Davies et al., 2008). Both yeast and human Pol η, as well as the other TLS DNA polymerases, Pol ι and Pol κ, interact with PCNA physically and functionally (Haracska et al., 2001a; Haracska et al., 2001b; Haracska et al., 2002). In human cells, Pol η is associated with replication forks during S phase, and forms nuclear foci following damage induced by UV or cisplatin, but not after induction of double-strand breaks by IR (Kannouche et al., 2001). Pol ι colocalises and interacts with Pol η in damage-induced nuclear foci. However, in Pol η-deficient XPV cells the percentage of cells with Pol ι foci is reduced suggesting that Pol η plays a role in the localization of Pol ι to foci (Kannouche et al., 2003).

Human DNA Pol η interacts with both unmodified and monoubiquitinated PCNA (Kannouche et al., 2004; Hoege et al., 2002). Pol η can bind to the interdomain connector loop of unmodified PCNA, through the PCNA-interacting peptide (PIP) located in the extreme C-terminal of Pol η. Pol η binds with greater affinity to monoubiquitinated PCNA (Kannouche et al., 2004). In *S. cerevisiae*, inactivation of the PIP domain of Pol η inhibits TLS (Haracska et al., 2001c). A second PIP domain, located just C-terminal to the polymerase-associated domain (PAD), has also been identified in human Pol η (Acharya et al., 2008). Mutational analysis, performed in XPV cells complemented with Pol η having mutations in the PIP domains, indicated

that both PIP domains are necessary for TLS (Acharya et al., 2008). Inactivation of one domain slightly impairs, but does not completely abolish, the function of Pol η. Mutation of both PIP domains eliminates the ability of Pol η to form nuclear foci, and to carry out TLS in vivo, and confers the same UV sensitivity as that seen in Pol η-deficient XPV cells (Acharya et al., 2008).

Human Pol η also contains a C_2H_2 ubiquitin-binding zinc finger (UBZ) domain, located between the catalytic domain of the DNA polymerase and the PIP domain (Bienko et al., 2005; Plosky et al., 2006). The solution structure of the UBZ domain of Pol η has been determined using nuclear magnetic resonance (Bomar et al., 2007). The UBZ domain consists of two short anti-parallel β-strands and a carboxy-terminal α-helix, with a zinc ion located between the α-helix and β-strands. The UBZ domain binds ubiquitin through its C-terminal α-helix, and together with the PIP domain, enhances the binding of Pol η to monoubiquitinated PCNA (Bomar et al., 2007). The role of this binding domain in the polymerase switch and in TLS is still under investigation. Bienko et al. (2005) demonstrated that expression of Pol η, carrying a C638A mutation in the UBZ domain, in XPV cells resulted in UV sensitivity, while a D652A mutant of Pol η failed to form foci in UV-treated cells (Bienko et al., 2005). An extensive mutational analysis of the UBZ domain of Pol η, in which five different residues in the domain were altered (Acharya et al., 2008) indicates that not all mutations in the UBZ affect the function of Pol η in TLS. The UBZ mutant proteins were able to form replication foci following UV-irradiation to the same extent in the presence of Ub-PCNA or unmodified PCNA (Acharya et al., 2008). This indicates that the binding of Pol η to PCNA only requires the PIP domains of Pol η, and that mono-ubiqiuitination of PCNA on lysine 164 is not strictly required for Pol η binding. The role of ubiquitination of PCNA may result from the fact that ubiquitin alters the conformation of PCNA, making it more accessible to the PIP domain of Pol η when replication is stalled at sites of damage (Acharya et al., 2008). Support for this hypothesis comes from a study in yeast, demonstrating that Pol η can only replace Pol δ at DNA replication forks in the presence of Ub-PCNA but not unmodified PCNA (Zhuang et al., 2008). The PIP domain of Pol η was required for this switch (Zhuang et al., 2008).

Deubiquitination of PCNA may cause dissociation of Pol η once TLS has taken place, and allow the recruitment of Pol δ and resumption of processive replication. PCNA is de-ubiquitinated by the de-ubiquinating enzyme USP1. The importance of USP1 in regulating the level of Ub-PCNA is supported by the observation that in UV-irradiated human cell lines, UPS1 undergoes autocleavage, allowing PCNA to be monoubiquitinated (Huang et al., 2006). It has also been shown that PCNA can be polyubiquitinated on lysine 164, as well being SUMOylated at two sites, K127 and K164 (Hoege et al., 2002). These modifications may offer an additional level of regulation of translesion synthesis by Pol η.

An important question with regard to the regulation of TLS is whether lesion bypass occurs directly at the arrested replication fork, or alternatively, whether the replication fork proceeds, leaving gaps in the nascent strand that are subsequently filled by TLS after fork passage. Whether this is related to different requirements for TLS on the leading and lagging strands (Yao et al., 2001) requires further

investigation. A recent study in chicken DT40 cells investigated the requirements for these two processes (Edmunds et al., 2008). PCNA mono-ubiquitiation was found be essential for filling of post-replicative gaps (Edmunds et al., 2008). In contrast, both the TLS DNA polymerase-interaction domain and the ubiquitin-binding domain of Rev1 were required to maintain fork progression at sites of damage (Edmunds et al., 2008). Rev1 interacts with a number of other Y family DNA polymerases, and may play a role in loading TLS DNA polymerases at the lesion site (Edmunds et al., 2008). Pol η itself is ubiquitinated (Bienko et al., 2005), which may also modulate its interaction with Rev1.

Activation of DNA Damage Responses in Pol η-Deficient Cells

It is now recognized that TLS is an integral component of the network of DNA damage responses in the cell. Both replication arrest which generates single-stranded DNA, and DNA strand break formation resulting from replication fork collapse, activate downstream DNA damage response (DDR) pathways mediated by the phosphoinositide 3-kinase (PI-3 K)-related protein kinases (PIKKs) (Cimprich and Cortez, 2008). The PIKK family consists of five serine-threonine kinases, including ataxia-telangiectasia mutated (ATM), ATM and RAD3-related (ATR), DNA-protein kinase catalytic subunit (DNA-PKcs), mammalian target of rapamycin (mTOR; also known as FRAP) and hSMG1 (Abraham, 2004; Bakkenist and Kastan, 2004; Branzei and Foiani, 2008; Cimprich and Cortez, 2008; Durocher and Jackson, 2001; Harper and Elledge, 2007). ATM, ATR, and DNA-PK, high molecular weight protein kinases with significant sequence homology, act as primary transducers of the DDR, by phosphorylation of a large number of downstream protein substrates to initiate signaling cascades that ultimately result in cell cycle arrest or in apoptosis (Abraham, 2004; Zhou and Elledge, 2000; Matsuoka et al., 2007). ATR is activated following replication fork arrest, while ATM and DNA-PK are activated by DNA strand breaks (Harper and Elledge, 2007).

Compared to normal cells, Pol η-deficient XPV cells show extended replication fork arrest after UV-irradiation, characterized by the formation of shorter nascent DNA strands, and the generation of extensive regions of single-stranded DNA (Cordeiro-Stone et al., 1999; Cordeiro-Stone et al., 1997). In addition, replication forks arrested at sites of UV-induced damage in Pol η-deficient XPV cells may collapse, generating DNA double-strand breaks (Limoli et al., 2002a) or more correctly, DNA double-strand ends (Shrivastav et al., 2008). XPV cells, but not normal cells, are hypersensitive to both UV radiation and cisplatin when grown in the presence of caffeine, a non-specific inhibitor ATM and ATR (Sarkaria et al., 1999), indicating that PIKK signaling may be altered in Pol η-deficient cells (Thakur et al., 2001; Arlett et al., 1975; Yamada et al., 2000; Yamada et al., 2003; Cleaver et al., 1999). The response of XPV cells to wortmannin, an inhibitor of DNA-PK and ATM (Sarkaria et al., 1998), is also altered (Limoli et al., 2002b).

To minimize the detrimental effects of DNA damage on genome stability, replication arrest at lesion sites activates S-phase checkpoints (Zhou and Elledge, 2000).

In Pol η-deficient XPV cells, extensive regions of single-stranded DNA (ssDNA) are generated as a result of fork arrest at sites of UV damage (Boyer et al., 1990; Cordeiro-Stone et al., 1997). S-phase progression is delayed in Pol η-deficient XP30RO cells following treatment with UV or cisplatin, and this effect can be reversed by expression of Pol η from an inducible promoter (Cruet-Hennequart et al., 2006; Cruet-Hennequart et al., 2008). ssDNA generated at stalled replication forks activates ATR in an ATR-interacting protein (ATRIP)- and RPA-dependent process (Binz et al., 2004; Zou and Elledge, 2003); for more details see "Function of TopBP1 in Genome Instability" by Miiko et al. and "Eukaryotic Single-Stranded DNA Binding Proteins and Genomic Stability" by Broderick et al., this book). RPA, the major ssDNA binding protein in eukaryotic cells, binds to ssDNA generated after replication stress (Binz et al., 2004). Following DNA damage the RPA2 subunit of the heterotrimeric RPA complex is phosphorylated on a number of N-terminal sites by PIKKs (Carty et al., 1994; Oakley et al., 2001; Oakley et al., 2003; Zernik-Kobak et al., 1997; Liu and Weaver, 1993; Olson et al., 2006; Patrick et al., 2005). ATR signaling is increased in UV- and cisplatin-treated Pol η-deficient human cells lines, as shown by increased phosphorylation of serine 33 in the RPA2 subunit of trimeric RPA, a known ATR phosphorylation site (Bomgarden et al., 2006; Cruet-Hennequart et al., 2006). ATR mediated phosphorylation of the cell cycle checkpoint kinase Chk1 is enhanced in Pol η-deficient cells (Bomgarden et al., 2006; Cruet-Hennequart et al., 2006). ATR-mediated phosphorylation of Chk1 leads to inhibition of both replication fork progression and the firing of new replication origins (Paulsen and Cimprich, 2007), and inhibition of cell cycle progression by phosphorylation and inactivation of the Cdk activator Cdc25A (Xiao et al., 2003).

In the absence of Pol η in XPV cell lines, replication arrest following UV radiation or cisplatin not only leads to activation of the ATR-mediated checkpoint, but also to the generation of DNA strand breaks and activation of ATM and DNA-PK, as evidenced by enhanced phosphorylation of key substrates including RPA2, H2AX and Nbs1 in Pol η-deficient cells (Cruet-Hennequart et al., 2006; Cruet-Hennequart et al., 2008). Using specific inhibitors of ATM and DNA-PK, UV- and cisplatin-induced phosphorylation of RPA2 on serines 4 and 8 in Pol η-deficient cells was found to be dependent on DNA-PK rather than ATM (Cruet-Hennequart et al., 2006, 2008). DNA-PK-dependent RPA2 hyperphosphorylation, by reducing the affinity of RPA for cisplatin-damaged DNA, and altering its interaction with key protein partners (Patrick et al., 2005; Wu et al., 2005), may be important in the processing of strand breaks generated by collapse of replication forks arrested for a prolonged period in the absence of Pol η (Cruet-Hennequart et al., 2006, 2008).

Extended replication arrest in Pol η-deficient cells following UV exposure leads to DNA strand breaks as measured by formation of γ-H2AX foci (Limoli et al., 2002a, 2000; Cruet-Hennequart et al., 2006). Homologous recombination is also activated in XPV cells after DNA damage (Limoli et al., 2005, 2000, 2002b). Induction of sister chromatid exchanges following UV irradiation is greatly enhanced in SV40-transformed XPV cells (Cleaver et al., 1999). The p53-status of Pol η-deficient cells may also influence the response to replication arrest (Cleaver

et al., 2002b; Limoli et al., 2002a, 2000, 2002b; Thakur et al., 2001; Limoli et al., 2005). Consistent with this, knockdown of Pol η using siRNA in cell lines with different p53 backgrounds showed that Pol η participates in p53 activation after camptothecin and IR-induced damage (Liu and Chen, 2006), while expression of p53 and the cell cycle inhibitor p21 regulates the extent of TLS in lung cancer cell lines (Avkin et al., 2006). The relationship between p53 status, Pol η expression and TLS in response to replication-blocking lesions requires further investigation.

Regulation of Pol η Expression

Given that Pol η is a low fidelity DNA polymerase during replication of undamaged DNA, but is required to allow TLS and replication fork progression at sites of DNA damage, the in vivo activity of Pol η needs to be tightly regulated. In general, *POLH* mRNA appears to be constitutively expressed in mammalian cells (Thakur et al., 2001). Yamada et al. (2000) showed that mouse *POLH* mRNA was not induced by UV irradiation, but increased at the onset of DNA synthesis, suggesting that expression of *POLH* is dependent on cell proliferation. *POLH* mRNA levels were elevated in more highly proliferating mouse tissues, including testis, thymus, liver and skin consistent with a requirement for Pol η in cells undergoing DNA replication (Yamada et al., 2000). Human *POLH* mRNA expression was detected in most tissues examined, except for very low or undetectable levels in peripheral lymphocytes, fetal spleen, and adult muscle (Thakur et al., 2001). An alternatively spliced form of the *POLH* transcript lacking exon 2, has also been identified, and comprises almost half of the *POLH* mRNA expressed in the testis and fetal liver (Thakur et al., 2001). The alternatively spliced form is also detectable in human skin tumors (Flanagan et al., 2007); however, the biological significance of the alternatively spliced form is not understood. Pol η protein is also expressed in skin tumor tissue (Flanagan et al., 2007). There is some evidence that human *POLH* gene expression is inducible following DNA damage. *POLH* mRNA expression was induced by cisplatin in three of five human non-small cell lung cancer lines examined, and the level of expression was associated with the sensitivity of the cell lines to cisplatin (Ceppi et al., 2009). A p53-binding site has been identified in the promoter of human *POLH*, and *POLH* mRNA expression can be up-regulated in a p53-dependent manner following ionizing radiation or camptothecin treatment (Liu and Chen, 2006).

In *S. cerevisiae*, the level of Pol η protein is regulated by proteolysis, through poly-ubiquitination of the protein and proteasomal degradation (Skoneczna et al., 2007). Following UV-irradiation, Pol η protein is stabilized and levels increase (Skoneczna et al., 2007). In *C. elegans* embryos, which are very resistant to DNA damage, Pol η protein is degraded following damage through CRL4-Cdt2-mediated proteolysis (Kim and Michael, 2008). This process may be important in removal of Pol η protein from sites of DNA damage once it has carried out TLS (Kim and Michael, 2008). While human Pol η is ubiquitinated (Bienko et al., 2005), the role

of regulated proteolysis in controlling the level of Pol η protein in human cells remains to be determined. Phosphorylation of human Pol η in an ATR- and protein kinase C (PKC)-dependent manner may also play a role in regulating Pol η activity in human cells (Chen et al., 2008). Clearly, further research is required in order to better understand how Pol η protein abundance and activity is regulated both in unstressed human cells and following DNA damage.

Overexpression of specialized DNA polymerases is a feature of many cancers (Albertella et al., 2005b). Given the role of Pol η in TLS at sites of DNA damage, and the skin cancer susceptibility of XPV patients lacking functional Pol η, the sequence of the coding exons of the *POLH* gene, and expression of *POLH* mRNA has been characterized in a series of normal skin samples and skin tumor tissues (Flanagan et al., 2007; Glick et al., 2006). No sequence changes specifically associated with skin tumors were detected in *POLH* DNA in any of the samples analyzed (Flanagan et al., 2007; Glick et al., 2006). However, individual tumors varied in the level of *POLH* mRNA expression when compared to the paired normal skin tissue, as determined by real-time PCR analysis, suggesting that differences in gene expression, rather than sequence changes may be the main mechanism by which *POLH* status varies between normal and skin tumors (Flanagan et al., 2007; Glick et al., 2006). *POLH* expression was found to be significantly down-regulated in human lung, and stomach cancers, but not in colorectal cancers, compared to the paired normal tissue (Pan et al., 2005). In contrast, a more recent study using real-time PCR found that *POLH* expression is down-regulated in a cohort of colorectal cancers compared to paired normal adjacent tissues (Bétous et al., 2009). A recent analysis of *POLH* mRNA levels in a series of tissues from normal individuals and from non-small cell lung cancer (NSCLC) patients, found that *POLH* expression did not differ significantly between the normal tissue and lung tumor samples (Ceppi et al., 2009). However, *POLH* expression in advanced NSCLC patients treated with platinum-based chemotherapy was found to be an independent factor associated with survival, with high *POLH* expression levels strongly associated with shorter survival (Ceppi et al., 2009). Thus, it may be of interest to further characterise the relationship between *POLH* gene expression, Pol η protein levels and the response of other tumors to platinum-based DNA damage, to determine whether higher Pol η expression is associated with increased tolerance of DNA damage induced by chemotherapeutic agents.

Concluding Remarks

DNA polymerase η plays a key role in translesion synthesis at UV-induced DNA damage. Since the original characterization of the protein in human cells, and the identification of *POLH* gene mutations as the cause of the skin cancer-prone disease xeroderma pigmentosum variant, considerable progress has been made in elucidating the structure and function of Pol η. The demonstration that Pol η plays a role in bypass of platinum-induced DNA adducts has increased interest in the role of the

protein in DNA damage tolerance in tumor cells. Further research is required into the regulation of Pol η expression, the role of Pol η in normal cells, for example in DNA replication at altered DNA structures in the genome, and its role in recombination. Further insights into the coordination of multiple TLS DNA polymerases and accessory proteins during TLS on the leading and lagging strand, and the integration of TLS with the DDR in human cells, are also required. Finally, inhibition of TLS mediated by Pol η or other Y-family DNA polymerases might provide a mechanism to potentiate the effects of certain DNA damaging agents used in cancer treatment.

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The Mitochondrial DNA Polymerase in Health and Disease

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Abstract Since mutations in mitochondrial DNA (mtDNA) have been shown to be a cause of many mitochondrial diseases as well as aging, it is important to understand the origin of these mutations and how replication proteins modulate this process. DNA polymerase γ (pol γ) is the polymerase that is responsible for replication and repair of mtDNA. Pol γ has three main roles in mtDNA maintenance and mutagenesis. As the only known DNA polymerase in mitochondria, pol γ is required for all replication and repair functions and is the main source of errors produced in human mtDNA. Pol γ is also sensitive to a host of antiviral nucleoside analogs used to treat HIV-1 infections, which can cause an induced mitochondrial toxicity. Finally, the gene for pol γ, *POLG*, is a genetic locus for several mitochondrial disease with over 150 genetic mutations currently identified.

Keywords DNA polymerase γ · Mitochondrial disease · Mitochondrial DNA replication · Mitochondrial DNA repair · Nucleoside analogs

Introduction

Mutations accumulate in mtDNA with age, and mutation of mtDNA has been shown to promote premature aging in mice (Dimauro and Davidzon, 2005). Point mutations, deletions and depletion of mtDNA have been observed in many human diseases (Wallace, 1999). Therefore, it is essential to understand the origins of mutations in human mtDNA. Mitochondrial DNA is replicated and repaired by DNA polymerase γ (Pol γ). Pol γ, encoded by the *POLG* gene, is the only DNA polymerase found in animal cell mitochondria and is involved in replication, mutagenesis, and repair of mtDNA as well is a target of antiviral nucleoside analogs

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that cause mitochondrial toxicity. DNA polymerase gamma has three main roles in health and disease:

1. Spontaneous mutagenesis – as the only DNA polymerase involved in mtDNA replication and repair, the origin of most spontaneous mutation is due to errors produced by pol γ.
2. NRTI induced mitochondrial toxicity – Pol γ is the only replicative DNA polymerase sensitive to a host of nucleoside analogs used to treat HIV infection and as a consequence patients being treated with antiviral therapies such as AZT, ddNs, D4T, 3TC and others, may develop an induced mitochondrial toxicity.
3. Mutations in the gene for Pol γ – The *POLG* gene is a locus for several mitochondrial diseases and more than 150 disease-associated mutations have been identified in the *POLG* gene from patients with mitochondrial disease.

The relevance of pol γ in each of these the health topics is discussed in this chapter.

Pol γ in mtDNA Replication

Of the 16 DNA polymerases in the eukaryotic cell, only pol γ is known to function in the mitochondria (Bebenek and Kunkel, 2004; Ropp and Copeland, 1996; Sweasy et al., 2006). Thus, pol γ is absolutely essential for mtDNA replication and repair. The holoenzyme of pol γ consists of a catalytic subunit (encoded by *POLG* at chromosomal locus 15q25) and a dimeric form of its accessory subunit (encoded by *POLG2* at chromosomal locus 17q24.1). The catalytic subunit is a 140 kDa enzyme (p140) that has DNA polymerase, 3′–5′ exonuclease and 5′ dRP lyase activities (Graziewicz et al., 2006). The accessory subunit is a 55 kDa protein (p55) required for tight DNA binding and processive DNA synthesis (Lim et al., 1999). The pol γ holoenzyme functions in conjunction with the mitochondrial DNA helicase, Twinkle, and the mtSSB to form the minimal replication apparatus in mitochondria (Korhonen et al., 2004) (Table 1). Other factors required for initiation of mtDNA replication and repair are listed in Table 1.

Mutations in mitochondrial DNA can arise from DNA damage from exogenous sources or from endogenous oxidative stress, which are believed to arise mostly from electron leakage in the electron transport chain during oxidative phosphorylation. Mutations can also arise as spontaneous errors of DNA synthesis during either DNA replication or repair events. As the only DNA polymerase known to exist in mammalian mitochondrial, pol γ is likely to produce these spontaneous errors. Comparison of mutation spectrum from in vivo sources with in vitro copied DNA by the highly purified human pol γ reveals that over 85% of mutation detected in vivo could be recapitulated in vitro by pol γ (Zheng et al., 2006). This indicates that spontaneous errors by pol γ account for the majority of base pair substitution mutations. Thus, understanding the fidelity of pol γ is critical.

Table 1 Gene products required for mtDNA replication and repair

Function	Gene	Protein	Size	Chromosome
Core replication	POLG	DNA polymerase γ	140 kDa	15q25
	POLG2	DNA polymerase γ accessory subunit	55 kDa	17q23-24
	SSB	Single stranded DNA binding protein	15 kDa	7q34
	PEO1 (Twinkle)	Helicase	77 kDa	10q24
Replication and repair accessory factors	DNA ligase III	Ligase	96 kDa	17q11.2-12
	RNase H1	RNA-DNA hybrid-specific RNase	32 kDa	19p13.2
	Topo I	Topoisomerase I	67 kDa	8q24.3
	Topo IIIα	Topoisomerase IIIα	112 kDa	17p12-11.2
	Fen-1	5′-3′ Flap endonuclease	43 kDa	11q12
	DNA2	5′-3′ DNA/RNA endonuclease/exonuclease	130 kDa	10q21.3-q22.1
	ExoG	5′-3′ exonuclease	41 kDa	3p21.3
DNA repair proteins	*UDG*	Uracil DNA glycosylase	27.5 kDa	12q23-q24.1
	OGG1	8-oxo-dG glycosylase	38 kDa	3p26.2
	NTH1	Thymine glycol glycosylase	34 kDa	16p13.3
	MUTYH	glycosylase	60 kDa	1p34.3-p32.1
	NEIL1	Fapy[a] glycosylase	44 kDa	15q4.2
	APE1	Ap[a] endonuclease	35 kDa	14q11.2-q12
	APE2	Ap endonuclease	57 kDa	Xp11.22
Transcription	mtRNA Pol	Core mitochondrial RNA polymerase	150 kDa	19q13.3
	mtTFA	Transcription factor	24 kDa	10q21
	mtTFB1	Transcription factor	39 kDa	6q25.1-q25.3
	mtTFB2	Transcription factor	45 kDa	1q44

[a] Ap – apurinic, apyrimidinic; Fapy – 2,6-diamino-4-hydroxy-formamido-pyrimidine.

The human catalytic subunit of pol γ has high base substitution fidelity that results from high nucleotide selectivity and exonucleolytic proofreading (Longley et al., 2001). Pol γ is also relatively accurate for base incorporation in non-iterated and short repetitive sequences where a misinsertion event occurs, on average, only once per 500,000 nucleotides synthesized (Longley et al., 2001). However, when copying homopolymeric sequences longer than four nucleotides, pol γ has lower frameshift fidelity, suggesting that homopolymeric runs in mtDNA may be particularly prone to frameshift mutation in vivo due to replication errors by pol γ. Inclusion of the p55 accessory subunit, which is important for processivity of pol γ, decreases frameshift and base substitution fidelity. Kinetic analyses indicate that

p55 lowers fidelity of replication by promoting extension of mismatched termini (Longley et al., 2001). Pol γ contains an intrinsic 3′–5′ exonuclease activity that contributes to replication fidelity. In human pol γ, substitution of Asp198 and Glu200 with alanine in the ExoI motif eliminated detectable 3′–5′ exonuclease function in vitro (Longley et al., 1998b). Comparing the in vitro rates of base substitution errors for the exonuclease-deficient and proficient forms of human pol γ indicated that the proofreading function contributes at least 20-fold to the fidelity of base selection (Longley et al., 2001).

Pol γ in Mitochondrial DNA Repair

DNA repair in mitochondrial is limited to base excision repair, which is well suited with a host of glycosylases (Table 1) to recognize base damage, as would occur during oxidative stress. Mitochondrial base excision repair can proceed via two pathways, single-nucleotide-BER (SN-BER) or long-patch BER (LP-BER) (Copeland and Longley, 2008). With either repair pathway, an oxidized or damaged base is recognized and cleaved by a specific glycosylase, leaving an abasic site that is cleaved on the 5′ end by AP endonuclease to generated a nick with a 5′deoxyribose phosphate (dRP) flap. During single nucleotide BER, the mitochondrial DNA polymerase, pol γ, fills the gap and cleaves the 5′dRP moiety prior to ligation (Longley et al., 1998a).

LP-BER activity in mitochondrial extracts and identification of proteins required for LP-BER in mitochondria has recently been described (Akbari et al., 2008; Liu et al., 2008; Szczesny et al., 2008). LP-BER requires an activity to remove the displaced 5′DNA commonly known as a 5′-flap structure and Liu et al found FEN-1 in their mitochondrial preparations that could carry out this activity (Liu et al., 2008). Furthermore, DNA2, originally identified as a yeast nuclear DNA helicase with endonuclease activity, has also been implicated in mitochondrial LP-BER, as well as having a possible role in mtDNA replication (Zheng et al., 2008). In this capacity, DNA2 functions with FEN-1 to process 5′ protruding flaps due to strand displacement synthesis during LP-BER prior to ligation by ligase III.

Mitochondrial Toxicity from Antiviral Inhibition of Pol γ

Nucleoside reverse transcriptase inhibitor (NRTI) therapy in HIV infected patients has been beneficial in extending life and slowing the progression of AIDS, but treatment with nucleoside analogs is accompanied by certain side effects. The most pronounced side effects from NRTI therapy are damage to the mitochondria and loss of mitochondrial function. Mitochondrial myopathies in patients on AZT (3′-azido-3′-deoxythmidine) therapy was first reported in 1990 (Dalakas et al., 1990). These patients had induced myopathies with ragged red fibers and reduced amounts of mitochondrial DNA (Arnaudo et al., 1991). Early investigations into

the observed mitochondrial toxicity implicated pol γ in the process of toxicity. Pol γ is unique among the cellular replicative DNA polymerases in that it is highly sensitive to inhibition by anti-HIV nucleotide analogs such as AZT-TP, dideoxynucleotides, and other antiviral nucleotide analogs (Kaguni et al. 1988; Longley and Mosbaugh, 1991; Martin et al., 1994; Hart et al., 1992; Parker et al., 1991; Lewis et al., 1994; Copeland et al., 1992; Eriksson et al., 1995; Nickel et al., 1992; Huang et al., 1990, 1992; Lim and Copeland, 2001; Johnson et al., 2001). The general inhibitory effect of NRTIs on polymerases is: HIV-RT >> pol γ > pol β > pol α = pol ϵ (Kakuda, 2000). Mitochondrial toxicity may be caused by (1) direct inhibition of pol γ activity without incorporation; (2) termination of the growing nascent DNA strand by incorporation of these chain-terminating analogs into mitochondrial DNA; (3) alteration of the fidelity of DNA synthesis of pol γ; (4) the persistence of these analogs in mtDNA due to inefficient excision; or (5) a combination of any of these effects. Kinetic studies indicate that the apparent in vitro hierarchy of mitochondrial toxicity for the approved NRTIs is: ddC (dideoxycitidine, zalcitabine) ≥ ddI (dideoxyinosine, didanosine) ≥ D4T (2′,3′-didehydro-2′,3′-dideoxythymidine, stavudine) > > 3TC (2′,3′- dideoxy-3′-thiacytidine, lamivudine) > PMPA (9-(R)-2-(phosphonomethoxypropyl)adenine, tenofovir) > AZT (zidovudine) > CBV(guanine analog, abacavir) (Lim and Copeland, 2001; Johnson et al., 2001). During in vitro chain elongation by pol γ, dideoxynucleotides and D4T-TP are utilized nearly as efficiently as natural deoxynucleotides, whereas AZT-TP, 3TC-TP, PMPA and CBV-TP are only moderate inhibitors of DNA chain elongation (Lim and Copeland, 2001; Johnson et al., 2001). Once incorporated the DNA polymerase may remove the terminal NRTI with its intrinsic exonuclease activity. However, we previously found that pol γ is inefficient in removing terminally incorporated dideoxynucleotides, D4T, AZT, and CBV from DNA (Lim and Copeland, 2001). This finding predicts persistence of these analogs in vivo following successful incorporation. In contrast, removal of 3′-terminal 3TC residues is 50% as efficient as natural 3′-termini, predicting reduced persistence and lower toxicity for this analog. In addition to the triphosphate form of these analogs, metabolic intermediates have the potential to inhibit pol γ or other cellular targets. The cellular conversion of AZT to AZT-TP has been shown to accumulate the monophosphate intermediate in vivo at millimolar concentration (Furman et al., 1986; Frick et al., 1988). We have shown that the pol γ exonuclease activity is inhibited by AZT-monophosphate at concentrations known to occur in cells (Lim and Copeland, 2001). Thus, although their greatest inhibitory effects are through incorporation and chain termination, persistence of these analogs in DNA and inhibition of exonucleolytic proofreading are also likely to contribute to mitochondrial toxicity. 3TC-TP is one of the analogs least likely to be incorporated and yet is one of those most efficiently removed. This may explain the low mitochondrial toxicity induced by 3TC in vivo. Although AZT-TP is one of the analogs least likely to be incorporated into DNA by pol γ, once incorporated it is not efficiently removed from DNA by the pol γ exonuclease function. The inefficiency of pol γ to remove AZT from DNA may help to explain some of the AZT-induced mtDNA depletion observed in vivo.

Based on sequence alignment of the bacterial DNA polymerases within family A, mutagenesis studies, and available three-dimensional structures, three amino acids, Tyr951, Tyr955 and Glu895, in human pol γ were studied for their role in NRTI selection (Lim et al., 2003). The function of these three residues accounts for the majority of the selection of incoming dNTPs. The cause of dideoxynucleoside and D4T sensitivity is mainly attributed to a single tyrosine in motif B, Tyr951, of human pol γ (Longley et al., 1998b; Lim et al., 2003). Substitution of this tyrosine residue with phenylalanine in the human enzyme reduces inhibition by dideoxynucleotide or D4T-TP by several thousand fold with only minor effects on overall polymerase function (Longley et al., 1998b; Lim et al., 2003).

Disease Mutations in the *POLG* Gene

The *POLG* gene is one of several nuclear genes that is associated with mitochondrial DNA depletion or deletion disorders (Table 2). To date, more than 150 disease mutations have been identified in the *POLG* gene and an up-to-date mutation database can be found at http://tools.niehs.nih.gov/polg/, which shows these mutations to be equally distributed over the length of the protein (Fig. 1). Disorders associated with *POLG* mutations have been defined to include the following, (1) Myocerebrohepatopathy spectrum disorder (MCHS), (2) Alpers syndrome, (3) Ataxia neuropathy spectrum disorder (ANS), (4) Myoclonus epilepsy myopathy

Table 2 Nuclear loci that affect the stability of mitochondrial DNA

Gene	Disorder	Chromosome locus	Function
POLG	PEO[a]/Alpers/ataxia	15q25	Mitochondrial DNA polymerase
POLG2	PEO	17q23-24	Pol γ accessory subunit
PEO1 (Twinkle)	PEO/ataxia	10q24	Mitochondrial DNA helicase
ANT1	PEO	4q34-35	Adenine nucleotide translocator
TP	MNGIE[a]	22q13.32	Thymidine phosphorylase
DGUOK	mtDNA depletion	2p13	Deoxyguanosine kinase
TK2	mtDNA depletion	16q22	Mitochondrial thymidine kinase
MPV17	mtDNA depletion	2p21-23	Mitochondrial inner membrane protein
SUCLA2	mtDNA depletion	13q12.2-q13.3	Succinate-CoA ligase
RRM2B	mtDNA depletion	8q23.1	P53-Ribonucleotide reductase, small subunit
OPA1	Dominant optic atrophy	3q28-q29	Dynamin-related GTPase

[a] MNGIE – Myoneurogenic gastrointestinal encephalopathy; PEO – Progressive external ophthalmoplegia.

sensory ataxia (MEMSA), (5) Autosomal recessive progressive external ophthalmoplegia (arPEO), and (6) Autosomal dominant progressive ophthalmoplegia (adPEO) (Wong et al., 2008) (Fig. 1). Also, alteration of the $(CAG)_{10}$ repeat in the 2nd exon of *POLG* has been implicated in male infertility.

MCHS includes myopathy or hypotonia, developmental delay, or dementia, and liver dysfunction (Wong et al., 2008). In addition, patients can have either a liver biopsy that excluded classical Alpers hepatopathy (Nguyen et al., 2006), or at least two of the following 8 findings: (1) neuropathy, (2) seizures, (3) elevated blood or cerebrospinal fluid lactic acid, (4) dicarboxylic aciduria, (5) renal tubular dysfunction with aminoaciduria, glucosuria, or bicarbonaturia, (6) hearing loss, (7) abnormal MRI with either cerebral volume loss, delayed myelination, or white matter disease, and (8) deficiency of either CIV (cytochrome c oxidase, COX) in isolation, or 2 or more electron transport complexes in skeletal muscle or liver biopsy (Wong et al., 2008). In some cases, patients came to diagnosis without, or before, the onset of liver dysfunction. In these cases, at least 3 of the 8 supportive diagnostic findings were required. Patients with *POLG* mutations meeting the diagnostic features for MCHS were first described in Ferrari et al. (2005), and de Vries et al. (2007).

Alpers syndrome or hepatocerebral degeneration is defined by the clinical triad of refractory, mixed-type seizures that often included a focal component, psychomotor regression that is episodic and triggered by intercurrent infection, and hepatopathy with or without acute liver failure (Wong et al., 2008). Although mtDNA deletion usually occurs in the very advanced stages in affected organs, mtDNA depletion analysis is not always a good predictor of the diagnosis or progression of the disease (Nguyen et al., 2006).

Ataxia Neuropathy Spectrum (ANS) includes an overlapping clinical spectrum of disorders centered around ataxia and neuropathy in the absence of significant muscle weakness or myopathy. ANS includes mitochondrial recessive ataxia syndrome (MIRAS) (Hakonen et al., 2005), spinocerebellar ataxia and epilepsy (SCAE), and the ataxia neuropathy spectrum (Tzoulis et al., 2006). Myoclonus Epilepsy Myopathy Sensory Ataxia (MEMSA) is an overlapping spectrum of disorders of myopathy, epilepsy, and ataxia in the absence of ophthalmoplegia with or without ragged red fibers (van Goethem et al., 2003b). ArPEO is the recessive form of progressive external ophthalmoplegia (van Goethem et al., 2001). Most patients have additional symptoms that include sensory ataxia, neuropathy, dysarthria, and ophthalmoplegia (SANDO) (van Goethem et al., 2003a). AdPEO is the dominant inherited form of progressive external ophthalmoplegia that can include parkinsonism (van Goethem et al., 2001).

The A467T mutation is the most common *POLG* mutation and has been found to be associated with all of the disease symptoms mentioned above. Previous studies have shown that the A467T pol γ possesses only 4% of the wild-type DNA polymerase activity and is compromised for its ability to interact with the p55 accessory subunit (Chan et al., 2005). The W748S mutation which has nearly always been found to be *in cis* with E1143G mutation is a frequent cause of ataxia-neuropathy syndrome (Hakonen et al., 2005). The E1143G is a single nucleotide polymorphism

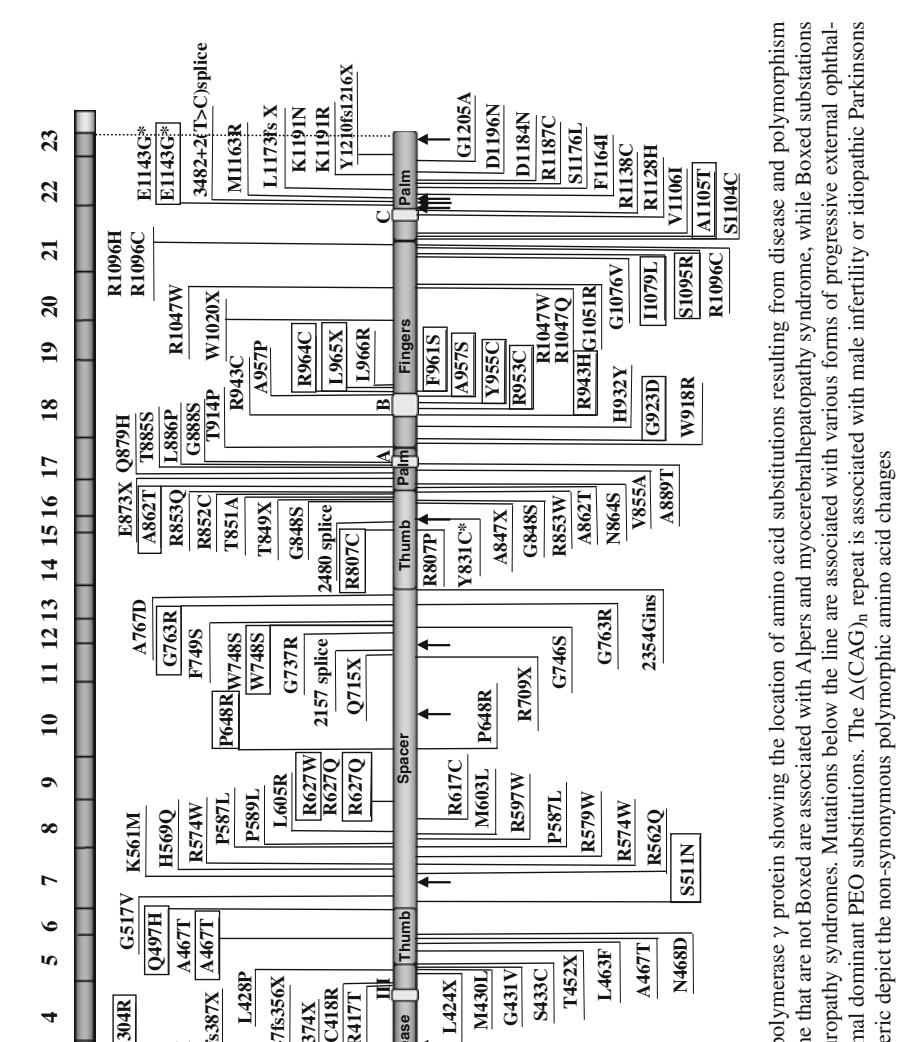

Fig. 1 Schematic diagram of human DNA polymerase γ protein showing the location of amino acid substitutions resulting from disease and polymorphism mutations. Disease substitutions above the line that are not Boxed are associated with Alpers and myocerebralhepatopathy syndrome, while Boxed substations above the line are associated with ataxia-neuropathy syndromes. Mutations below the line are associated with various forms of progressive external ophthalmoplegia where Boxed mutations are autosomal dominant PEO substitutions. The $\Delta(CAG)_n$ repeat is associated with male infertility or idiopathic Parkinsons disease. *Arrows* and substitutions with an asteric depict the non-synonymous polymorphic amino acid changes

(SNP), which is found in 4% of European populations. The W748S mutations has intrinsic lower polymerase activity as well as a demonstrated lower affinity for DNA (Chan et al., 2006). We have found that the E1143G SNP can modulate the deleterious effect of the W748S mutation (Chan et al., 2006). This finding raises the possibility that other SNPs could potentially affect *POLG* enzymatic activity.

Four adPEO mutations, G923D, R943H, Y955C and A957S that are found in and around motif B in the active site of the DNA polymerase were characterized biochemically (Graziewicz et al., 2004). Two of the substitutions, R943H and Y955C, change side chains that interact with the incoming dNTP and pol γ with these substitutions retained less than 1% of the wild-type polymerase activity and display a severe decrease in processivity. The significant stalling of DNA synthesis and extremely low catalytic activities of both mutant enzymes are the two most likely causes of the severe clinical presentation in R943H and Y955C heterozygotes (Graziewicz et al., 2004). The substitution of Tyr955 to cysteine also increases nucleotide misinsertion replication errors 10–100 fold in the absence of exonucleolytic proofreading (Ponamarev et al., 2002). For the majority of the disease substitutions that have been studied in vitro, the biochemical defects correlate with the severity and age of onset found in patients (Chan and Copeland, 2009). Further analysis of disease substitutions as well as structural analysis should aid in the continued understanding of disease mutations in the *POLG* gene.

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Centromeres: Assembling and Propagating Epigenetic Function

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Abstract The faithful replication of DNA and the accurate segregation of genomic material from one generation to the next is critical in the maintenance of genomic stability. This chapter will describe the structure and assembly of an epigenetically inherited locus, the centromere, and its role in the processes by which sister chromatids are evenly segregated to daughter cells. During the G2 phase of the cell cycle kinetochores are assembled upon the chromatids. During mitosis, kinetochores attach chromosome(s) to the mitotic spindle. The kinetochore structure serves as the interface between the mitotic spindle and the chromatids and it is at the kinetochore where the forces that drive chromatid separation are generated. Unattached chromosomes fail to satisfy the spindle assembly checkpoint (SAC), resulting in cell cycle arrest. The centromere is the locus upon which the kinetochore assembles, and centromeres themselves are determined by their unique protein composition. Apart from budding yeast, centromeres are not specified simply by DNA sequence, but rather through chromatin composition and architecture and are thus epigenetically determined. Centromeres are built on a specific nucleosome not found elsewhere in the genome, in which histone H3 is replaced with a homologue – CENP-A or CenH3. This domain is flanked by heterochromatin and is folded to provide a 3-dimensional cylinder-like structure at metaphase that establishes the kinetochore on the surface of the mitotic chromosomes. A large family of CENtromere Proteins (CENPs) associates with centromeric chromatin throughout the cell cycle and are required for kinetochore function. Unlike the bulk of histones, CENP-A is not assembled concurrently with DNA synthesis in S-phase but rather assembles into the centromere in the subsequent G1 phase. The assembly of CENP-A chromatin following DNA replication and the re-establishment of this network of constitutive proteins have emerged as critical mechanisms for understanding how the centromere is replicated during the cell cycle.

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Abbreviations

APC/C	Anaphase Promoting Complex-Cyclosome
BUB	Budding Uninhibited by Benzimidazole
CAD	CENP-A Distal Complex
CAF1	Chromatin Assembly Factor
CATD	CENP-A Targeting Domain
CCAN	Constitutive Centromere-Associated Network
CENP	Centromere Protein
HAT1	Histone AcetylTransferase catalytic subunit
HFD	Histone Fold Domain
hFLEG1	human Fetal Liver Expressing Gene 1
Hir1	HIRA like histone chaperone
KMN	Knl1-Mis12-Ndc80 complex
MAD	Mitotic-Arrested Deficient
MCC	Mitotic Checkpoint Complex
MPS1	Multipolar Spindle 1
NAC	Nucleosome Associated Complex
PEV	Position Effect Variegation
PRC2	Polycomb Repressive Complex 2
RZZ	ROD-ZW10-Zwilch Complex
SAC	Spindle Assembly Checkpoint

Introduction

The centromere is a chromosomal locus present in a single copy per chromosome. It is essential for the stable inheritance of the genome but it is itself epigenetically inherited. The functional identity of the centromere derives from interplay of specific and global chromatin components that provide unique centromere function and also specify a mechanism for replicating centromeres in the cell cycle. This is accomplished through the assembly and maintenance of a core set of constitutive centromere-associated proteins that are anchored to the DNA fiber through a specialized nucleosome that contains a centromere-specific histone H3 homologue, CENP-A or CenH3.

For higher organisms the centromere displays properties of an epigenetic locus with the information stably passed from one cell cycle to the next without being directly encoded or templated by the underlying DNA sequence. The essence of centromere identity and function is the specialized chromatin that forms it. The details of many of the issues in centromere biology have been recently summarized in several excellent reviews (Cleveland et al., 2003; Allshire and Karpen, 2008; Cheeseman and Desai, 2008b; Musacchio and Salmon, 2007b; Henikoff and Ahmad, 2005). In this chapter, we aim to provide an overview of the factors that

specify centromeres as genomic loci, the specific biochemistry of the specialized chromatin that comprises it and a summary of the functions of the kinetochore, the mitotic product of centromere function, in chromosome movement and cell cycle control.

Specifying the Centromere

Centromeric DNA

Centromere DNA organization varies widely among eukaryotes (see Cleveland et al. 2003 for review). The simplest centromere is found in the budding yeast *S. cerevisiae,* a so-called point centromere built upon a small 125 bases of centromeric DNA (Fitzgerald-Hayes et al., 1982). A diffuse centromere has been found to exist in nematodes like *C. elegans,* where the kinetochore forms along the entire length of the mitotic chromosome. Such chromosomes are said to be *holocentric*. More typical is the regional centromere, in which the centromere is associated with a discrete DNA locus. The best studied example of this type of centromere are those of *S. pombe* where a central core that specifies centromere function is flanked by long arrays of repetitive DNA (Clarke, 1998). In higher eukaryotes regional centromeres form on long stretches of repetitive DNA sequences that span several hundreds to thousands of kilobase pairs (Gieni et al., 2008). Centromeres in humans are comprised of arrays of repetitive satellite DNA that is characteristic of multicellular organisms. The primate centromeric repeat is termed alpha satellite DNA (or alphoid DNA) and consists of a basic repeating unit of 171 bp. Individual alpha satellite repeats or monomers show variation around a consensus sequence of up to 60% across the entire genome. Higher order repeats of 2–16 alpha satellite monomers are found in chromosome specific families and these higher order repeats are arranged in tandem to form arrays ranging from ~200–5,000 kb (Schueler et al., 2001). While such organization is typical of both plant and animal centromeres, the sequences that make up centromeric DNA are rapidly evolving and diverge extensively even among closely related species (Henikoff et al., 2001). Indeed, centromere DNA in *Drosophila* is organized as islands of transposable elements in simple satellite DNA with no specific sequences detectable as common to all centromeres of the karyotype (Sun et al., 1997).

Does the centromeric DNA sequence specify centromere function? In the budding yeast, centromere function is dependent on specific DNA binding proteins such that single point mutations can abolish centromere activity (Bouck and Bloom, 2005). Only one conserved DNA binding sequence has been identified in centromeres of higher organisms, a small 17 base pair binding site for the sequence specific DNA binding protein, CENP-B (Masumoto et al., 2004). Mutations of CENP-B Boxes caused reduced efficiency of artificial chromosomes to form on alphoid DNA and those de novo events were shown to be dependent on CENP-B

(Ohzeki et al., 2002; Okada et al., 2007). However, CENP-B is nonessential, demonstrating that it does not play a role in the maintenance or normal replication of centromeres (Hudson et al., 1998). Rather, the model that has emerged for centromere formation in higher cells has focused on epigenetic determinants as discussed below.

Evidence for Epigenetic Behavior at the Centromere

Strong evidence for epigenetic inheritance at the centromere comes from the fact that the centromere displays various epigenetic behaviors. Position effect variegation (PEV) is an effect observed when a gene or reporter is positioned next to heterochromatin which leads to gene expression states being stochastically inherited (Ekwall, 2007). In fission yeast there is striking evidence of PEV in the form of centromeric silencing of nearby genes. Swi6 was one of the first centromeric proteins identified in the fission yeast and when mutations of this were introduced it had an effect on centromeric silencing (Ekwall et al., 1995). Extreme cases of silencing of a centromere locus itself, observed in human cells, demonstrate that centromeric DNA is not sufficient to specify centromere formation. Certain derivatives of Robertsonian translocation (a type of translocation in which the long arms of non-homologous arcocentric chromosomes fuse at the centromeres) in humans maintain two loci of centromeric DNA (known as a dicentric chromosome) but exhibit stable segregation at mitosis (Earnshaw and Migeon, 1985). In some of these cases, the centromeres are close enough to functionally cooperate (Sullivan and Willard, 1998). However, in numerous examples, one of the sites will be inactivated, allowing chromatid separation to occur normally (Sullivan and Schwartz, 1995; Sullivan and Willard, 1998). A complementary example demonstrates that centromeric DNA is not necessary to form centromeres on human chromosomes – the formation of neocentromeres (Marshall et al., 2008). This involves the formation of a new centromere on non-centromeric DNA. These centromeres have been observed in both humans and plants. Studies of neocentromeres have found that these loci possess full complements of functional centromeric proteins (Saffery et al., 2000; Warburton, 2004). Repositioning of centromeres is another epigenetic phenomenon which is found at the centromere. This involves two epigenetic mechanisms, the first being inactivation of the original centromere and the formation of a neocentromere. Finally the inhibitor of histone deacetylase HDAC Trichostatin A (TSA) has been used to demonstrated epigenetic effects at centromeres in *S. pombe* (Ekwall et al., 1997). These experiments document epigenetic influences on the centromere which are mediated by histone modification. Human cell studies with TSA have pointed to a role for histone acetylation in the formation of neocentromeres (Nakano et al., 2003). Taken together, these observations argue that centromeric DNA itself is not the dominant determinant of centromere function but, rather, epigenetic mechanisms determine the functional identity of the locus.

Chromatin and Centromere Determination

While the DNA sequences of eukaryotic centromeres vary widely, one common feature found in all is a distinctive, centromere-specific nucleosome based on a histone H3 variant known as CENP-A, or CenH3 (Earnshaw and Rothfield, 1985; Meluh et al., 1998; Palmer et al., 1991; Sullivan et al., 2001; Henikoff et al., 2000; Oegema et al., 2001). This essential centromere component lies at the foundation of a distinctive chromatin structure that is thought to underlie the functional properties and the epigenetic determination of the centromere.

CENP-A, The Centromere Specific Histone

CENP-A was originally identified from auto antisera derived from patients with limited systemic sclerosis – CREST syndrome (Tan et al., 1980). These sera recognized three centromere specific antigens: CENP-A, -B and -C (Earnshaw and Rothfield, 1985). CENP-B and CENP-C were cloned using autoantibodies and shown to be proteins that associate with alpha-satellite DNA and the inner kinetochore plate respectively (Earnshaw, 1987; Saitoh et al., 1992). CENP-A, however, was isolated from nucleosomal preparations following digestion with micrococcal nuclease (Palmer and Margolis, 1985), and was shown to co-purify with nucleosomal core particles (Palmer, 1987). Partial amino acid sequence of bovine CENP-A suggested homology to histone H3 (Palmer et al., 1991) which was confirmed by direct isolation of the cDNA for CENP-A (Sullivan, 1994) and generalized as a common feature of eukaryotes by identification of Cse4 as the yeast homologue of the centromere protein (Stoler et al., 1995).

Human CENP-A contains a histone H3-related histone fold domain (HFD) that is required for targeting CENP-A to the centromere (Sullivan, 1994). In histone H3, this HFD forms an interface with histone H4 to assemble the canonical heterotetrameric core of nucleosome structure. The HFD, contact with DNA and homodimerization are equally necessary for correct CENP-A localization (Keith et al., 1999; Shelby et al., 1997; Vermaak et al., 2002). A region of the CENP-A HFD was found to confer centromeric targeting to histone H3, and was termed the CENP-A targeting domain (CATD) (Black et al., 2004; Black et al., 2007b). This CATD is essential for CENP-A function (Black et al., 2007a). *Drosophila* CENP-A^{CID}-targeting domain is even smaller, consisting of the C-terminus of α1, all of loop 1 and the N-terminus of α2 (15 amino acids) (Vermaak et al., 2002). Thus assembly of centromere-specific CENP-A nucleosomes requires core histone structures rather than unique sequence elements of CENP-A's divergent N-terminus.

The N-terminal tail of CENP-A exhibits a surprisingly high level of divergence between species in terms of both length and composition (Henikoff and Dalal, 2005). Analysis of centromere protein evolution indicates that CENP-A and other components of centromeric chromatin are rapidly and adaptively evolving (Meraldi et al., 2006; Talbert et al., 2004). A meiotic drive mechanism, in which centromeres

of different strengths affect their heritability at the level of positioning within the spindle in female meiosis, has been proposed to account for the rapid co-evolution of centromere proteins and DNA in higher organisms (Talbert et al., 2004). The N-terminal domain of Cse4, the budding yeast CENP-A homologue, contributes an essential role in interaction with other centromere proteins (Chen et al., 2000) and in human cells CENP-A is phosphorylated by Aurora kinases in G2 and M (Kunitoku et al., 2003; Zeitlin et al., 2001), though little is known about what role(s) the N-terminus plays in most organisms.

CENP-A Nucleosomes

In vitro nucleosome assembly studies have shown that CENP-A can replace histone H3 in octamers containing H4, H2A and H2B (Yoda et al., 2000; Furuyama et al., 2006). Concordantly, purification of CENP-A containing nucleosomes from both human and *Drosophila* cells have shown that the majority of CENP-A is located in nucleosomes comprised as [CENP-A – H4 – H2A – H2B]$_2$ octamers (Blower et al., 2002; Foltz et al., 2006). Atomic force microscopy of reconstituted centromeric nucleosomes from *Drosophila* suggests that individual tetramers of [CENP-A – H4 – H2A – H2B] may exist (Dalal et al., 2007). Reconstituted CENP-A containing nucleosomes have a more compact binding surface with H4 than H3 nucleosomes, observed in both [CENP-A – H4]$_2$ tetramers and [CENP-A – H4 – H2A – H2B]$_2$ octamers (Black et al., 2004). CENP-A nucleosomes are also more readily destabilized than canonical nucleosomes, a property that has been proposed to ensure that misplaced CENP-A nucleosomes are easily removed (Conde e Silva et al., 2007). In budding yeast Cse4-containing nucleosomes are found in which the H2A and H2B histones are replaced by a non-histone protein (Scm3) that co-localizes with CENP-A^{Cse4} at centromeres (Mizuguchi et al., 2007). The precise nature of the CENP-A nucleosomes and nucleosome like complexes that exist, and the extent to which they are conserved evolutionarily, remains to be fully elucidated.

Organization of CENP-A Within Centromeres

CENP-A containing nucleosomes are the marker for the location and functionality of the centromere, but what is the context in which they are found? Using stretched chromatin fibers from human and *Drosophila* cells, CENP-A and H3 nucleosomes are found to occupy mutually exclusive domains, alternating across several hundred kb at the locus (Blower et al., 2002). Core histones H4, H2B and H2A are distributed throughout the domain, indicating their common use between the CENP-A- and H3-containing nucleosomes. Recently, interspersion motifs have been proposed in both fission and budding yeast (Gregan et al., 2007; Yeh et al., 2008), suggesting that alternating conventional and centromeric histones are a conserved motif. ChIP experiments using neocentromeres demonstrate that CENP-A domains in human chromosomes discontinuously span >100kb domains (Alonso et al., 2007; Lo et al., 2001). The interspersed CENP-A domains are thought to fold

or coil into a cylindrical-like structure, with the CENP-A containing nucleosomes orientated to chromosome surface and the H3 domains facing the inter-chromatid space (Sullivan et al., 2001).

The formation of centromeric chromatin is dependent on its association with flanking domains of heterochromatin. Classically associated with morphologically defined heterochromatin, a centromere-specific role for the molecular mechanisms that mediate heterochromatic silencing of chromosomal domains has been most clearly documented by studies in *S. pombe* (Ekwall, 2007). In *S. pombe*, centromere function was shown to be dependent on the RNA interference pathway, which is required for silencing and modification of the heterochromatin that flanks the CENP-A nucleosome-containing central core domain of the centromere (Volpe et al., 2002). Transcription from within the centromere initiates an RNAi-based regulatory loop essential for the establishment and maintenance of pericentric heterochromatin (Grewal and Elgin, 2007). The RNA machinery is involved in the localization of HP1 to the pericentric heterochromatin by coordinating the molecular machinery for modification of histones within these domains (Maison et al., 2002; Pal-Bhadra et al., 2004).

Clr3 is a homologue of human class II histone deacetylase and it deacetylases lysine 14 of histone H3. Both the RNAi and Clr3 pathways recruit Clr4 (homologue of human SUV39 H1 and H2) which causes the trimethylation of lysine 9 of histone H3 and this goes on to form the binding site which the chromodomain of Swi6 recognizes (Nakayama et al., 2001). Swi6 is a homologue of the heterochromatin protein 1 (HP1) and its chromodomain binds to histone H3 trimethylated lysine 9 (Hall et al., 2002). Mutations of either lysine 9 or 14 of H3 cause delocalization of Swi6 which leads to chromosome missegregation (Mellone et al., 2003).

In humans, the centromere is associated with the pericentric heterochromatin domains which cause the recruitment of HP1 through the methylation of the lysine 9 of histone H3 (Fischle et al., 2005). Deletion of Suv39, the H3 lys9 methyltransferase, causes improper centromere formation and function. Knockout studies in mice have shown an increase in genome instability and increased incidence of lymphomas (Peters et al., 2001). Studies carried out in Suv39H1 and H2 null mice demonstrated 4-fold increase in defects of chromosome alignment and segregation defects. These results confirm the requirement of histone lysine methylation for the recruitment of cohesin to the pericentric heterochromatin (Heit et al., 2006). A direct role for heterochromatin in recruitment of CENP-A has been documented in *S. pombe*, where the RNAi pathway is required for CENP-A deposition on naïve templates, but is dispensable for its maintenance (Folco et al., 2008).

These investigations have directly documented a role for epigenetic mechanisms in centromere formation and function. They detail the role(s) played by histone modification pathways in heritable phenotypes of their target loci. In addition, the pathways for maintenance of epigenetic information are brought in focus by these observations. For the vast majority of species, the pericentric heterochromatin of the centromere is composed of arrays of repeated DNA sequence. In budding yeast CAF1 (chromatin assembly factor 1) is found in a region of the kinetochore where it interacts with Hir1 (HIRA like histone chaperone) and it is necessary for a proper centromere chromatin structure (Sharp et al., 2002). In fission yeast the Hir1

Table 1 Histone post-translational modifications in active, inactive and CEN chromatin

	Active	Inactive	CEN	Notes
H3K9Ac	+			
H3K4Me	+			
H3K4Me2	+		+	"Poised", but not always active
H3K4Me3	+			Found at pericentromeric heterochromatin
H3K9Me		+		
H3K9Me2		+		Found at pericentromeric heterochromatin
H3K9Me3		+		Found further from CEN than H3K9Me2
H4K5Ac	+			
H4K8Ac	+			
H4K12Ac	+			
H4K16Ac	+			

homologue, Hip1, is required for epigenetic silencing of pericentric heterochromatin (Blackwell et al., 2004). Finally the human homologue of HirA was found to be essential for the recruitment of HP1 to the heterochromatin in humans (Zhang et al., 2007). HirA homologues appear to play a role as chaperones in the pericentric chromatin across different species (Loyola and Almouzni, 2004). Taken together with the considerations of CENP-A targeting described below, it is clear that the mechanisms that modify nucleosomes and specify their incorporation during or after DNA replication play a key role in maintaining the functional and molecular identity of the centromere across successive generations.

Thus, the large-scale context of CENP-A chromatin is critical for its function. Within the domain of CENP-A nucleosomes themselves there is further specialization in histone modification. Centromeric chromatin in *Drosophila* lacks many characteristic heterochromatin-associated modifications but surprisingly also lacks marks associated with classical euchromatic chromatin (Sullivan and Karpen, 2004) – see Table 1. Concurrently, centromeres do maintain some modifications that are normally found enriched at both active and inactive regions of the chromosome. These observations have led to the suggestion that the chromatin present at the centromere is distinct from chromatin both flanking the centromere (pericentric heterochromatin), and at the chromosome arms. This form of chromatin has been coined centrochromatin, or CEN chromatin (Blower et al., 2002; Sullivan and Karpen, 2004).

Assembly of CENP-A Chromatin and the Constitutive Centromere-Associated Network of Proteins

CENP-A Assembly in the Cell Cycle

The assembly of centromeric chromatin does not occur during S-phase, as occurs for the bulk of canonical histones (Shelby et al., 2000; Sullivan et al., 2001). In human cells, CENP-A mRNA and protein accumulate late in G2 after DNA replication,

and CENP-A deposition can take place in the absence of DNA replication (Shelby et al., 2000, 1997). Precisely when does CENP-A assemble? Use of a novel covalent protein labeling approach has shown directly that CENP-A synthesized in one cell cycle does not assemble into centromeres until passage through mitosis and entry in to the G1 phase of the subsequent cell cycle (Jansen et al., 2007). During *Drosophila* development, CENP-A^{CID} assembly occurs during anaphase of mitosis in the syncytial embryonic nuclear divisions, which cycle between S- and M-phases without G1 or G2 phases (Schuh et al., 2007). *S. pombe* CENP-A can assemble in both S-phase and G2 (Takayama et al., 2008). There appear to be no absolute restrictions on the timing of CENP-A assembly, suggesting that the process is integrated with other cell cycle and chromosomal replication pathways.

Using a FRAP technique, loading of CENP-A was observed exclusively in G1 in HEp2 human cells expressing GFP-CENP-A (Hemmerich et al., 2008). Fluorescent centromeres bleached at late mitosis/early G1 showed recovery after 30 min with a slow but steady increase in fluorescence over the following 2 h. The total number of fluorescent centromeres were also monitored during FRAP at the same stage of the cell cycle. The number of fluorescent centromeres in a cell decreased accordingly following the bleaching of a region containing 10 centromeres. This number increased to pre-bleach numbers after 1 h, thus indicating that all bleached centromeres had acquired new GFP–CENP-A molecules (Hemmerich et al., 2008). The maximum FRAP recovery of CENP-A was <50%, suggesting centromere incorporation without exchange of already loaded molecules. Using sister telophase cells as controls for one another, the rate of exchange between loaded centromeric CENP-A in G1 was assayed by FRAP. The complete set of centromeres in one daughter cell were bleached, resulting in recovery to ~40% pre-bleach fluorescence after 2 h. An area containing 5–10 centromeres was then photobleached for a second time to ask if molecules could exchange at this later time in G1. Little or no recovery was observed at the double-bleached centromeres, indicating that centromeric CENP-A did not exchange, while FRAP still occurred in the second daughter cell, demonstrating that CENP-A incorporation was still active at that time (Hemmerich et al., 2008).

Heritability and Dynamics of Centromeric Chromatin Proteins

A key question for understanding the basis of epigenetic inheritance is how molecular information is passed from mother to daughter cells in a locus-specific fashion. One possibility is that the epigenetic information can be assembled in each cell cycle on the basis of tissue-specific DNA binding proteins. Developmental biology has provided a broad mechanistic view of how stable epigenetic states, manifest as programs of gene expression, are coupled to histone modifications. For example, tissue specific gene silencing can be regulated during development through the binding of large protein complexes, such as the Polycomb Repressive Complex 2 (PRC2), in proximity to target genes. The binding of these complexes induces gene

repression by causing changes to the local chromatin structure, mainly affecting histone modifications. The majority of Polycomb target genes are marked by histone modifications. A bivalent modification system, which involves both repressive H3K27me3 modifications and H3K4me3 activating modifications in embryonic stem cells, is predicted to provide the potential for genes cycling between active and inactive states during development (Barski et al., 2007; Bernstein et al., 2006; Mikkelsen et al., 2007; Pan et al., 2007; Zhao et al., 2007). Perhaps a similar mechanism exists for the maintenance of the centromeric locus? Alternatively, molecules present at the locus can be inherited locally, directly maintaining epigenetic marks at defined chromosomal sites. Evidence for the latter is seen in the slow rate of turnover of histones in general and, specifically, of CENP-A at centromeres (Regnier et al., 2005; Shelby et al., 2000). Is parental CENP-A "inherited" in equal abundance by both daughter centromeres or is CENP-A segregated exclusively at one daughter centromere while the sister centromere was replenished with "new" CENP-A? Direct observation of a cadre of CENP-A molecules fluorescently labeled in G1 suggests strongly that pre-existing CENP-A is equally distributed to daughter centromeres following passage through each mitosis (Jansen et al., 2007). Therefore, despite the synthesis of new CENP-A during the cell cycle, CENP-A already loaded into centromeric chromatin by late G1 is redistributed equally to daughter centromeres, with each daughter receiving a "half-dose" of CENP-A. This results in the surprising conclusion that cells execute mitosis with a "half dose" of CENP-A at the centromere, and a newly synthesized "half dose" which is not yet assembled into the centromeric chromatin, heightening the relevance of the question of precisely how CENP-A chromatin is configured following DNA replication.

Targeting CENP-A to the Centromere

A role for histone chaperones is indicated for assembly of CENP-A. Human RbAp46, a histone chaperone (Verreault et al., 1996), is highly similar to *S. pombe* Mis16, a gene originally identified in a screen for chromosome missegregation (Hayashi et al., 2004). The RbAp46/RbAp48 complex is essential for CENP-A localization to centromeres (Hayashi et al., 2004). Components of the human Mis18 protein complex have also been shown to be essential for the structure and function of the centromere/kinetochore. The hMis18α/hMis18β/Mis18 Binding Protein 1 (Mis18BP1) complex accumulates specifically at the centromere in late telophase-G1 (Fujita et al., 2007), co-incident with CENP-A assembly into the centromere. RNAi depletion of hMis18α resulted in loss of centromeric CENP-A signal, while the total cellular level of CENP-A remained constant (Fujita et al., 2007). The Mis18 protein complex was also shown to interact directly with the histone H3–H4 chaperone RbAp46/48, but does not directly interact with CENP-A, however, RbAp48 has previously been observed to interact directly with CENP-A^{CID} in vitro and facilitate

the assembly of centromeric chromatin (Furuyama et al., 2006). As RbAp46 binds to histone acetyltransferase catalytic subunit (Hat1), the effect of suppressing histone deactylase on CENP-A assembly in the absence of hMis18α was analyzed. results indicate that histone deactylase inhibitors do indeed enhance the recruitment of newly synthesized CENP-A to the centromere (Fujita et al., 2007). These results suggest that general nucleosome modification and assembly machinery is used in centromeric chromatin assembly by adaptation through specific protein factors.

Genetic analysis in fission yeast has revealed a number of proteins necessary for proper assembly and maintenance of CENP-A (Cnp1) chromatin. Mis6, Mis16-18 and Ams2 have all been identified as essential for the assembly of CENP-A^{Cnp1} to centromere, with defects in of any one resulting in loss of CENP-A^{Cnp1} signal at the centromere (Chen et al., 2003; Hayashi et al., 2004; Takahashi et al., 2000). Hrp1 and Sim4 are also required for CENP-A^{Cnp1} recruitment to the centromere (Pidoux et al., 2003; Walfridsson et al., 2005). Recent characterization of the *S. pombe* Scm3 ($Scm3^{Sp}$), the *sim1* gene product, has provided valuable insight into CENP-A targeting. $Scm3^{Sp}$ localizes to centromeres of *S. pombe* and is present in central domain chromatin. However, during mitosis it is absent from the central core, returning during late anaphase (Pidoux et al., 2009; Williams et al., 2009). CENP-A^{Cnp1} does not target to centromeres in *scm3* mutants, while $Scm3^{Sp}$ maintains its centromeric localization in *cnp1-1* mutants (Pidoux et al., 2009). However, centromeric targeting of $Scm3^{Sp}$ is dependent on the constitutive centromeric Mis6-Sim4 complex and also on Mis16-Mis18 as $Scm3^{Sp}$ mis-localized in *mis16* and *mis18* mutants (Pidoux et al., 2009; Williams et al., 2009). This places $Scm3^{Sp}$ upstream to CENP-A^{Cnp1} loading, but under control of Mis16-Mis18.

In *S. cerevisiae*, Scm3 is required for targeting of both Ndc10 and CENP-A^{Cse4} at the centromere (Collins et al., 2005; Ortiz et al., 1999). Ndc10 and $Scm3^{Sc}$ are also co-dependent, as Ndc10 is required for the centromere association of $Scm3^{Sc}$, while $Scm3^{Sc}$ is also required for Ndc10 localization (Mizuguchi et al., 2007). In contrast to other CENP-A^{Cse4} interacting partners, *SCM3* is essential and in the absence of $Scm3^{Sc}$, CENP-A^{Cse4} fails to localize to centromeric DNA. It has been proposed that $Scm3^{Sc}$ is a core constituent of unconventional, CENP-A^{Cse4} nucleosomes, replacing H2A-H2B, as $Scm3^{Sc}$ in vitro displaces H2A-H2B dimers from CENP-A^{Cse4} histone octamers while in vivo the core centromere chromatin appears to lack both H2A and H2B (Mizuguchi et al., 2007).

A *Drosophila* genome-wide RNAi screen for mis-localization of CENP-A^{CID} identified both CENP-C and CAL1, as essential factors for the correct assembly of newly synthesized CENP-A^{CID} at the centromere (Erhardt et al., 2008). CAL1 had been previously identified in a screen for mitotic defects (Goshima et al., 2007). Thus, studies in a number of organisms have revealed a variety of components that are required for the assembly and/or maintenance of the centromere-specific nucleosome. Understanding how the pathways defined by these proteins are organized and regulated, which ones serve conserved functions and, mechanistically, what those functions are remains a challenge of current research.

Assembly of the Constitutive Centromere-Associated Network, CCAN

In addition to CENP-A, there is a large set of proteins that are uniquely localized to centromeres throughout the cell cycle (Cheeseman and Desai, 2008b). These proteins comprise a distinctive chromatin-associated complex that mediate the functions of centromeric chromatin and may play a role in the heritability of the centromere. Biochemical and in vivo studies involving CENP-A and other molecular "starting points" have elucidated this large group of chromosome proteins. Their identification and functional characterization provides not only a key advance in understanding a complex genetic locus, but also a picture of the state of the art of chromosome biology and the challenges faced by researchers bringing genetic, molecular and cellular approaches to bear on a network of protein complexes whose discrete functions are, for the most part, yet to be identified.

In vertebrates, a set of centromere proteins co-localize with CENP-A throughout the cell cycle and co-purify with CENP-A nucleosomes using monoclonal antibodies or affinity-based molecular probes (Foltz et al., 2006; Obuse et al., 2004; Okada et al., 2006). A recent review of this set of proteins has suggested the unifying nomenclature "constitutive centromere-associated network" (CCAN) (Cheeseman and Desai, 2008b). This network is comprised, in addition to CENP-A, of CENP-C and 14 interacting proteins (CENP-H, CENP-I, CENP-K–U, CENP-W). CENP-C is an essential component of the inner kinetochore and is presently the only protein in this network besides CENP-A that has been identified in all eukaryotes (Heeger et al., 2005; Meluh and Koshland, 1995; Moore and Roth, 2001; Saitoh et al., 1992).

Initial biochemical studies isolated CENP-A chromatin and interacting proteins – the "CEN-complex" (Obuse et al., 2004). Known centromere proteins, CENP-A, CENP-B, CENP-C, CENP-H and CENP-I/hMis6 were specifically found in the CEN-complex as were a series of known proteins not previously reported as centromere proteins, such as uvDDB-1, XAP8, hSNF2H, FACTp180, FACTp80/SSRP1, PcG proteins (BMI-1, RNF1, RNF2, PHP2 and HPC3), KNL5, and racGAP. Only uvDDB-1 and BMI-1 were confirmed to localize to centromeres, however, no functional studies were carried out (Obuse et al., 2004). Multiple tandem affinity purifications have since been used to define the most proximal complex directly recruited by CENP-A-containing centromeric nucleosomes, the nucleosome associated complex (CENP-A NAC), and a set of seven CENP-A distal proteins (CAD) recruited to centromeric nucleosomes through their interaction with the CENP-A NAC (Foltz et al., 2006). These studies establish a degree of structural hierarchy in the centromere. Found to be uniquely associated with the CENP-A nucleosomes using this approach were CENP-U (also named CENP-50) (Minoshima et al., 2005) and a set of three proteins CENP-M, CENP-N and CENP-T, which had been previously identified (Obuse et al., 2004) but not established as centromere components. Also associated with CENP-A nucleosomes were CENP-B, human fetal liver expressing gene 1 (hFLEG1), nucleophosmin- 1 and the FACT complex members.

Expressing CENP-M, CENP-N and CENP-T putative centromeric proteins as fluorescent fusions confirmed their localization to the centromere, while subsequent affinity purifications using a localization and affinity purification (LAP) tag to identify the binding partners of CENP-M, CENP-N or CENP-U, in which each protein was co-purified with each of the others led to the conclusion that the CENP-A NAC was comprised of previously identified centromere components CENP-H (Sugata et al., 2000) and CENP-C (Saitoh et al., 1992), in addition to CENP-M, N, T and U(50) (Foltz et al., 2006). Suppression of *CENP-M*, *CENP-N* or *CENP-T* by siRNA disrupted recruitment of the CENP-A NAC. Reduction of *CENP-M* or *CENP-N* resulted in their reciprocal loss from centromeres as well as loss of CENP-H. Reduction of *CENP-T* eliminated CENP-M at centromeres, thus presumably also CENP-N and CENP-H. Eliminating detectable CENP-U did not affect centromeric recruitment of CENP-A, CENP-B, Hec1, CENP-E, CENP-F, Mis12 or Aurora B. However, it was shown to eliminate a tagged form of both CENP-O and CENP-P from interphase and mitotic centromeres, suggesting that these components are interdependent for localization and/or maintenance at centromeres. Disruption on CENP-M, -N and -T also resulted in mitotic defects, with the depletion of CENP-T producing the most severe phenotype. The defects were mainly failures in chromosome congression that yielded an increased proportion of cells in prometaphase or pseudometaphase, suggesting the NAC components are required for mitotic progression.

Further tandem affinity purifications involving the NAC components yielded a set of proteins associated with each, establishing a CENP-A distal (CAD) component of the centromere. As CENP-B, hFLEG1, nucleophosmin-1 and the FACT complex members were absent in these purifications they were assumed to be not tightly associated with the NAC components. CENP-I, CENP-K, CENP-L, CENP-O, CENP-P and CENP-Q were also purified with each of the CENP-M, CENP-N and CENP-U-containing complexes. CENP-R and CENP-S were associated with the CENP-M and CENP-U complexes. CENP-K, CENP-L and CENP-Q have been shown to be constitutively associated with centromeres (Okada et al., 2006).

Affinity purifications from both human and chicken (DT40) cells using cell lines expressing epitope-tagged CENP-H and CENP-I identified other constitutive CENPs (Okada et al., 2006). Centromere proteins CENP-K to R, CENP-U were found to constitutively localize to centromeres as components of a complex with CENP-H and CENP-I (Okada et al., 2006). Interestingly the purifications in this screen did not isolate CENP-M, CENP-L, CENP-S or CENP-T in human cells. The interactions detectable between various CENPs are dependent on the level of stringency used in the purifications. Some interactions detected using a single purification step were not detected following a more stringent tandem affinity purification or LAP. For example, CENP-H and CENP-I were not present in significant amounts in a tandem affinity purification of human LAP–CENP-O, but were present in a one-step immunoprecipitation. In contrast, both methods isolated a large amount of CENP-O, CENP-P, CENP-Q, CENP-R and CENP-U (Okada et al., 2006). This suggests that some protein-protein interactions are stronger than others, that some complexes are more stable than others or that there are distinct sub-complexes

within the centromere. It is also possible that the centromere protein interactions remaining undetectable in a more stringent purification are either less stable or of a more transient nature.

Biochemical and in vivo studies in vertebrates assessing interdependencies, mitotic defects and lethality have divided the CENPs into subclasses of a constitutive centromere associated network (CCAN) (Cheeseman and Desai, 2008b). There are three distinct protein classes within the set of CENP-H–I-associated proteins, as was suggested by the phenotypic analysis and the purifications: an essential CENP-H class (CENP-H, -I, -K and -L), and an essential CENP-M class (CENP-M) and a non-essential CENP-O class (CENP-O, -P, -Q and –U) (Okada et al., 2006). Depletion of components of the first subclass of proteins, comprising CENP-H, CENP-I and CENP-K, causes cell-cycle arrest, kinetochore-assembly defects and severe chromosome missegregation (Foltz et al., 2006; Fukagawa et al., 2001; Izuta et al., 2006; Liu et al., 2003; Mikami et al., 2005; Nishihashi et al., 2002; Okada et al., 2006). The CENP-O class forms a stable complex, which is required in chicken cells for proper kinetochore function (Hori et al., 2008). Kinetochore localization of CENP-O, -P, -Q and -U was shown to be interdependent, yet all proteins correctly localized in CENP-R deficient cells. All proteins were also required for recovery from spindle damage, with phosphorylation of CENP-U essential for the recovery (Hori et al., 2008). In human cells, depletion of individual members of the CENP-O subclass of proteins causes defects in chromosome segregation that are severe enough to cause cell lethality (Foltz et al., 2006; McAinsh et al., 2003; Okada et al., 2006).

Members of the CENP-H-I complex may contribute to the efficient incorporation of newly synthesized CENP-A into centromeres in DT40 cells. The percentage of cells with CENP-A–GFP foci was observed to be significantly lower in *CENP-H*-, *CENP-I*-, *CENP-K*- and *CENP-M*-deficient DT40 cells, however, endogenous CENP-A levels were unaltered in CENP-H–I complex mutant cells by western blot analysis. CENP-A–GFP was also incorporated into non-centromeric chromatin in *CENP-M* and *CENP-K* mutants (Okada et al., 2006). In contrast with this, CENP-I has previously been thought to not be required for CENP-A targeting in DT40 cells (Nishihashi et al., 2002). These data suggest that centromere proteins may play a role in the recruitment and/or deposition of newly synthesized CENP-A at the centromere. As the localization of CENP-H and CENP-I also requires CENP-A, the loading of newly synthesized CENP-A is either dependent on preexisting CENP-A nucleosomes, with the CENP-H–I complex acting as an intermediate, or preexisting centromeric protein complexes. It is unknown is whether the CENPs (H-I complex) that play a role in “new” CENP-A deposition are the CENPs that are already present at the centromere and thus recruit newly synthesized CENP-A to a “centromere” defined by their presence, or whether they are also newly synthesized and are deposited at the centromere coincident with CENP-A deposition. As the CENP H-I subclasses of centromere proteins have a degree of hierarchical dependence, it is possible that the formation of sub-complexes of “new” CENPs is necessary prior to their deposition at the centromere.

Of the CCAN components, only CENP-S and CENP-T together with the recently identified CENP-W contain histone fold domains, similar to CENP-A. CENP-W was identified as a CENP-T interacting factor. Under stringent conditions, CENP-T can be separated from the rest of the CCAN and is distinct from the CENP-H-containing complex as purifications of affinity-tagged CENP-T do not contain CENP-H or CENP-O complex proteins (Hori et al., 2008). However, in addition to some histone proteins, a novel 11 kDa protein was identified and named CENP-W due to its co-purification with CENP-T and its constitutive localization to centromeres (Hori et al., 2008). The CENP-T/CENP-W complex directly associates with nucleosomal DNA and with canonical histone H3, but not with CENP-A, in centromeric regions similarly to CENP-C (Hori et al., 2008). The DNA binding activities of the complex requires their histone-fold domain. Some studies were carried out to assign hierarchical importance to the complex in order to elucidate its role in the assembly of the centromere and the mitotic assembly of the kinetochore. Phenotypic analysis of CENP-T/W and CENP-C deficient DT40 cell lines indicates that CENP-T/W and CENP-C function in separate pathways in the assembly of the kinetochore, while in CENP-W-deficient cells, kinetochore localization of other CCAN proteins, with the exception of CENP-C, was abolished suggesting CENP-W functions upstream of other CCAN proteins in centromere assembly. Kinetochore localization of CENP-T complex occurs upstream of the CENP-H complex as CENP-T and CENP-W were still visible on mitotic chromosomes in cells depleted of CENP-H, thus the T/W complex functions also upstream of the CENP-H complex.

What these studies make clear is that the complex of proteins required to form a centromere and to confer its ability to nucleate kinetochore assembly and function is indeed complex. Teasing apart the associations and functions of these proteins, how functions are distributed within the network and how this complex is replicated will require an integration of biochemical and cellular methods, coupled with traditional and in vitro genetic approaches. Just as clearly, identifying a consistent set of functional assays for centromere behavior is necessary to systematically integrate findings from numerous organisms and experimental perspectives. In addition to the chromatin structure and replication issues, the key functional output of the centromere is through the kinetochore in mitosis and meiosis.

Kinetochore Function, Assembly and Signaling in the Spindle Assembly Checkpoint

Ultimately, the function of the centromere is to nucleate the assembly of the mitotic kinetochore to provide the cell a means of connecting chromosomes to spindle fibers and regulating this association. Chromosome missegregation often leads to aneuploidy or polyploidy, which has been recognized as a common characteristic of cancer cells and possible cause of tumorigenesis (Weaver and Cleveland, 2005). To avoid loss of genomic information during mitosis, each pair of sister chromatids

connects to spindle microtubule through their kinetochores – macromolecular structures assembled onto the centromeres at mitosis (Liu et al., 2006), that monitor the attachment state and activate signaling pathways to prevent anaphase onset before all chromosomes have achieved merotelic (attachement to opposite poles of the spindle) attachment (Cheeseman and Desai, 2008a). Defects in chromosome biorientation activate the spindle assembly checkpoint (SAC) which is required to block sister chromatid separation until all chromosomes are properly attached to the mitotic spindle. The SAC prevents cells entering anaphase by inhibiting the ubiquitination of cyclin B and securin by the anaphase-promoting complex/cyclosome (APC/C) ubiquitin ligase. The checkpoint proteins, including BubR1, Bub1, Bub3, Mad1, Mad2 and CENP-E, target the essential APC/C activator Cdc20. A single unattached kinetochore can generate a robust SAC activity thus providing the necessary time to correct chromosome attachment problems.

Kinetochore Structure and Function

The major function of kinetochore protein complex in mitosis is: (1) kinetochore specification, (2) kinetochore assembly, (3) microtubule binding and (4) monitoring and attachment regulation of kinetochore-microtubule structure (Cheeseman and Desai, 2008a; Logarinho and Bousbaa, 2008). In higher eukaryotes, kinetochore proteins with MT-binding function can be dissected into: proteins that form the core attachment site (Ndc80/HEC1) and are responsible for attachment chromosome to spindle microtubules; proteins that control microtubule dynamics (CLASP, APC/EB1, MAP125) and promote kinetochore motility (dynein and CENP-E) to generate or to transduce the forces that are required for chromosome segregation (Logarinho and Bousbaa, 2008). The SAC is tightly integrated with these proteins, allowing kinetochore activity and function to directly modulate the generation of diffusible anaphase inhibitors. Thus, the irreversible transition from metaphase to anaphase is delayed until every kinetochore is attach to the mitotic spindle and can be faithfully segregated into a daughter cell.

Kinetochore assembly, structure and function is dynamically regulated during the cell cycle (Cheeseman and Desai, 2008a). Kinetochores have a trilaminar morphology that consist of: the inner kinetochore that forms the interface with chromatin; the outer kinetochore, a 50–60 nm thick region, which forms the interaction surface for spindle microtubules; and the central domain, the region in between outer and inner kinetochore. Kinetochore assembly is nucleated by centromeric chromatin and its associated protein complexes – CCAN, NAC and CAD, starts with CENP-A chromatin, which forms a foundation for kinetochore structure in yeast, *C. elegans, D. melanogaster* and vertebrates. In vertebrates, the network of constitutively associated centromere chromatin proteins plays an essential role while additional proteins specifically required for mitotic events are recruited to kinetochores starting from late G2 phase, through all stages of mitosis until the onset of anaphase (Cheeseman and Desai, 2008a; Logarinho and Bousbaa, 2008).

The Core Microtubule-Attachment Site – KMN Network

The core microtubule-attachment site is compromised by the components of KNL1-Mis12-Ndc80 complex (KMN) network that is conserved widely in the eukaryotic kingdom (Cheeseman and Desai, 2008a). By association with the Mis12 complex KNL1 generates a binding site for the Ndc80 complex, which allows both KNL1 and Ndc80 bind to microtubules. The CCAN plays an important role in localizing the Ndc80 complex, and the amount of CCAN and KMN complexes at kinetochores does not change significantly from late prophase until late anaphase, irrespectively of microtubule-attachment status (Cheeseman and Desai, 2008a), in contrast to SAC components.

Members of the CCAN that have been shown to play a role in kinetochore assembly include CENP-C and CENP-H/I/K (Cheeseman and Desai, 2008a). CENP-C is upstream of most other kinetochore proteins, including the Mis12 complex, whereas CENP-H, -I, and -K are not required for the mitotic localization of CENP-A and CENP-C, but contribute to the localization of outer kinetochore proteins, including components of the mitotic checkpoint (SAC), the Ndc80 complex and the coiled-coil-domain-containing protein CENP-F (Cheeseman and Desai, 2008a; Liu et al., 2006, 2003). Moreover, CENP-H has been reported to associate with Ndc80 during mitosis and both proteins are under control of Mis12 and CCAN (Cheeseman and Desai, 2008a).

Although Ndc80 plays a key role in generating the robust chromosome-microtubule attachment, Ndc80-depleted cells still exhibit weak kinetochore-microtubule interactions. Other candidates that have microtubule-binding activities that could participate in forming these attachments in Ndc80-depleted cells are the motor proteins dynein and CENP-E (Cheeseman and Desai, 2008a). Depletion of CENP-E reduces number of kinetochore-microtubule attachment, but it does not cause dramatic chromosome misalignment or segregation defects, although a subset remains at the poles and is missegregated (Cheeseman and Desai, 2008a; Schaar et al., 1997). Taken together, the data indicate that microtubule attachment by kinetochores occurs through multiple molecular linkages and that their coordination is required for robust, regulated interaction of chromosomes with spindle microtubules.

Controlling Dynamics of Kinetochore-Microtubule Attachment and Chromosome Movement

Kinetochores bind dynamically to microtubules, allowing them to undergo both polymerization and depolymerization and influencing these reactions significantly to coordinate chromosome movement. The major microtubule-associated proteins that are responsible for polymerization of microtubules at kinetochores are cytoplasmic linker protein (CLIP)-associating protein (CLASP), MAP215 and CLIP170. MAP215 is essential for spindle assembly and chromosome segregation in all

eukaryotes. EB1 protein binds and stabilizes the microtubule lattice. A complementary class of microtubule destabilizing proteins, microtubule depolymerases, are comprised by kinesin-13 (MCAK) and kinesin-8 family members (Cheeseman and Desai, 2008a).

To maintain chromosome attachment on dynamic microtubules kinesins function as couplers to depolymerizing microtubules and in vertebrate cells this function is accomplished by CENP-E and Dam1 complex in yeast (Cheeseman and Desai, 2008a; Schaar et al., 1997; McAinsh et al., 2003). The Dam1 complex consists of nine or more subunits, including Spc34p, all of which are essential and oligomerize to form a ring around the microtubule attached to kinetochore (McAinsh et al., 2003). These two proteins are the example of linkage the length changes of microtubules with motility and chromosome movement.

A balance of forces toward spindle poles and the spindle equator is accomplished, in part, by the complementary activities of two motor proteins that are localized to kinetochores – CENP-E and dynein, plus and minus end directed motors, respectively. During early stages of kinetochore attachment, dynein translocates laterally associated kinetochores toward the vicinity of spindle poles. CENP-E translocates along the kinetochore fiber of an already bi-oriented chromosome to move a mono-oriented chromosome towards the spindle equator. Dynein and the associated proteins LIS1 and ROD-ZW10-Zwilch (RZZ) complex also contribute a poleward force at end-on attached kinetochores that allows chromosome alignment and segregation (Cheeseman and Desai, 2008a; Schaar et al., 1997; Yang et al., 2007; Musacchio and Salmon, 2007a). Integrating these mechanochemical motor activities with microtubule dynamics to accomplish proper chromosome alignment is an intricate task that coordinates a number of diverse molecular mechanisms.

Spindle Assembly Checkpoint (SAC): Maintaining Fidelity of Chromosome Segregation

Kinetochores that are unattached to microtubules recruit additional proteins to facilitate microtubule interactions and signal cell-cycle arrest (Cheeseman and Desai, 2008a). This surveillance strategy is accomplished by the spindle assembly checkpoint (SAC), also known as mitotic checkpoint (Musacchio and Salmon, 2007a). SAC delays chromosome segregation until the last chromosome has correctly attached to the spindle. However, it is still not known in detail how this unattached chromosome can generate a checkpoint signal and inhibit the anaphase promoting complex-cyclosome (APC/C) (Musacchio and Salmon, 2007a; Medema, 2009).

Core components of the SAC proteins involve the evolutionarily conserved MAD (mitotic-arrested deficient) Mad1, Mad2, Mad3 (BubR1 in humans) and BUB (budding uninhibited by benzimidazole) Bub1 and Bub3 proteins (Musacchio and Salmon, 2007a; Logarinho and Bousbaa, 2008). Additional proteins of the SAC involve the kinases multipolar spindle-1 (Mps1) and Aurora B (Ipl1 in *S. cerevisiae*), proteins that regulate SAC activity in higher eukaryotes like CENP-E, dynein,

dynactin, CLIP170, LIS1, and the RZZ (ROD-ZW10-Zwilch) complex (Musacchio and Salmon, 2007a; Logarinho and Bousbaa, 2008).

The SAC targets Cdc20, a co-factor of the APC/C ubiquitin ligase, that is required for the ubiquitinylation of several key cell cycle regulators, including cyclin B and securin. The degradation of these substrates is required for anaphase onset. The SAC negatively regulates the ability of Cdc20 to activate the APC/C and thus inhibits the downstream proteasome-dependent degradation of key mitotic substrates until all chromosome have become bi-orientated between separated spindle poles on the metaphase plate (Musacchio and Salmon, 2007a).

Kinetochore localization of SAC proteins is hierarchical, meaning that recruitment of some proteins depends on prior recruitment of others (Logarinho and Bousbaa, 2008; Vigneron et al., 2004). In this cascade, kinetochore localization of Aurora B/INCENP is the first regulatory step that stimulates the formation of a kinetochore complex, consisting of Mps1, Bub1, and CENP-E and probably also Bub3 and BubR1 (Vigneron et al., 2004). This complex will subsequently promote the recruitment to the kinetochore Mad1/Mad2, Cdc20 and APC/C. Checkpoint components Mad1 and Mad2 represent the downstream proteins of the checkpoint signaling pathway due to the fact that they do not regulate the kinetochore localization of either Cdc20 or APC/C. The latter two do not depend on each other in order to be localized at these chromosome structures (Vigneron et al., 2004).

During prometaphase Cdc20 and all SAC proteins localize to unattached kinetochores dynamically (Musacchio and Salmon, 2007a; Howell et al., 2000). That provides a catalytic platform that accelerates the production of the mitotic checkpoint complex (MCC), which consist of three SAC proteins Mad2, BubR1/Mad3, Bub3 in association with Cdc20 itself (Musacchio and Salmon, 2007a). The MCC binds and potently inhibits the APC/C. Its formation is inhibited by microtubule attachment and chromosome bi-orientation (Musacchio and Salmon, 2007a). Coupled with dissociation of the complex, the kinetochore-dependent formation of the MCC links its concentration to the state of chromosome attachment in the cell, producing a robust monitoring system that ensures that anaphase does not occur in the presence of unattached chromosomes.

The SAC pathway will be active as long as there are unattached kinetochores or improper attachment unable to produce enough tension between sister chromatids (Logarinho and Bousbaa, 2008). Once the last chromosome, during the unperturbed mitosis, achieves proper bipolar attachment to the spindle, the checkpoint signaling is silenced, Cdc20 release triggers APC/C activation and cyclin B and securin start to be degraded, which promotes anaphase onset and subsequent exit from mitosis (Logarinho and Bousbaa, 2008; Musacchio and Salmon, 2007a).

In addition to kinetochore-microtubule attachment, the SAC can be also inactivated by tension, since the attachment is normally destabilized at low kinetochore tension and stabilized by high tension between sister chromatids. This tension-based strategy allows discriminating incorrect attachments, such as syntelic and merotelic attachment to the kinetochores (Musacchio and Salmon, 2007a). Dissecting the precise contributions of attachment versus tension per se remains a critical but daunting task.

One of the challenges in dissecting centromere kinetochore function is the fact that centromere proteins may play more than one role in the entire structure. The perfect example of dual role in function is BubR1 kinase protein. BubR1 serves as a checkpoint in mitotic checkpoint complex (MCC) and has been shown to be essential for proper kinetochore microtubule attachment through an association with CENP-E as well (Logarinho and Bousbaa, 2008; Musacchio and Salmon, 2007a; Yao et al., 2000). One of the possible explanations for these distinct functions of BubR1 could be multiple conformations of the protein. It has been shown that BubR1 is differentially phosphorylated depending on the status of microtubule attachment and tension (Huang et al., 2008).

In addition to its monitoring function of the microtubule attachment condition, BubR1 plays a role together with Mad2 component with relaying the checkpoint signal from kinetochore to APC/C, thus providing the actual inhibition signal and preventing untimely anaphase onset (Musacchio and Salmon, 2007a; Kulukian et al., 2009). BubR1 can function as a pseudo-substrate inhibitor of the APC/C, forming independently of unattached kinetochores, in G2 phase, when functional kinetochores have not yet assembled (Medema, 2009; Kulukian et al., 2009). It has been shown that APC/C inhibitor can form in the absence of kinetochores – it just happens more slowly (Kulukian et al., 2009). During prometaphase, after assembly of kinetochores, Mad2/Mad1 complex is recruited and remains stably bound until kinetochore attaches to the spindle. The Mad2/Mad1 complex promotes formation of a Mad2/Cdc20 intermediate that can transfer Cdc20 into a complex with BubR1 and therefore block APC/C activity, much more efficiently than in G2 phase (Kulukian et al., 2009). These interrelated yet distinct molecular activities underscore the challenge in precisely identifying the mechanistic roles of the individual elements of the centromere/kinetochore complex.

Summary

Centromeres are unique chromosomal loci, contributing a well-defined genetic function but with a very ambiguous relationship to the DNA that they are associated with. Understanding how CENP-A is replenished at centromeres following DNA replication is a critical question and one that echoes similar questions at other loci where distinctive nucleosomes are assembled and modified. The answer is proving to be complex, requiring the dissection of chromatin contextual contributions through heterochromatin, specific nucleosome assembly pathways and the role of the CCAN, which appears to both require CENP-A for assembly and to influence assembly of CENP-A. Many of the activities of CENP-A chromatin and the CCAN remain to be elucidated and it is feasible that this platform for kinetochore assembly has direct influence on the function of the kinetochore and the SAC in mitosis and meiosis. Understanding how the kinetochore generates and regulates chromosome movement is essential for the development of novel chemotherapeutic agents that work through the spindle and the SAC. The centromere thus provides a key experimental tool for unraveling epigenetic inheritance as well as for designing new strategies to promote human health.

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Nucleotide Excision Repair in Higher Eukaryotes: Mechanism of Primary Damage Recognition in Global Genome Repair

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Abstract Nucleotide excision repair (NER) is one of the major DNA repair pathways in eukaryotic cells that counteract the formation of genetic damage. NER removes structurally diverse lesions such as pyrimidine dimers, arising upon UV irradiation, and bulky chemical adducts, arising upon exposure to carcinogens and some chemotherapeutic drugs. NER defects lead to severe diseases, including some forms of cancer. In view of the broad substrate specificity of NER, it is of interest to understand how a certain set of proteins recognizes various DNA lesions in the contest of a large excess of intact DNA. This review focuses on DNA damage recognition, the key and, as yet, most questionable step of NER. Understanding of mechanism of this step of NER may give a key contribution to study of similar processes of DNA damage recognition (base excision repair, mismatch repair) and regulation of assembly of various DNA repair machines. The major models of primary damage recognition and pre-incision complex assembly are considered. The model of a sequential loading of repair proteins on damaged DNA seems most reasonable in the light of the available data. The possible contribution of affinity labeling technique in study of this process is discussed.

Keywords Damage recognition · Nucleotide excision repair · Photoaffinity labeling · Repair factors

Abbreviations

AAF	acetylaminofluorene
Antr	anthracene
BER	base excision repair
BHD	beta-hairpin domain
CPD	cyclobutane pyrimidine dimers

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DBD	DNA binding domain
dsDNA	double-stranded DNA
ERCC1	excision repair cross-complementing rodent repair deficiency, complementation group 1
FAP-dCTP	exo-N-[2-N-(N-(4-azido-2,5-difluoro-3-chloropyridine-6-yl)-3-aminopropionyl)-aminoethyl]-deoxycytidine-triphosphate
FAP-dUTP	5-{N-[N-(4-azido-2,5-difluoro-3 chloropyridine-6-yl)-3-aminopropionyl]-trans-3-aminopropenyl-1}-deoxyuridine-triphosphate
FAP	fluoro-arylazido
Flu	fluoresceinyl
CS	Cockayne syndrome
GFP	green fluorescent protein
GG-NER	global genome repair
NER	nucleotide excision repair
PCNA	poliferating cell nuclear antigen
RPA	replication protein A
ssDNA	single-stranded DNA
TFIIH	transcription factor IIH
TTD	tichothiodistrophy
UbL	ubiquitin-like
UV-DDB	UV-damaged DNA-binding protein
UvrABC	UV resistant protein ABC
XP	xeroderma pigmentosum

Introduction

Cellular DNA is permanently damaged by endogenous reactive metabolites and exogenous factors. The genetic stability of the organism is maintained by several repair mechanisms (Lindahl and Wood, 1999; Hoeijmakers, 2001; Schärer, 2003; Sancar et al., 2004), among them nucleotide excision repair (NER) plays an important role. NER is the pathway that removes the most types of damages generated in DNA by exogenous agents. Endogenous damage as a rule is producing helix-distorting ("bulky") DNA lesions. Defects in NER bring to serious inherited recessive diseases, such as xeroderma pigmentosum (XR), Cockayne syndrome (CS), and tichothiodistrophy (TTD) (Bootsma et al., 1998; Berneburg and Lehmann, 2001). Patients with XP syndrome are highly sensitive to sunlight, and many of them develop skin cancer. NER is an intricate process and depends on the coordinate functions of more than 30 polypeptides (Wood et al., 2001; Gillet and Schärer, 2006). There are two repair pathways, which differ only in the step of recognition of the DNA lesion: global genome repair (GG-NER), which removes lesions from total genomic DNA, and transcription-associated repair, which is restricted to removing

lesions from the transcribed strand of active genes and is tightly associated with the function of RNA polymerase II (Sweder and Hanawalt, 1993). A stop of RNA polymerase II provides a signal for the assembly of a complex of repair proteins. In the case of GG-NER, damage recognition is the most sophisticated step. The mechanism of NER in eukaryotes understood in some detail, though many aspects of damage recognition, protein assembly and regulation remain to be elucidated. One of the limited stages for understanding of the detail NER mechanism is that the crystal structures of only a few of the NER participants have been solved so far (Min and Pavletich, 2007; Liu et al., 2008) and there is no one structure of mammalian NER proteins except of replication protein A (RPA) though in its proteolyzed form (Bochkareva et al., 2002). On the other hand it is quite dynamic machinery which is making its assembly on DNA around the damage and the crystal structure of individual proteins or their complexes with DNA is not able to provide complete information concerning the function of this machine. A study of NER system was really advanced by using confocal electron microscopy in living cell (Hoogstraten et al., 2002; Rademakers et al., 2003; Solimando et al., 2009). This analysis permitted to show the time resolved interaction of specific NER proteins with DNA damages induced by UV-light and to explain some details of spatial organization of NER system. One of the most astonishing properties of NER system is its substrate versatility while preserving selectivity for damaged DNA sequences. This property is the remarkable feature of NER in comparison for example with base excision repair (BER) system. The last process is initiated by specific DNA glycosilases, which catalyze rather selective excision of specifically modified base of DNA. The knowledge gained in the last few years in understanding of molecular mechanisms of mammalian global genome repair was summarized in the recent reviews (Gillet and Schärer, 2006; Maillard et al., 2008; Sugasawa, 2008; Shuck et al., 2008). The purpose of this review is to summarize the most central points in the damage recognition and assembly of NER machine for the following study and to show the contribution of affinity labeling techniques to understand mechanism of nucleotide excision repair.

The Order of Protein Assembly in GG-NER

The molecular mechanism of GG-NER is studied for the few decades and in general it is well understood. The removal of a lesion is a sequential process and includes the following steps (Fig. 1): (i) lesion recognition and unwinding of DNA duplex around the damaged area; (ii) cleavage of the damaged DNA strand at the 3′- and 5′-ends of the lesion; (iii) gap filling and ligation of resulting nick. GG-NER was reconstructed in vitro (Aboussekhra et al., 1995; Mu et al., 1995; Araujo et al., 2000). Six core repair factors proved to be necessary and sufficient for repairing most lesions: XPC-hHR23B, XPA, RPA, TFIIH, XPG, and XPF-ERCC1. In addition, the UV-damaged DNA-binding protein (UV-DDB), plays a role in the recognition of certain adducts, particularly cyclobutane pyrimidine dimers (CPD) (Wakasugi et al., 2001).

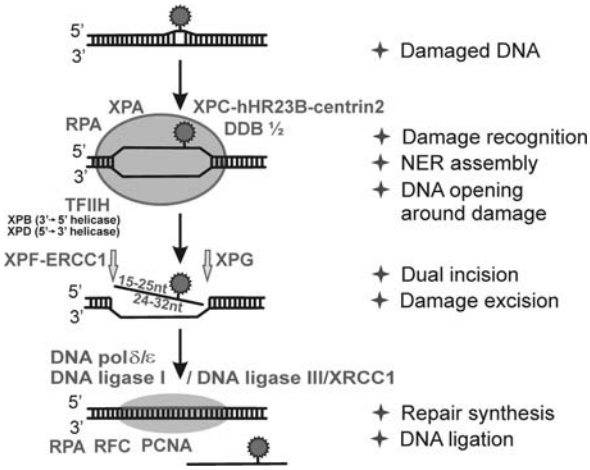

Fig. 1 Global genome nucleotide excision repair (GG-NER)

The mechanism of initial DNA lesion recognition in the GG-NER is still a matter of debate, mainly raised by the fact that none of the core proteins displays a high enough specificity to function as a unique sensor of damage. Three models were proposed for NER complex assembly on damaged DNA: a repairosome model, a concerted binding model, and a sequential binding model.

The repairosome model suggests that the cell contains a repairosome, a complex of six or more proteins that continuously scans the genome for lesions. The model was supported by copurification of some repair factors from yeast and human cell extracts (Mu et al., 1996; Sancar, 1996; Kong and Svejstrup, 2002). The model obviates the problem of a rapid and well-concerted assembly of many proteins at a damaged site. However, the model is considered unfeasible now for several reasons. First, the repair factors copurified from cell extracts readily dissociate under sufficiently mild conditions (Mu et al., 1996; Sancar, 1996; Wood, 1996). Second, it was impossible to reconstruct a stable preincision complex containing both XPC-hHR23B and XPG in vitro (Wakasugi and Sancar, 1998, 1999). Third, a functional repairosome was estimated to be about 3 MDa (Houtsmuller et al., 1999), which hinders genome scanning for lesions at the chromatin level and disagrees with the velocity of diffusion of fluorescently labeled proteins to a damaged site in living cell (Hoogstraten et al., 2002; Rademakers et al., 2003).

The concerted binding model suggests that proteins moderately specific for damaged DNA – XPC-hHR23B, XPA, and RPA – form a ternary complex whose specificity for damaged DNA is elevated owing to additive effects. It is known that XPA and RPA form a tight complex whose affinity for damaged DNA is higher than the affinity of either individual factor. Gel retardation assays revealed the XPC-RPA-DNA and XPA-RPA-DNA complexes, but not XPC-XPA-RPA-DNA (He et al., 1995; Li et al., 1995; Krasikova et al., 2008). Footprinting revealed a stimulating effect of XPA, RPA, and, especially, XPA-RPA on DNA cleavage in

complex with XPC (Wakasugi and Sancar, 1999). However, these findings do not necessarily imply the formation of a common DNA-protein complex. The concerted binding model cannot be totally rejected but requires further experimental verification.

The sequential binding model suggests a certain order in which the repair factors bind to damaged DNA. The model was recently supported by the results of experiments with fluorescently labeled proteins in living cell. The green fluorescent protein (GFP), used as a label, was covalently bound to a repair factor (Hoogstraten et al., 2002; Rademakers et al., 2003; Hoogstraten et al., 2008). GFP-XPC, GFP-ERCC1, GFP-XPB, and GFP-XPA freely moved in the nuclei of intact cells and migrated to a damaged site and were temporally immobilized on damaged DNA in UV-irradiated cells. The diffusion coefficients of these proteins were directly proportional to their molecular weights, which disagrees with the presumable existence of a large repairosome in the cell. It is possible that individual proteins form larger complexes at the damaged site. The complexes may be dynamic and unstable, which prevents their detection with known methods.

Advantages of Sequential Assembly of the Repair Complex

Preassembly of proteins into a repairosome is convenient, as repair can be started at any moment. However, dynamic dissociation and association of proteins increases the number of possible combinations, making the system more flexible and multifunctional. Proteins involved in other DNA metabolic pathways can rapidly switch between the two or more pathways. It is known that all NER factors are involved in other processes as well, including transcription (TFIIH and CSB), recombination repair (ERCC1-XPF), base excision repair (XPC and XPG), replication, homologous recombination, and checkpoint-dependent signalling (RPA). RPA possesses domains binding with many other proteins involved in various repair pathways, suggesting competitive interactions with such proteins rather than a preexistence of many complexes specific to each process (Fanning et al., 2006; Pestryakov and Lavrik, 2008).

Models of DNA Damage Recognition

The sequential binding model implies that certain proteins are responsible for recognizing a lesion and recruiting other repair proteins to the damaged site. It is still unclear which of the NER factors the first to recognize a lesion is. As candidates for this role, the proteins with higher affinity to damaged than to intact DNA are regarding: XPC-hHR23B, XPA, UV-DDB, and RPA. The subunit compositions and main properties of these repair factors are summarized in the Table 1. The GG-NER system recognizes a lesion in the presence of a vast excess of intact DNA, and none of the above factors has sufficient affinity for a lesion to ensure this (Hey et al., 2002). The required specificity is probably achieved in a repair complex via cooperative

Table 1 Protein factors involved in DNA damage recognition during GG-NER

Protein factor	Subunites	Properties, activity	Function in GG-NER
XPC-hHR23B-Cen2	XPC (p125)	DNA binding	Initial damage recognition
	HR23B (p58)		
	Centrin2 (p18)	Ca^{2+}-binding protein	
UV-DDB	DDB1 (p125)	DNA binding	Recognition of some lesions (CPD)
	DDB2 (p48)		Chromatin remodeling
XPA	XPA (p36)	Contains a Zn^{2+} finger	Complex assembly
		DNA binding	Recognition and/or verification of lesions?
RPA	RPA70 (p70)	Contains a Zn^{2+} finger	Stabilization of ssDNA regions
		DNA binding	Damage recognition?
	RPA32 (p32)	DNA binding	
	RPA14 (p14)	Heterotrimer assembly	
		DNA binding	
TFIIH	XPB (p89)	DNA-dependent ATPase, $3' \rightarrow 5'$ helicase	DNA duplex unwinding Complex assembly Lesion detection
	XPD (p80)	DNA-dependent ATPase, $5' \rightarrow 3'$ helicase	
	p62	Core subunit	
	p52	Core subunit	
	p44		
	p34		
	CDK7 (p40)	Protein kinase	
	Cyclin H (p36)		
	MAT1 (p32)		
	p8		

effects. Two main models of damage recognition have been proposed: “XPC first” and “XPA first”.

In the “XPC first” model, XPC-hHR23B regards as the primary sensor of lesion that initiates the NER pathway by recruiting TFIIH and other factors (Sugasawa et al., 1998; Batty et al., 2000; Sugasawa et al., 2002). Experiments addressing the order of repair factor assembly on damaged DNA revealed that a lesion-containing plasmid preincubated with XPC-hHR23B is more quickly repaired in a cell extract than a plasmid preincubated with the XPA-RPA complex (Sugasawa et al., 1998). This finding agrees with a model that XPA-RPA is not involved in primary damage recognition, but plays a role in further steps, helping TFIIH to unwind the DNA duplex (Evans et al., 1997). Further studies showed that the recruiting of XPC-hHR23B and TFIIH to a lesion does not depend on ATP, while ATP is required for XPA recruiting (Riedl et al., 2003); i.e., XPA is not included in the preincision repair

complex until the duplex is opened by TFIIH in the presence of ATP. However, there is evidence that damaged DNA duplexes preincubated with XPA-RPA are more quickly repaired than substrates preincubated with XPC-hHR23B (Wakasugi and Sancar, 1999). Based on this finding, XPA was assumed to ensure the primary recognition of certain DNA lesions, therefore the "XPA first" model can not be refused completely.

Studies with a method of local UV irradiation of cells in combination with a labeling with fluorescent antibodies showed that the XPC-hHR23B complex recognizing a lesion is especially important for the involvement of other NER factors in repair (Volker et al., 2001). This approach permits to analyze dynamics of protein loading to the DNA damage. XPC-hHR23B accumulates on damaged DNA in the cell regardless of whether XPA is present. At the same time, XPA migrates to a lesion only in the presence of XPC-hHR23B. XPC-hHR23B, but not XPA, is essential for TFIIH binding to a photoinduced lesion. Further experiments with fluorescently labeled XPA (GFP-XPA) demonstrated that the involvement of RPA and XPG in the repair complex precedes the XPA involvement and is independent of XPA (Rademakers et al., 2003). Study of the dynamics of GFP-tagged XPC using photobleaching techniques showed that XPC exhibited a distinct dynamic behavior and subnuclear distribution compared with other NER factors (Hoogstraten et al., 2008). Moreover, the authors proposed a novel regulatory mechanism for XPC. Under unchallenged conditions, XPC is continuously exported from and imported into the nucleus, which is impeded when NER lesions are present. XPC is omnipresent in the nucleus, allowing a quick response to genotoxic stress.

One more model, the "RPA first", was advanced to explain the results of protein modification with a DNA duplex containing a psoralene adduct (Reardon and Sancar, 2002). Only two RPA subunits (RPA70 and RPA32) and one TFIIH subunit (XPD) were modified in a NER system reconstructed in vitro, while contacts of XPC and XPA with the psoralene residues were not detected. The result suggests a key role or RPA in damage recognition, but can also be explained by an RPA binding to the intact DNA strand. In the latter case, RPA can be close to the psoralene residue and is accessible for modification. In addition, it is possible that XPC and XPA lack proper acceptor amino acids in the sites contacting the psoralene residue in DNA. Another interpretation assumes that there is no strict order of repair complex assembly, in particular, no universal protein to recognize a lesion. A model advanced in line with this interpretation implies that the lesion is initially recognized by any of the three proteins: XPA, RPA, or XPC. The cooperative effect of XPA (which is capable of RPA and TFIIH binding), RPA, and XPC (capable of TFIIH binding) leads to the formation of a quaternary complex on damaged DNA. At the next step, the specificity of the complex is kinetically verified on the basis of the helicase activity of TFIIH: the reaction is terminated when the complex is nonspecific or stimulated when the complex is specific. This is a universal mechanism of DNA scanning for lesions, allowing for the formation of an efficient repair complex for DNA lesions of any type (Reardon and Sancar, 2003; Maltseva et al., 2006; Kesseler et al., 2007; Krasikova et al., 2008).

XPC-hHR23B Complex as a Potential Sensor of Helix Distortion

XPC (125 kDa) forms a stable ternary complex with hHR23B, a homologue of yeast Rad23 (van der Spek et al., 1996), and centrin 2 (Araki et al., 2001). The interaction of XPC with hHR23B involves the evolutionarily conserved C-terminal domain of XPC. It is known that hHR23B stabilizes XPC (Ng et al., 2003), in particular, protects XPC from proteolytic degradation. In vitro, hHR23B stimulates the XPC activity in NER (Sugasawa et al., 1996). The XPC-binding domain (56 amino acid residues) of hHR23B is sufficient for the effect (Masutani et al., 1997). Human cells have two Rad23 homologues, hHR23A (50 kDa) and hHR23B (58 kDa), which have 57% identity and 76% similarity on the protein level (van der Spek et al., 1996). The two proteins are each capable of forming a stable complex with XPC and equally stimulate its DNA repair activity in cell extracts and a reconstructed system (Sugasawa et al., 1997). However, the total amount of XPC is associated with HR23B in human cells (van der Spek et al., 1996).

Each of the two homologues, hHR23B and hHR23A, has an ubiquitin-like (UbL) domain at the N terminus and two ubiquitin-binding domains (UBA1 and UBA2) (van der Spek et al., 1996; Masutani et al., 1997). Ubiquitin is a highly conserved polypeptide (76 amino acid residues) found in all eukaryotes. A special ubiquitin ligase system covalently attaches one or more ubiquitin molecules to an acceptor protein, which is consequently involved in various processes such as protein degradation and translocation, DNA repair, and cell cycle control. It was recently found that hHR23B inhibits XPC ubiquitination, thereby protecting XPC from ubiquitin-dependent degradation (Sugasawa et al., 2005).

The third protein partner centrin 2 is a small acid Ca^{2+}- binding protein of 18 kDa. Centrin 2 is involved in the cell cycle control, in particular, the localization of basal bodies and division of microtubules. Centrin 2 is not essential for DNA repair in vitro, but increases the affinity of the XPC-hHR23B dimer for damaged DNA and stimulates the excision of lesions in cell extracts (Nishi et al., 2005).

Jumping back and forth: XPC-hHR23B has high affinity for certain lesions such as (6-4)-photoproducts and acetylaminofluorene adducts (Sugasawa et al., 1998; Batty et al., 2000; Kusumoto et al., 2001). Purified XPC binds to single- and double-stranded DNA and sustains the excision of a lesion in the absence of hHR23B, which lacks DNA-binding activity (Sugasawa et al., 1996; Reardon et al., 1996; van der Spek et al., 1996). A key role in XPC-hHR23B binding to DNA is played by damage-induced destabilization of the DNA double helix rather than by damage itself (Sugasawa et al., 2001; Buterin et al., 2005). For instance, XPC-hHR23B efficiently interacts with DNA containing short mismatched regions regardless of whether a lesion is present (Sugasawa et al., 2002). XPC also displays a strong preference for single-stranded over double-stranded oligonucleotide (Maillard et al., 2007b). This observation agrees with the presence of a putative single-stranded DNA-binding motif discovered in XPC (Maillard et al., 2007b). Furthermore, XPC exhibits more efficient binding to undamaged single-stranded oligonucleotides compared to damaged ones. These findings are consistent with an indirect mode of bulky lesion recognition that proposes the XPC-hHR23B final localization not on

the damage but on the undamaged strand opposite to the damage. Further support for this model of DNA damage recognition has been provided by the crystal structure of a complex of yeast orthologue XPC-hHR23B, Rad4-Rad23, with a fragment of damaged DNA bearing CPD lesion within a three base-pair mismatch (Min and Pavletich, 2007). Based on these and other data it was suggested that XPC is sensitive to the abnormal strand fluctuations. This protein is likely able to probe the thermodynamic property of nucleic acid substrates without direct sensing of lesion (Blagoev et al., 2006; Maillard et al., 2007a, 2008). Scanning microscopy revealed that XPC-hHR23B binding induces a DNA bend (Janicijevic et al., 2003). The resulting structure probably provides a signal for recruiting other repair factors, in particular, TFIIH, which interact with XPC through its C-terminal domain (Yokoi et al., 2000).

Interaction of the XPC protein with abnormally oscillating damaged DNA may be used as universal property to recognize different DNA lesions. For example, some of these lesions, such as 5-formyluridine, can be repaired by BER and NER pathways (Kino et al., 2004). It was shown that XPC facilitates recognition of base lesion by various DNA glycosylases (Miao et al., 2000; Shimizu et al., 2003; D'Errico et al., 2006). Therefore, this protein can recruit proteins involved in base excision repair or into other repair pathways (Maillard et al., 2008).

Role of UV-DDB in DNA Damage Recognition

The UV-damaged DNA-binding protein (UV-DDB) was discovered as a protein demonstrating preference affinity for UV-irradiated DNA. This protein binds with high affinity to (6-4)-photoproducts and also interacts with DNA duplexes containing CPDs (Fitch et al., 2003b) while XPC does not show this interaction. UV-DDB is heterodimer protein consisting of two subunits, DDB1 (127 kDa) and DDB2 (48 kDa) (Keeney et al., 1993). Mutations of the small DDB2 subunit distort CPD repair and lead to group E xeroderma pigmentosum (XP-E) syndrome (Rapic Otrin et al., 2003). Although DNA repair is possible in the absence of UV-DDB in vitro, ample experimental evidence testifies that UV-DDB is necessary for DNA repair, especially in the case of CPD. UV-DDB binding to DNA bends DNA by 55° (Fujiwara et al., 1999). This finding supports the hypothesis that UV-DDB plays an accessory role in recognizing the lesions that have no effect on the DNA double helix and, consequently, are poorly recognized by other repair factors. UV-DDB efficiently recognizes such lesions and binds to DNA to bend it. DNA is thereby destabilized, and the resulting distortion is easily recognized by other proteins, e.g. XPC-hHR23B (Fitch et al., 2003b). XPC and UV-DDB interact physically (Sugasawa et al., 2005) and UV-DDB promotes recruitment of XPC to UV-damaged sites in vivo as was shown by using local UV irradiation technique (Fitch et al., 2003b; Wang et al., 2004; Moser et al., 2005).

Another possible role of UV-DDB is associated with chromatin remodeling. UV-DDB tightly binds to chromatin soon after UV irradiation (Wakasugi et al.,

2002). DDB1 and DDB2 form a complex with ubiquitin ligase components in UV-irradiated cells (Groisman et al., 2003). Presumably, UV-DDB plays a role in modifying histones and other proteins in the vicinity of a lesion. Such a modification changes the chromatin structure so that the lesion becomes accessible for other repair factors, in particular, XPC-hHR23B (Fitch et al., 2003b).

DDB2 can be ubiquitinated. Ubiquitination provides a signal for ubiquitin-dependent degradation of DDB2 soon after UV irradiation (Rapic Otrin et al., 2002; Fitch et al., 2003a). XPC is also a target for ubiquitin ligase associated with UV-DDB, but XPC ubiquitination is reversible and does not lead to degradation, unlike that of DDB2 (Sugasawa et al., 2005). Moreover, DDB2 ubiquitylation in vitro leads to UV-DDB loss of the damaged DNA-binding activity, whereas ubiquitylated XPC still retains its DNA-binding capacity. Based on these findings, Sugasawa and co-authors proposed that ubiquitylation may assist UV-DDB dissociation from the lesion, thereby promoting the lesion transfer from UV-DDB to XPC and the subsequent initiation of NER (Sugasawa et al., 2005). Ubiquitin ligase associated with UV-DDB also induces ubiquitylation of histones, suggesting its roles in chromatin remodeling around the sites where repair occurs (Kapetanaki et al., 2006; Wang et al., 2006). However, in summary, the role of UV-DDB in damage recognition in NER and contribution of both subunits of heterodimer is still far from understanding.

Roles of XPA and RPA in NER

XPA is a small (calculated molecular mass is 31 kDa) Zn-binding protein that is capable to form homodimers in solution (Yang et al., 2002) whereas in vivo studies demonstrate XPA diffusion rather in monomeric form (Rademakers et al., 2003). XPA interacts with various repair factors: RPA (Li et al., 1995), ERCC1 (Li et al., 1994), and TFIIH (Park et al., 1995). RPA is a stable heterotrimer of three subunits: RPA70 (70 kDa), RPA32 (32 kDa), and RPA14 (14 kDa) (for more details see (Nasheuer et al., 2002, 2007; Wold, 1997) and Broderick et al., in this book). The main function of RPA is to stabilize single-stranded DNA (ssDNA) and to physically interact with other proteins involved in DNA metabolism. RPA is the most abundant ssDNA-binding protein in human cells. It is known that RPA is involved in all main processes of DNA metabolism, including replication, repair, and homologous recombination (Nasheuer et al., 2002, 2007; Wold, 1997). The mode of the RPA-ssDNA interaction is regulated by local RPA concentration at ssDNA region. RPA binds to ssDNA platform of 8 or 30 nt (Blackwell et al., 1996). In the latter case, the size of the binding site is approximately the same as the size of the open DNA duplex produced during NER.

Both XPA and RPA have higher affinity for alterations of the DNA double helix such as mismatches, bubbles, and small loops (Missura et al., 2001). These proteins were found to cooperate in NER. Indeed, the affinity of the XPA-hRPA complex to the damaged DNA is more than one order of magnitude higher in comparison to that

of hRPA alone (Schweizer et al., 1999), RPA stabilizes the XPA binding to DNA as well (Wang et al., 2000). However, the affinity of the XPA-RPA complex for damaged DNA is one order of magnitude lower than that of XPC-hHR23B (Hey et al., 2001, 2002). These proteins are presumably involved in verification of a lesion. XPA was found to prefer cruciform DNA duplexes (Missura et al., 2001; Camenisch et al., 2006). Furthermore, XPA displays high affinity for ds-ssDNA junctions with 3′- and/or 5′-ssDNA overhangs, including the junctions with either a 3′- or 5′-ssDNA branch (Yang et al., 2006; Maltseva et al., 2006) and Y-structure junction (Yang et al., 2006). This finding made it possible to assume that XPA recognizes the DNA bends arising during DNA damage and repair. For a long time XPA functions were completely attributed to NER. However, several recent studies suggested that XPA may play additional roles in cellular DNA damage responses via its phosphorylation and possible involvement in DNA damage checkpoints (Bomgarden et al., 2006; Wu et al., 2006; 2007).

As discussed above, RPA recognizes and stabilizes the DNA intermediates containing ssDNA regions. The open DNA duplex, which is formed as DNA is unwound in the damaged site, has both of these structural elements (DNA bends and ssDNA regions) and, consequently, can be efficiently recognized by the RPA-XPA complex. In this context, RPA and XPA play a structural role and ensure a proper three-dimensional structure of the DNA intermediate before the excision of a lesion, rather than being involved in DNA damage recognition. As was shown recently (Coin et al., 2008) using reconstituted in vitro NER assay, XPA catalyzes the rearrangement of TFIIH subunit composition together with the arrival of the downstream NER factors, which promote the incision/excision of the damaged oligonucleotide. Therefore, XPA is important at the different stage of NER process.

It is known that RPA binds to ssDNA in a polar manner (de Laat et al., 1998b; Lavrik et al., 1998; Kolpashchikov et al., 2001; Fanning et al., 2006; Pestryakov and Lavrik, 2008). The polarity is due to a consecutive binding of DNA binding domains DBD-A, DBD-B, DBD-C of RPA70 and DBD-D of RPA32 to ssDNA in the direction from 5′ to 3′. The binding polarity determines the orientation of RPA subunits on ssDNA. RPA70 is initially positioned at the 5′ end of a ssDNA region, and then its DNA binding domains (A, B, and C) are spread toward the 3′ end. The orientation of RPA32 (DBD- D) at the 3′ end depends on the size of the ssDNA region, which modulates the conformational changes of RPA (Kolpashchikov et al., 1999, 2001; Lavrik et al., 1999; Pestryakov et al., 2003). This mode of RPA binding can be realized when one RPA molecule is bound to ssDNA in an extended conformation interacting with ssDNA of 30 nt in length. The 14-kDa subunit of RPA (RPA14) contains only one domain; this OB-fold domain (DBD E) appears to be primarily a structural component of the RPA complex (Henricksen et al., 1994) though contacts between it and ssDNA have recently been detected (Perstryakov et al., 2007; Salas et al., 2009). Therefore, RPA14 possesses DNA binding activity. It is also known that this subunit is indispensable for correct position of RPA on ssDNA and consequently RPA14 is essential to ensure a polar RPA binding (Weisshart et al., 2004). Another kind of RPA binding to ssDNA is tight interaction of several RPA molecules with the 8–10 nt binding platform for each molecule. RPA binds 8–10 nt of DNA

when the protein has globular conformation which is not able to provide "polarity" in its binding to DNA and therefore if necessary asymmetry of DNA-protein complex. Therefore, extended RPA conformation seems the most appropriate to form NER preincision complex. The RPA polarity is probably significant in the preincision NER complex for the positioning on DNA and stimulation of repair nucleases XPG and EXCC1-XPF (de Laat et al., 1998a, b).

In the open DNA complex, RPA is predominantly associated with the non-damaged strand and protects it from endonuclease attack (de Laat et al., 1998a; Hermanson-Miller and Turchi, 2002). NMR analysis of the binding of short DNA fragments with purified proteins showed that XPA increases the preferential binding of RPA with intact DNA (Lee et al., 2003). In addition, XPA suppresses the DNA-unwinding activity of RPA (Patrick and Turchi, 2002). Unlike the majority of other repair factors, RPA remains bound to DNA after the lesion is excised, stabilizing the single-strand gap and recruiting RFC and PCNA (Gomes and Burgers, 2001) for the next NER step, gap filling DNA synthesis.

Photoreactive DNA Intermediates as a Tool to Study NER Assembly

NER is an intricate multistep process that is driven by a complex of proteins and is regulated by numerous protein-protein and DNA-protein interactions. In view of the intricate and dynamic character of the process, it is necessary to develop new approaches to detection of transition states and unstable intermediate complexes arising in the course of repair. One of such approaches is affinity modification with chemically reactive DNA repair intermediates (Khodyreva and Lavrik, 2005; Dellavecchia et al., 2004; Schweizer et al., 1999). This approach is able to follow to the architecture of intermediates DNA structures formed in NER process as well as to clarify outstanding question of this process concerning a role of individual polypeptides of this protein machine at various NER stages.

Verification of Photoreactive dNMP Analogues as NER Substrates

One of the most astonishing characteristics of the NER pathway is its extraordinarily wide substrate specificity, being able to recognize and repair a large number of structurally unrelated lesions (Gillet and Schärer, 2006). Bulky photoreactive nucleotide analogues introduced into DNA were demonstrated to be substrates for the bacterial NER system and were recognized and processed by UvrABC proteins (DellaVecchia et al., 2004). Using photoreactive DNA duplexes containing arylazido-modified nucleotides and UvrB mutants, the mechanism of the transfer of damaged DNA from UvrA to UvrB, prior to the incision by UvrC, was studied. These data suggested that the transfer of damaged DNA from UvrA to UvrB proceeds in a three-step process: (1) UvrA and UvrB bind to the damaged site, with

UvrA in direct contact to the DNA; (2) a transfer reaction to UvrB occurs with UvrB contacting mostly the non-damaged DNA strand; (3) lesion engagement of the damage recognition pocket of UvrB takes place with the concomitant release of UvrA. Following this results, it was suggested that dNMP moiety carrying photoreactive bulky substituents modulates a damage of DNA duplex and creates a target to recognize by mammalian NER system (Maltseva et al., 2007) and it was tested whether oligonucleotides containing bulky arylazido substituents serve as NER substrates to further analyze loading of NER factors on damaged DNA (Fig. 2).

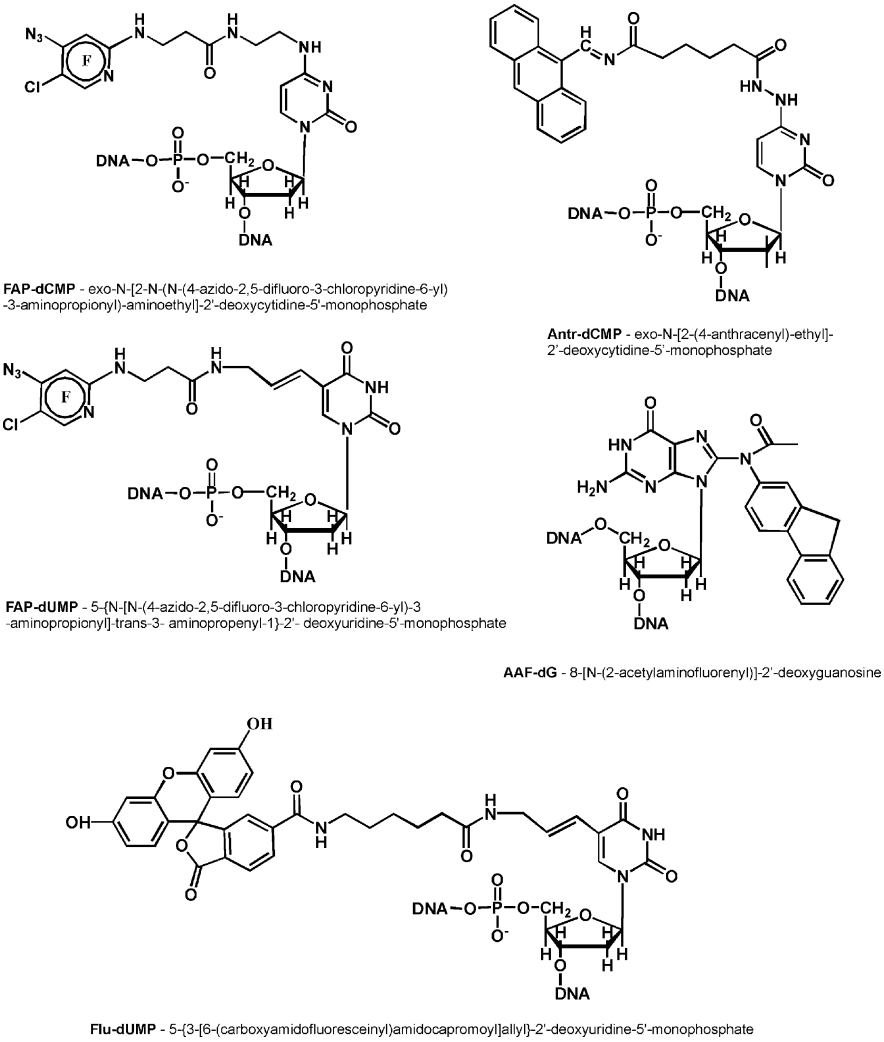

Fig. 2 Nucleotide analogues mimicking the bulky lesions processed by NER

To determine whether base-substituted arylazido derivatives of dU and dC are recognized and excised by the NER machinery, oligonucleotides containing these adducts were incorporated into a plasmid (Maltseva et al., 2007) and NER repair was investigated using HeLa whole cell extracts with the acetylaminofluorene adduct of dG (AAF-dG, see Fig. 2 for structure) (Heflich and Neft, 1994) for comparison. The 5′-phosphorylated oligomer containing FAP-dCMP or FAP-dUMP residue was annealed with single-stranded plasmid DNA and closed circular plasmids were produced, purified and incubated with HeLa whole cell extracts (Shivji et al., 1999; Gillet et al., 2005). After detection of the excised oligonucleotides containing the lesion, the resulting excision pattern indicated that FAP-dCMP and FAP-dUMP were processed by the NER machinery albeit with lower efficiency than AAF-dG (Fig. 3).

The structural properties of NER substrates probably play a key role in damage recognition and removal: the efficiency of DNA repair can vary over several orders of magnitude and there appears to be a general correlation between the efficiency of repair of a given lesion and the amount of helical distortion it causes (Gunz et al., 1996; Geacintov et al., 2002; Mocquet et al., 2007). Incorporation of a photoreactive group via the exocyclic amino group of cytosine (e.g. FAP-dCTP), in contrast to the C5 position of both cytosine and uracyl (e.g. FAP-dUTP), might interfere with the formation of the canonical Watson–Crick hydrogen bonds and cause partial dsDNA destabilization thereby enhancing the efficiency of processing by NER. Based on this presumption it was expected that FAP-dCMP should be repaired more effectively than FAP-dUMP. Surprisingly, FAP-dUMP containing DNA was processed more efficiently by cell extracts than FAP-dCMP. To increase

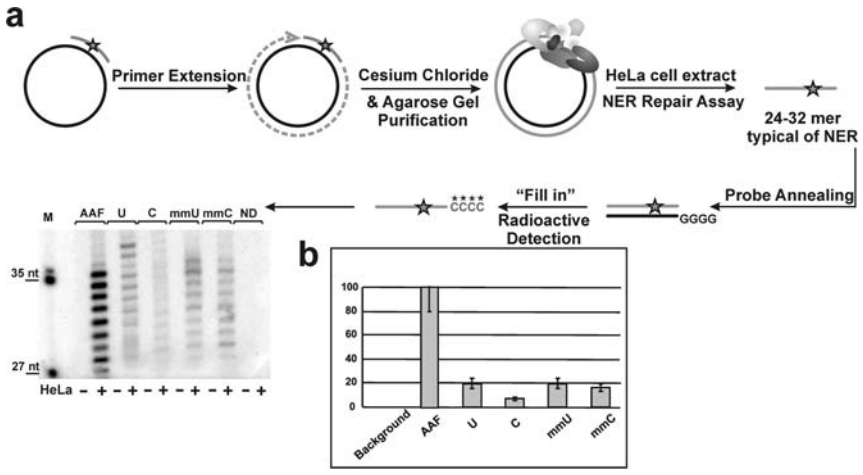

Fig. 3 Photoreactive arylazido derivatives of dNMP when introduced into DNA are processed by the NER system. Scheme of preparation of photoreactive plasmid DNA and repair pattern of the photoreactive dNMP residues upon in vitro NER assay (**a**). Integrated radiointensity of the excised fragments (normalized on dG-AAF) (**b**)

duplex DNA distortions, photoreactive nucleotides were introduced into the central position of a small bubble (three mispaired nucleotides) of identical sequences. This distortion led to the intensification of damage processing in the case of FAP-dCMP, but did not visibly influence on FAP-dUMP processing. It was shown that substrates containing three base mismatches without covalent modification were not processed by the NER complex, although they were bound by the XPC-hHR23B protein, the initial damage recognition factor in NER (Buterin et al., 2005). Consequently, both FAP-dCMP and FAP-dUMP residues are mimics of NER targets.

Crosslinking of XPC-hHR23B to Photoreactive DNA is Moderated by XPA and RPA

Photocrosslinking has served to determine how the initial DNA damage recognition factor in NER, the heterodimeric protein complex XPC-hHR23B, interacts with damaged DNA. Oligonucleotides bearing photoreactive FAP-dCMP or FAP-dUMP in ssDNA form (ssC, ssU) paired to complementary oligonucleotides (dsC, dsU) fully base-paired and in the context of a three nucleotide mismatch (mmC, mmU) were incubated with XPC-hHR23B and irradiated with UV light. Crosslinking of the XPC-hHR23B complex with all photoreactive oligomers results in modification of the XPC (p125) subunit that was confirmed by Western blot analysis but DNA-protein adducts of the hHR23B (p58) polypeptide were not observed. Photocrosslinking and DNA binding efficiency of XPC decreased in the presence of Mg^{2+} and depended on the photoreactive dNMP analogue and on the structure of DNA probe. The most efficient crosslinking was observed with ssDNA. mmC was a more effectively crosslinked to XPC than dsC whereas mmU and dsU displayed the same crosslinking yields. This result correlates with the efficiency of the excision reaction processed by the NER system with damaged DNA in HeLa cell extract, in agreement with the notion that the NER repair rate for a given lesion correlates with the binding efficiency to the initial damage recognition factor XPC-hHR23B. Therefore, in the case of bulky arylazido group attached to the base via long linker, the interaction of XPC with these groups has been detected.

Damage recognition in the NER process is coordinated by numerous protein-protein and protein-DNA interactions. To analyze mutual interactions of XPC-hHR23B and one of the other NER factors – XPA or RPA – with damaged DNA two proteins were added simultaneously to avoid an influence of mixing order on final complex formation of proteins with DNA. XPA was shown to stimulate slightly the XPC labeling in the presence of Mg^{2+}, whereas in the absence of Mg^{2+} no influence of XPA on photocrosslinking of XPC was detected. Mutual effects of XPC and RPA were more complicated than those of XPC and XPA, and depend on the structure of the DNA and the presence of Mg^{2+}. As expected, RPA replaced XPC on ssDNA regardless of the presence of Mg^{2+}. Despite its very low affinity for dsDNA, RPA stimulated XPC crosslinking to this DNA in a Mg^{2+}-dependent manner: in the absence of Mg^{2+} stimulation was significantly higher than in the presence of

Mg^{2+}, resulting in more than two fold increase of XPC crosslinking to photoreactive lesions. Both proteins crosslinked more efficiently to mmC DNA than to dsC duplex. In the absence of Mg^{2+} there was no visible influence of one protein on crosslinking of other one to mmC DNA. In the presence of Mg^{2+}, mutual inhibition of both proteins labeling by mmC DNA was observed: the photocrosslinking yields of XPC and RPA decreased when both proteins were present in the reaction mixture in comparison to each protein individually. The capacity of RPA to affect XPC interaction with damaged DNA depended on the DNA structure and Mg^{2+} concentration could reflect diverse functions of RPA at the different stages of the NER process.

Undamaged Strands are Strongly Required for XPC-hHR23B Crosslinking to Damaged DNA Duplexes

Photoreactive nucleotides crosslinked with proteins allow the analysis for changes in the topography at the site of DNA damage. Therefore, photoreactive arylazido or thio group in one of the strands of DNA duplex with bulky anthracene (Antr) group in the opposite DNA strand (see Fig. 2 for structure) were combined to analyze contacts of XPC-hHR23B, XPA, and RPA in cases of damage on one DNA strand and damages on both strands of the DNA duplex (Maltseva et al., 2008). The 48-mer oligonucleotiodes contained photoreactive FAP-dCMP or ^{4}S-dUMP within the DNA strand.

The photocrosslinking efficiency was dependent on the kind of photoreactive dNMP residue and on the structure of the DNA probes. For all proteins investigated, the most efficient crosslinking was determined in the case of ssDNA and for the DNA duplex containing FAP-dCMP residue in one of the strands. Crosslinking efficiency was much lower for DNA duplex containing ^{4}S-dUMP in one of the DNA strands, most probably, because ^{4}S-dUMP is not distorting the DNA helix, therefore ^{4}S-dUMP containing DNA duplex mimics undamaged DNA. The yield in photocrosslinked XPC with the thio group in the undamaged strand was slightly increased when bulky anthracenyl residue was located in the opposite strand and ^{4}S-dUMP was mispaired. Insertion of bulky anthracene group in DNA results in the helix distortion although in less extent than for FAP group (Petruseva et al., 2008). The results on crosslinking of XPC-hHR23B to FAP-dCMP containing 48-mers were similar to those reported for 60-mers (Maltseva et al., 2007). It means that the length of the model DNA duplex has no or not much influence in this range on the interaction with XPC.

The yield of photocrosslinking products was decreased dramatically when the anthracene group was located at the position opposite to FAP-dCMP. According to gel shift experiments, binding affinities of the XPC-hHR23B complex to both doubly modified duplex and dsDNA containing the FAP-damage were similar, and therefore, it was concluded that the crosslinking efficiency was sensitive to

geometry at the site of the DNA-protein complex containing the juxtapositioned damages. Similarly, it has been shown by the Naegeli laboratory that in comparison to singly damaged duplexes, NER activity in HeLa cell extract toward doubly damaged dsDNA substrates was strongly inhibited (Buterin et al., 2005). The idea of XPC binding via a short open ssDNA stretch opposite to the lesion has also been formulated (Maillard et al., 2007b). This fact was directly confirmed with the crystal structure of the yeast XPC orthologue Rad4 bound to 24-mer DNA duplex containing a CPD lesion within a stretch of three nucleotide mismatches (Min and Pavletich, 2007). In the structure resolved, Rad4 was found to bind to DNA in two parts. The first one contains the N-terminal α/β domain and a beta-hairpin domain (BHD1) and binds undamaged 11-mer fragment of dsDNA. Two additional BHD domains (BHD2/3) bind the DNA around the CPD lesion. The hairpin of the BHD3 domain inserts into the DNA helix through the major groove, expelling the two residues containing the CPD lesion. Rad4 does not directly interact with the CPD lesion, which is disordered in the crystal structure. The protein instead specifically interacts with the two nucleotides opposite the CPD in the undamaged strand through a groove formed by the BHD2 and BHD3 domains. The authors propose that Rad4 binds damaged DNA by an induced fit mechanism that could probe the DNA helix for its propensity to adopt an open conformation that allows binding of unpaired nucleotides by the BHD2/3 domains. This suggestion is in a good agreement with the multistep recognition model for NER, which postulates that lesions which thermodynamically destabilize duplex DNA are favored substrates for NER (Geacintov et al., 2002). On the other hand the X-ray structure of the Rad4-Rad3 protein complex with DNA fragment shows the only one, probably, the most stable configuration of this structure. However, the dynamic mechanism of damage recognition by XPC remains unclear and demands analysis by various techniques in solution. The overall result on photocrosslinking of the XPC-hHR23B recognition dimer to damaged DNA shows that the method is appropriate to follow the dynamic architecture of DNA-protein complex formation, and, that it is sensitive to structural variations at the DNA damage site.

Both RPA and XPA crosslinked to ssDNA more efficiently than to dsDNA. FAP-dCMP containing DNAs were more efficient probes than the ones containing ^{4}S-dUMP. Duplexes containing ^{4}S-dUMP crosslinked to XPA with extremely low yields. Interestingly that DNA duplex with the bulky substituents FAP and Antr in both strands crosslinked to XPA more efficiently than DNA containing only the FAP residue. This was opposite to the results when RPA was the crosslinking target. Based on the results of EMSA, both XPA and RPA bind with higher affinities to the duplex with one bulky group than to the duplex with two bulky groups. The difference in crosslinking efficiency of XPA and RPA with these DNAs suggests an effect of the protein-DNA complex topography, which is different for duplexes with one or two bulky groups. As in the case of XPC-hHR23B, the overall results show that recognition complexes of damaged DNA with protein factors XPA and RPA can be analysed by photocrosslinking, and that the efficiency of crosslinking reflects spatial differences at the site of DNA damage.

Localization of NER Factors on Undamaged Strand of Damaged DNA Duplex

Use of lesion-imitating photoreactive groups allows covalent fixation of the polypeptide in direct contact with photoreactive DNA damage, though in the case of distant linker carrying photoreactive group, the protein bound to the opposite DNA strand may be also modified. To detect what protein factors of the NER system are in contact with the undamaged DNA strand in the course of damage recognition, DNA duplexes bearing 5I-dUMP in the different position of undamaged strand and fluoresceinyl substituted analogue of dUMP (Flu-dUMP) (see Fig. 2 for structure) as a lesion in the damaged strand were constructed (Krasikova et al., 2008). It is suggested that a photoreactive group bound with a linker of "zero" length should react only with amino acid residues of proteins which are in close contact with DNA (Meisenheimer and Koch, 1997). Flu-dUMP was used as bulky group; being inserted into DNA, this analogue of dNMP is the damage recognized and repaired by the NER system (Nakano et al., 2005). To enhance helix distortion, mismatched pair was built in the adjacent position to bulky substitute in DNA duplex.

The affinity of NER factors to DNA structures was evaluated by gel retardation assay. The data indicate that any one of the NER factors taken separately does not demonstrate noticeable preference in binding to damaged DNA, which is necessary for discrimination of DNA damages in the massive body of undamaged DNA. Therefore, the discrimination mechanism is more complicated and requires participation of several proteins simultaneously and/or other factors governing discrimination.

To evaluate the effect of RPA on XPC-hHR23B binding to the studied DNA duplexes, DNA was titrated with XPC-hHR23B heterodimer in the absence or in the presence of RPA (Krasikova et al., 2008). The data demonstrated stimulation of XPC-hHR23B binding to various DNA structures by RPA. Simultaneous presence of XPA and XPC-hHR23B results in mutual stimulation of their binding to DNA. Interactions between XPC and XPA, XPC and RPA in the course of their binding to UV-damaged DNA duplex were also detected by footprinting (Wakasugi and Sancar, 1999). However, the mechanism of interaction of these proteins remains unclear.

Modification of XPC-hHR23B by 5I-dUMP containing DNA duplexes was observed in the presence of RPA. The products of labeling corresponding to XPC-hHR23B-DNA complex were absent in the mixtures containing XPC-hHR23B and DNA. The data indicate that RPA stimulates an interaction between XPC-hHR23B and DNA and support data obtained by gel retardation. The intensity of RPA modification decreased simultaneously with appearance of the products of XPC-hHR23B modification. The stimulating effect of RPA manifested itself most clearly in the case of DNA bearing non-complementary nucleotides in the vicinity of the damage (Krasikova et al., 2008).

To clarify the suggestion of a role of protein–protein contacts in RPA-induced stimulation of XPC-hHR23B interaction with DNA, experiments on photoaffinity modification of XPC-hHR23B were performed in the presence of an RPA mutant

form depleted of all domains responsible for protein–protein interactions. The absence of any effect of this mutant RPA on XPC-hHR23B modification indicates that the mutant form of RPA is unable to stimulate the interaction of XPC-hHR23B with DNA. The data indicate that the observed stimulating effect is based on protein–protein interactions between RPA and XPC-hHR23B. The specificity of interaction between RPA and XPC-hHR23B was demonstrated in experiments on photoaffinity modification of XPC-hHR23B in the presence of a prokaryotic RPA analogue – SSB protein of *E. coli*. Replacement of RPA by SSB in the reaction mixture resulted in complete disappearance of the stimulating effect of XPC-hHR23B labeling.

Since it was found that RPA preferentially binds to the undamaged strand of DNA duplex (Hermanson-Miller and Turchi, 2002), it was of interest to study protein localization on undamaged DNA strand and to evaluate its effect on the interaction of XPC-hHR23B factor with various DNA sites. Modification of proteins by DNA duplexes different in position of a 5I-dUMP residue in the undamaged strand was analyzed. Study of the effect of RPA on XPC-hHR23B labeling by DNA structures bearing damage, non-complementary nucleotides, and photoreactive analogue at various positions of the undamaged strand showed that modification profile (dependence of modification intensity on position of photoreactive analogue of these proteins was different). Moreover, the data on localization of XPC-hHR23B factor on damaged DNA obtained by affinity modification were in accordance with the X-ray data (Fig. 4).

So, data presented demonstrate a negligible preference in binding of all studied NER factors taken separately, including that of XPC-hHR23B to damaged DNA compared with native DNA duplex. At the present time XPC-hHR23B is considered to be the most probable factor providing primary recognition of DNA damages. However some of the data show that RPA and XPA stimulate the interaction of XPC-hHR23B with damaged DNA. The effect of RPA and XPA on XPC-hHR23B binding to DNA was practically independent on the type of DNA duplex, whereas the effect on photoinduced cross-linking manifested itself to the maximal degree in

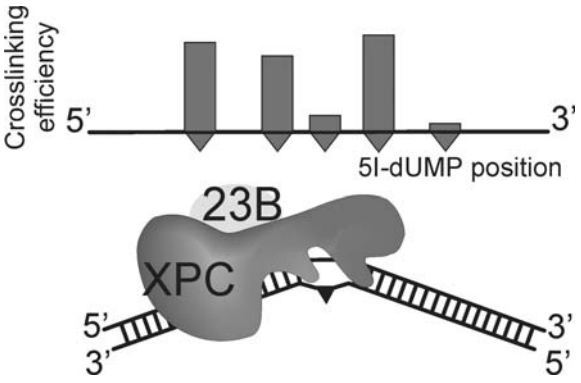

Fig. 4 Intensity of UV-induced crosslinking of XPC-hHR23B to 5I-dUMP at the definite positions of undamaged strand correlates with the points of DNA-protein contacts within crystal structure of the complex of Rad4-Rad23, yeast orthologue of XPC-hHR23B, with damaged DNA

case of DNA bearing a bulky substituent. Analogous results on the effect of these proteins on the efficiency of XPC-hHR23B modification were obtained, studying the interaction of NER factors with 60-mer DNA duplexes bearing bulky photoreactive nucleotide derivatives as damage (Maltseva et al., 2007). In summary, despite the common opinion that XPC-hHR23B is a factor providing primary recognition of

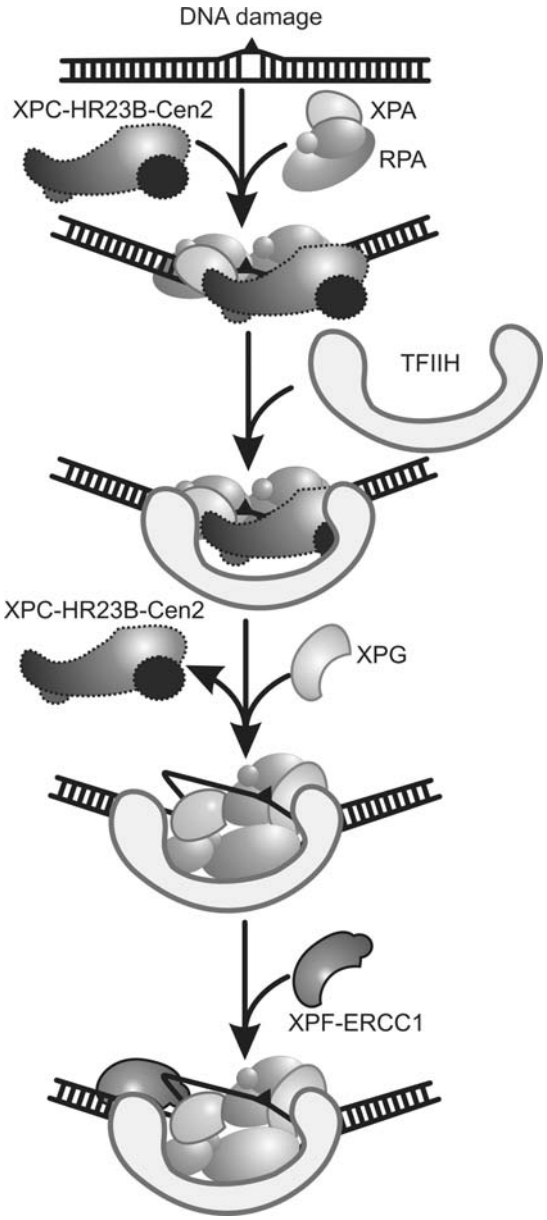

Fig. 5 Hypothetical model of assembly of pre-incision complex on damaged DNA during nucleotide excision repair

damage in DNA, contribution of RPA and XPA in this process cannot be excluded. The data reviewed most likely indicate cooperative binding of proteins participating in formation of pre-incision complex to the damaged DNA (Reardon and Sancar, 2003; Kesseler et al., 2007). A hypothetical model of formation of this complex accounting for possible cooperative interactions of RPA, XPA, and XPC–hHR23B factors is presented (Fig. 5).

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Nonhomologous DNA End Joining (NHEJ) and Chromosomal Translocations in Humans

Michael R. Lieber, Jiafeng Gu, Haihui Lu, Noriko Shimazaki, and Albert G. Tsai

Abstract Double-strand breaks (DSBs) arise in dividing cells about ten times per cell per day. Causes include replication across a nick, free radicals of oxidative metabolism, ionizing radiation, and inadvertent action by enzymes of DNA metabolism (such as failures of type II topoisomerases or cleavage by recombinases at off-target sites). There are two major double-strand break repair pathways. Homologous recombination (HR) can repair double-strand breaks, but only during S phase and typically only if there are hundreds of base pairs of homology. The more commonly used pathway is nonhomologous DNA end joining, abbreviated NHEJ. NHEJ can repair a DSB at any time during the cell cycle and does not require any homology, although a few nucleotides of terminal microhomology are often utilized by the NHEJ enzymes, if present. The proteins and enzymes of NHEJ include Ku, DNA-PKcs, Artemis, DNA polymerase μ (Pol μ), DNA polymerase λ (Pol λ), XLF (also called Cernunnos), XRCC4, and DNA ligase IV. These enzymes constitute what some call the classical NHEJ pathway, and in wild type cells, the vast majority of joining events appear to proceed using these components. NHEJ is present in many prokaryotes, as well as all eukaryotes, and very similar mechanistic flexibility evolved both convergently and divergently. When two double-strand breaks occur on different chromosomes, then the rejoining is almost always done by NHEJ. The causes of DSBs in lymphomas most often involve the RAG or AID enzymes that function in the specialized processes of antigen receptor gene rearrangement.

Keywords Double-strand DNA breaks · DNA repair · DNA recombination · NHEJ · Ku · Artemis · DNA-PKcs · DNA-dependent protein kinase · DNA polymerase μ · DNA polymerase λ · XLF · Cernunnos · XRCC4 · DNA ligase IV · Incompatible DNA ends · Polymerase slippage · Template-independent synthesis

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Abbreviations

AID	activation-induced deaminase.
CpG	the DNA sequence where the dinucleotide is 5′ CG 3′. These sites are distinctive because the C in such a sequence is methylated at the 5 position of the cytosine.
CSR	class switch recombination, the process by which the Ig heavy chain isotype is changed from IgM to IgG, A or E by DNA recombination using switch regions. This process requires AID.
DNA-PKcs	this is a serine/threonine protein kinase that is stimulated by free DNA ends. It phosphorylates itself and Artemis, the nuclease of NHEJ.
HR	homologous recombination.
icr	intermediate cluster region of the bcl-2 gene.
MBR	major break point cluster region of the bcl-2 gene.
mcr	minor break point cluster region of the bcl-2 gene.
MLL	mixed-lineage lymphoma.
MTC	major translocation cluster of the bcl-1 gene.
NHEJ	nonhomologous DNA end joining.
PCNA	proliferating cell nuclear antigen.
Pol λ	DNA polymerase λ.
Pol μ	DNA polymerase μ.
RAG	recombination activating gene.
RSS	recombination signal sequence. This is the sequence at which the RAG1:RAG2:HMGB1 complex, also called the RAG complex, binds in a sequence-specific manner to initiate the double-strand breaks that begin the V(D)J recombination process. The RSS has two components: CACAGTG, called the heptamer, and ACAAAAACC, called the nonamer, and the hetamer and nonamer are separated by either 12 nonconserved base pairs (then designated as a 12-RSS) or 23 base pairs (then designated as a 23-RSS). The RAG complex cuts 5′ to the first C of the CACAGTG.
SHM	somatic hypermutation, which is a process of point mutagenesis at the Ig genes, primarily in the assembled VJ or VDJ exons, which encode the variable domain exon of the light and heavy chains, respectively. SHM, like CSR, requires AID.
Switch regions	repetitive zones upstream of the constant domains exons for the heavy chains genes, Igγ, Igα, and Igε.
V(D)J	refers to the variable, diversity or joining segment (subexons) that must be assembled during the process of V(D)J recombination so that a complete variable domain exon can be generated at the immunoglobulin (Ig) and T-cell receptor (TCR) loci.
XLF	XRCC4-like factor (also called Cernunnos). This protein is part of the XLF:XRCC4:DNA ligase IV complex.

XRCC4 XRCC4 cross-complementation group 4. This protein is part of the XLF:XRCC4:DNA ligase IV complex.

Frequency and Causes of Double-Strand Breaks

In dividing primary initial passage mammalian fibroblasts, estimates are that there are about ten double-strand breaks (DSBs) per day per cell, based on metaphase chromosome and chromatid breaks (Lieber et al., 2003; Lieber and Karanjawala, 2004; Martin et al., 1985). Estimates in nondividing cells are difficult to make because methods for assessing DSBs outside of metaphase are subject to even more caveats of interpretation.

The causes of double-strand breaks in wild type cells include replication across a nick, which would give rise to chromatid breaks during S phase (Fig. 1). A second cause is oxidative free radicals. During the course of normal oxidative respiration, mitochondria convert about 0.1–1% of the oxygen to superoxide (O^2–) (Chance et al., 1979), and superoxide dismutase in the mitochondrion (SOD2) or cytosol (SOD1) can convert this to hydroxyl free radical, which may react with DNA to cause single-strand breaks. It is not entirely clear whether one free radical can trigger a DSB, but clearly two closely space lesions on anti-parallel strands can cause a DSB. About 10^{22} free radicals are produced in the human body each hour.

A third cause is inadvertent action by nuclear enzymes of DNA metabolism. These include failures of type II topoisomerases, which transiently break both strands of the duplex. If the topoisomerase fails to rejoin the strands, then a DSB results. Failures of type I topoisomerases (spontaneous or due to inhibition) cause single-strand breaks (SSBs), which can result in a DSB upon DNA replication.

Still within this same category of inadvertent action by nuclear enzymes are the lymphoid-specific enzymes for antigen receptor gene rearrangement, which include the RAG complex (composed of RAG1 and 2) and activation-induced deaminase (AID). In humans, these account for about half of all of the chromosomal translocations that results in lymphoma, as will be discussed later.

A fourth cause of DSBs is natural ionizing radiation of the environment. These include gamma rays and X-rays. At sea level, each person is hit by about 300 million ionizing radiation particles per hour. As these traverse the body, they create free radicals primarily from water along their path. When the particle traverses close to a DNA duplex, clusters of free radical damage double- and single-stranded DNA at a ratio of about 1:25. About half of the ionizing radiation that strikes each of us comes from outside the earth. Orders of magnitude more such radiation would reach the surface of the earth were it not for the atmosphere, because most of the particles encounter the ozone of the upper atmosphere first. Another half of the radiation that strikes us comes from the decay of radioactive elements, primarily metals in the earth.

The Discussion above relates to mitotic cells. Meiotic cells have an additional form of double-strand breakage which is physiologic and is caused by an enzyme called Spo11 (Zickler and Kleckner, 1999), which creates DSBs in order to cause

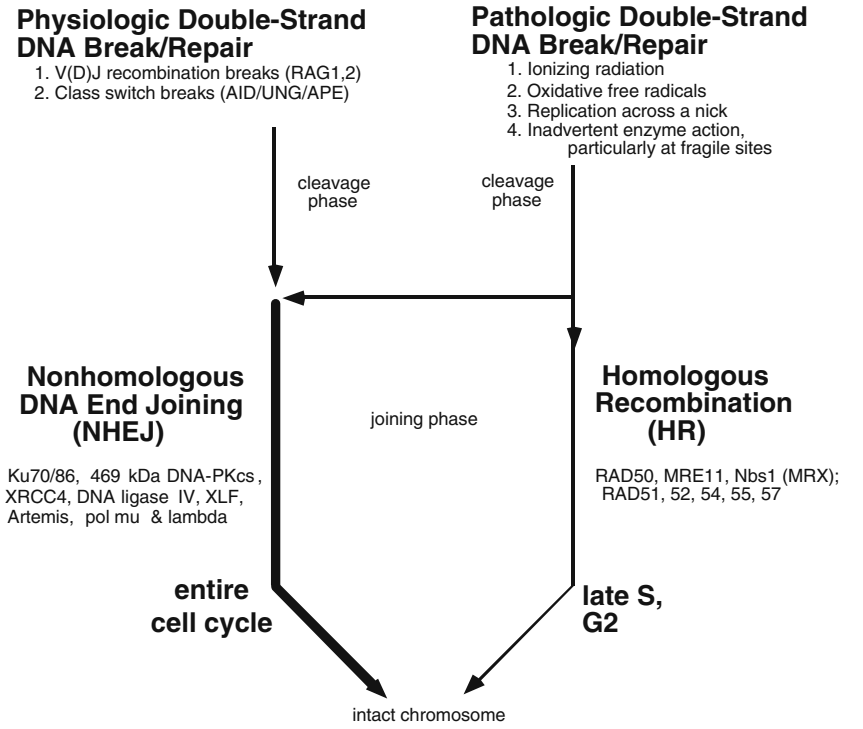

Fig. 1 Diagram of the Causes and Known Repair Pathways for Double-Strand Break Repair. Four prominent causes of pathologic double-strand breaks in living cells are listed. Among multicellular eukaryotes, physiologic double-strand breaks are found only the vertebrate immune system. V(D)J recombination is present in all true vertebrates and is initiated by an endonucleases complex composed of RAG1 and 2 (Lieber et al., 2006). Class switch recombination is present in only a subset of these vertebrates and is initiated by cytidine deaminase called activated-induced deaminase (AID). In human cells, DSBs that arise in late S or G2 of the cell cycle are often repaired at long regions (>100 bp) of homology using homologous recombination (though single-strand annealing also can occur) (Sonoda et al., 2006). However, in human cells the dominant pathway for the repair of double-strand breaks is called NHEJ, and this repair pathway can function at any time during the cell cycle. NHEJ does not use long stretches of homology, but the processing of the DNA ends can, in a minority of cases, be influenced by alignment of a few nts of homology called terminal microhomology (typically 1–4 nt in length). It should be noted that NHEJ proceeds even if there is no terminal microhomology. Important protein components of the repair pathways are listed

cross-overs between homologues during meiotic prophase I. But it is not clear that NHEJ occurs in vertebrate meiotic cells because one group reports the lack of one of Ku70 in spermatogonia. Human spermatogonia remain in meiotic prophase I for about 3 weeks, and human eggs remain in meiotic prophase I for 12–50 years; hence, these cells can rely on homologous recombination (HR) during these long periods. Given the error-prone nature of NHEJ, reliance on HR may be one way to minimize alterations to the germ cell genome at frequencies that may be deleterious.

Vertebrate Nonhomologous DNA End Joining

The discussion of NHEJ here relates to the mechanism in vertebrate organisms. In most invertebrate organisms, there is no DNA-PKcs and no Artemis, though the evolutionary emergence of these two components remains an active area of study. The nuclease functions of Artemis seem to be accomplished by the RAD50:MRE11:NBS1 complex in invertebrate animals, yeast, and in plants (Chen et al., 2001).

In vertebrate cells, the first protein thought to bind at a DSB is Ku, because it is abundant (~400,000 molecules per cell) and it binds tighter to duplex DNA ends than any other protein in the cell (K_D ~1 nM) (Falzon et al., 1993). Ku consists of Ku70 and Ku86 and forms a toroidally-shaped heterodimer that has a hole large enough to admit DNA (Walker et al., 2001). Ku can load onto DNA only at DNA termini and can thread onto the duplex to internal positions (deVries et al., 1989).

Ku has no confirmed enzymatic function, though claims of helicase activity, ATPase activity, or end bridging have been made at various points without confirmation. The primary function of Ku seems to be to improve the stability of the NHEJ enzymes at the DNA termini so that they can carry out their functions for longer periods (Lieber, 2008). Improvements in stability have been reflected in improved equilibrium dissociation constants determined using surface plasmon resonance and electrophoretic mobility shift assays (EMSA) on acrylamide gels as well as with qualitative methods such as co-immunoprecipitation and atomic force microscopy (Yaneva et al., 1997; West et al., 1998). Hence, Ku seems to function by marking the DNA terminus as a node for the DNA polymerases, nuclease and ligase of NHEJ. In that sense, it functions somewhat like tool belt proteins such as PCNA (Lieber, 2008).

Most DNA repair processes involve a nuclease to remove damaged DNA, a DNA polymerase to fill-in new DNA, and a ligase to restore the integrity of each strand of the duplex. In vertebrate NHEJ, the ligase is DNA ligase IV (Grawunder et al., 1997; Wilson et al., 1997; Schar et al., 1997; Teo and Jackson, 1997), and it is supported by two apparently nonenzymatic components called XRCC4 and XLF (or Cernunnos) (Ahnesorg et al., 2006; Buck et al., 2006). The DNA polymerases are Pol μ and Pol λ, though in double mutants of these two, it appears that other DNA polymerases can also participate (Bertocci et al., 2006; Lieber, 2006). The nuclease functions appear largely covered by a complex of Artemis and DNA-PKcs (Ma et al., 2002) although DNA-PKcs appears to have additional functions related to cell cycle components, chromatin, and others.

The DNA Ligase IV Complex

In the absence of DNA ligase IV in *S. cerevisiae*, NHEJ does not occur (Wilson et al., 1997; Daley et al., 2005b; Schar et al., 1997; Teo and Jackson, 1997). Hence, the other ligase in *S. cerevisiase*, DNA ligase I, cannot substitute for ligase IV. However, if there are several nucleotides of terminal microhomology between the two ends, then some joining can occur. The amount of joining may depend on the length of

terminal microhomology, but it is usually greater than 6 nucleotides (Ma et al., 2003). In the nonrepetitive yeast genome, such microhomology is quite unusual. Hence, the alternative or backup role of such microhomology-mediated end joining (MMEJ) is likely to be of limited survival utility for naturally-occurring DSBs.

In vertebrates, XRCC4 seems to stabilize ligase IV. Other possible roles may be possible, but are still under investigation. XLF (or Cernunnos) seems to stimulate the ability of ligase IV to ligate a more diverse array of DNA end configurations (Gu et al., 2007a, b) (see below). The stoichiometry of the XLF:XRCC4:ligase IV complex is thought to be 2:2:1 (Li et al., 2008; Ahnesorg et al., 2006), but this is difficult to measure, and additional studies are warranted.

DNA ligase IV can ligate fully incompatible DNA ends, namely DNA ends that do not share even one nucleotide of terminal microhomology (e.g., a blunt end joined to a 5′ overhang) (Gu et al., 2007a). Though the efficiency of this joining is low, ligase I and III appear to be even less efficient at this. Importantly, XLF can markedly stimulate the ability of the XRCC4:ligase IV complex to carry out incompatible DNA end joining (Gu et al., 2007b).

Two ends are partially compatible when they share one or more nucleotides of terminal microhomology but they can not be ligated without resection of some excess DNA flaps or fill-in of some gaps on one or both DNA strands of the duplex. Joining by the XLF:XRCC4:ligase IV complex is more efficient when there is one or more nucleotides of microhomology shared between the DNA ends. Ku is able to make joining much more efficient if there is no microhomology and more efficient if there are one or two nucleotides of microhomology (Gu et al., 2007a, b). If there are 4 or more nucleotides of microhomology, then the stimulation by Ku is relatively small (<2-fold in some cases).

In addition to the flexibility mentioned above, ligase IV can ligate one strand when the other strand is in a configuration that cannot be ligated, and it can ligate some single-stranded DNA sequences (Ma et al., 2002). Overall, the ligase IV complex is perhaps the most flexible ligase known for tolerating a wide range of substrates.

The DNA Polymerases of NHEJ

In *S. cerevisiae*, POL4 is responsible for a substantial amount of fill-in synthesis at NHEJ junctions (Wilson and Lieber, 1999; Daley et al., 2005a; Daley and Wilson, 2007), though other DNA polymerases such as POL3 (pol delta) also contribute. POL4 is a member of the POLX DNA polymerase family, a family that is a subset of all DNA polymerases (Tseng and Tomkinson, 2002; Burgers et al., 2001; Hübscher et al., 2000; Nasheuer et al., 2006).

The human homologues of POL4 are both Pol μ and Pol λ. Both Pol μ and Pol λ have BRCT domains located within their N-terminal portions. The other two members of the POLX family in humans are terminal transferase (TdT) and DNA polymerase β (Pol β). TdT also has a BRCT domain within its N-terminal portion. Pol β is distinctive among POLX DNA polymerases for its lack of a BRCT domain.

Ku is able to recruit Pol μ and Pol λ by binding to their BRCT domains, and removal of this BRCT domain eliminates the ability of Ku to recruit either DNA polymerase (Gu et al., 2007a).

Pol μ and Pol λ are both capable of fill-in synthesis. But remarkably, Pol μ also has a robust ability for template-independent synthesis (Ramadan et al., 2003, 2004; Gu et al., 2007a). This template-independent synthesis is useful for generating terminal microhomology when none exists.

Pol μ and Pol λ are both prone to slippage on the template strand, which would generate direct repeats (Bebenek et al., 2003). In addition, when Pol μ generates overhangs via template-independent synthesis, it is able to fold that strand onto itself and continue polymerization. This would generate inverted repeats. At NHEJ junctions, both direct and inverted repeats are commonly seen (Jaeger et al., 2000).

There is some evidence that Pol μ, in the context of Ku, may be able to cross the discontinuity in the template strand between two DNA ends (Nick McElhinny et al., 2005; Moon et al., 2007). Importantly, without Ku, there is no evidence that Pol μ can use a discontinuous template; that is, cross from one DNA end to another DNA end.

Therefore, Pol μ and Pol λ are both very flexible. Pol μ has the additional flexibility of template-independent synthesis and possibly of synthesis across a discontinuous template strand. These features make Pol μ and Pol λ among the most flexible DNA polymerases known.

Artemis, DNA-PKcs and the Nuclease of NHEJ

In vertebrates, most and perhaps all of the nuclease activity for NHEJ is due to Artemis (Ma et al., 2004). Artemis forms a complex with DNA-PKcs, and this complex is relatively stable even at 500 mM NaCl (Ma et al., 2002). DNA-PKcs is a serine/threonine protein kinase, but it is only active when bound to duplex DNA ends (Anderson and Carter, 1996). The range of DNA ends that activate DNA-PKcs kinase activity is quite diverse (Falzon et al., 1993). Purified Artemis has 5′ exonuclease activity. But Artemis additionally acquires 5′ endonuclease, 3′ endonuclease, and hairpin opening activity when it is in a complex with DNA-PKcs:DNA complexes (Ma et al., 2002). The 5′ endonuclease activity of the Artemis: DNA-PKcs complex prefers to nick 5′ overhangs at the junction of the single- to double-strand portions. The 3′ endonuclease activity of the Artemis:DNA-PKcs complex nicks 3′ overhangs preferentially ~4 nt into the single-stranded overhang from the double- to single-strand transition (Ma et al., 2002). The hairpin opening activity of the Artemis:DNA-PKcs complex preferentially nicks the hairpins two nucleotides past the tip of the hairpin. These diverse endonuclease properties are somewhat unified by a model in which the Artemis:DNA-PKcs complex binds four nucleotides of single-stranded DNA adjacent to the single- to double-strand transition, and nicks 3′ of it (Ma et al., 2002). This explains the hairpin nicking preference because the last two base pairs of a perfect hairpin remain unpaired, thereby providing the four nucleotides of single-strandedness at the hairpin tip.

The primary phosphorylation target of DNA-PKcs is itself. DNA-PKcs autophosphorylates itself at more than 13 sites. This causes a conformational change of DNA-PKcs while bound to a DNA end based on studies of DNA end accessibility to other enzymes, such as ligases.

DNA end activated DNA-PKcs also phosphorylates Artemis at 11 sites within the C-terminus, and removal of the C-terminus of Artemis gives it endonuclease activity on some substrates even in the absence of DNA-PKcs (Ma et al., 2005; Niewolik et al., 2006). Based on this, it appears that the conformational change in Artemis that converts it from an exonuclease to an endonuclease is conconmittant with Artemis phosphorylation. However, if one interferes with the Artemis phosphorylation by adding a DNA-PKcs inhibitor (after the DNA-PKcs has already phosphorylated itself), then the Artemis still acquires its endonucleolytic properties (Goodarzi et al., 2006). Therefore, the conformational change in Artemis induced by DNA-PKcs appears to be the primary event that permits Artemis to acquire endonucleolytic activity.

Concluding Comments on Vertebrate NHEJ

The nuclease, DNA polymerase, and ligase components of NHEJ bind to the Ku:DNA end, and in that sense, the Ku marks the DNA end as a node for recruitment of these other NHEJ proteins. The nuclease, DNA polymerases and ligase are all among the most flexible enzymes of their type. This flexibility permits NHEJ to join a very diverse range of DNA ends that have various sequences, various overhang configurations, and various degrees of oxidative damage.

Chromosomal Translocations

Types of Translocations and Relation to Cancer

Genetic changes in neoplasms can arise from a variety of causes including numerical changes in chromosome number; structural changes in chromosomes (translocations, inversions and deletions); focal mutations (e.g., point mutations); DNA methylation, which can cause transcriptional shutdown; and reduction to homozygosity due to homologous recombination or due to loss of a chromosomal segment.

Changes in chromosome structure (translocations, inversions, or deletions) are among the most common change in many neoplasms, particularly neoplasms of the hematopoietic system. In acute leukemias, oncogenic translocations often result in the formation of fusion proteins with capabilities beyond those of the original constituent proteins (Gelb and Medeiros, 2002). Examples include fusions of the MLL gene in 3–5% of acute myeloid leukemia with many different partner genes (Gelb and Medeiros, 2002), and the E2A-Pbx1 fusion in 6% of acute lymphoblastic leukemia.

In chronic leukemias and lymphomas, oncogenic translocations often activate a gene that drives cell proliferation (e.g., an oncogene) (Gelb and Medeiros, 2002). Many of these translocations involve one of the antigen receptor loci. While these generalizations are useful for formulating a framework, there are many exceptions.

In order for a chromosomal translocation to occur, two independent breaks typically occur on different chromosomes, thereby generating two DNA ends at each break, or four DNA ends total (Lieber, 1998). If the correct DNA ends get rejoined, then no translocation occurs. But if ends from different chromosomes are joined, then a translocation arises.

Interstitial deletions are mechanistically similar to translocations, but in this case, both breakpoints are on the same chromosome, and the region between the breaks is deleted. Chromosomal inversions can also arise this way, but where the DNA ends of the chromosomal segment in the middle of two breaks are joined.

NHEJ in Chromosomal Structural Changes

Nearly all chromosome structural changes involve double-strand DNA breaks. When the breaks become rejoined, NHEJ is responsible for the vast majority of the joining (Ferguson and Alt, 2001). If a key component of NHEJ is missing, such as DNA ligase IV, then one of the other two ligases (ligase I or III) can do the joining (Ferguson and Alt, 2001), although the efficiency of joining seems to be lower and there appears to be more nucleotide loss from the junctions (Han and Yu, 2008). However, in human spontaneous neoplasms, NHEJ is nearly always intact, and apparently responsible for the vast majority of joining.

Causes of DSBs That Initiate Translocations

As mentioned in the first section of this review, there are many causes of DSBs. In considering the causes underlying a particular chromosomal translocation, it is useful to examine the sequence features and distribution of the translocation. For translocations that create a fusion protein (such as a novel transcription factor that changes the regulation of the cell), the fusion protein is often a specific set of exons from each original protein. In these cases, the DSB often can occur anywhere within the intron following the last essential exon. Upon examination of many patient breakpoints, if breakpoints are distributed evenly across the entire intron, then the simplest explanation involves nonspecific causes of DSBs, such as oxidative free radicals or natural ionizing radiation.

However, even for some fusions, such as E2A joined to Pbx1, a subset of the breakpoints can be tightly clustered in a manner that is 50–300-fold more focused than random (Wiemels et al., 2002) (Fig. 2).

For translocations that do not generate a fusion protein but rather upregulate a gene such as bcl-2 or cyclin D1, many or even most of the breakpoints are focused

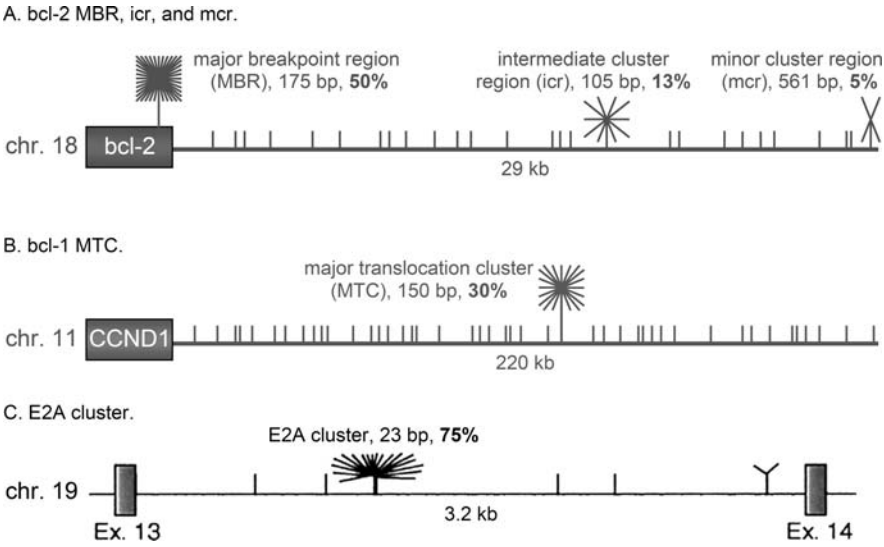

Fig. 2 Schematics of breakpoint cluster regions. Schematics of the bcl-2, bcl-1, and E2A regions illustrate clustering of breakpoints within the various identified cluster regions. The breakpoints that do not fall into cluster regions are plotted randomly for illustrative purposes, as most of them are never sequenced and often only mapped to general regions by Southern blotting. The *panel* (**a**) depicts relative proportions of breakpoints at the bcl-2 MBR, icr, and mcr cluster regions. The third exon of the bcl-2 gene contains the MBR (within the 3′ UTR region), while the centromeric 29 kb contains the icr and mcr. Short lines above the gene diagram mark the approximate locations and relative abundance of patient breakpoints. The 175 bp MBR, 105 bp icr, and 561 bp mcr account for about 50, 13, and 5% of bcl-2 translocation breakpoints, respectively. The major translocaton cluster, bcl-1 MTC, represented in *panel* (**b**), is located about 110 kb from CCND1, the gene for the cyclin D1 oncoprotein. The 150 bp MTC contains about 30% of breakpoints, whereas the remaining 70% of events are distributed widely over the surrounding 220 kb. The *panel* (**c**) shows a diagram of intron 13 of the E2A gene, taken from (Wiemels et al., 2002a). Seventy five percent of breakpoints occur in the 23 bp E2A cluster, while the surrounding 3 kb only account for 25%

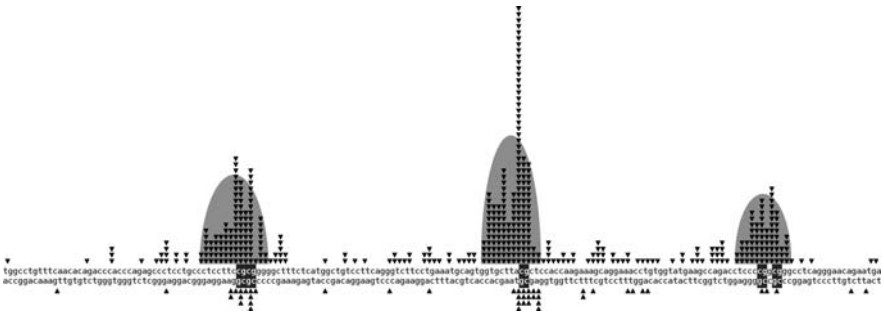

Fig. 3 Proximity of Patient Chromosome 18 Breakpoints in Follicular Lymphoma Relative to CpG Sites within the Major Breakpoint Region. Each breakpoint is represented as a triangle adjoining the breakpoint site, with the top strand sequences running telomeric to centromeric, with der(14) breakpoints *above*, and der(18) breakpoints *below*

by a factor of roughly 100-fold over random (Tsai et al., 2008) (Fig. 3). In neoplasms with such intense focusing, some of the patient breakpoints are scattered across wide zones outside of the focused zone. However, in most cases, as in the bcl-2 or bcl-1 translocations, the clinical features of the neoplasm are not highly dependent on the location of the DSB (Jaeger et al., 2000; Welzel et al., 2001).

There are two general mechanisms that have been proposed for intense focusing of translocation breakpoints in hematopoietic neoplasms, particularly those of the lymphoid lineage (Tsai et al., 2008).

Mistakes of V(D)J Recombination

The RAG complex is expressed in pre-T and pre-B cells, and consists of RAG1, RAG2 and HMGB1. It normally functions in antigen receptor gene rearrangement by cutting at heptamer/nonamer sequences, for which the consensus is flexible. The heptamer/nonamer sequences are often called recombination signal sequences (or RSS). The flexibility of the RSS sequence means that there are locations in the genome that can look similar to an RSS, but not located at the antigen receptor loci. These have been called pseudo RSSs, cryptic RSSs, or misrecognition sites, all of which refer to aberrant, non-receptor sites of RAG complex cutting (Lewis et al., 1997). In some T-cell acute lymphoblastic leukemias/lymphomas, these pseudo RSSs are cut and joined to a T-cell receptor (TCR) locus. The enhancers at the TCR locus then upregulate the gene. If this gene can serve as a first step on a pathway to neoplasia, then the resulting translocation is seen in the resulting tumor. This is precisely what occurs in many types of T-ALL (T-cell acute lymphoblastic lymphoma).

DSBs that arise due to misrecognition of a pseudo RSS are referred to as DSBs of the V(D)J-type. Recall that chromosomal translocations require two DSBs. In most cases, the DSB at the oncogene by a V(D)J-type mechanism joins to a DSB at one of the antigen receptor loci. Therefore, one could think of this as the joining of ends from two DSBs in which both were of the V(D)J-type (Tsai et al., 2008).

In some cases, both DSBs are sites of pseudo RSS, such as in the interstitial deletion between SCL and SIL. Both DSBs are still V(D)J-type, but the distinction from above is that in the above case, one V(D)J-type event is at an antigen receptor locus and the other is at a pseudo RSS (Raghavan et al., 2001).

Sequential Action by AID and the RAG Complex

For many translocations that occur in pre-B cells during V(D)J recombination, translocation breakpoints are often tightly clustered into small zones (e.g., the major breakpoint region (MBR) in bcl-2 translocations, or the major translocation cluster (MTC) in bcl-1 translocations) but do not have features of a pseudo RSS. The mechanism for breakage at these zones has been unclear.

Recently, we reported the striking statistical observation that the peaks of the translocations occur at CpG sequences (Tsai et al., 2008). CpG is unique because the C in such dinucleotides can be methylated. Methyl C is distinctive from C

because when it deaminates spontaneously, regular C becomes a U, but methyl C becomes a T. U:G mismatches are relatively efficiently repaired back to the original sequence. But T:G mismatches are poorly repaired. The poor repair of T:G mismatches that arise from meCpGs explains why 25–50% of the point mutations at the p53 gene are located at CpG sites. This also explains the evolutionary depletion of CpG from the vertebrate genome, except in regions where the CpG is unmethylated or of functional importance (i.e., at promoters, which thus give rise to CpG islands).

Our finding of some translocations centered at CpG sites suggests a mechanism (Tsai et al., 2008). We find this CpG propensity only in lymphoid malignancies where there is RAG expression at the time of the translocation (Fig. 4). Nonlymphoid neoplasms and lymphoid neoplasms where the translocation occurs before or after the B lineage window of RAG expression do not appear to suffer DSBs by this mechanism. In other words, breaks at CpG sites only appear to occur in pro-B and pre-B cells and not in all other B cell stages of differentiation and not in other hematopoietic lineages.

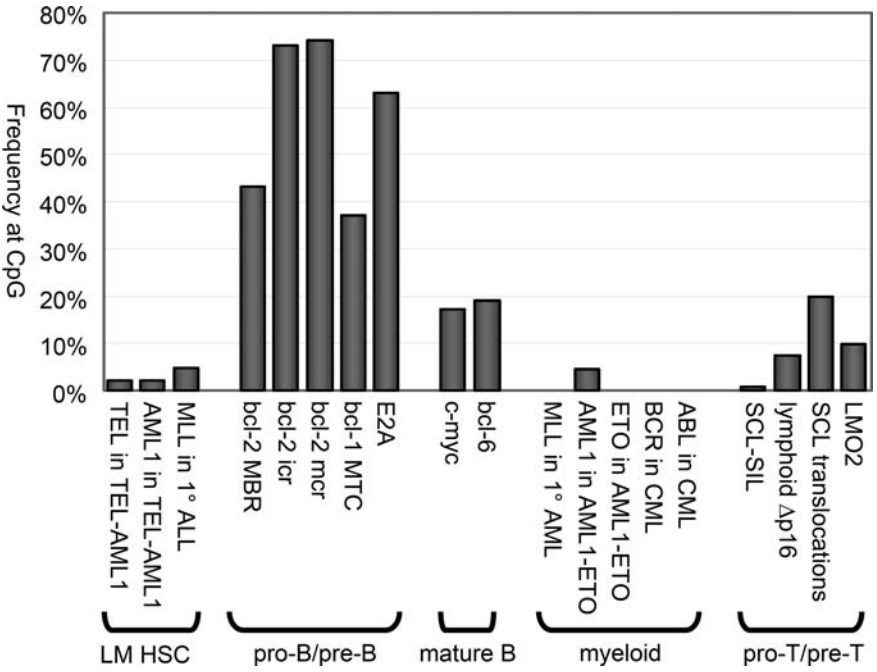

Fig. 4 Frequencies of Breakpoints at CpG in Various Chromosomal Rearrangements. Percentages of breakpoints at CpGs are plotted for various chromosomal rearrangements, organized by cell lineage and stage of development when the rearrangement occurs. Frequencies for pro-B/pre-B rearrangements far exceed those from other lineages and stages

What is this RAG-dependent mechanism? We have shown previously that the RAG complex can nick small bubble structures, and we now know that such nicking applies even to 1 bp mismatches (Tsai et al., 2008). This propensity of the RAG complex to nick small bubble structures, even ones as small as 1 bp, may have derived from the fact that the RAG complex has to convert a nicked species to a hairpin form during V(D)J recombination. Therefore, the RAG complex may readily bind duplexes that have DNA distortions, and in the course of such binding, it appears that the RAG complex can nick the top or bottom strand of an isolated bubble structure at efficiencies that are within 10-fold of the nicking at a duplex RSS (Tsai et al., 2008; Raghavan et al., 2005a; Raghavan and Lieber, 2006; Raghavan et al., 2007). The ability of the RAG complex to nick at a 1 bp mismatch means that any meC in a meCpG site that deaminates to a T can be subsequently nicked by the RAG complex. We call this a DSB of the CpG-type to distinguish it from other mechanisms

The ability of the RAG complex to nick 1 bp sites of deaminated meC would predict that one could have a RAG-dependent mechanism in both pre-B (and pro-B) and pre-T cells (Tsai et al., 2008). However, we only observe this CpG localization of breakpoints in pro-/pre-B cell translocations. This means that one additional factor must participate. Moreover, this additional factor must overlap with the temporal expression of the RAG complex. There is only one enzyme that converts C to U or methyl C to T and is specific to B cells. This enzyme is activation-induced deaminase (AID). AID is most highly expressed in germinal center B cells located in the lymph nodes, spleen, and Peyer's patches of the GI tract. The deaminase activity of AID triggers the somatic hypermutation (SHM) and class switch recombination (CSR) processes. Surprisingly, a percentage of mammalian pre-B cells prematurely express substantial levels of AID. Hence, AID and the RAG complex are concurrently present in some pre-B cells (Tsai et al., 2008).

Our model then is that the B lineage specificity of the CpG-type of DSB is due to AID acting at meC within meCpG. Then the RAG complex nicks both the top and bottom DNA strands, resulting in a DSB (Tsai et al., 2008) (Fig. 5).

If one DSB of the translocation occurs by the CpG-type mechanism, how does the other DSB occur? For the t(14;18) translocation of follicular lymphoma, which is the most common translocation in human lymphoma, the other DSB occurs via a V(D)J-type mechanism at an RSS at the immunoglobulin heavy chain (IgH) locus. For the t(11;14) translocation of mantle cell lymphoma, the CpG-type DSB occurs in the major translocation cluster (MTC) about 100 kb from the cyclin D1 gene, and the other DSB is a V(D)J-type mechanism at the IgH locus (Tsai et al., 2008).

For the t(1;19) translocation involving the E2A gene on chromosome 1 and the Pbx1 gene on chromosome 19, the E2A break is of the CpG-type, occurring typically within a 23 bp window on the 3.2 kb intron 13, while the Pbx1 break appears to be random, occuring anywhere within the 232 kb intron 1 (Wiemels et al., 2002).

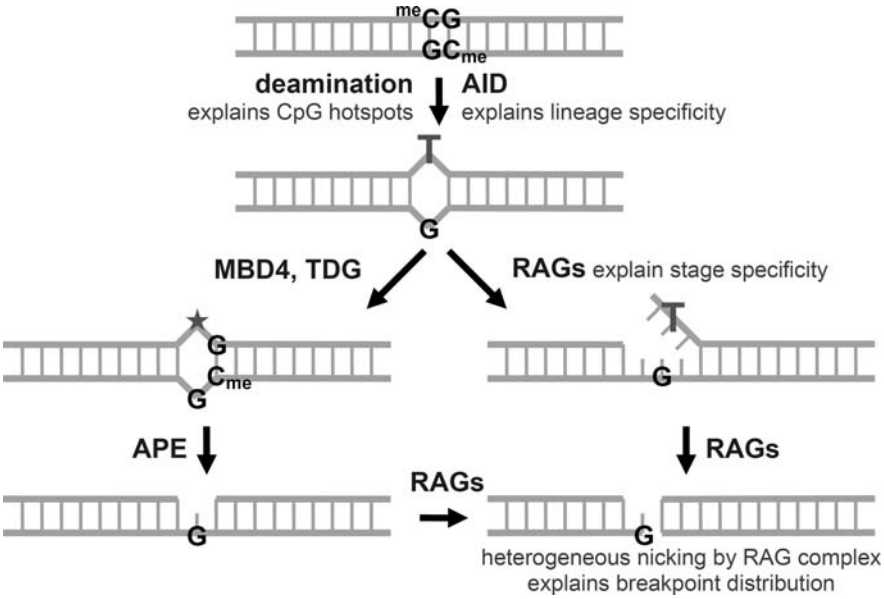

Fig. 5 Proposed Mechanism for CpG-type Double-strand Breakage. Deamination at a methylcytosine within a CpG creates a T:G mismatch which persists due to catalytic inefficiency of methyl-CpG binding domain protein 4 (MBD4) and thymine DNA glycosylase (TDG) in cleaving the thymine glycosidic bond, leaving an abasic site (*asterisk*). Single-strand breaks are generated either by the normal base excision repair pathway of glycosylase and AP endonuclease (APE) activity, or by RAG nicking the mismatch directly, resulting in a poorly ligatable flap. Heterogeneous RAG nicking of the remaining strand creates a double-strand break close to the original site of the T:G mismatch. We observe the following features at CpG-type translocation hotspots: (1) an extremely high degree of focusing to CpGs, (2) a breakpoint distribution consistent with a structure-specific endonuclease, (3) specificity to the pro-B/pre-B stage, and (4) specificity to the B-cell lineage. Involvement of the RAG complex explains points 2 and 3, while involvement of AID explains point 4 and is strongly suggestive for point 1

Source of Single-Strandedness at Sites where AID Acts on meC Sites

In order for AID to convert C to U or meC to T, the DNA must be single-stranded (Bransteitter et al., 2003). So how does single-strandedness arise? We do not know with certainty. However, the bcl-2 MBR and the bcl-1 MTC do contain regions of distinctive reactivity with chemical probes that require either some degree of single-strandedness or substantial departures from the B-form duplex (Raghavan et al., 2004a; Tsai et al., 2009).

Concluding Comments

Chromosomal translocations and related pathologic genome rearrangements in somatic cells require two DSBs, and the mechanism for the two breaks does not need to be the same. Once the breaks are formed, then NHEJ generally does the joining of the four DNA ends.

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Fluorescence-Based Quantification of Pathway-Specific DNA Double-Strand Break Repair Activities: A Powerful Method for the Analysis of Genome Destabilizing Mechanisms

Michael Böhringer and Lisa Wiesmüller

Abstract This chapter provides instructions for the application of a fluorescence-based assay to examine different DNA double-strand break (DSB) repair pathways in primary mouse embryo fibroblasts (MEFs). The assay relies on targeted DSB formation in one of a series of repair substrates and subsequent repair-mediated reconstitution of the *EGFP* reporter. We present protocols for efficient introduction of extra-chromosomal repair substrate together with I-*Sce*I endonuclease expression vector and subsequent measurement of DSB repair events down to frequencies of 0.001%. Concomitant transfection of plasmid and siRNA enables assessment of DSB repair under conditions of knockdown of protein expression, allowing to evaluate the contribution of single factors. Since the proteins of interest frequently have dual roles in DSB repair surveillance and checkpoint control, our assay procedure concomitantly corrects for transfection efficiencies, growth-, death-, and expression-related changes and also integrates the examination of the cell cycle status.

Keywords DNA double strand break repair pathways · Screening · siRNA · Biomarker · Prediction of therapeutic responsiveness

Background

Implications of Pathway-Specific DNA Double-Strand Break Repair Activities

DNA double-strand break (DSB) repair and its surveillance have received increasing attention due to the identification of defects in patients with combined immunodeficiency diseases or chromosome breakage syndromes and in individuals with

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increased breast and ovarian cancer risk (Gennery, 2006; Walsh and King, 2007; Taniguchi and D'Andrea, 2006). Removal of DSBs is taken care of by two principal repair pathways: Non-homologous end-joining (NHEJ) and homologous DSB repair (Krejci et al., 2003). One of the first, if not the initial step in both pathways is the recognition of DSBs by a protein complex comprising MRE11, RAD50, and NBS1 (MRN). The MRN complex is involved in virtually all aspects of DNA end metabolism, including tethering of the two DNA substrates, DSB processing, and checkpoint signaling via activation of the phosphatidylinositol 3-kinase related kinases ATM (*Ataxia telangiectasia* mutated) and ATR (ATM- and Rad3-related protein) (Paull and Lee, 2005). In the NHEJ pathway, KU70 and KU80 bind the DSB, followed by recruitment and activation of the catalytic subunit of the DNA protein kinase (DNA-PK_{cs}), which mediates synapsis and recruits XRCC4 and DNA ligase IV for break sealing together with Cernunnos/XLF (for more details see review Lieber (2008) and the chapter "Nonhomologous DNA End Joining (NHEJ) and Chromosomal Translocations in Humans" by Lieber et al., this book). End processing may involve nuclease activities of Artemis and gap filling polymerase μ. Recently, in vitro and in vivo evidence was also provided for the existence of a non-canonical NHEJ pathway that may involve poly(ADP-ribose)polymerase-1 (PARP-1) and ligase III (Wang et al., 2005; Audebert et al., 2004; Yan et al., 2007; Corneo et al., 2007).

Pathways that utilize sequence homology for repair are subdivided into two types, based on whether homologous associations arise from strand annealing activities (single strand annealing, SSA) or from strand exchange (homologous recombination, HR) activities (Krejci et al., 2003). CTip is likely to be responsible for initial DNA resection at DSBs to enable homologous DSB repair (Sartori et al., 2007). Next, human single-stranded DNA binding protein, replication protein A (RPA), protects the ssDNA overhang of the resected DSB (Levy et al., 2009; see also chapter "Eukaryotic Single-Stranded DNA Binding Proteins and Genomic Stability" by Broderick et al., this book). During the following and central step in HR, RAD51 forms a nucleoprotein filament with the 3′ overhanging ssDNA, thereby replacing RPA, and catalyzes homologous pairing and strand exchange. The RAD51 paralogs XRCC2, XRCC3, RAD51B, RAD51C and RAD51D, as well as RAD54 and in yeast also RAD52 support RAD51 during HR. The translesion synthesis DNA polymerase η interacts with RAD51 and shows D loop extension activity (McIlwraith et al., 2005). SSA does not rely on RAD51 but requires RAD52. RAD52 facilitates pairing of the processed ssDNA tails before removal of the heterologous overhangs by the structure-specific endonuclease ERCC1-XPF (Krejci et al., 2003; Ralhan et al., 2007).

When unregulated, DSB repair processes may end up with loss of heterozygosity, sister chromatid exchanges (SCEs), and chromosomal aberrations which may accelerate the multistep process of tumorigenesis. Thus, NHEJ is frequently associated with the gain or loss of nucleotides at the break site. SSA occurs between repeated sequences and ultimately creates a deletion, and, therefore, is always mutagenic (Stark et al., 2004). HR is considered the least error-prone pathway, because sister chromatids are the preferred template. However even during HR, exchange between

divergent sequences creates mutations. Moreover, HR factors can contribute to the formation of pathological structures at replication forks such as reversed-fork four-way structures (Klein, 2006). Interestingly, the products of the breast and ovarian cancer susceptibility genes *BRCA1*, *BRCA2*, *BRIP1*, *PALB2*, *p53*, *CHEK2*, *ATM*, *NBS1*, and *RAD50* are critical components in the hierarchy of surveillance mechanisms that ensure error-free repair of DSBs: BRCA1 and BRCA2, inhibit the mutagenic repair pathways of microhomology-mediated NHEJ and SSA, respectively. The Fanconi anemia gene products FANCJ/BACH1/BRIP1 and PALB2 bind BRCA1 and BRCA2 and help to channel repair into HR. Further downstream, p53 represses excessive and aberrant HR. ATM together with the MRN complex as well as the downstream kinase Chk2 coordinate DSB repair, surveillance, and checkpoint reponses (Baldeyron et al., 2002; Stark et al., 2004; Gatz and Wiesmüller, 2006; Riches et al., 2008; Taniguchi and D'Andrea, 2006; Zhang et al., 2009). Taken together, both loss of proper pathway choice and decreased fidelity of DSB repair can cause tumor formation, in particular of the breast and the ovary.

Fluorescence-Based DSB Repair Analysis

Our preceding work has led to the development of a fluorescence-based assay for the quantitative and qualitative analysis of distinct DSB repair mechanisms (Akyüz et al., 2002). The methodology relies on a test system, which measures DSB repair processes on the basis of cellular fluorescence signals from the expression of enhanced GFP (EGFP) in mammalian cells. The use of a reporter gene product emitting fluorescent light rather than conferring antibiotic resistance provides a fast readout and, therefore, enables application even on short-lived primary cells from man and mouse (Keimling et al., 2008). This property also allows distinguishing between early repair-related and late growth regulatory or apoptotic activities. Thus, this fast readout system is superior to systems relying on clonal outgrowth of cells under selection pressure, which obviously does not allow to separate between growth-/death- and repair-related effects (Willers et al., 2002). This is critical for the analysis of proteins with dual roles in DSB repair and checkpoint control such as p53. Inclusion of the rare-cutting I-*Sce*I endonuclease enables to specifically cleave the reporter gene and, thereby, to exclude indirect effects on DSB repair due to activities in other repair pathways such as nucleotide excision repair. The assay system comprises a series of different plasmids, designed for the qualitative and quantitative analysis of NHEJ, SSA, and HR pathways (Fig. 1). Direct comparison of different pathways on the basis of the fast EGFP reporter each is another improvement compared to previously performed analysis of one pathway via the reporter and other pathways semi-quantitatively via PCR analysis of the DNA products (Weinstock et al., 2006). Indeed, parallel use of DNA substrates that allow to distinguish between DSB repair mechanisms initiated by RAD51-dependent strand invasion, i.e. mostly gene conversion (HR), non-conservative, homologous DSB repair events, i.e. mostly SSA, and mutagenic, i.e. microhomology-mediated, NHEJ successfully demonstrated interference of BRCA1 with both NHEJ and SSA

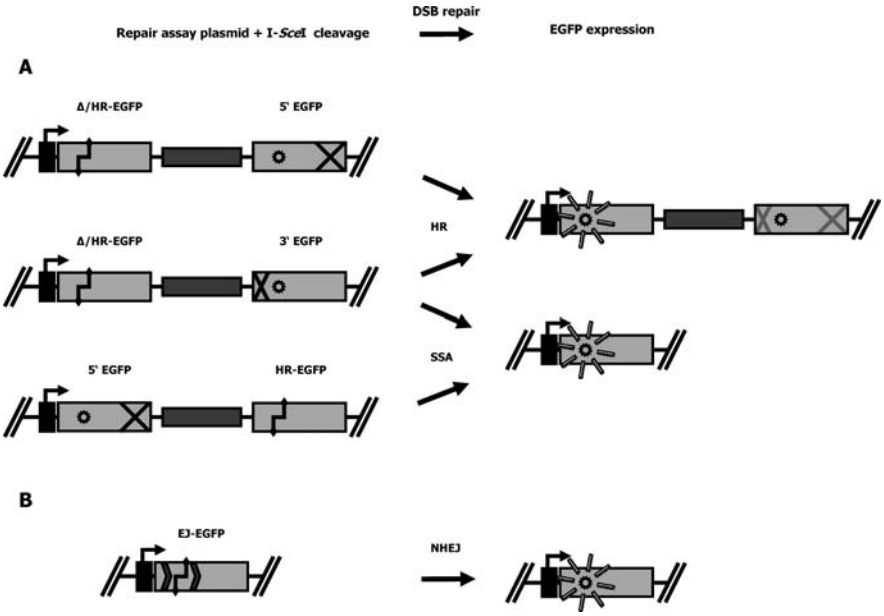

Fig. 1 Schematic of repair assay substrates and products. To analyze DSB repair, the previously established, fluorescence-based test system is used (Akyüz et al., 2002). The assay is based on the restoration of a functional *EGFP* sequence through genetic exchange. The so-called acceptor *EGFP* is cleaved by meganuclease I-*Sce*I at the nucleotides encoding the chromophore amino acids (*circle*) followed by rejoining of the cleaved DNA termini (NHEJ) or homologous sequence exchange with the uncleaved donor *EGFP* (HR, SSA). The frequency of DSB repair events is monitored by FACS analysis as the fraction of green fluorescing cells within the transfected cell population. Thus, to measure DSB repair, the cells are simultaneously transfected with I-*Sce*I expression vector, one of the DSB repair plasmid constructs, and pBS (control vector) or wt*EGFP* plasmid (for transfection efficiency) each, further cultivated for DSB repair to take place and, then, subjected to flow cytometry. To correct for plasmid uptake, translational, transcriptional, growth- and death-related changes, the amount of green fluorescent cells in repair assays (with pBS) are individually normalized by use of the correspondingly determined transfection efficiency (with wt*EGFP* plasmid). The left part of the chart displays different repair plasmid constructs and the right part DSB repair products from HR, SSA, and NHEJ, respectively. *Light gray*: EGFP, *black*: CMV promoter, *dark gray*: spacer. (**a**) Plasmid constructs for detection of homologous DSB repair. Δ-EGFP and HR-EGFP acceptor genes contain an I-SceI restriction site (↱). The surrounding sequences share short (Δ-EGFP) or long (HR-EGFP) homologies with the donor sequences, because the I-*SceI* recognition sequence replaces either 46 bp (Δ-EGFP) or 4 bp (HR-EGFP) within the wtEGFP sequence. Donor genes are either an N-terminally mutated (5′EGFP) or a C-terminally truncated (3′EGFP) *EGFP* gene. 5′EGFP encodes a non-fluorescent EGFP variant devoid of the C-terminal chromophore environment (big cross); in 3′EGFP stop codons replace the start methionine (small cross). (**b**) Plasmid for the detection of microhomology-mediated NHEJ with 5 bp homologous sequences (❱ ❱) flanking the I-SceI cleavage site

and BRCA2 with SSA. Moreover, multi-substrate testing was also demonstrated to detect breast cancer predisposing DSB repair defects (Keimling et al., 2008). When we applied HR substrates differing in the length of homologies adjacent to the targeted DSB, we discovered that the tumor suppressor p53 and the mismatch repair protein MLH1 counteract low-fidelity HR processes involving short homology tracts (Gatz and Wiesmüller, 2006; Siehler et al., 2009).

Thus, the fluorescence-based DSB repair assay provides researchers with a method (i) to characterize the role of genes/gene variants in DSB repair through application on cells differing in the respective genotype. Further, the assay serves (ii) to detect functional changes in DSB repair of whole organisms ex vivo and, therefore, may represent a useful biomarker for pathological dysfunction. Depending on the specific defect identified, the change in DSB repair may be associated with combined immunodeficiency diseases, chromosome breakage syndromes, or increased breast and ovarian cancer risk with known and, even more importantly, with unknown gene mutations, may enable candidate selection for the identification of the specific gene defect, and could also be relevant for therapeutic responsiveness to novel therapeutics (Ralhan et al., 2007; Keimling et al., 2008). Moreover, in screening approaches with siRNA- or expression-libraries (iii) this assay can be useful for the identification of the exact genetic change underlying a specific repair defect.

Materials

Solutions and Reagents

all protocols

- Ca^{2+}/Mg^{2+}-free phosphate-buffered saline (PBS)

transfections (and cell culture)

- Mouse embryonic fibroblasts (MEFs). In our laboratory we used primary fibroblasts from BALB/c and C57BL/6 mice
- MEM Alpha Modification (α-MEM) with L-Glutamine (e.g. PAA Laboratories, Pasching, Austria)
- Fetal Bovine Serum (FBS), (e.g. FBS Gold, PAA Laboratories, Pasching, Austria)
- siRNA: 10–20 μM siRNA solution (e.g. from Qiagen, Hilden, Germany)
- Plasmid mix (0.5–1 μg/μl) with equal amounts of repair assay plasmid (for details see Fig. 1), I-*SceI* expressing vector and "filling" plasmid pBS (Stratagene, Heidelberg, Germany). Replace pBS by wild-type (wt) EGFP expressing vector to determine transfection efficiency in a split sample each.
- HEPES-buffered saline (HBS): 20 mM HEPES, 150 mM NaCl, pH 7.4

- Hiperfect transfection reagent (Qiagen, Hilden, Germany)
- Fugene HD (Roche, Penzberg, Germany)

processing cells for flow cytometry

- Trypsin-EDTA
- PBS-EDTA: PBS supplemented with 0.2% (w/v) $Na_2EDTA \cdot 2H_2O$

cell cycle analysis

- 96% Ethanol
- Propidium iodide staining solution: 20 μg/ml propidium iodide, 80 μg/ml DNase-free RNase, 0.1% (v/v) Triton X-100 in PBS, prepared freshly

Equipment

- Laminar flow hood
- Humidified CO_2 incubator
- Benchtop centrifuge with swing-out rotor
- Flow cytometer equipped with 488 nm argon laser (e.g. FACSCalibur, BD Biosciences, Heidelberg, Germany)
- Plasticware: 6-well tissue culture plates, sterile and non-sterile round-bottom 96-well plates, FACS tubes, adhesive foliage

Protocols

Transfection of MEFs with Repair Assay Plasmids

Without further treatment this method routinely yields a transfection efficiency of 10–20%.

(1) 24 h before transfection, plate out 125,000 cells per well of a 6-well plate in 2 ml α-MEM supplemented with 10% fetal bovine serum each to reach 80–90% confluency the next day.
(2) Optional step for specific drug treatment of cells: one hour before transfection replace growth media by media supplemented with active component or inhibitor.
(3) To start preparing the transfection mix, add 1.25 μg of plasmid mix to each well of a round-bottom 96-well plate, then add 100 μl α-MEM without FBS and antibiotics, mix.

(4) Add 5 μl Fugene HD to each well, mix immediately by pipetting up and down several times, incubate exactly 15 min. Then add transfection mix to cells, while shaking the plate.
(5) The following day, process cells for flow cytometry measurement (see separate protocol below).

Co-transfection of MEFs with siRNA and Repair Assay Plasmids

Note: A total amount of 24 pmol siRNA is used in this protocol to roughly give a 10 nM final concentration. This method routinely yields a transfection efficiency of 3–10%.

(1) 24 h before transfection, plate out 100,000 cells per well of a 6-well plate in 2 ml α-MEM supplemented with 10% fetal bovine serum each to reach 60–70% confluency at the time of transfection.
(2) To start preparing the transfection mix, add 1.25 μg plasmid mix to each well of a round-bottom 96-well plate (e.g. Sarstedt, Nümbrecht, Germany), add 100 μl HBS buffer and 24 pmol siRNA, then mix with a plate shaker.
(3) Prepare the Hiperfect mix by adding Hiperfect reagent to HBS buffer, vortex immediately for 20 s, then incubate for 5 min. For each transfection sample use 85 μl HBS and 15 μl Hiperfect reagent.
(4) Prepare the final transfection mix by adding 100 μl of the Hiperfect mix to each well with the DNA/siRNA put in before, mix well by pipetting up and down several times, incubate 5–10 min.
(5) Add the transfection mix to cells, one by one, with shaking.
(6) 24 h after transfection, add fresh media to cells.
(7) 48 h after transfection, process cells for flow cytometry measurement (see separate protocol below).

Note: The Fugene HD transfection method for DSB repair analysis can be combined with the Hiperfect-transfection method for siRNA-mediated knockdown, which will improve transfection efficiencies compared with concomitant plasmid and siRNA transfection. Thus, transfect siRNA with Hiperfect first and then introduce plasmids by use of Fugene HD on two consecutive days.

Processing of Cells for Flow Cytometric Quantification of EGFP-Positive Cells

(1) Remove medium from treated cells in 6-well plates.
(2) Wash cells once with PBS.
(3) Wash cells once with Trypsin-EDTA.
(4) Add 250 μl Trypsin-EDTA, incubate until cells are detached, then stop tryptic digest by adding 75 μl FBS.

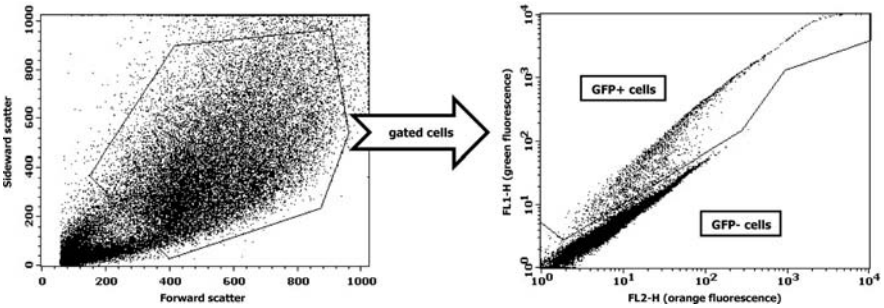

Fig. 2 Flow cytometry analysis of MEFs transfected with wtEGFP expressing vector. The *left window* shows gating of cells in the forward (FSC) vs. sideward scatter (SSC) dot plot, to select vital cells with a well-defined size and shape (*central area* on the diagonal), thereby eliminating debris and clumps. This gated population is then subjected to fluorescence analysis. The *right window* shows a representative example for the discrimination of EGFP expressing, green fluorescent (GFP+) from non-expressing (GFP-) cells by use of the diagonal gating method in the green (FL1) vs. orange (FL2) fluorescence dot plot. The plotted events are pulse heights detected through the 530/30 nm (FL1) vs. 585/42 nm (FL2) bandpass emission filters. GFP+ cells are found in the comet above the GFP- cells, which lie on the diagonal

(5) Transfer each sample to one well of a 96-well round bottom plate, centrifuge for 5 min at 500×*g*, pour out supernatant (with speed) and pat dry plate on a paper towel.
(6) Add appropriate volume of PBS-EDTA (100–250 µl), resuspend pellet by pipetting up and down several times, and quantify green fluorescent cells using a flow cytometer equipped with a 488 nm laser.
(7) Plot live cells from the forward scatter (FSC) vs. sideward scatter (SSC) gate with FL1 (530 nm, green fluorescence) vs. FL2 (585 nm, orange fluorescence) without compensation between FL1 and FL2.

Cells which express EGFP will appear above the FL1-FL2 diagonal of non-EGFP expressing cells (Fig. 2).

Processing Cells for Cell Cycle Analysis in 96-Well Plate

(1) Centrifuge cell suspension in 96-well plate, e. g. remaining transfected cells after FACS measurement, 5 min at 500×*g*, pour out supernatant (with speed) and pat dry on a paper towel.
(2) Add 50 µl PBS-EDTA, resuspend pellet by pipetting up and down several times.
(3) Transfer suspension to 96-well plate, wells pre-filled with 150 µl EtOH (96%) on ice, and leave on ice for 15–30 min.
(4) At this point cover plate with self-adhesive foliage (e.g. MultiAmp Clear Adhesive Film, Applied Biosystems, Darmstadt, Germany) and store at –20°C, or proceed with propidium iodide staining (steps 5 and 7–12).

(5) For propidium iodide staining re-adapt the frozen 96-well plate to room temperature and centrifuge at 500×*g* for 5 min.
(6) Remove self-adhesive foliage, centrifuge again 5 min at 500×*g*, pour out supernatant (with speed) and pat dry on a paper towel.
(7) Wash pellets by adding 200 μl PBS, resuspend by repeatedly pipetting up and down.
(8) Centrifuge 5 min at 500×*g*, pour out supernatant (with speed) and pat dry on a paper towel.
(9) Add propidium iodide staining solution (usually 100–150 μl) and resuspend by pipetting up and down several times.
(10) Incubate plate 30 min at 37°C.
(11) Measure cellular DNA content in samples with flow cytometer in the FL2 channel (585 nm) and plot pulse width vs. pulse area.
(12) Analyse cell cycle phase distribution directly in FL2-area histogram and/or with appropriate software (e.g. ModFit from Verity Software House, Topsham, ME, USA).

General Notes

(1) To enable detection of statistically significant differences between DSB repair frequencies in different cell types or after Introduction of different siRNAs, assay conditions should be optimized such that at least 5–10 fluorescing cells are counted per sample. Low plasmid transfection efficiencies, high toxicities, and low proliferation rates are the major obstacles that may lead to values below the detection limit.
(2) The sensitivity of the assay can be raised by transfection of and subsequent measurement of large cell numbers (e.g. transfection of 1,250,000 instead of 125,000). Negative controls performed in parallel with cells transfected with I-*Sce*I expressing vector and inactive DSB repair plasmid devoid of acceptor or donor gene will, then, exclude detection of false positives.

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Apoptosis: A Way to Maintain Healthy Individuals

Chiara Mondello and A. Ivana Scovassi

Abstract Apoptosis, the best known form of programmed cell death, is tightly regulated by a number of sensors, signal transducers and effectors. Apoptosis is mainly active during embryonic development, when deletion of redundant cellular material is required for the correct morphogenesis of tissues and organs; moreover, it is essential for the maintenance of tissue homeostasis during cell life. Cells also activate apoptosis when they suffer from various insults, such as damage to DNA or to other cellular components, or impairment of basic processes, such as DNA replication and DNA repair. Removal of damaged cells is fundamental in maintaining the health of organisms. In addition, apoptosis induction following DNA damage is exploited to kill cancer cells. In this chapter we will review the main features of developmental and induced apoptosis.

Keywords Aneuploidy · Apoptosis · Cancer · Development · Genome instability

Abbreviations

AIDS	acquired immune deficiency syndrome
AIF	apoptosis inducing factor
APC/C	anaphase promoting complex/cyclosome
ced	cell death abnormal
CIMD	caspase independent mitotic death
DISC	death inducing signaling complex
DS	Down Syndrome
eEF1A1	eukaryotic translation elongation factor-1 α1
ES	embryonic stem
PARP-1	poly(ADP-ribose)polymerase-1
SAC	spindle assembly checkpoint
TRAIL	tumor necrosis factor-related apoptosis-inducing ligand
17-AAG	17-allylaminogeldamycin

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Introduction

Not always cell death is a detrimental process, in fact cells are endowed with mechanisms of programmed cell death, the best known of which is apoptosis, which can be activated either in physiological or pathological conditions and free the organism from undesired cells. During embryonic development, activation of apoptosis allows remodelling of the growing organism, while, in a wider number of situations, it plays a role in eliminating damaged cells. To maintain a healthy individual, the integrity of the genome of the cells must be preserved. Several processes are deputed to this task, such as mechanisms contributing to faithful DNA replication, to the removal of DNA lesions, and to the control of the correct timing and development of the cell cycle. If these mechanisms fail, a wide range of genetic alterations is generated, from point mutations to variations in the number of chromosomes or in their structure, leading to highly altered cells. Apoptosis is involved in the removal of these cells, which could compromise the organismal homeostasis. The ability of apoptosis to eliminate damaged cells is also exploited in cancer treatment; in fact, cell exposure to drugs activating apoptosis can lead to the massive death of cancer cells. In this chapter, we aim at describing the apoptotic process and its role in the maintenance of healthy individuals.

Apoptosis: General Considerations

Apoptosis is the best known form of programmed cell death, defined for the first time by Kerr, Currie and Wyllie (Kerr et al., 1972) as an event reminiscent of the falling of leaves from trees. Apoptosis is activated in a number of developmental conditions and during cell life can be triggered by "external" stimuli, among which different types of damage either to DNA or to different cellular components (Scovassi, 2006) (see below).

Apoptotic cells can be easily identified by typical morphological changes, such as shrinkage, chromatin aggregation and loss of plasma membrane integrity, which ultimately originate the apoptotic bodies, consisting of chromatin fractions sorrounded by a thin layer of blebbed plasma membrane. Apoptosis triggers cell death without rising any inflammatory response, given that apoptotic bodies are eliminated by phagocytosis through the recognition of peculiar "eat-me signals" of various nature (Gregory and Brown, 2005).

Concomitantly to the above described morphological hallmarks, typical biochemical reactions take place, including controlled and organized DNA and protein digestion, operated respectively by specific endonucleases and proteases (Scovassi and Torriglia, 2003; Counis and Torriglia, 2006). Among the latter enzymes, the best known are *caspases* (*c*ystein *asp*artate prote*ases*), so called because they contain a cystein residue in their catalytic site and cleave their substrates after an aspartic acid (Kumar, 2007). Caspases exist as inactive zymogens that are proteolytically converted into active enzymes in response to apoptotic stimuli (Shi, 2004). Apoptotic caspases include "initiator" caspases (caspase-2,-8,-9 and -10) which start

the proteolytic cleavage and "effector" caspases (-3,-6 and -7) that dismantle the cell by digesting a number of proteins (Scovassi, 2005; Giansanti and Scovassi, 2008a). Some targets of caspases are involved in the maintenance of cell structures, others control DNA metabolism (Luthi and Martin, 2007; Li and Yuan, 2008); the best known is the DNA repair protein poly(ADP-ribose)polymerase-1 (PARP-1), whose proteolysis is considered as a hallmark of apoptosis (Soldani et al., 2001; Soldani and Scovassi, 2002; Scovassi and Diederich, 2004) (see "Introduction").

Apoptosis occurs mainly through two different pathways: the extrinsic and the intrinsic one, the former being mediated by soluble molecules that interact with outer membrane receptors, and the latter triggered by mitochondria-converging stimuli. Indeed, these pathways are not completely distinct and partially overlap, both converging to the activation of effector caspases (Fadeel and Orrenius, 2005; Green, 2005; Letai, 2008). As illustrated in Fig. 1, the extrinsic pathway is triggered by external stimuli leading to the activation of initiator caspase-8, whereas intrinsic signals converge to mitochondria and promote the activation of caspase-9.

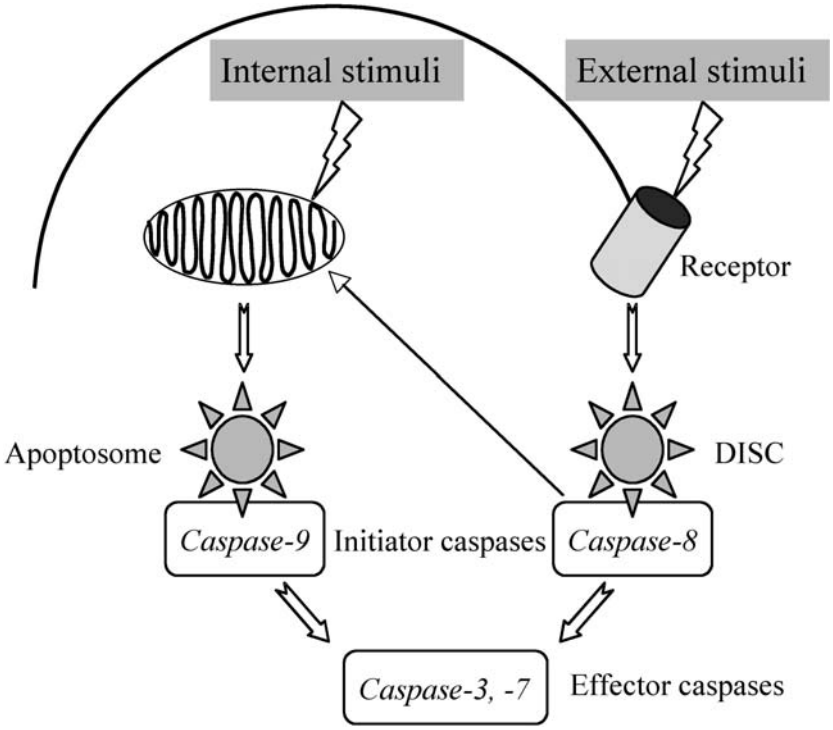

Fig. 1 Simplified scheme of apoptotic pathways. *Right*: *Extrinsic pathway*. External stimuli are sensed by receptors that promote the formation of DISC (*d*eath *i*nducing *s*ignaling *c*omplex), which includes caspase-8. *Left*: *Intrinsic pathway*. Internal stimuli converge to mitochondria; these organelles release factors leading to the assembly of the apoptosome, which is formed also by caspase-9. Both initiator caspase-8 and -9 activate effector caspase-3 and -7, which are responsible for further DNA and protein degradation. The intercorrelation between the two pathways is exemplified by the path connecting caspase-8 to mitochondria

Mitochondria can be considered as a central target of apoptogenic conditions, being also disturbed by caspases mainly involved in the extrinsic pathway. The final step of both ways is the activation of effectors caspases, which cleave several proteins and promote DNA degradation (Scovassi, 2005; Giansanti and Scovassi, 2008b).

Apoptosis During Embryogenesis and Development

Developmental Apoptosis and Model Organisms

During embryonic development, apoptosis takes part in several processes, including the separation of the digits, metamorphosis and atrophy of tissues and organs, sexual differentiation and tissue turnover, when deletion of redundant cellular material is required (Jacobson et al., 1997; Vaux and Korsmeyer, 1999; Zakeri and Lockshin, 2002). The so-called "developmental apoptosis" is involved in the regulation of cell numbers in response to feeding, in regeneration and removal of non-self cells. It also participates in the maintenance of cellular homeostasis in germ cells (Penaloza et al., 2008).

These features have been elucidated in animal models with a conveniently small number of cell types, such as the nematode *Caenorhabditis elegans*, which is characterized by the selective elimination of 131 out of 1,090 cells during embryo development (Ellis et al., 1991). Genetic studies in this organism provided the first evidence for the gene products essential in regulating apoptosis, i.e. *ced* (cell death abnormal*)-3, ced-4, ced-9*, the latter being a negative regulator of the others (Metzstein et al., 1998). The evidence for a genetic control of life and death in *C. elegans* represented the starting point in the definition of apoptosis in more complex multicellular organisms and made it possible to progress in the apoptosis field. The considerable efforts in this field were rewarded in 2002 with the Nobel Prize in Physiology and Medicine to Sydney Brenner, Robert Horvitz and John Sulston for their discoveries concerning genetic regulation of organ development and programmed cell death in *C. elegans*.

Also fresh water polyp *Hydra,* one of the simplest metazoans, represents a useful model for studying the evolution of developmental death in multicellular organisms. As recently reviewed (Bottger and Alexandrova, 2007), the morphology of apoptotic cells identified during Hydra development is indistinguishable from that of higher animals; apoptosis proceeds through caspase activation and is mediated by Bcl-2 family members (Bottger and Alexandrova, 2007), thus supporting the evolutionary conserved nature of apoptosis (Degterev and Yuan, 2008).

Cell proliferation and physiological cell death are highly coordinated and tightly controlled during normal development. Cell loss can occur in response to stress and damage, thus promoting "compensatory proliferation" in surrounding cells to maintain tissue homeostasis, as it occurs in tissue regeneration. The control mechanisms of this kind of proliferative process have been depicted in *Drosophila melanogaster* (Fan and Bergmann, 2008). The authors speculate that compensatory proliferation might have a pathological relevance for tumor development, when

apoptosis-resistant cells secrete mitogenic stimuli able to promote uncontrolled proliferation. The switch from death to life relies extensively on targeted degradation of cell death proteins by the ubiquitin-proteasome pathway (Steller, 2008). Since this insect offers the unique possibility to carry out genetic screenings of genes that determine life/death of specific cell populations during development and adulthood, it has also been instrumental for the identification of the mammalian factors involved in the balance between proliferation and death (Hay and Guo, 2006).

Apoptosis and Germ Cells

As for the induction of apoptosis to preserve genome integrity, the best example is represented by germ cells, which cannot tolerate a high level of DNA damage, given that it can affect future generations (Hong et al., 2007). Germline apoptosis, which occurs as part of a developmental program, shares with somatic apoptosis key components of the core apoptotic machinery. The main features of germline apoptosis have been reviewed by Gartner et al. (2008), who defined a "physiological" pathway of apoptosis and a "stress-induced" germline apoptosis in the nematode *C. elegans*, the latter triggered by certain environmental insults or pathogens. This evidence enforces the notion that many apoptotic regulators are highly conserved, from worms to mammals (Oberst et al., 2008).

A tuned regulation of cell death occurs in mammalian oocytes. According to a precise developmental program, the vast majority of oocytes acquired during embryonic development do not survive beyond birth and are gradually depleted over the organism's lifetime through the process of apoptosis, possibly as a result of limited amounts of trophic factors (Tilly, 2001). Also pathological insults or chemotherapy promote oocyte death, leading to premature oocyte depletion and sterility (Kim and Tilly, 2004). Attention has been paid to the apoptotic features of dying oocytes, including caspase activation (Nutt et al., 2005), PARP-1 proteolysis (Ghafari et al., 2007) and expression of Bcl-2 family members (Kim and Tilly, 2004). Moreover, it has recently been observed that fetal oocytes possess and are able to activate several players of forms of cell death other than apoptosis (De Felici et al., 2008).

During male germ cell development, the growing number of cells requires the equilibrium with the number of Sertoli cells, which ensure the nutritional environment necessary for correct development (Johnson et al., 2008). This goal is achieved through the activation of the apoptotic process in an orchestrated manner (Boekelheide et al., 2000). Apoptosis also plays an important role in removing abnormal germ cells: during germ cell development, DNA integrity has to be preserved for successful fertilisation, as well as for further embryonic and fetal development (Matulis and Handel, 2006). Damaged spermatocytes originate from environmental stress, gene mutation or chromosomal abnormalities and can trigger infertility unless they are not efficiently eliminated by apoptosis (Matulis and Handel, 2006).

A further class of cells that play a crucial role in embryogenesis is represented by embryonic stem (ES) cells, which are characterized by high proliferation

rate, specialisation and ability to differentiate. ES cannot cope with high levels of damage, given that once altered they can affect the development of the organism. Since ES cells are hypersensitive to DNA damaging agents, apoptosis is activated to eliminate damaged cells from the population, thus ensuring the preservation of genome integrity (Tichy and Stambrook, 2008).

Apoptosis in Neurons and Lymphocytes

Neurons normally die in large numbers during development, possibly to counteract the paucity of specific neurotrophic factors that are required for their survival. Cecconi et al. (2008) recently reviewed the concept that deletion of unwanted cells regulates the correct morphogenesis and cell number in developing central nervous system. In fact, mice defective for apoptotic factors (e.g. caspases) display prominent neuronal defects, including the lack of neural tube closure. Remarkably, apoptosis is not the exclusive form of cell death involved in neural tube defects: also autophagy, that is both distinct from and intercorrelated with apoptosis (Maiuri et al., 2007; Thorburn, 2008), has proven to be active in neural development (Cecconi and Levine, 2008).

Defects in the control of inappropriate death of neurons are associated to a number of neurodegenerative disorders (Mattson, 2000; Vila and Przedborski, 2003), including Huntington (Gil and Rego, 2008), Parkinson (Andersen, 2001) and Alzheimer diseases (Takuma et al., 2005), which are characterized by extensive neuron loss.

Neuron loss can be driven by an imbalance in gene dosage, as it occurs for Down Syndrome (DS), where a reduction in both the number and the density of neurons in the brain is correlated with trisomy of chromosome 21 (Sawa, 1999). The additional copy of chromosome 21 leads to extra copies of a number of genes, among which a crucial role is recognized to that coding for amyloid precursor protein, already involved in neuron apoptotic death in Alzheimer disease (reviewed by Wiseman et al., 2009).

Deletion of unwanted cells is also critical for the development and maintenance of the innate and adaptive immune system: circulating neutrophils are continuously eliminated and further replaced; the vast majority of developing thymocytes dies before their conversion to mature T cells in order to remove self-reactive and potentially autoimmune lymphocytes (negative selection process). All these clearance processes occur through apoptosis without inflammation, due to the rapid and efficient removal of apoptotic bodies by phagocytes (Erwig and Henson, 2007). Interestingly, apoptotic cell clearance defects can contribute to the development of autoimmunity (Rosen and Casciola-Rosen, 1999; Erwig and Henson, 2007; Rovere-Querini et al., 2008).

Excessive apoptosis of lymphocytes in adults is a hallmark of immunodeficiency syndromes (Rosen and Casciola-Rosen, 1999; Zhang et al., 2005; Bidere et al., 2006; Erwig and Henson, 2007; Giovannetti et al., 2008; Rovere-Querini et al., 2008). This was first demonstrated in acquired immune deficiency syndrome

(AIDS) patients by the pioneering work of Gougeon and Montagnier (Gougeon and Montagnier, 1992, 1993), who found a correlation between HIV-associated T-cell depletion and progression to AIDS. A body of evidence supports the hypothesis that cell dysfunction and depletion in AIDS are due to deregulated programmed cell death (Gougeon, 2003).

Apoptosis and DNA Damage

Apoptosis Regulators in Response to DNA Damage

The cellular response to DNA damage may involve three distinct processes (i) cell cycle arrest, which allows time for the repair of DNA damage; (ii) DNA repair, and (iii) cell death, triggered either by unsuccessful DNA repair or too heavy levels of DNA damage. A number of damage sensors cooperate with factors that initiate an apoptotic response. The most active "guardian angels" of DNA integrity are P53 and PARP-1. P53 plays a central role in maintaining genome integrity by controlling the initiation of DNA repair, cell cycle arrest, senescence and apoptosis (Rodier et al., 2007; Riley et al., 2008). After the pioneering work of M. Oren showing that the product of the *p53* suppressor gene regulates apoptosis (Yonish-Rouach et al., 1991), accumulating evidence supported the relevance of P53-mediated apoptosis in suppressing tumorigenesis, and possibly driving cancer cells to death (Meulmeester and Jochemsen, 2008; Pietsch et al., 2008; Riley et al., 2008; Vazquez et al., 2008). Reactivation of the P53 pathway in tumors with reduced or null P53 activity (about 50% of all tumors) could represent an efficient anticancer strategy (Rodier et al., 2007; Vazquez et al., 2008). Other members of the P53 family, namely P63 and P73, are involved in apoptosis induction. In the absence of functional P53, P73 activation by damage sensor kinases can lead to P53-independent apoptosis (Pietsch et al., 2008).

The other "angel" PARP-1 (Jeggo, 1998) carries out the poly(ADP-ribosylation) reaction, i.e. a post-translational modification of proteins that mediates, in eukaryotic cells, cell proliferation and death, DNA repair and replication, and transcription (Scovassi 2002; Schreiber et al., 2006; Hassa, 2009). In the presence of DNA strand breaks, PARP-1 promotes the rapid synthesis of polymers of ADP-ribose from NAD and their subsequent transfer to nuclear acceptor proteins, including PARP-1 itself, thus modifying DNA-protein and protein-protein interactions due to the negative charge of poly(ADP-ribose) (Scovassi et al., 1998). This prompt response to DNA damage guarantees the accessibility of damaged DNA to repair factors (Oei et al., 2005; Frouin and Scovassi, 2008). When DNA damage persists, activation of PARP-1 is no more beneficial for the cell, and the protein is inactivated through the proteolysis operated by apoptotic caspases to avoid excessive NAD consumption (Scovassi et al., 1998; Scovassi and Poirier, 1999; Soldani et al., 2001; Chiarugi, 2002; Soldani and Scovassi, 2002; Scovassi and Diederich, 2004). Under mild and moderate damage in vivo, PARP-1 favours DNA repair, thus

preserving genome stability; in contrast, in the presence of high levels of DNA damage, it promotes the apoptotic elimination of heavily damaged cells, which, loosing genome integrity, could compromise organismal goodness. The relevance of PARP-1 in driving cell death is also supported by the observation that its overactivation initiates a nuclear signalling that propagates to mitochondria and triggers the release of the Apoptosis Inducing Factor (AIF). These events identify a peculiar form of caspase-independent/PARP-1-dependent apoptosis (Hassa, 2009). The surveillance network against DNA damage implies the interaction of PARP-1 with P53 (Malanga and Althaus, 2005; Won et al., 2006; Kanai et al., 2007), thus supporting the existence of cooperation between these proteins in maintaining genome integrity.

Since several types of DNA damage, induced by a variety of agents, including oxidants and free radicals, gamma and UV radiation, can trigger apoptosis (Scovassi 2006), it has been envisaged that damaged/unrepaired DNA could be the basis of anticancer therapies (Call et al., 2008). In fact, drugs that cause DNA damage are among the most effective compounds used in chemotherapy today and kill tumor cells by apoptosis. For example, anti-topoisomerase drugs promote the stabilisation of the so-called "cleavable complex" which is constituted by DNA, topoisomerase and drug (Degrassi et al., 2004; Montecucco and Biamonti, 2007). The presence of this structure is an obstacle to DNA replication ad causes DNA double strand breaks, thus promoting accumulation of cells in the G2 phase of cell cycle and consequent death (Negri et al., 1993, 1995; Bernardi et al., 1995; Kaufmann, 1998; Sordet et al., 2003). The ability of topoisomerase II inhibitors to trigger cells to apoptosis has been exploited for the development of several classes of anticancer drugs that are widely employed for the clinical treatment of human malignancies (McClendon and Osheroff, 2007).

Apoptosis and Aneuploidy

An early event in tumorigenesis involves the abrogation of integrated DNA damage response resulting in increased genome instability and enhanced mutation rate that facilitate cancer formation. Genome instability can be viewed as a condition leading to the accumulation of different types of genetic alterations. A specific subset of genome instability is chromosomal instability, which is associated with the genesis of structural chromosomal aberrations or with variations in the chromosome number. The number of chromosomes can differ from the species specific one for multiples of an entire set of chromosomes (polyploidy), or for the gain or loss of a limited number of elements (aneuploidy).

That aneuploidy could be associated with cancer was first hypothesized at the beginning of the twentieth century by the German scientist Theodor Boveri (Boveri, 1914), who was the first to envisage that chromosomes are not all equivalent, but each of them has its individuality. Boveri realized that aneuploidy is a common feature of cancer cells and proposed that an abnormal chromosome number could alter the cellular behavior leading to tumorigenesis.

Since Boveri's intuition, a huge amount of data has shown that aneuploidy is a prevalent condition in cancer; however, it is still a matter of debate whether aneuploidy is a cause or a consequence of cancer. Despite there are scientists strongly supporting one or the other opinion (Duesberg, 2007; Ricke et al., 2008), a large body of evidence indicates that both of them can be true in different circumstances (for a review see Chandhok and Pellman, 2009).

In somatic cells, aneuploidy is generated during mitosis. For a faithful separation between daughter cells, sister chromatids must attach through their kinetochores to microtubules of the bipolar mitotic spindle. A defect in this attachment activates a checkpoint known as mitotic or spindle assembly checkpoint (SAC), which prevents progression to anaphase until proper attachments are not fulfilled. SAC is also activated by treatment with drugs that either inhibit microtubule polymerization (e.g. nocodazole and vinca alkaloids) or prevent microtubule dynamics by stabilising microtubules themselves (e.g. taxanes) (Checchi et al., 2003). SAC was first discovered in yeast and then it was found that proteins taking part in this checkpoint are highly conserved in humans as well (Rieder and Maiato, 2004). Major human SAC proteins are MAD2, BUB3, BUBR1/MAD3, and Cdc20. Defects in SAC can contribute to chromosomal instability and aneuploidy observed in human tumors, allowing cells to continue mitosis under inappropriate conditions (Cahill et al., 1998; Lengauer et al., 1998; Rieder and Maiato, 2004)

How can cells protect themselves from the risk of aneuploidy due to an aberrant mitosis? One of the main defense mechanism is apoptosis. There is evidence that SAC proteins intervene in the regulation of cell death due to mitotic failure (Suijkerbuijk and Kops, 2008).

Cells exposed to agents interfering with the proper function of the mitotic spindle arrest in mitosis and can die during this cell cycle phase (Fig. 2). Niikura et al. (2007) showed that depletion of BUB1 by RNA interfering, together with the exposure of the cells to mitotic drugs (such as cold shock, treatment with nocodazole, 17-allylaminogeldamycin (17-AAG) or paclitaxel) induces DNA fragmentation during early mitosis. This form of death is independent of caspase activation and p53, while depends on p73, AIF and endonuclease G. This type of death, named by the authors CIMD (Caspase Independent Mitotic Death), is strictly dependent on low levels of BUB1; when BUB1 is completely absent, cells prematurely exit mitosis and CIMD does not occur. Interestingly, treatment with spindle poisons of colon cancer cells lines induces CIMD in several cell lines characterized by chromosomal instability, but not in those characterized by microsatellite instability and this is related to low levels of BUB1 in the former, but not in the latter (Niikura et al., 2007)

MAD2 or BUBR1 depletion lead to premature mitotic exit, linked to abnormal chromosome segregation followed by aneuploidy (Meraldi et al., 2004). Upon treatment with 17-AGG of MAD2 depleted cells, a high frequency of cells with abnormal nuclei and micronuclei was observed by Niikura et al. (2007), suggesting that MAD2 depletion, in contrast to BUB1 reduction, can lead to cell death through mitotic catastrophe (Fig. 2), which is characterized by the

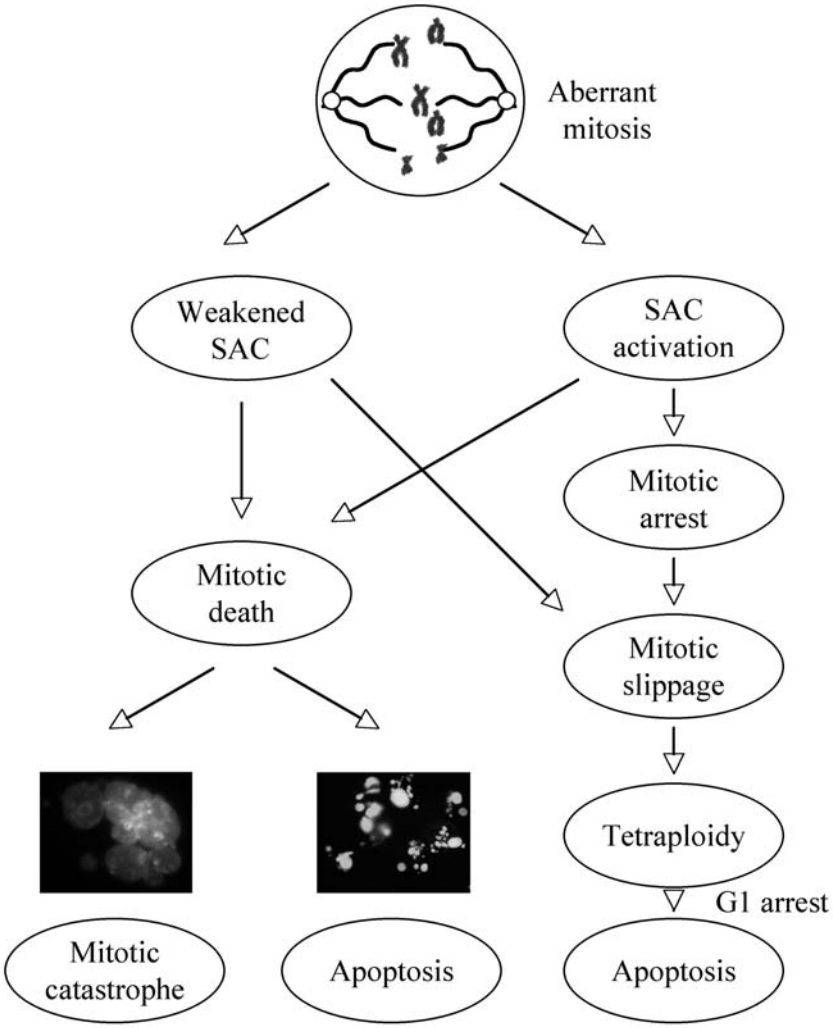

Fig. 2 Possible and interconnected cell death pathways induced by an aberrant mitosis. Examples of nuclear morphologies of cells undergone mitotic catastrophe or apoptosis are shown. SAC: spindle assembly checkpoint

formation of multinucleated and giant cells containing uncondensed chromosomes (Vakifahmetoglu et al., 2008).

A functional SAC is required for arsenite-induced apoptosis. In p53 proficient cells, arsenite trioxide induces G1-arrest (Yih and Lee, 2000; States et al., 2002; Liu et al., 2003), while, in the absence of p53, it induces mitotic arrest, which results in

apoptosis, as shown by the colocalisation of phospho-Histone H3 and active caspase 3 in arrested cells (McNeely et al., 2008). Apoptotic death is mediated by BUBR1; in fact, BUBR1 depletion by RNA interfering prevents caspase activation and PARP-1 cleavage in arsenite-treated cells. In cell lines derived from different types of cancer, a clear correlation between functional activation of SAC and arsenite-induced mitotic arrest and apoptosis was found (Wu et al., 2008).

After prolonged treatment with spindle interfering drugs, cells accumulate in mitosis, however the arrest is not permanent and a reduction in the proportion of mitotic cells is progressively observed, paralleled by an increasing amount of dead cells showing nuclear fragmentation and sub-G1 DNA content. Kim et al. (2005) showed that, in HeLa cells exposed to nocodazole, the escape from the mitotic checkpoint is associated with caspase activation and degradation of BUBR1. In the absence of BUBR1 cleavage, mitotic arrest is prolonged, cell death is reduced and aneuploidy is increased. These results, together with those showing that also BUB1 can undergo caspase degradation (Baek et al., 2005; Perera and Freire, 2005), highligth multiple intersections between activation of the apoptotic enzyme caspases and mitotic checkpoint proteins. Recently, Kim et al. (2008) have shown that tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) stimulates caspase activation and mitotic checkpoint protein degradation, potentiating cell death induced by spindle poisons.

Cells arrested in mitosis for a prolonged time, can adapt and exit. Adapted cells enter G1 with a 4 N DNA content, since they do not undergo cytokinesis, and show nuclear fragmentation (Panvichian et al., 1998). Mitotic checkpoint slippage can occur either because of a faulty checkpoint, or because of proteasome cyclin B1 degradation, in the presence of an active checkpoint (Brito and Rieder, 2006). Cyclin B1 generally drives cells into mitosis together with Cdk1 and, in normal conditions, cells exit mitosis when cyclin B1 is degraded by the anaphase promoting complex/ciclosome (APC/C) (Kotani et al., 1999). If mitosis is protracted, degradation by APC/C is prevented; however, a slow but continuous proteasomal degradation of cyclin B1 occurs until cells exit mitosis. Under normal conditions, these cells undergo a second arrest in the cell cycle and then die by apoptosis (Fig. 2). P53 is a major player in determining the death of the adapted cells and evidence has been reported that its activation requires signaling via BUBR1-mediated phosphorylation (Ha et al., 2007).

Sustained inhibition of the mitotic spindle motor protein Eg5 induces apoptosis through SAC activation and mitotic slippage, both processes are mediated by activation of the pro-apoptotic gene *Bax* (Tao et al., 2005).

If tetraploidy occurs because of mitotic slippage, alternative pathways not implying G1 arrest can lead to cell death. Among these, it is worth mentioning a novel type of caspase-independent cell death, observed in CHO cells, proved to eliminate abnormal tetraploid cells and to inhibit tumorigenesis through the downregulation of eEF1A1 (eukaryotic translation elongation factor-1 α1), a typical house keeping gene product required for the maintenance of cell growth and/or survival. This form of cell death cooperates with P53-dependent apoptosis in avoiding tetraploidy (Kobayashi and Yonehara, 2009).

Concluding Remarks

Living cells maintain genome stability, which is absolutely essential for healthy survival, through complex surveillance and repair systems. Cell cycle arrest, DNA repair and apoptosis may be independent/intercorrelated events aiming at counteracting a dangerous DNA status. If repair is successful, cell cycle arrest is reversed and cells divide, if not, cells survive with DNA defects or die by apoptosis, which normally eliminates cells with damaged DNA or deregulated cell cycle (Jaattela, 1999). Drugs inducing apoptotic death are exploited for cancer treatment and it is more and more important to identify cellular targets that can promptly trigger apoptosis when attacked. It is also important to underline that, in recent years, other forms of cell death besides apoptosis have been discovered (Jaattela, 2004; Lockshin and Zakeri, 2004; Fink and Cookson, 2005; Degterev and Yuan, 2008). Among them, the best known is autophagy, a genetically controlled mechanism that promotes cell death when extensively activated (Levine and Kroemer, 2008). A novel function of autophagy in limiting chromosomal instability has been recently depicted (Mathew et al., 2007), supporting the hypothesis that autophagy, as well as apoptosis, exerts a protective role against DNA damage (Mathew and White, 2007). Taken together, the observations reported in this chapter indicate that regulation of cell death is as important as the regulation of cell life, for the maintenance of a healthy individual.

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The Use of Transgenic Mice in Cancer and Genome Stability Research

Sarah Conmy and Heinz-Peter Nasheuer

Abstract The development of effective cancer therapeutics is an important goal of modern biomedical sciences. To identify potential cancer therapeutic targets, the processes involved in tumorigenesis must be understood at all levels, which requires the development of model systems accurately mimicing tumor development. Cancer is the general name given to a variety of complex diseases characterised by uncontrolled cell proliferation. Cancer development is dependent not only on the changes occurring within the transformed cells, but also on the interactions of the cells with their microenvironment. The majority of our current understanding of carcinogenesis comes from the in vitro analysis of late-stage tumor tissue removed from cancer patients. While this has elucidated many genomic changes experienced by cancer cells, it provides little information about the factors influencing early-stage cancer development in vivo. Also certain hallmarks of cancer, such as metastasis and angiogenesis, are impossible to study in vitro. The mouse has become an important model for studying the in vivo aspects of human cancer development. Transgenic mouse models have been engineered to develop cancers, which accurately mimic their human counterparts, and have potential applications to test the effectiveness of novel cancer therapeutics. One of the most promising transgenic mouse models of human cancer arises from mice engineered with genomic instability. These transgenic models have been shown to develop human-like cancers and have the potential to provide insights into the molecular events occurring in earliest stages of tumorigenesis in vivo.

Keywords Transgenic mice · Cancer · Genome stability

Abbreviations

Apc	adenomatous polyposis coli
ATM	Ataxia-telangiectasia-mutated

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CIN	chromosomal instabilities
Cre/loxP	recombinase systems (Cre recombinase with loxP recognition site)
DDR	DNA damage responses
ES	embryonic stem
FAP	Familial Adenomatous Polyposis
Flp/FRT	recombinase systems (Flp recombinase with FRT recognition site)
HR	homologous recombination
Min	multiple intestinal neoplasia
MIN	microsatellite instabilities
NOD/SCID	non-obese diabetic/severely compromized immunodeficient
OIS	oncogene-induced senescence
Rb	retinoblastoma gene (tumor suppressor gene)
SCID	severely compromized immunodeficient
Terc	telomerase RNA component also called hTR
TERT	telomerase reverse transcriptase subunit
TSG	tumor suppressor gene
tTA	transcriptional transactivator
WT1	Wilms' tumor gene 1
WT1-TCR	WT1-specific T cell receptor

Introduction

The term "cancer" provides the general name for a variety of complex diseases, characterized by uncontrolled cell proliferation. Cellular transformation is a multistep process and requires the accumulation of multiple genomic alterations, each giving the cell a specific growth advantage and allowing clonal expansion and tumor formation (Cahill et al., 1999). The use of in vitro cell culture systems has allowed the identification of genes which are mutated in cancer cells, such as loss of function tumor suppressor genes (TSGs) or gain of function oncogenes, and this has been crucial for our current understanding of cancer development. We are coming to understand however that the malignant transformation of cells is dependent not only on the genetic alterations seen in the transformed cell, but on the transformed cells interactions with its microenvironment, which includes neighboring non-transformed cells and immune cells. Moreover, it is almost impossible to study microenvironment effects and other hallmarks of cancer (Hanahan and Weinberg, 2000), such as metastasis and angiogenesis in vitro. Obvious ethical constraints prevent the testing of novel therapeutics in humans and so the development of systems, which efficiently model human tumorigenesis, is becoming more and more important.

The house mouse, *Mus musculus,* has been the model of choice for the study of cancer for decades due to its; (i) small size and short life span, (ii) large breeding capacity and short gestation period, (iii) physiological and molecular similarities to humans, (iv) sequenced genome and (v) the existence of techniques which allow the genetic manipulation of the mammalian organism. The engineering of transgenic mice to develop human-like cancers has been an important breakthrough in the field

of cancer research. Transgenic mice have provided models, which accurately mimic human cancers (Zhou et al., 2006) and allow monitoring of transformed cells from the earliest stages of cancer initiation and have promising applications in the development of cancer therapeutics. Transgenic mice have also been engineered with genome instabilities (Maser et al., 2007) and these mice were shown to develop human-like cancers. This provides evidence for the mutator phenotype hypothesis (Loeb, 1991) and expands the potential use of transgenic mice to studying the effects of genome instability on cancer progression. In this review some of the advances and limitations of current methods utilized for the generation of transgenic mice will be discussed. Moreover, the roles of these transgenic mice in the fields of cancer and genome stability research, highlighting some of the key findings regarding human tumorigenesis that have been uncovered through the use of transgenic mouse models, will be outlined. Furthermore, the mutator phenotype hypothesis, which predicts that early in tumorigenesis cells experience genome instability leading to malignant transformation, will be discussed.

Evolution of Mouse Models of Cancer

Spontaneous and Carcinogen-Induced Cancers in Mice

Early mouse models of cancer were produced by extensive inbreeding, which resulted in mouse strains that were prone to developing spontaneous or mutagen-induced cancers. An example of a chemical mutagen used was ethylnitrosourea (ENU), which induces mutations in the germ cells of male mice. The use of ENU in one study resulted in mice that had increased incidences of developing spontaneous intestinal adenomoas and adenocarcinomas (Moser et al., 1990) these mice were coined multiple intestinal neoplasia (Min) mice. The Min mouse was later shown to have a mutation in the adenomatous polyposis coli (*Apc*) gene (Su et al., 1992). The *Apc* gene product is a tumor suppressor, which indirectly regulates the transcription of genes controlling cell proliferation. The *Apc* gene has been shown to be mutated in the germline of humans suffering from the rare inherited genetic disease Familial Adenomatous Polyposis (FAP) (Nishisho et al., 1991). FAP sufferers have a predisposition to developing colorectal cancer. Transgenic mice models of colorectal cancer have since been engineered, including those with mutated *Apc* genes, and they provide useful systems for the analysis of human colorectal cancers (as reviewed in Heyer et al., (1999)).

Xenograft Models

Spontaneously occurring murine tumors have been useful in vivo systems, which allowed the identification of oncogenes and tumor suppressor genes (Moser et al., 1990). However, murine tumors are not identical to human tumors, and because of this the xenograft model was developed, which allows the in vivo analysis of

human tumors. Xenograft models are produced by grafting human tumor cells or tissue onto immunocompromized or nude mice. An example of a xenograft model, which has been successfully used in the study of human cancer, is the non-obese diabetic/severely compromized immunodeficient (NOD/SCID) mouse. NOD/SCID mice have been optimized for the effective incorporation of human leukaemias and lymphomas (Hudson et al., 1998). The NOD/SCID mouse model of human leukaemia has been well validated and has been used to study the effects of various human leukaemia therapeutics. An example of a therapeutic tested in NOD/SCID mice is the effects of WT1-TCR gene-transduced T-cells on leukaemias (Xue et al., 2005). WT1-TCR gene-transduced T-cells injected into NOD/SCID mouse models of leukaemia were shown to result in elimination of leukaemia cells (Xue et al., 2005).

Transgenic Mice

Transgenic mice have foreign DNA inserted into the genome of every cell in that mouse. Transgenic mouse models of cancer have many advantages over xenograft models of cancer (as reviewed in Gopinathan and Tuveson (2008)); (i) they have genetic alterations similar to human tumors, (ii) they allow the analysis of cancer development during the various stages of malignant transformation and (iii) they have been developed to accurately reproduce the genetic instability and physiological features of human tumors (Maser et al., 2007; Zhou et al., 2006). But transgenic mice are not without their limitation, different genotypes in mice and human cells have shown to exhibit the same phenotypes. For example, loss of both alleles of BRCA1 gene necessary for tumor formation in mice whereas in humans tumor formation is seen with loss of just one *BRCA1* allele (Hennighausen, 2000). Nevertheless, transgenic mice models have proven to be extremely useful systems for the generation of information regarding human cancer development. The remainder of this review will concentrate on the various types of transgenic mice currently available, how they are generated and the applications of these transgenic mice in cancer and genome stability research.

Production of Transgenic Mice

The two most commonly used methods for the production of transgenic mice are: (i) pronuclear injection and (ii) the genetic engineering of embryonic stem (ES) cells. Both methods have advantages and limitations as outlined below.

Pronuclear Injection

The principle steps involved in the production of transgenic mice via the pronuclear injection method are: (i) isolation of fertilized oocytes, (ii) injection of a DNA construct into the large male pronucleus of the oocyte, (iii) implantation of the injected

oocytes into a pseudopregnant mouse and (iv) genotypic analysis of the offspring to identify transgenic mice (Ittner and Gotz, 2007, see Fig. 1a). This method relies on the random integration of the DNA construct into the genome of the injected oocyte and so the promoter used is key to ensure transcription of the transgene in tissues of interest. The limitations of the pronuclear injection method are; (i) random integration of the transgene DNA construct occurs only in 20–30% of cases (Ittner and Gotz, 2007), (ii) integration of multiple copies of the DNA construct is often seen, resulting in varying levels of gene expression in the transgenic offspring and (iii) in some cases integration of the DNA construct can interrupt the function of essential genes. In spite of these drawbacks the pronuclear injection method is commonly used in the production of transgenic mice.

Genetically Engineered Mouse Embryonic Stem Cells

The ES cell-mediated production of transgenic mice involves a gene targeting approach (Joyner, 1993). Gene targeting exploits the fact that DNA constructs introduced into a cell can recombine with an endogenous homologous sequence in the host genome, by a process known as homologous recombination (HR). The steps involved in ES cell-mediated production of transgenic mice include: (i) isolation of pluripotent ES cells from a mouse blastocyst, (ii) introduction of the DNA construct into the ES cell, (iii) selection for ES cells which have undergone HR and incorporated the DNA in an appropriate manner, (iv) injection of selected ES cells into another mouse blastocyst and (v) implantation of the blastocyst into pseudopregnant female mouse (Joyner, 1993, see Fig. 1b). Resultant offsprings, which have successfully incorporated the transgenic cells, are known as chimeras. If the transgenic cells are incorporated into the germ cells of offsprings they can be bred to produce transgenic mice homozygous for the target transgene.

Types of Transgenic Mice

Traditional Transgenic Mouse Models

Gene trap and gene targeting technologies were traditionally used to produce germ line mutations in mice, leading to activation of oncogenes or inactivation of tumor suppressor genes. Gene trapping involves the random integration of a DNA construct into the genome of the host cell. Gene targeting, outlined above, relies on HR occurring between the insert DNA and an endogenous homologous sequence. While these traditional transgenic models with germline mutations were useful, they were not without their limitations, which include embryonic lethality caused by germline mutations in some tumor suppressor genes, for example *APC, RB* and *BRCA1,* and widespread uncontrolled tumor formation due to constitutive oncogene expression (Jonkers and Berns, 2002).

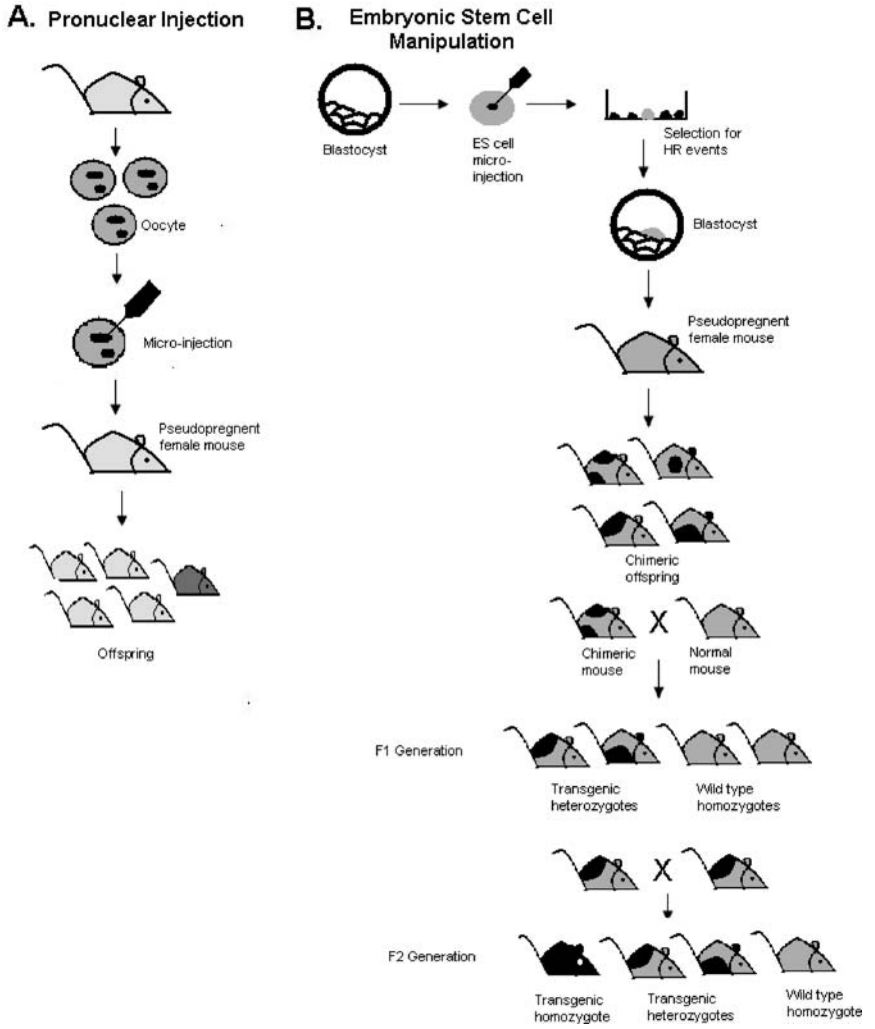

Fig. 1 Pronuclear injection vs. Embryonic stem (ES) cell-mediated production of transgenic mice. (**a**) In the pronuclear injection procedure, fertilized oocytes are removed from female mice. The large male pro-nucleus is injected with foreign DNA. The oocytes are reintroduced into a pseudo-pregnant female mouse and allowed to develop. Approximately 20–30% of the offspring will have incorporated the foreign DNA and are said to be transgenic. (**b**) To produce transgenic mice using ES cells, pluripotent ES cells are removed from a mouse blastocyst. Then DNA is micro-injected into the ES cell. The DNA is allowed to incorporate into the host cell and then rare cells, in which homologous recombination (HR) has occurred, are selected. The selected ES cells are injected into mouse blastocysts, which are reintroduced into pseudopregnant female mice. The resultant off-spring are chimeras (i.e. they are composed of normal and transgenic cells). Crossing of chimeric offsprings with normal mice will give rise to F1 hybrids and additional breeding will finally yield homozygous transgenic mice

Conditional Transgenic Mouse Models

To overcome the limitations posed by traditional transgenic mouse models, conditional models have been produced in which genes can be manipulated in a time and tissue specific manner. These conditional models include site-specific recombinase systems and transcriptional transactivation systems. Site-specific DNA recombinase systems include Cre/loxP and Flp/FRT systems (Jonkers and Berns, 2002). These systems require insertion of 34 base pair recombinase recognition sites (loxP or FRT) into the introns of genes of interest. For the systems to be effective these inserted sites must not affect the normal genes function in the absence of recombinase expression. Tissue specific expression of the DNA recombinase (Cre or FLP) then induces the excision of regions of the gene of interest, which have been floxed with the recombinase recognition sequences. Site-specific recombinase systems can be used to inactivate TSGs or activate oncogenes (as reviewed in Jonkers and Berns (2002)).

While recombinase systems allow the tissue and time-dependent manipulation of gene function they do have one limitation in that the change in gene function is irreversible. Other systems have been developed which allow the reversible control of gene expression. These systems are double regulatory systems and rely on an effector transgene or transcriptional transactivator (tTA) to induce transcription of the target gene. The tTA is also subject to regulation by an inducer molecule (such as doxycycline). Examples of transcriptional transactivation systems include the tetracycline-regulatable systems (as reviewed in Jonkers and Berns (2002)).

Uses of Transgenic Mice in Cancer Research

As cancers arising in transgenic mice develop through transformation of normal cells, in their normal microenvironment, and progress through the various stages of tumor development seen in human cancers, transgenic mice are emerging as key model systems in the field of cancer research. One of the key advantages of transgenic mice models of cancer is that they allow analysis of certain hallmarks of cancer, such as metastasis and angiogenesis (Zhou et al., 2006; Hanahan and Folkman, 1996), which are impossible to study in other systems, such as in vitro cell culture systems.

Mouse Models of Metastasis and Tissue Invasion

Transgenic mice with Cre-mediated conditional mutations of *p53* and *Rb* tumor suppressor genes in prostate epithelium cells have been produced (Zhou et al., 2006). These transgenic mice efficiently model human prostate cancer showing metastasis similar to human prostate cancer. The *p53* and *Rb*-deficient tumors were also shown to have 16 of the 18 known gene expression alterations commonly seen in human

prostate cancers. These transgenic mouse models are very promising tools for studying prostate tumor biology, and have implications in a pre-clinical trail setting for the development of prostate cancer therapeutics.

Mouse Models of Angiogenesis

Angiogenesis is the process by which capillaries are stimulated to sprout from existing blood vessels. As tumor masses form, cells require the ability to stimulate angiogenesis in order to acquire sufficient oxygen and nutrients to allow survival (Hanahan and Weinberg, 2000). Transgenic mice have been useful in the elucidation of the mechanisms of angiogenesis. The advantage of using transgenic mice in the analysis of angiogenesis is that tumor cells can be analyzed at varying stages of development, including very early pre-neoplastic stages. The "angiogenic switch" hypothesis, which suggests that initiation of angiogenesis is a necessary step in early tumor development, was postulated following analysis of pre- and post-neoplastic lesions form a range of transgenic mouse tumors (Hanahan and Folkman, 1996). It was found that angiogenesis is initiated in the early stages of cancer development, before the formation of solid tumors. The effects of inhibitors of angiogenesis on tumor progression have been studied in transgenic mouse models of cancer. For example, endostatin, an angiogenic inhibitor, has been shown to inhibit tumor growth in transgenic mice (O'Reilly et al., 1997) providing additional evidence of the essential role of angiogenesis in tumor progression.

Transgenic Mice Models in the Development of Cancer Therapeutics

A debate is ongoing into the relative effectiveness of xenograft and transgenic mouse in a pre-clinical trial setting for the development of cancer therapeutics (Gopinathan and Tuveson, 2008; Richmond and Yingjun, 2008). One of the main advantages of using xenograft models is the ease of model development, compared with the production of transgenic mice, which is technically challenging and largely more expensive. One might also argue the advantage of modeling therapeutics targeted to human tumor tissues in the case of xenograft models, compared with therapies targeted to murine tumor tissues in the case of transgenic mouse models. But, the fact of the matter is, although the effects of therapeutics on tumors cell elimination in xenograft models have yielded positive results in a pre-clinical setting, often the same therapeutic property is not seen in clinical trials (Twombly, 2002). The reasons for these differences in therapeutic effectiveness could be explained by subtle variations in species with regards to drug metabolism, absorption and biodistribution. Other reasons, which include the lack of a fully functional immune system in xenograft models and a non-native tumor microenvironment, are also possible causes and have been eradicated in transgenic mouse models. Further studies using

transgenic mice in pre-clinical trials is needed before a definitive conclusion can be made regarding this debate, but the development of transgenic mice which accurately mimic the molecular and physiological characteristics of human tumors will surely increase the use of transgenic mice in therapeutic trials.

Uses of Transgenic Mice in Genome Stability Research

Genome instability refers to an accelerated rate of mutagenesis (Lengauer et al., 1998). There are two main types of genomic instability seen in human cancers (Cahill et al., 1999; Lengauer et al., 1998), (i) microsatellite instabilities (MIN), which are mutations occurring at a nucleotide level and include base mutations and insertion or deletion of nucleotides and (ii) chromosomal instabilities (CIN), which include chromosome duplications, deletions and translocations. Whether this genome instability is required for the malignant transformation of cells or occurs later in the process of tumor development remains an ongoing debate.

The development of cancer has been described as an evolutionary process, where cells must overcome "selection barriers" in order to survive and transform (Cahill et al., 1999). Recent studies have shown that these barriers may include the DNA damage responses (DDR) (Bartkova et al., 2005; Gorgoulis et al., 2005) and oncogene-induced senescence (OIS) (Bartkova et al., 2006; Di Micco et al., 2006). These pathways are shown to be activated in cells in the early stages of tumorigenesis in response to a yet unknown "replication stress" and they act to alleviate the replication stress or induce cell death in the case of severe stress. Cells, which are able to overcome these barriers, are thought to be cells, which have the capability to transform.

It has been hypothesized that in order to become transformed cells must adopt a "mutator phenotype" (Loeb, 1991), which allows generation of the genomic instabilities seen in human cancers. Loeb suggested that genomic instability associated with cancer could be induced by defects in the cellular machinery, which acts, under normal circumstances, to ensure genome stability by ensuring accurate DNA replication, chromosomal segregation and repair of damaged DNA. Transgenic mice with defects in the machinery responsible for ensuring genome stability (Sotillo et al., 2007; Wang et al., 2005) have been engineered and provide evidence for this hypothesis. Transgenic mice with defects in spindle assembly checkpoint due to inducible overexpression of *Mad2* were developed (Sotillo et al., 2007) and shown to have increased incidences of CIN. Transgenic mice overexpressing *Mad2* were also shown to have increased levels of tumor formation, but interestingly *Mad2* overexpression was not required for tumor maintenance, further evidence of the possibility that genome instability is an early event in malignant transformation.

One limitation associated with the use of transgenic mice as models of human cancers is that murine cancers do not tend to show the same level of genome instability as seen in human cancers (Atkin, 1986). This difference is thought to be, at least in part, due to the fact that murine cells have much longer telomeres than human cells and that murine somatic cells have the ability to express telomerase,

the enzyme responsible for the elongation of the telomeres (Prowse and Greider, 1995). Evidence for the roles of telomerase in ensuring genome stability, and thus preventing tumorigenesis, comes from studies of telomerase deficient transgenic mice. Cells from transgenic mice deficient in the telomerase RNA subunit (*Terc*$^{-/-}$ mice) were shown to be susceptible to chromosome alterations, including end-to-end fusions (Blasco et al., 1997). The genomic instabilities seen in aging mice did not result in tumor formation, consistent with the hypothesis that telomerase activity, which is known to be up-regulated in many human cancers, is required to elongate short telomeres and ensure cell viability. Telomerase is, however, also up-regulated in murine tumors, even in the presence of long telomeres (Blasco et al., 1996). This suggested that telomerase may play another role in tumorigenesis besides telomere elongation. Further evidence for the role of telomerase in tumorigenesis independent of telomere length, comes from the study of early generation *Terc*$^{-/-}$ mice, which have long telomeres. These mice when exposed to carcinogens had a lower susceptibility to developing tumors than wild type mice expressing telomerase (Gonzalez-Suarez et al., 2000). While telomerase-deficient transgenic mice are not cancer prone, they do exhibit phenotypes similar to pre-mature aging diseases (Blasco et al., 1997), suggesting a role of telomerase in the prevention of aging.

To further analyze the role of telomerase in tumorigenesis, transgenic mice over-expressing the telomerase reverse transcriptase subunit (TERT) in basal kerationcytes, known as K5-mTERT mice, were developed (Gonzalez-Suarez et al., 2001). These mice were shown to be more prone to developing carcinogen-induced skin cancer, than wild type mice. Interestingly these K5-mTERT mice also lived longer than the wild type mice (Gonzalez-Suarez et al., 2005) consistent with the idea that telomerase plays a role in the prevention of premature aging.

Transgenic mice with altered telomerase expression, as outlined above, provided insights into the role of telomerase in tumorigenesis, but alterations in telomerase activity alone is not sufficient to produce transgenic mice which accurately model human tumorigenesis. Transgenic mice triply deficient in *Terc*, *ATM* (Ataxia-telangiectasia-mutated protein, an essential DNA damage response protein) and the tumor suppressor gene *p53*, were shown to develop lymphomas (Maser et al., 2007). The lymphomas of these transgenic mice were shown to accurately mimic the morphological characteristics and genomic instabilities seen in human lymphomas. These models confirm the fact that disruption of genome stability in mouse cells can selectively induce tumor formation, even in the absence of oncogene activation, providing evidence for the mutator phenotype hypothesis (Loeb, 1991). Transgenic mice engineered to experience genomic instability are therefore a promising model for the study of human cancer and in the future could provide models for the effective development of cancer therapeutics.

Outlook

The potential use of transgenic mice in the area of cancer and genome stability research in the future is dependent on the engineering of models, which more accurately mimic human tumorigenesis. Evidence provided by Maser et al.,

(2007) suggests that the efficient modeling of human tumors requires the development of transgenic mice with genome instabilities characteristic of human cancers. Hopefully these models will help to enhance our understanding of the molecular events leading to the initiation of cellular transformation in vivo. The potential uses of transgenic mice engineered to model human cancers, at both a genomic and physiological level, are endless. Most importantly they will serve as model systems for the testing of novel therapeutics, in an attempt to alleviate cancer, a disease, which instils so much personal hardship on nearly every family in our modern day society.

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