REPLICATION DEPENDENT MECHANISMS OF GENOME MAINTENANCE

By

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To my parents

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LIST OF ABBREVIATIONS

9-1-1	RAD9-HUS1-RAD1
53BP1	p53 Binding Protein 1
Aph	aphidicolin
ATM	Ataxia Telangiectasia Mutated
ATR	ATM- and RAD3-Related
ATRIP	ATR-interacting protein
BRAF	v-raf murine sarcoma viral oncogene homolog B1
BRCA	breast and ovarian cancer gene
BRCT	BRCA1 C-terminal
BrdU	bromodeoxyuridine
BSA	bovine serum albumin
CC	coiled-coil
CDC25	cell division cycle 25
CDC45	cell division cycle 45
CDC6	cell division cycle 6
CDK	cyclin-dependent kinase
CDT1	CDC10-dependent transcript 1
CHK1	checkpoint kinase 1
CHK2	checkpoint kinase 2
CINP	CDK2-interacting protein
CRD	Checkpoint protein Recruitment Domain

CS	Cockayne's Syndrome
CSN	COP9 Signalasome
CUL4	cullin 4
DAPI	4'6-diamidino-2-phenylindole
DDB1	damaged DNA binding protein 1
DDB2	damaged DNA binding protein 2
DDK	DBF4-dependent kinase
DDR	DNA Damage Response
DNA	deoxyribonucleic acid
dNTP	deoxyribonucleotide triphosphate
DSB	double strand break
dsDNA	double stranded DNA
DUSP	ubiquitin specific protease domain
FAT	FRAP-ATM-TRRAP
FATC	FAT C-terminal
FHA	forkhead associated
G1/G2	gap phase 1/gap phase 2
GGR	global genomic repair
gH2AX	H2AX phospho-serine 139
GINS	Go (5), Ichi (1), Nii (2), San (3): Sld5, Psf1, Psf2, Psf3
HA	hemagglutinin
HEAT	Huntingtin, Elongation factor 3, A subunit of protein phosphatase 2A and TOR1

HU	hydroxyurea
IP	immunoprecipitation
IR	ionizing radiation
KAP1	KRAB domain-associated protein 1
MCM	minichromosome maintenance
MDC1	mediator of DNA damage checkpoint 1
Mec1	mitotic entry checkpoint protein 1
MMS	methyl methane sulfonate
MRE11	meiotic recombination 11
MRN	MRE11-RAD50-NBS1
NBS1	Nijmegen breakage syndrome
NER	nucleotide excision repair
NLS	nuclear localization signal
ORC	origin recognition complex
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate buffered saline
PCNA	poliferating cell nuclear antigen
PCR	Polymerase Chain Reaction
PIKK	PI-3 kinase-related kinase
Pre-RC	pre-replication complex
RNF8	ring finger protein 8
RNR	ribonucleotide reductase
ROS	reactive oxygen species

RPA	replication protein A
RFC	replication factor C
SCF	SKP1-CUL1-F box protein ubiquitin ligase
SDS	sodium dodecyl sulfate
SI	sensitivity index
SKP2	S-phase kinase-associated protein 2
ssDNA	single stranded DNA
siRNA	small interfering RNA
TCR	transcription-coupled repair
TopBP1	Topoisomerase II-beta binding protein 1
Ubox	ubiquitin ligase domain
UV	ultraviolet radiation
XAB2	XPA binding protein 2
XP	Xeroderma Pigmentosum

CHAPTER I

INTRODUCTION

The basic unit of life, a cell, is anything but basic. It requires multiple, enormously complex networks of signaling pathways to maintain existing conditions and complete the fundamental task of passing on an exact copy of its genetic information to the next generation of cells. The genetic information of the cell encoded by the DNA is referred to as the genome. The process by which the cell replicates involves a highly ordered sequence of events called the cell cycle, in which the genetic information of the cell is duplicated and segregated into two identical daughter cells. Replication of the genome must be an exact process to maintain cellular viability and prevent changes to the genetic information that can produce an array of human genetic diseases, including cancer.

Cells are continually faced with an abundance of insults that can disrupt the integrity of the genome. Challenges to genome integrity come from environmental mutagens, byproducts of cellular respiration, and errors during nucleic acid metabolism, including DNA replication. Cells must recognize and repair the DNA damage caused by these mutagens in order to faithfully reproduce the genetic information. To accomplish this task, cells have evolved genome surveillance machinery that responds to these diverse genotoxic insults and ensures that the integrity of the genetic information is maintained. The necessity of these genome surveillance mechanisms is exemplified by

the observations that disruption of these protective pathways is associated with a variety of developmental defects and genetic diseases [1, 2].

In this chapter, I will discuss how cells faithfully duplicate their genetic information, the role of the ATR-mediated DNA damage response in preserving genome integrity, and the importance of these genome surveillance pathways in the prevention, diagnosis, and treatment of cancer.

DNA Replication

Duplication and segregation of the genetic information is regulated by an ordered series of events termed the cell cycle. The cell cycle is divided into four canonical phases. Exactly one copy of the genome is produced during S-phase (DNA synthesis phase), and in M-phase (mitosis) one copy of the genome in segregated into each of two daughter cells. Separating these phases of the cell cycle are two gap phases (G1 and G2), during which the cell grows and monitors the internal and external environment to ensure conditions are suitable for duplication and division. Progression through the cell cycle is directed by fluctuations in the activities of specific cyclin-dependent kinases (CDKs) [3]. CDK activity is regulated by three distinct mechanisms. First, CDK activation requires the association with a cyclin binding partner, whose availability is strictly controlled during the cell cycle by transcription factors and ubiquitin-mediated proteolysis. Second, phosphorylation of the CDK-cyclin complex can inhibit or promote CDK activity. And third, CDK-cyclin complexes can be inactivated if bound by CDK inhibitors. Once activated, phosphorylation of target proteins by various CDK-cyclin complexes directs

the major events of the cell cycle. Those events specifically required for duplication of the genome can be divided into two discrete steps: origin licensing and initiation.

Replication origin licensing

DNA replication is regulated by the recruitment of the replication machinery to chromosomal origins of replication. These origins have conserved and identifiable sequences in budding yeast. However, a limited number of sites consistently function as origins in human cells and the mechanisms that select these sites remain unclear [4-6]. Replication origins in metazoans typically lack identifiable sequences, and increasing evidence suggests replication may initiate from several potential origins within a zone of initiation [7].

Licensing of an origin occurs through the sequential recruitment of specific proteins during late M and G1 phases, resulting in the formation of a pre-replication complex (pre-RC). The origin recognition complex (ORC) is a six-subunit protein complex that identifies the sites of replication initiation by binding to origins [6]. ORC binding at origins of replication is required for the subsequent and independent recruitment of CDC6 and CDT1, which cooperatively promote loading of the MCM2-7 helicase complex and formation of the pre-RC [8-10]. Origins are licensed for replication upon loading of the MCM helicase, and considered competent for replication initiation upon entry into S phase [11].

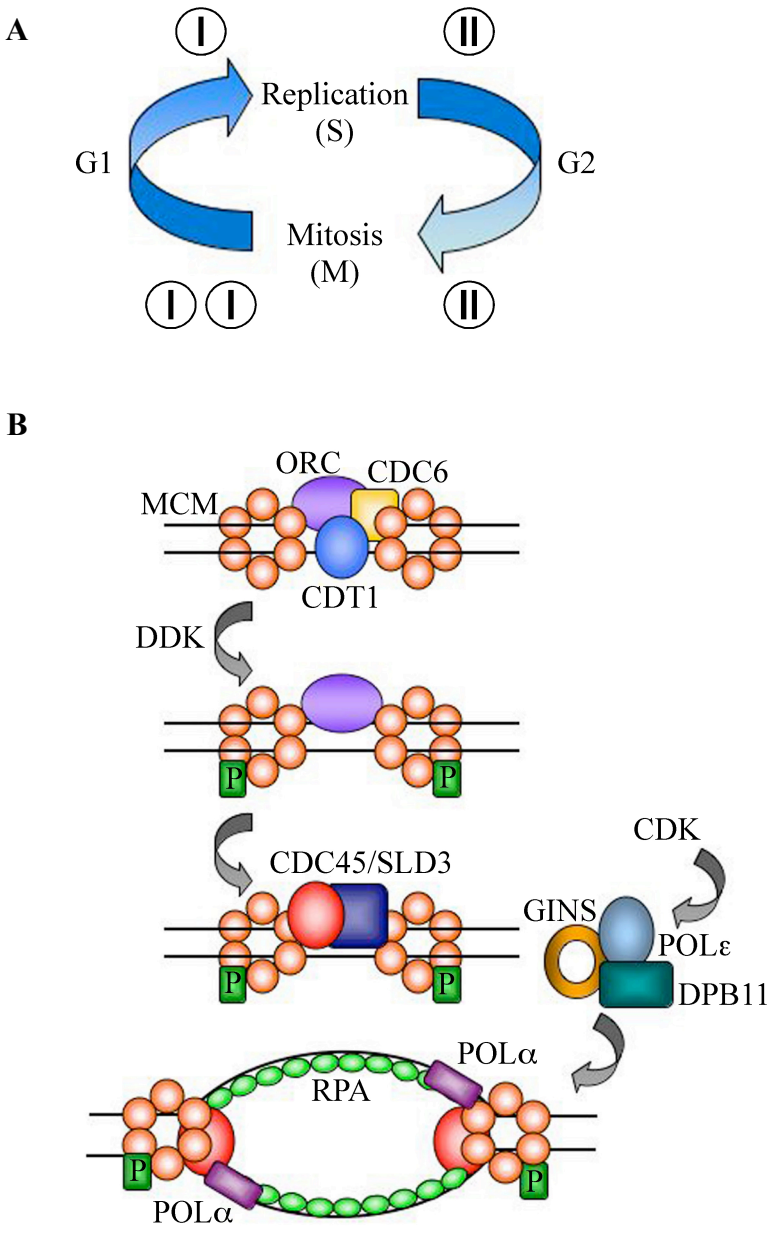

Figure 1.1. Initiation of DNA replication. **(A)** Representation of the cell cycle, showing the nucleus and the replication of the genome during DNA synthesis (S) phase. The duplicated genome is segregated into two identical daughter cells during mitosis. **(B)** Pre-replication complexes containing ORC, CDT1, CDC6, and the MCM2-7 helicase are loaded onto origins of replication by the end of G1 phase. Upon entry into S phase, activation of the DDK and CDK kinases is necessary to promote the association of proteins that facilitate origin unwinding and replisome loading. The recruitment of the replication machinery initiates duplication of the genome in a bi-directional manner. Yeast proteins are shown.

Initiation of DNA replication

The formation of pre-RCs marks potential sites for initiation of DNA replication, but additional proteins must associate with an origin to begin DNA synthesis. Unwinding of the DNA at origins requires activation of the MCM2-7 helicase and loading of the replication machinery, two events that require CDK- and DDK (CDC7/DBF4-dependent kinase)-mediated phosphorylations. CDK-dependent phosphorylation promotes the formation of a multiprotein complex containing GINS at the G1/S boundary, and its association with origins [12, 13]. Phosphorylation of the MCM2-7 complex by DDK recruits a CDC45-containing complex to origins [14-16]. The association of these proteins with origins of replication allows loading of the replisome and activation of the MCM2-7 helicase [13, 16-18].

The ultimate goal of the replication initiation machinery is to assemble the replisome, including RPA, PCNA, and DNA polymerases, at origins of replication [12]. After local unwinding and recruitment of the ssDNA binding protein RPA, an RNA/DNA primer is formed by DNA primase/DNA polymerase alpha. This primer is extended by DNA polymerases epsilon and delta as the replisome commences DNA replication in a coordinated and bi-directional fashion. Thousands of origins fire during S phase to ensure the rapid and accurate duplication of the genome. The timing of origin firing during S phase is largely correlated with the association of the replication initiation factors, as well as the surrounding chromatin structure [14, 15, 19, 20]. ATR and CHK1 (discussed below) also appear to influence regulatory mechanisms that control the timing of origin firing by inhibiting the kinase activity of CDK and DDK [6, 21].

Mechanisms to ensure a single round of replication

Multiple origins of replication fire simultaneously during S phase to rapidly duplicate the genetic information. To ensure that reproduction is accurate, it is essential that no segment of the genome be duplicated more than once. Thus, mechanisms exist to ensure that multiple initiations from the same origin (a process termed re-replication) are prevented once cells enter S phase. During late M and G1, low CDK activity allows pre-RC assembly, but initiation is inhibited. When cells enter S phase, increased CDK activity allows initiation of DNA replication but prevents new pre-RC assembly. This separation into two distinct phases ensures that no origin can initiate more than once during a cell cycle.

The mechanisms that restrict origin firing to once per cell cycle inhibit the licensing of replication origins [6]. After MCM2-7 is loaded at origins, ORC, CDC6, and CDT1 are no longer required for initiation of DNA replication [22-24]. Thus, these components of the pre-RC can be inactivated upon entry into S phase to inhibit further origin licensing without compromising replication initiation. In mammalian cells, re-replication is prevented primarily through destruction or functional inhibition of CDT1. Two independent ubiquitin ligase complexes target CDT1 for proteosomal degradation during S phase, SCF^{SKP2} and $DDB1\text{-}CUL4^{CDT2}$. The binding of Geminin also functionally inhibits CDT1 during S and G2 phases of the cell cycle. A role for ORC or CDC6 in preventing re-replication in mammalian cells is less clear, but dissociation of ORC subunits from origins of replication, or the nuclear export or degradation of CDC6 may also be important [25].

Challenges to DNA replication and genome integrity

Genome maintenance requires the accurate completion of DNA replication. Cells are continually challenged by endogenous and exogenous DNA damaging events, as well as by the complex task that DNA replication presents. Reactive oxygen species (ROS) are normal byproducts of metabolism that can cause lipid peroxidation, protein damage, and a variety of DNA lesions, including single-strand and double-strand DNA breaks, base adducts, and DNA crosslinks [26]. ROS can also arise from environmental sources of damage such as ultraviolet (UV) and ionizing radiation (IR), but these mutagens directly induce DNA lesions as well. UV can produce DNA adducts and crosslinks, while IR can generate base modifications, single-strand breaks, and DNA double-strand breaks (DSBs). Double-strand breaks can arise endogenously during rearrangements of immunoglobin loci in B cells, rearrangement of the T-cell receptor loci in T cells, during meiotic recombination in germ cells, or from aberrant activity of cellular enzymes such as DNA topoisomerases [2, 27]. Also challenging the accurate completion of DNA replication are DNA secondary structures, repetitive sequences, fragile sites, and tightly associated proteins, which can impede replication fork progression and are associated with increased chromosomal rearrangements [28-31].

Some of these lesions, such as interstrand crosslinks, inhibit progression of both the replicative helicase and polymerases, and thus require removal of the lesion before replication can continue. Many lesions, including base adducts and intrastrand crosslinks, will block DNA polymerases without affecting the progression of the helicase. Replication stress agents such as aphidicolin and hydroxyurea also selectively inhibit polymerase activity without disrupting helicase progression. Aphidicolin prevents

RNA/DNA primer formation by inhibiting DNA polymerase alpha/primase, while HU indirectly stalls polymerases by depleting cellular dNTP pools. These physical or functional barriers to DNA polymerase activity result in the uncoupling of polymerase and helicase activities, and generate stretches of ssDNA as the helicase continues to unwind duplex DNA in the absence of any polymerization [32-34].

Repair mechanisms such as base excision repair, nucleotide excision repair, and mismatch repair are key for repairing these DNA damaging events before replication begins. Lesions that are encountered by the replication machinery and stall fork progression can be dealt with by two primary mechanisms. Post-replicative repair pathways simply bypass the DNA damage. Special translesion synthesis polymerases can temporarily replace the replicative polymerase when it encounters a lesion. These polymerases have greater flexibility in base-pairing properties that allow nucleotide incorporation across from damaged bases, thus preventing prolonged replication fork stalling that can result in the formation of DSBs. However, lesion bypass occurs at the expense of an increased error rate, because translesion polymerases have low fidelity and no proofreading activity [2, 35]. Also activated by stalled replication forks is the DNA damage response (DDR), which will be the focus of the remainder of this chapter due to its relevance for the context of my thesis work.

The DNA Damage Response

With the abundance of challenges that cells face to maintain the integrity of their genetic information, the evolution of cellular responses to deal with these challenges and

maintain genome integrity is of critical importance. Eukaryotic cells have thus developed genome surveillance machinery, collectively referred to as the DNA damage response (DDR), which functions to maintain genome integrity by coordinating DNA replication, cell cycle progression, DNA repair, apoptosis, and cellular senescence [36].

The DDR is a signal transduction cascade regulated by the phosphoinositide-3-kinase-related protein kinases (PIKKs). The PIKK enzymes are large proteins that share a common domain structure (Figure 1.2). The kinase domain is located near the C-terminus and is flanked by two regions of sequence similarity among all PIKKs called the FAT (FRAP, ATM, TRRAP) and FATC (FAT C-terminus) domains. The FAT domain consists of HEAT (Huntingtin, Elongation factor 3, A subunit of protein phosphatase 2A and TOR1) repeats that are weakly conserved among the family members. The FATC domain is a small domain required for PIKK kinase activity; however, the precise functions of the FAT and FATC domains remain unclear. Since these domains flank the PIKK kinase domain, it has been suggested that they may interact and participate in kinase regulation [37]. The N-terminus of each protein contains additional HEAT repeats that may serve as a protein-protein interaction surface [38].

Ataxia-telangiectasia mutated (ATM) and ATM-and Rad3 related (ATR) are two PIKKs that function at the apex of DDR signaling pathways. In addition to sharing similar domain architectures, these kinases phosphorylate an overlapping set of target proteins to coordinate cell cycle and DNA repair activities. ATM and ATR preferentially phosphorylate serine and threonine residues that are followed by a glutamine in hundreds of target proteins [36]. However, these kinases differ in the type of DNA damage to which they respond. ATM is activated primarily by infrequently occurring DSBs. ATR

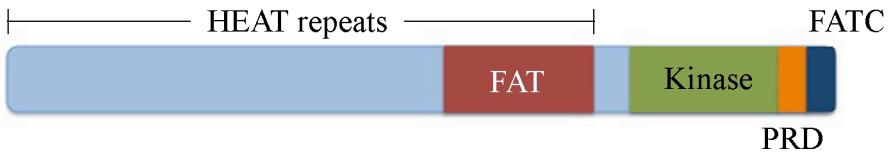

Figure 1.2. Functional domains of the PIKKs ATR and ATM. The PIKK kinase domain is flanked by two regions of sequence similarity termed the FAT and FATC domains. The FAT domain consists of an alpha-helical HEAT repeat structure, and the FACT domain is required for kinase activity. The precise functions of the FAT and FACTC domains are unclear. Situated between the kinase domain and the FATC domain is a PIKK regulatory domain (PRD). This region of ATR interacts with TopBP1 and is necessary for ATR activation [39]. In ATM, acetylation of the PRD by Tip60 is required for kinase activation [40].

is activated by a variety of DNA lesions including base adducts, crosslinks, DSBs, and compounds that directly promote replication stress such as hydroxyurea and aphidicolin. Unlike ATM, ATR is essential for the viability of replicating somatic cells [41, 42]. ATR is activated during every cell cycle to regulate origin firing, repair damaged replication forks, and prevent mitotic entry in the presence of DNA damage (Figure 1.3) [43-45]. Disruption of ATR results in an accumulation of DSBs during S phase, cell cycle arrest or apoptosis, and early embryonic lethality in mice [41, 42].

Recognition of replication stress and DNA damage by ATR

Recruitment of ATR to sites of replication stress and DNA damage promotes kinase activation. While ATR is reported to have some affinity for nucleic acids, the primary mechanism of lesion recognition is dependent on protein-protein interactions mediated by a common DNA structure. Importantly, the diverse ATR-activating DNA lesions have in common the ability to expose single-stranded DNA (ssDNA), often as a consequence of stalling the replicative polymerases. The relative insensitivity of the replicative helicase to these lesions causes an uncoupling of polymerase and helicase activities, resulting in ssDNA gaps [32]. End resection of DSBs can also generate ssDNA. Thus, the common ATR-activating signal among these diverse lesions is the formation of ssDNA [46, 47].

The ssDNA serves as a platform for the recruitment of proteins required for ATR activation. Initially, ssDNA is rapidly coated by the heterotrimeric ssDNA binding protein RPA. Several observations have highlighted the importance of RPA-coated ssDNA in ATR activation [48]. The extent of ssDNA generated by a lesion influences

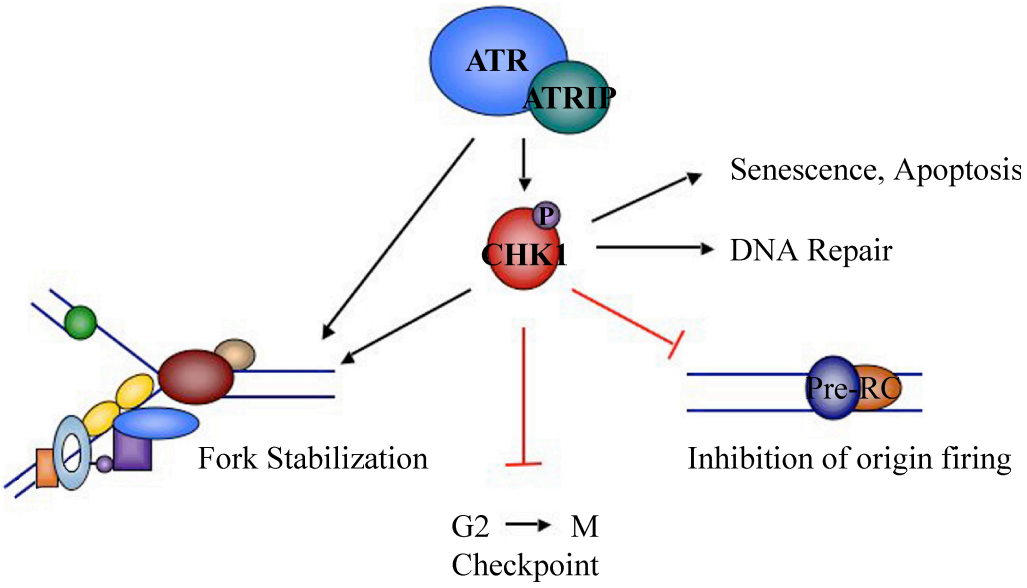

Figure 1.3. ATR-dependent genome maintenance activities. Activation of ATR in response to replication stress and DNA damage results in the phosphorylation and activation of a key effector protein, CHK1. ATR and CHK1 cooperate to phosphorylate numerous downstream target proteins to genome integrity.

the amount of DDR activation observed, with larger regions producing greater ATR activation [32]. Additionally, mutation or knockdown of RPA in both mammalian cells and *S. cerevisiae* impairs activation of ATR (Mec1) [49-51]. These defects may be partially reflective of the important role that RPA has in the recruitment of ATR to DNA lesions in eukaryotic organisms.

RPA is involved in the independent recruitment and co-localization of the two sensor proteins in the ATR signaling pathway. ATR recruitment to RPA-ssDNA requires ATR-interacting protein (ATRIP) [42, 47]. These proteins are obligate binding partners whose stability and functions are interdependent [42]. An evolutionarily conserved RPA binding surface in ATRIP called the checkpoint protein recruitment domain (CRD) binds an N-terminal domain of RPA70 [52]. Deletion of the ATRIP CRD severely compromises the localization of ATR-ATRIP to DNA lesions; however, it has only mild effects on ATR signaling [52, 53]. The absence of a significant signaling defect may be explained by additional RPA interactions, alternative mechanisms of ATR-ATRIP recruitment, or possibly ATR activation with only transient localization to ssDNA gaps.

Recruitment of the RAD9-RAD1-HUS1 (9-1-1) complex is also required for ATR activation. The 9-1-1 complex is a heterotrimeric ring similar to the replicative sliding clamp PCNA, and is loaded onto ssDNA-dsDNA junctions by the RAD17-RFC2-5 clamp loader [49, 54-56]. *In vitro*, the 9-1-1 complex is preferentially loaded at ssDNA gaps at the free 5' DNA end and a 5' primer end appears to be the relevant checkpoint-activating structure [56, 57]. Single stranded DNA gaps with a 5' primer end can be found at the ATR-activating structures formed by resection of DSBs, deprotection of telomeres, nucleotide excision repair, and at stalled replication forks. A checkpoint recruitment

domain on RAD9 with sequence similarity to the ATRIP CRD binds the same RPA70N binding surface as ATRIP and may help direct 9-1-1 loading or retain it at the 5' junction [58]. Recent work in *Xenopus* egg extracts indicates topoisomerase binding protein 1 (TopBP1) is also required for loading the 9-1-1 complex [59]. TopBP1 promotes the hyperloading of DNA polymerase alpha, which generates the 5' primer end necessary for 9-1-1 loading. TopBP1 has an additional function in 9-1-1 loading that is distinct from DNA polymerase alpha recruitment, which may be to concentrate RAD17-RFC and 9-1-1 on the DNA for efficient loading. Thus, multiple protein interactions promote the assembly of two checkpoint complexes (ATR-ATRIP and 9-1-1) at ssDNA gaps formed as a consequence of many types of DNA lesions.

Activation of ATR kinase activity

The localization of ATR to sites of replication stress and DNA damage promotes kinase activation by concentrating it with an activating protein. TopBP1 directly stimulates ATR kinase activity [60]. TopBP1 is not required for the localization of ATR to sites of damage or for the basal kinase activity of ATR [39, 61]. However, TopBP1-mediated activation of ATR is required for the damage-induced phosphorylation of all ATR substrates, and is essential for cell viability in the absence of exogenous DNA damage [39]. TopBP1 is recruited to lesions independently of ATR-ATRIP, and may be retained or appropriately positioned through an interaction with the phosphorylated C-terminal tail of RAD9 [62-65]. The concentration of these proteins at sites of replication stress and DNA damage is hypothesized to facilitate an interaction between TopBP1 and the ATR-ATRIP complex, which allows TopBP1 to promote activation of ATR kinase

activity. The requirement for the independent recruitment of two distinct complexes to sites of replication stress and DNA damage may prevent the inappropriate activation of ATR-ATRIP [66].

CHK1 is a prominent mediator of ATR-dependent DDR signaling

The phosphorylation of downstream target proteins is necessary for ATR to coordinate replication, cell cycle progression, and DNA repair. The most prominent substrate of ATR is another kinase, checkpoint kinase 1 (CHK1). CHK1 is activated by ATR-mediated phosphorylation of serines 317 and 345 [67, 68]. ATR-dependent phosphorylation of CHK1 requires the adaptor protein Claspin, which facilitates sustained CHK1 activation through interactions with the phosphorylated form of RAD17, one component of the 9-1-1 clamp loader [69, 70]. Intermolecular interactions between the C-terminus and kinase domains of CHK1 suggest the presence of an auto-inhibitory domain [71, 72]. This is further supported by the findings that deletion of the CHK1 C-terminus or mutation of the ATR-mediated phosphorylation sites to phospho-mimetic residues abolishes the intermolecular interaction and activates the kinase in the absence of DNA damage [72-74]. Thus, ATR-mediated phosphorylation of CHK1 may disrupt an inhibitory, intermolecular interaction that activates the kinase and allows phosphorylation of target proteins.

Several studies suggest that CHK1 mediates many of the ATR-dependent cellular functions. Disruption of CHK1 in mice leads to early embryonic lethality [75, 76]. CHK1-deficient cells also display many phenotypes similar to those observed with loss of ATR, including the accumulation of DSBs in the presence and absence of replication

stress [77-79]. The ATR-CHK1 signaling pathway is important during S phase for regulating late origin firing, arresting cell cycle progression, and preventing replication fork collapse [44, 45]. The essential functions of ATR and CHK1 may be related to their fork-stabilization activity [80]. The existence of endogenous DNA damaging events and inherent difficulties in the replication process may necessitate ATR and CHK1 to stabilize stalled replication forks and promote replication restart during every cell cycle. The essential function of ATR (Mec1) in yeast includes regulating nucleotide levels through activation of ribonucleotide reductase (RNR) [81, 82]. Whether ATR regulates nucleotide levels in higher eukaryotes remains unclear.

Replication-dependent genome maintenance activities of ATR and CHK1

CHK1 is phosphorylated by ATR at sites of replication stress and DNA damage, and is subsequently released from chromatin to transmit the damage signal throughout the nucleus by phosphorylating target proteins [83]. ATR-mediated regulation of cell cycle progression is largely accomplished through CHK1 phosphorylation of CDC25 phosphatases, which function to activate CDKs by removing inhibitory phosphorylations [84]. As described earlier, CDK2 is important for the initiation of DNA replication by promoting the recruitment of proteins that facilitate origin unwinding and replisome loading [6]. Activation of the DDR in S-phase inhibits late origin firing to slow DNA synthesis while the damaged DNA is repaired [85-88]. One mechanism through which this may be accomplished involves inhibition of CDK2 activity. ATR-mediated phosphorylation and activation of CHK1 results in the phosphorylation of additional downstream target proteins, including CDC25A. Phosphorylation of CDC25A inhibits

the phosphatase activity of this protein, and allows the accumulation of a phosphorylated and inactivated form of CDK2 that cannot promote initiation at late replicating origins [89]. ATR signaling through CHK1 is also critical for replication progression in the absence of exogenous DNA damage to regulate origin firing, but the precise mechanisms by which this is accomplished remain unclear [44, 90].

In addition to slowing DNA synthesis in the presence of DNA damage, ATR and CHK1 promote genome integrity by stabilizing stalled replication forks and facilitating the recovery of stalled forks to ensure complete replication of the genome. One mechanism by which ATR accomplishes this is to prevent dissociation of the replisome from the DNA. In the absence of yeast ATR (Mec1), the association of DNA polymerases alpha and epsilon with a stalled replication fork was significantly reduced [91, 92]. In higher eukaryotes, chemical inhibition of ATR signaling or mutation of ATRIP prevented completion of DNA synthesis, and resulted in the release of MCM2-7 and PCNA from chromatin after aphidicolin treatment [93, 94]. CHK1-deficient cells also show release of PCNA from chromatin and an inability to resume DNA synthesis in the presence of this replication stress [78, 79, 86]. These findings suggest that components of the replisome dissociate from chromatin in the absence of ATR signaling. The requirement for ATR in re-loading polymerase epsilon onto chromatin after treatment with DNA damaging agents also indicates that ATR has a role in restarting replication from a collapsed fork [95].

Several replisome components are phosphorylated by ATR in response to replication stress, including RFC, RPA1, RPA2, the MCM complex, and DNA polymerases [96-101]. The functional significance of these phosphorylation events is

largely unknown. Recently, the phosphorylation of MCM2 was shown to be a docking site for polo-like kinase 1 (PLK1). PLK1 recruitment to a stalled replication fork promotes the firing of adjacent origins by recruiting CDC45, thus ensuring that replication near the stalled fork is completed [102].

Regulation of mitotic entry by ATR and CHK1

ATR-induced inhibition of mitotic entry in the presence of DNA damage is also mediated largely by CHK1-dependent phosphorylation of the phosphatase CDC25C. CDK1/cyclin B is rendered inactive in G2 phase of the cell cycle by inhibitory phosphorylations mediated by WEE1 and MYT1 [103, 104]. Activation of CDK1/cyclin B and entry into mitosis requires removal of these phosphorylations by CDC25C. Phosphorylation of CDC25C by CHK1 inhibits the phosphatase activity of this protein, either directly, or indirectly by facilitating an association with 14-3-3 proteins. This interaction masks the nuclear localization site of CDC25C and restricts the protein to the cytoplasm [105-108]. PLK1-mediated phosphorylation of CDC25C also activates the phosphatase activity of this protein. ATR-dependent phosphorylation, either directly or indirectly through CHK1, inhibits PLK1 activity as another mechanism of CDC25C inhibition [109, 110]. Additionally, CHK1 phosphorylation of WEE1 can also increase WEE1 activity [111, 112]. The regulation of CDC25C, PLK1, and WEE1 activities by the DDR cooperate to prevent activation of CDK1/cyclin B and thus inhibit entry into mitosis (Figure 1.4).

This signaling pathway likely predominates for the initiation of the G2 checkpoint by ATR but additional mechanisms may contribute to the maintenance of the checkpoint

response. Transcriptional mechanisms in particular may be important, since phosphorylation of p53 by ATR results in the transcriptional activation of Gadd45, p21, and 14-3-3 sigma, all of which inhibit CDK1. Binding of cyclin B to CDK1 is also required for its activity, and repression of the CDK1 gene by p53 may also contribute to the persistent inhibition of mitotic entry [113]. Innumerable proteins appear to be involved in the G2 checkpoint response, but the precise mechanisms by which many of these additional proteins promote cell cycle arrest is unknown.

Activation of the DDR kinase ATM

Another PIKK at the apex of the DDR signaling cascade is ATM. As stated earlier, ATM is very similar to ATR in protein structure and sequence, and both kinases phosphorylate an overlapping set of target proteins preferentially on serine and threonine residues that are followed by a glutamine. Unlike ATR, ATM is not essential for the viability of replicating cells. Mutations in ATM are present in approximately 0.5-1.0% the population, resulting in a neurodegenerative and cancer predisposition disorder called ataxia-telangiectasia [114-116].

ATM is activated by DNA double strand breaks to arrest cell cycle progression and facilitate DNA repair [117]. Reminiscent of ATR activation, ATM is recruited to DSBs by a binding partner to facilitate co-localization with an activator protein. The recruitment of ATM to DSBs is dependent on an interaction with NBS1 [118]. The co-localization of ATM/NBS1 with MRE11/RAD50 at sites of damage results in activation

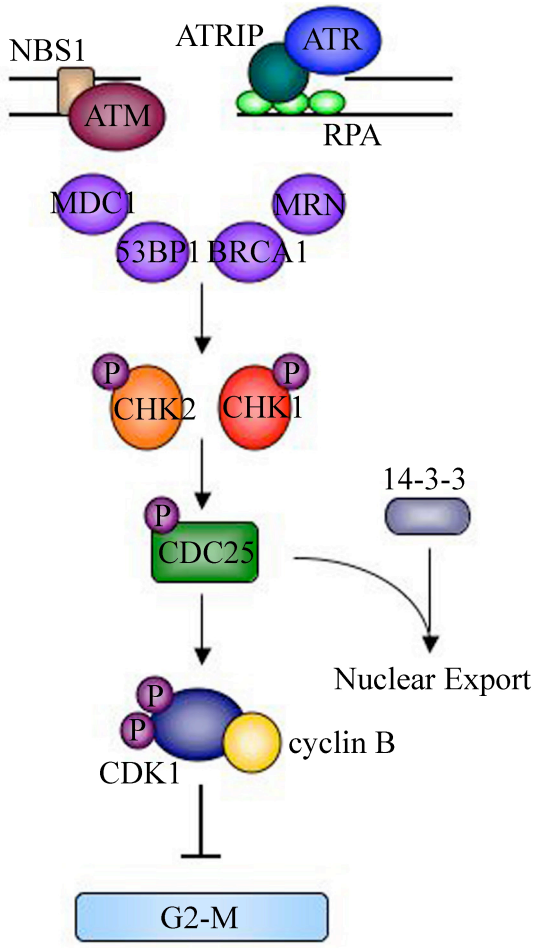

Figure 1.4. Inhibition of mitotic entry by ATM and ATR in the presence of DNA damage. The formation of DSBs directly activate ATM, and resection of the DSB to reveal ssDNA results in ATR activation as well. The G2 checkpoint is initiated by phosphorylation of the ATM and ATR target proteins CHK2 and CHK1. These kinases then phosphorylate and inactivate the CDC25 phosphatase. This prevents dephosphoryaltion of CDK1/cyclin B, which is required for activation of this complex and entry into mitosis.

of ATM kinase activity [119, 120]. Upon activation, ATM undergoes a transition from a dimeric to a monomeric form, and acetylation of ATM is required for this transition [40, 121]. The autophosphorylation of ATM at serine 1981 correlates with formation of the monomeric form, however it is unclear whether this event is necessary for ATM activation [119, 121-123].

ATM activation results in the phosphorylation of histone H2AX (γH2AX) at sites of DSBs, which is the initial signal for accumulation of additional DDR proteins. This phosphorylation event mediates the recruitment of MDC1 (mediator of DNA damage checkpoint 1) through its BRCT domain. MDC1 is among the first proteins to accumulate at DSBs, and the assembly and retention of MDC1 are required for retention of most other proteins at the DSB [124]. MDC1 directly interacts with NBS1 (Nijmegen Breakage Syndrome) to localize the MRN complex (MRE11-RAD50-NBS1), resulting in the recruitment and activation of additional ATM proteins [125]. ATM phosphorylation of MDC1 promotes association of the ubiquitin ligase RNF8 through an interaction with its FHA domain. The ubiquitin ligase activity of RNF8 is required for the subsequent recruitment of 53BP1 and BRCA1 [126-128]. RNF8-mediated ubiquitination at DSBs is thought to promote a chromatin restructuring event, revealing methylated histones that can localize 53BP1 through its tudor domains. The assembly of these DDR proteins at DSBs promote amplification of the DNA damage signal by ATM and increases the efficiency of DSB repair.

The primary effector protein of ATM is another kinase, checkpoint kinase 2 (CHK2). CHK2 displays many similarities in functions to the ATR effector kinase CHK1, but these two protein kinases have no sequence homology outside the protein

kinase domain. ATM-mediated phosphorylation of threonine 68 promotes CHK2 autophosphorylation and activation [129-131]. Like CHK1, CHK2 also mediates the cell cycle arrest function of ATM largely through phosphorylation and inactivation of CDC25 phosphatases (Figure 1.4) [45].

Crosstalk between the ATR and ATM pathways

ATM and ATR phosphorylate an extensive and overlapping set of target proteins, and have some redundancy in cell cycle progression mechanisms. The distinct DNA structures that these kinases recognize (ssDNA for ATR and DSB for ATM) suggest unique roles for these proteins in response to DNA damaging agents. However, the ability of each of these structures to be converted into the other suggests some interdependency of these pathways as well. ATR activation and recruitment in response to IR is dependent on ATM and the nuclease activity of MRE11, which process DSB ends to generate RPA-coated ssDNA. ATM-dependent regulation of ATR activation in response to IR is restricted to S and G2 phases of the cell cycle, suggesting that the MRE11-mediated DSB resection is mediated by CDKs [132]. This is consistent with data from budding yeast indicating that ATR (Mec1) activation after DSB induction by HO endonuclease requires CDK1-dependent DSB resection [133]. Additionally, the ATM-mediated phosphorylation of the ATR activating protein TopBP1 places ATM upstream of ATR in a common signaling pathway [134]. ATM can also be activated by replication stress. The collapse of a stalled replication fork can leave the unprotected DNA susceptible to nuclease cleavage, resulting in the formation of an ATM-activating

DSB. The activities of ATM and MRE11 may also be important to prevent replication fork stalling prior to DSB formation [95, 135].

The DDR and tumorigenesis

The biochemical data indicates the DDR has a critical role in preventing genome instability by regulating DNA replication, cell cycle progression, and DNA repair activities. The biological significance of these genome surveillance pathways is evident from the observations that disruptions to DDR pathways result in developmental defects, premature ageing, and genetic diseases including cancer (Table 1.1).

The DDR is an inducible barrier to tumorigenesis at its earliest stages of development [136, 137]. Genome instability is evident in pre-cancerous lesions by the presence of constitutive DDR signaling and DNA damage. The activation of oncogenes and inactivation of tumor suppressors promotes replication stress and DDR activation through inappropriate origin firing, premature termination of fork progression, and the generation of DSBs. DDR activation serves to restrict the proliferation of cells with mutated or unstable genomes by inducing cell cycle arrest, senescence, or apoptosis. The progression to a malignant disease is thus often associated with inactivation of DDR pathways, typically by mutations in the transcription factor p53, to overcome the growth restriction imposed by the DDR.

Understanding the mechanisms that promote genome instability and DDR activation in pre-cancerous lesions is an important avenue of investigation. It may produce biomarkers for early identification of disease and suggest the most effective

treatments based on the genetic and functional profiles of the tumor. DDR pathways are also activated by many chemotherapeutic agents. Further insight into the mechanisms and functions of the DDR can facilitate a better understanding of the etiology and progression of cancer, as well as how to more effectively treat it.

Table 1.1. DDR genes associated with inherited disorders.

Gene	Function	Disease
ATM	Checkpoint signaling, DNA repair	Ataxia-Telangiectasia, breast cancer predisposition
ATR	Checkpoint signaling, DNA repair	Seckel syndrome
BLM	Checkpoint signaling, DNA repair	Bloom's syndrome, cancer predisposition
BRCA1, BRCA2	Checkpoint signaling, DNA repair	Familial breast and ovarian cancer
FANCD2	Checkpoint signaling, DNA repair	Fanconi Anemia, cancer predisposition
MRE11	Checkpoint signaling, DNA repair	Ataxia-Telangiectasia Like Disorder
NBS1	Checkpoint signaling, DNA repair	Nijmegen breakage syndrome, cancer predisposition
TP53, CHK2	Checkpoint signaling	Li-Fraumeni syndrome, cancer predisposition

CHAPTER II

MATERIALS AND METHODS

Cell Culture

U2OS, HeLa, Phoenix-amphotropic, and HEK293 cell lines were maintained in Dulbecco's modified Eagle medium supplemented with 7.5% fetal bovine serum at 37°C in 5% CO_2. The RPE-hTERT cell line was maintained in Dulbecco's modified Eagle/F12 medium supplemented with 10% fetal bovine serum and 0.2% sodium bicarbonate at 37°C in 5% CO_2.

DNA constructs and transfections

The DDB1 construct resistant to degradation by siRNA-mediated silencing was created using site-directed mutagenesis. The following primer and its complement were used to introduce selected wobble base pair mutations: 5'-GGAGAGCAAGGATCTACTCTTTATCTTGACAGC-3'. Calcium phosphate transfections of the Phoenix-amphotropic packaging cell line were performed to produce retroviruses. Following infection of U2OS cells with the viral media, stable cell lines were selected using puromycin. C-terminal HA-tagged CDT1 expression constructs were generated by PCR in the pLPCX vector. Mutagenesis was performed using the following primers: 5'-TATGAAGCTTATGGAGGCTCGCCGCGCTACCGACGCAGCTGCGCGCCGCCGC-3' and 5'-CAAGCTGGCCTGCCGGGCCCCCAGC-3'.

Fragile site analysis

Fragile sites were induced by treatment of U2OS cells with 0.1μM aphidicolin for 24 hours. Metaphase cells were enriched by treating with Demecolcine solution (Sigma) for 1 hour at 37°C. Cells were then incubated in a hypotonic solution (3:1 0.566% KCl:0.8% NaCit) for 15 minutes at 37°C, fixed by multiple washes with Carnoy fixative (3:1 methanol:acetic acid), and dropped onto slides. Slides were baked at 90°C for 30 minutes and stained with Giemsa. Metaphase spreads were scored for chromosomal gaps and breaks, and common fragile sites were identified based on the idiogram in Richards [138].

RNAi constructs and transfections

All small interfering RNA oligonucleotides (siRNA) used in Chapter III were purchased from Dharmacon, Inc. The siRNA duplexes were as follows: non-targeting siRNA sense strand, 5'-AUGAACGUGAAUUGCUCAAdTdT; DDB1 siRNA sense strand, 5'-GCAAGGACCUGCUGUUUAUUU; CUL4A siRNA sense strand, 5'-GAACCCAUAUUAUUAGUGAUU; DDB2 siRNA sense strand, 5'-GAUAUCAUGCUCUGGAAUUUU; XPC siRNA sense strand, 5'-GCAAAUGGCUUCUAUCGAAUU; XPA siRNA sense strand, 5'-GGAGACGAUUGUUCAUCAAUU; CDT1 siRNA sense strand, 5'-GCGCAAUGUUGGCCAGAUCUU. The SKP2 depletions were achieved using a SMARTpool of four gene-silencing siRNAs. Transfections were performed with 100nM siRNA using Oligofectamine reagent according to the manufacturer's instructions (Invitrogen). For the RNAi screen in Chapter IV, HeLa cells were transiently transfected

with the shRNA plasmids using Lipofectamine 2000 (Invitrogen). Individual siRNA oligonucleotides targeting the candidate genome maintenance genes were purchased from Qiagen. The siRNA transfections of U2OS and RPE-hTERT cells in Chapters IV and V were accomplished using HiPerFect (10nM, Qiagen) and Oligofectamine (100nM, Invitrogen), respectively.

RNAi screen

Plasmid-based shRNAs were selected from a genome-wide shRNA library commercially available from Open Biosystems [139], and maintained by the Vanderbilt Microarray Shared Resource (http://array.mc.vanderbilt.edu/). Selected shRNA plasmids were provided in 96-well plates as frozen cultures of *E. coli* in low salt LB broth with 8% glycerol, carbenicillin (100μg/ml), and zeocin (25μg/ml). Liquid cultures of 1.5ml low salt LB were inoculated in 96-well blocks with 10μl of the glycerol stock, and incubated at 37°C, 200rpm for ~24 hours. Plasmids were isolated using the QIAprep 96 Turbo Miniprep Kit according to the manufacturer's instructions (Qiagen). A non-targeting RNAi molecule was used as a negative control, and an *RPA1* RNAi molecule was used as a positive control for genetic disruptions that activate DDR pathways. Additionally, several internal positive controls were present among the genes screened (i.e. *BRIP1*, *CHK1*, *DDB1*, and *POLA*).

HeLa cells were transfected with 200ng of the shRNA plasmids in 96-well plates using Lipofectamine 2000 (Invitrogen). Cells were split into two plates 24 hours after transfection, and 0.1μM aphidicolin was added to one plate for 24 hours prior to fixing cells for analysis at 96 hours after transfection. After immunostaining for KAP1 S824

phosphorylation, the wells of the 96-well plates were manually scored as positive or negative for DDR activation. KAP1 pS824 is visible as pan-nuclear staining in cells where the DDR is activated. This robust level of phosphorylation and the absence of non-specific staining allowed positive wells to be readily identified.

Quantitative analysis of DDR activation was then obtained by examining the percentage of cells with γH2AX staining. Four individual siRNAs were obtained (Qiagen) for each gene target that induced KAP1 phosphorylation in the shRNA screen. U2OS cells were transfected in duplicate with 10nM of each gene-silencing siRNA in 24-well plates using HiPerFect (Qiagen). Transfected cells were split into 12-well plates containing glass coverslips at 24 hours, and fixed for analysis at 96 hours after transfection. The percentage of cells with γH2AX staining after siRNA-mediated gene silencing in the absence and presence of 0.1μM aphidicolin (24 hour treatment prior to analysis) was manually determined with 3-4 counts of ≥100 cells from each transfection. Statistical significance was determined using a two-tailed, unpaired t test comparing each gene-silencing siRNA to the non-targeting control. The *p* values were adjusted to minimize type I error using a false-discovery rate of 0.05.

Immunofluorescence

For γH2AX and MRE11 immunostaining after silencing of *DDB1*, cells grown on glass coverslips were fixed and permeabilized with 100% methanol at -20°C for 15 minutes. After rinsing twice with PBS, cells were incubated in 100% acetone at -20°C for 30 seconds. The cells were then air dried for 1 minute, rinsed six times with PBS, and blocked for 15 minutes at room temperature with 5% BSA in PBS. Primary antibodies

recognizing γH2AX (Bethyl Laboratories) or MRE11 (GeneTex) were diluted in 1% BSA/PBS and incubated on cells for 20 minutes at 37°C, 5% CO_2. After washing three times with PBS, cells were incubated in the secondary antibodies, fluorescein (FITC)-conjugated goat anti-rabbit IgG or Rhodamine Red-X-conjugated donkey anti-mouse IgG (Jackson ImmunoResearch Laboratories, Inc.), diluted in 1% BSA/PBS for 20 minutes at 37°C, 5% CO_2. Cells were washed and counterstained with 4'6-diamidino-2-phenylindole (DAPI).

The analysis of RPA34 foci formation in *DDB1*-silenced cells was accomplished by fixing cells with 3% paraformaldehyde at room temperature for 10 minutes and permeabilizing with 0.5% Triton X-100 for 10 minutes on ice. After blocking at room temperature for 15 minutes with 5% BSA/PBS, the RPA34 antibody (Neomarkers) was diluted in 1% BSA/PBS and incubated on cells for 20 minutes at 37°C, 5% CO_2. Following three washes with PBS, cells were incubated in FITC-conjugated goat anti-mouse secondary antibody (Jackson ImmunoResearch Laboratories, Inc.), diluted in 1% BSA/PBS, for 20 minutes at 37°C, 5% CO_2. The cells were washed and counterstained with DAPI.

To analyze KAP1 pS824 by immunostaining for the shRNA screen, cells were plated in BD Falcon Optilux 96-well clear-bottom plates, fixed with 3% paraformaldehyde for 10 minutes at room temperature, and permeabilized with 0.5% triton X-100 for 10 minutes on ice. After blocking at room temperature for 15 minutes with 5% BSA/PBS, the cells were incubated with KAP1 pS824 (Bethyl Laboratories) diluted in 1% BSA/PBS for 1 hour at 37°C, 5% CO_2. Following three washes with PBS, cells were incubated with an Alexa fluor 546-conjugated goat anti-rabbit secondary

antibody (Invitrogen) diluted in 1% BSA/PBS for 20min at 37°C, 5% CO_2. The cells were then washed and counterstained with DAPI.

The γH2AX immunostaining for the RNAi screen followed the same fixation, permeabilization, and blocking protocol as that described for KAP1 pS824, with the exception that the cells were grown on glass coverslips. The antibody recognizing γH2AX pS139 (Upstate Biotechnology) was diluted in 1% BSA/PBS and incubated on cells for 1 hour at 37°C, 5% CO_2. Following three washes with PBS, cells were incubated with a Rhodamine red-conjugated goat anti-mouse IgG antibody (Jackson ImmunoResearch Laboratories, Inc.) diluted in 1% BSA/PBS for 20min at 37°C, 5% CO_2. After three washes in PBS the cells were counterstained with DAPI. All images were obtained with a Zeiss Axioplan microscope equipped with a Zeiss camera and software.

HU sensitivity

U2OS cells were transfected with siRNA and split into four 96-well plates the day after transfection. Three days after transfection cells were incubated in media with (2 plates) or without (2 plates) 3mM HU for 24 hours, followed by 24 hours in fresh growth media. Cell viability was quantified using the WST-1 cell proliferation reagent (Roche).

The sensitivity of gene-silencing siRNAs to HU was calculated by two separate methods. First, cell viability after HU treatment was expressed as the ratio of the mean absorbance at 450nM for the two HU-treated wells to the mean 450nM absorbance of the two untreated wells. Ratios for each gene-silencing siRNA were then normalized to the mean ratio of the non-targeting siRNAs within each plate to control for plate-to-plate

variation. The $\log_2$ of these viability ratios was calculated for determination of statistical significance.

Secondly, the effect of siRNA silencing on HU sensitivity was calculated as an index of antagonism or sensitivity (SI) using a previously described method, which accounts for the individual effects of the siRNA and the HU on cell viability [140]. For this calculation, the viability effect of the untreated siRNA compared to the untreated non-targeting control siRNA is designated Rc/Cc. The effect of HU on the viability of control transfected cells is designated Cd/Cc. The expected combined effect of the siRNA and HU on cell viability is therefore Rc/Cc*Cd/Cc. The observed combined effects of HU and the siRNA on cell viability compared to untreated control-transfected cells is designated Rd/Cc. The SI for each gene-silencing siRNA was determined by subtracting the observed combined effects of HU and siRNA from the expected total viability effect (SI = (Rc/Cc*Cd/Cc)-(Rd/Cc)). The mean viability ratio and sensitivity index values were calculated from three independent transfections, and statistical significance was determined using a two-tailed, unpaired t test comparing each gene-silencing siRNA to the non-targeting siRNA.

HU Recovery

U2OS cells were transfected with siRNAs targeting genome maintenance genes that exhibited both γH2AX foci formation and HU sensitivity after silencing. Three days after siRNA transfection cells were treated with 2mM HU for 16 hours to arrest cell cycle progression in early S phase. The cells were released into nocodazole-containing media (0.33ng/μl) to capture those reaching mitosis, and harvested at 16 hours. Mitotic cells

were quantified by flow cytometry analysis of propidium iodide (25μg/ml)- and histone H3 pS10-stained cells. Where indicated, cells were pulsed with 10μM of the thymidine analog bromodeoxyuridine (BrdU) in cell culture medium for 20 minutes prior to harvesting, and DNA synthesis was monitored by flow cytometry analysis of propidium iodide- and BrdU-stained cells. All cells quantified by flow cytometry were also treated with 0.1mg/ml RNase A for at least 30min prior to analysis.

Histone H3 phospho-S10 immunostaining

Both adherent and detached cells were harvested and washed once with PBS. Cells were pelleted by centrifuging at 1800*xg* for five minutes, and resuspended in 300μl cold PBS. Cells were fixed by adding 700μl cold 100% ethanol and incubated at -20°C for at least one hour. The cells were pelleted, the supernatant was removed by pipetting, and the cells were resuspended in 1ml cold PBS to rehydrate. After incubating on ice for 10 minutes, cell were again pelleted, the PBS was removed by pipetting, and the cells were resuspended in 1ml cold 0.25% triton X-100 in PBS to permeabilize. After incubating on ice for 15 minutes, the cells were rinsed once with 1% BSA/PBS (without pipetting). The cells were then resuspended in 100μl of room temperature 1% BSA/PBS containing the Histone H3 phospho-S10 antibody (DC-Bethyl Laboratories, diluted 1:2000). The samples were incubated at room temperature for 1.5 hours, and the cells were resuspended every 15 minutes by flicking the tube. The cells were washed twice with 1% BSA/PBS, and resuspended in 100μl of 1% BSA/PBS containing the fluorescein (FITC)-conjugated goat anti-rabbit IgG diluted 1:75 (Jackson ImmunoResearch Laboratories, Inc.). The samples were incubated at room temperature for 1 hour

protected from light, and resuspended every 15 minutes by flicking tube. After washing once with 1% BSA/PBS and once with PBS, the DNA was labeled by incubating in 25μg/ml propidium iodide in PBS containing 0.1mg/ml RNase A for 30 minutes at 37°C. The cells were filtered and analyzed using a flow cytometer.

BrdU labeling and immunostaining

Equal numbers of cells were labeled by incubation in DMEM containing 10μM BrdU at 37°C and 5% CO_2 (do not wash cells prior to incubation with BrdU). Fifteen to twenty minutes is sufficient to label replicating cells; for the analysis of re-replication in *DDB1*-silenced cells the labeling was extended to two hours. After labeling, the cells were pelleted by centrifuging at 1000rpm for 5 minutes, and washed once with PBS. Cells were resuspended in 300μl cold PBS, and 700μl cold 100% ethanol was added to fix the cells. After incubating at -20°C for at least one hour, cells were centrifuged at 1800*xg* for 5 minutes and the supernatant was removed using a pipette. The cell pellets were loosened by vortexing briefly, and 1ml of 2N HCl/0.5% triton X-100 was slowly added to the cells, a few drops at a time, while maintaining a vortex. Samples were incubated at room temperature for 30 minutes to denature the DNA. The cells were centrifuged, the supernatant was removed by pipetting, and the cells were resuspended in 1ml 0.1M $Na_2B_4O_7$ x 10 H_2O, pH 8.5, to neutralize the acid. The cells were centrifuged, the supernatant was remove, and the cells were resuspended in 1ml of 0.5% Tween 20/1% BSA/PBS. For indirect immunostaining, 20μl of anti-BrdU antibody (BD Biosciences) was added per 0.5 x 10^6 cells and samples were incubated at room temperature for 1.5 hours. Cells were resuspended every 15 minutes by flicking the tube.

The cells were centrifuged and resuspended in 100μl of 0.5% Tween 20/1% BSA/PBS containing fluorescein (FITC)-conjugated goat anti-mouse IgG diluted 1:50 (Jackson ImmunoResearch Laboratories, Inc.). Samples were incubated at room temperature for one hour protected from light, and the cells were resuspended every 15 minutes by flicking the tube. After immunostaining, the cells were washed once with PBS and the DNA was stained with propidium iodide as described above prior to analysis by flow cytometry.

Yeast two-hybrid

A two-hybrid screen was performed with full-length ATRIP cloned into pDAB1 containing the DNA-binding domain of GAL4. This bait was used to screen a B-cell cDNA library using the PJ694A yeast strain. Of the approximately 330,000 transformants screened, eleven interacting clones were identified that specifically interacted with the ATRIP bait and did not interact with two unrelated test baits. Two of the eleven interacting clones encoded full-length CINP. The two-hybrid assay was also used to map the interacting surfaces between ATRIP and CINP. Full-length CINP was cloned into pDAB1 containing the DNA-binding domain of GAL4, and used as bait to screen the pACT-ATRIP cDNA fragment library described previously [141]. The pACT-ATRIP fragment plasmids from 14 positively selected yeast colonies were rescued and sequenced. This screen was performed by Gloria Glick.

Co-immunoprecipitation of CINP with ATR-ATRIP

HEK293 cells were transiently transfected with Flag-tagged ATR or ATRIP and HA-tagged CINP, or Flag-tagged CINP alone. Cells were lysed in CHAPS lysis buffer (50mM Tris, pH 7.5, 0.15M NaCl, 0.75% CHAPS, 5 μg/ml aprotinin, 5 μg/ml leupeptin, 1 mM NaF, 20 mM μ-glycerolphosphate, 1 mM sodium vanadate, 0.5 mM dithiothreitol, and 0.2 mM phenylmethylsulfonate). Flag purifications were separated by SDS-PAGE and co-precipitating proteins were identified by immunoblotting. For endogenous co-precipitations, HeLa cells were lysed in CHAPS lysis buffer or nuclear extracts were prepared from HEK293 cells by dounce homogenization. CINP immunoprecipitations from HeLa whole cell lysates and HEK293 nuclear extracts were separated by SDS-PAGE and co-precipitating proteins were identified by immunoblotting.

G2 checkpoint assay

U2OS cells were transfected with siRNA and the integrity of the G2 checkpoint was examined as described previously [142], with the following modifications. Nocodazole (0.33ng/μl) was added one hour after irradiation, and mitotic cells were quantified 17 hours after irradiation by flow cytometry analysis of propidium iodide (25μg/ml)- and histone H3 pS10-stained cells. Transfections were performed in triplicate, and 10,000 cells were counted per experiment.

Cell lysis

For the immunoblot analyses performed after silencing of *DDB1* and *CINP*, cells were lysed in Igepal lysis buffer for 30 minutes on ice (20mM Tris, pH 7.5, 0.1M NaCl,

1mM EDTA, 0.5% Igepal CA 630, 5 μg/ml aprotinin, 5 μg/ml leupeptin, 1 mM NaF, 20 mM μ-glycerolphosphate, 1 mM sodium vanadate, 1 mM dithiothreitol, and 1 mM phenylmethylsulfonate). Cell lysates were cleared by centrifugation at 13,000*xg*, 4°C, for 20 minutes and the supernatants were transferred to new pre-chilled microfuge tubes. Protein concentrations of the lysates were determined using the Bio-Rad Protein Assay Solution according to the manufacturer's instructions.

Antibodies

The antibody dilutions used for analysis by immunoblot (IB) and immunofluorescence (IF) are indicated below in Table 2.1. The antibodies are diluted in 1% nonfat dry milk (NFDM)/TBST for immunoblot analysis and incubated at room temperature for 1-1.5 hours unless indicated otherwise. All antibodies used for immunofluorescence are diluted in 1% BSA/PBS. See the *Immunofluorescence* section of this chapter for incubation times.

Table 2.1. Antibody sources and dilutions for immunoblotting and immunofluorescence.

Antibody	Species	Source	Catalog Number	Application	
				IB	IF
ATM	Rabbit	Novus Biologicals	NB100-104H2	1:2500	
ATM pS1981	Rabbit	Rockland, Inc.	601-401-400	1:600	
ATR	Goat	Santa Cruz Biotechnology	sc-1887	1:500	
ATRIP (403)	Rabbit	DC; Bethyl Laboratories	NA	1:3000	
ATRIP pS224	Rabbit	DC; Bethyl Laboratories	NA	1:2000 in 1% BSA	
BrdU	Mouse	BD Biosciences	555627		
CDT1 (H-300)	Rabbit	Santa Cruz Biotechnology	sc-28262	1:750	
CHK1 (G-4)	Mouse	Santa Cruz Biotechnology	sc-8408	1:750	
CHK1 pS317	Rabbit	Cell Signaling	23445S	1:2000	
CHK1 pS345	Rabbit	Cell Signaling	2341S	1:500 in 1% BSA 4C O/N	
CHK2	Rabbit	DC; Bethyl Laboratories	NA	1:2000	
CHK2 pT68	Rabbit	Cell Signaling	2661S	1:1000 in 5% NFDM 4C O/N	
CINP	Mouse	Vanderbilt Monoclonal Antibody Core	NA	1:1000	
CUL4A	Rabbit	Yue Xiong	NA	1:2000	
Cyclin A	Rabbit	Santa Cruz Biotechnology	sc-751	1:750	1:100
DDB1	Mouse	BD Biosciences	612488	1:500	
DDB2 (H-127)	Goat	Santa Cruz Biotechnology	sc-16295	1:500	
Flag M5	Mouse	Sigma	P-4042	1:2000	
GAPDH	Mouse	Chemicon	MAB374	1:40,000	
γH2AX (pS139)	Rabbit	DC; Bethyl Laboratories	NA		1:1000
γH2AX (pS139)	Mouse	Upstate Biotechnology	05-636		1:10,000
HA	Mouse	Covance	MMS-101P	1:1000	
KAP1 pS824	Rabbit	Bethyl Laboratories	IHC-00073		1:300
MCM2 (BM28)	Rabbit	BD Biosciences	610700	1:3500	
MCM2 pS108	Rabbit	DC; Bethyl Laboratories	NA	1:10,000	
MRE11 (12D7)	Mouse	GeneTex, Inc.	GTX70212		1:250
p21 (EA10)	Mouse	Calbiochem	OP64	1:500	
p53 (DO-1)	Mouse	Santa Cruz Biotechnology	sc-126	1:500	
p53 pS15	Rabbit	Cell Signaling	92845	1:1000 in 5% BSA 4C O/N	
RPA34 (9H8)	Mouse	Neomarkers	MS-691-P1		1:100
SKP2 p45	Mouse	Zymed	32-3300	1μg/ml	
XPA	Mouse	Neomarkers	MS-650-P0	1:1000	
XPC	Mouse	Novus Biologicals	NB100-477	1:3000	