

## Chapter 2

# DNA Damage Signaling Downstream of ATM

Fred Bunz

**Abstract** ATM is the apical signaling molecule that triggers diverse cellular responses to double-strand DNA breaks. Directly and indirectly, ATM initiates a two-tiered cascade of protein kinase activation, composed of upstream phosphatidylinositol 3-kinase-like kinases, mediator proteins, and checkpoint kinases. Together, these proteins signal a broad network of downstream effectors that modulate virtually every aspect of cell growth and death. This review will focus on the signaling molecules required for the diverse ATM-dependent responses to DNA damage, with an emphasis on the extensively characterized pathways that suppress proliferation and promote DNA repair.

**Keywords** DNA damage • ATM • ATR • Checkpoints • Signaling network

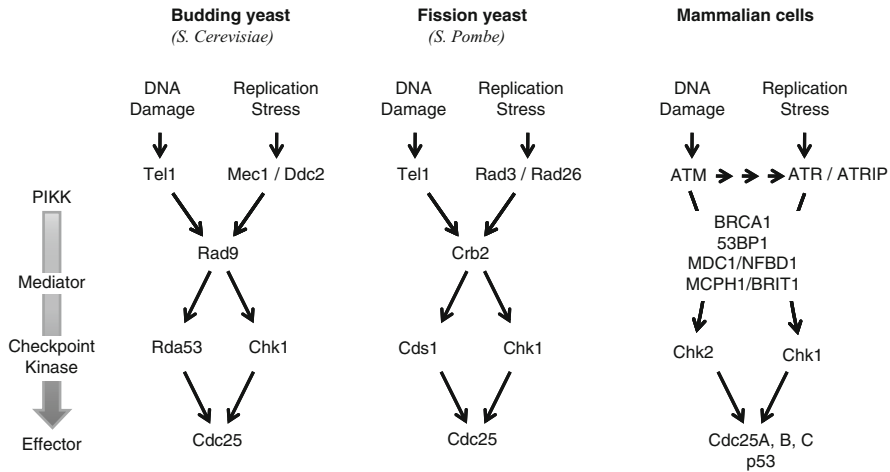
## 2.1 Introduction

Damage to genomic DNA stimulates a profound and functionally diverse cellular response that affects fundamental cellular processes. As described in previous chapters, ATM plays a central role in initiating the collective responses to a particularly lethal form of DNA damage, the double-strand DNA break (DSB). Elegant biochemical experiments have demonstrated the fundamental mechanisms by which ATM kinase activity is activated at DSB sites (Bakkenist and Kastan 2004). The transduction of ATM signals arising from focal sites on damaged chromosomes to the many cellular compartments that mount responses to DSBs will be the focus of this review.

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F. Bunz (✉)

Associate Professor, Department of Radiation Oncology and Molecular Radiation Sciences,  
Sidney Kimmel Comprehensive Cancer Center, Johns Hopkins University School of Medicine,  
David H. Koch Cancer Research Building (CRB2), Room 453, 1550 Orleans Street,  
CRB II, Room 462, Baltimore, MD 21231, USA  
e-mail: fredbunz@jhmi.edu



**Fig. 2.1** Conserved DNA damage signaling pathways. The general organization of the two-tiered DNA damage signaling cascade was defined by genetic studies in budding and fission yeasts. Double-strand DNA breaks activate orthologs of ATM, while DNA replication intermediates trigger the activation of orthologs of ATR and its binding partner ATRIP. Mediator proteins facilitate the activation of the checkpoint kinases by the upstream PIKKs. In mammalian cells, a family of BRCT domain-containing proteins appears to provide the functions of the single mediator proteins in the yeasts. Downstream signals converge on cell cycle regulatory proteins including Cdc25 protein phosphatases. Mammalian cells contain additional regulators of cell cycle checkpoints, including p53. Both the PIKKs and the checkpoint kinases have many additional downstream substrates. See text for details

Studies of the DNA damage pathways in evolutionarily divergent yeasts have revealed a two-tiered kinase cascade that is conserved in human cells (Fig. 2.1). DNA lesions directly activate apical serine/threonine protein kinases that are structurally related to the phosphatidylinositol 3-kinase and are accordingly known as the (PI3-kinase-like kinase) PIKK family. Identified as the gene mutated in ataxia-telangiectasia patients in 1995, ATM is one of six human PIKKs (Lavin et al. 2005). A second PIKK known to be functionally conserved in human cells is the ataxia-telangiectasia and Rad3-related (ATR) kinase. In both humans and yeasts, upstream PIKK proteins activated by DNA damage phosphorylate downstream serine/threonine protein kinases known as checkpoint kinases (Bartek and Lukas 2003; McGowan and Russell 2004; Reinhardt and Yaffe 2009). Chk1 and Chk2, the human orthologs of the yeast checkpoint kinases, transduce DNA damage signals from lesions and stalled replication forks to spatially and functionally distinct compartments of the cell (Stracker et al. 2009). An emerging class of phosphoprotein-interacting proteins cumulatively play the role of yeast Rad9, the first identified checkpoint protein, and mediate the activation of the checkpoint kinases by the PIKKs (Mohammad and Yaffe 2009). By illuminating the relationship between PIKKs, mediator proteins and the checkpoint kinases, genetic analyses of yeast have provided the theoretical framework for understanding the functional organization of the DNA damage response signaling pathways in human cells.

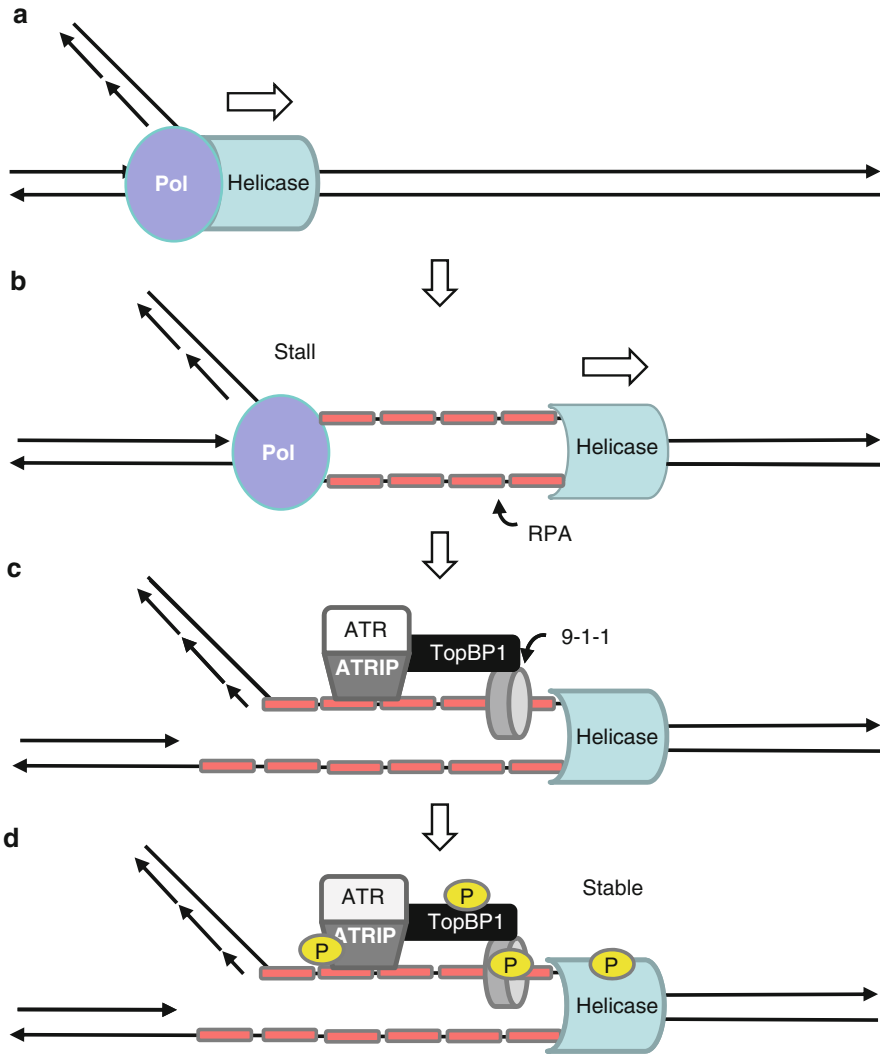
## 2.2 Ataxia-Telangiectasia and Rad3-Related

ATR is a large serine/threonine protein kinase with significant homology to ATM and the other members of the PIKK family (Abraham 2001; Cimprich and Cortez 2008). Both ATM and ATR preferentially phosphorylate serine residues that are followed by glutamine (SQ sites), and therefore target overlapping sets of substrates. However, ATM and ATR respond to distinct stimuli and thus have nonredundant functions. ATM is primarily activated by DSBs. ATR responds to DSBs but is additionally activated by a wide range of DNA lesions and DNA structures caused by environmental and therapeutic agents that inhibit or impede DNA replication, including ultraviolet (UV) radiation, DNA cross-linking agents and antimetabolites that interfere with nucleotide metabolism (Abraham 2001; Osborn et al. 2002).

From a genetic perspective, the ATM and ATR signaling pathways exhibit important differences that suggest distinct roles in the cell. Both ATM and Chk2 are encoded by tumor suppressor genes that confer cancer predisposition – most prominently an increased risk of breast cancer – when inactivated by germline mutations. The complete loss of ATM signaling in patients with ataxia-telangiectasia or in ATM-knockout mice largely eliminates the response to DSBs and leads to extreme sensitivity to ionizing radiation (Shiloh 2006) but does not obviously impair unperturbed cell growth. In contrast, both ATR and Chk1 are essential genes that are required for cell viability and proliferation (Liu et al. 2000; Brown and Baltimore 2003). Only rare hypomorphic ATR alleles are tolerated in the germline; it is unclear if these predispose carriers to cancer (O’Driscoll et al. 2004). While ATR and CHK1 mutations have been found in small number of mismatch repair-deficient cancers, these genes do not have the requisite characteristics of established tumor suppressors.

Inhibition of ATR activity in the presence of low levels of DNA replication stress causes DNA breaks at defined loci known as fragile sites (Casper et al. 2002). Detected at the cytogenetic scale, fragile sites are thought to represent structurally distinct regions in the genome where DNA replication forks tend to stall. The requirement of ATR for normal cell growth, its activation by DNA replication inhibitors and its role as a suppressor of fragile site expression all support a critical role for ATR in the stabilization of DNA replication forks (Fig. 2.2).

Recent studies have determined the fundamental mechanism by which ATR is activated at stalled replication forks (Zou and Elledge 2003; Kumagai and Dunphy 2006; Cimprich and Cortez 2008; Mordes et al. 2008). The molecular machines that replicate genomic DNA include multiprotein DNA polymerase complexes that function to synthesize nascent strands and DNA helicase complexes that unwind the DNA template ahead of the advancing fork. Critical to the activation of ATR is the decoupling of polymerase and helicase activities. When a polymerase complex runs out of nucleotide substrate or if it encounters a DNA lesion, such as a strand break or an adduct, DNA synthesis can stall (Zegerman and Diffley 2009). In this case, the helicase complex can continue unwinding DNA, exposing a stretch of single-stranded DNA that would otherwise be rapidly replicated. The single-stranded DNA at the lagging strand of the stalled fork



**Fig. 2.2** Activation of ATR. (a) Advancing DNA replication forks are driven by the coordinated activities of multiprotein DNA polymerase (Pol) and DNA helicase complexes. (b) DNA polymerases that synthesize the leading and lagging strands may run short of nucleotides, or encounter a DNA lesion that impairs DNA synthesis. In such cases, the polymerase complexes will pause and become uncoupled from the helicase complexes, which continue unwinding DNA ahead of the stalled fork. Regions of single-stranded DNA are rapidly coated with the trimeric replication protein A complex (RPA). (c) ATR is recruited to the RPA-coated single-stranded DNA via association with ATRIP. The Rad9–Rad1–Hus1 (9-1-1) complex is loaded at the single strand–double strand DNA junction by the ATP-dependent Rad17 clamp loader. The TopBP1 protein associates with both the 9-1-1 complex and the ATR–ATRIP complex, which are all required for ATR activation. (d) Activated ATR phosphorylates numerous proteins assembled at the stalled fork (phosphates shown in yellow), which apparently serves to increase fork stability and eventually facilitate resumption of DNA synthesis. Thus activated, ATR also phosphorylates Chk1 and many other downstream substrates

becomes rapidly coated with the heterotrimeric single-strand DNA-binding protein complex known as replication protein A (RPA) (Fanning et al. 2006). The junction between RPA-coated single-stranded DNA and adjacent double-stranded DNA creates a structure that is recognized by the protein complexes that function to restart stalled forks. The RPA-coated single-strand DNA attracts binding by the ATR-interacting protein (ATRIP), the binding partner of ATR (Cortez et al. 2001). A heterotrimeric ring-like protein complex composed of Rad9-Rad1-Hus, known as the 9-1-1 sliding clamp, is loaded onto the single-strand DNA/double-strand DNA junction by an ATP-dependent clamp loader derived from the replicative replication factor C (RFC) complex (Parrilla-Castellar et al. 2004). The 9-1-1 complex and ATR function together to recruit the topoisomerase-interacting protein TopBP1 (Lee et al. 2007). The binding of ATR–ATRIP to RPA-coated single-strand DNA and the recruitment of TopBP1 are all required for activation of ATR kinase activity at stalled, unwound replication forks (Kumagai and Dunphy 2006; Mordes et al. 2008).

ATR is known to phosphorylate several proteins involved in DNA replication, including subunits of RFC and RPA, proteins in the minichromosome maintenance (MCM) complex required for replication initiation and fork progression, and several DNA polymerases (Matsuoka et al. 2007; Cimprich and Cortez 2008). The consequences of individual ATR-dependent phosphorylation events remain poorly understood, but the overall effect of ATR on the replication fork appears to be to stabilize the protein complexes of the replisome and thereby maintain fork integrity and velocity (Cimprich and Cortez 2008; Wilsker et al. 2008). The role of ATR–ATRIP as both a sensor of stalled replication forks and as a stabilizer of the multi-protein complexes at the fork would appear to represent, in essence, a feedback circuit that ensures the efficient replication of the genome.

## 2.3 The Checkpoint Kinases

The human homologs of the yeast checkpoint kinases serve as transducers of the signals generated by upstream PIKKs (Bartek and Lukas 2003). PIKKs are large kinases that, once activated, appear to remain closely located with DNA breaks and stalled replication forks. The smaller, more mobile checkpoint kinases are the mechanism by which signals originating at chromatin are transmitted throughout the cell to spatially and functionally diverse effectors (Smits 2006; Stracker et al. 2009). The checkpoint kinases preferentially phosphorylate R-X-X-S/T sites flanked by hydrophobic residues, and like the PIKKs have overlapping sets of substrates. In addition to the well-described ATM–Chk2 and ATR–Chk1 pathways, an additional complex consisting of the stress-responsive kinases p38MAPK and MK2 have been found to share substrate preferences with Chk1 and Chk2, and to function in the activation of checkpoints (Reinhardt and Yaffe 2009). The cross-specificity between Chk1, Chk2, and MK2 substrates has made it difficult to evaluate the respective roles of these kinases in downstream pathways. Recent studies

suggest that Chk1 plays a primary role in cell cycle arrest pathways (Jallepalli et al. 2003; Wilsker et al. 2008) and is the most relevant checkpoint kinase in terms of therapeutic responses (Xiao et al. 2006; Wilsker et al. 2008).

### 2.3.1 *Chk1*

The most extensively characterized of ATR's many substrates is the checkpoint kinase Chk1. The phosphorylation and activation of Chk1 requires a binding partner called Claspin that mediates the ATR–Chk1 physical interaction (Kumagai and Dunphy 2000; Chini and Chen 2004; Liu et al. 2006). Associated with the clamp loading complex at active replicons, Claspin is phosphorylated when replication forks stall (Wang et al. 2006). The association of Claspin with Chk1 is mediated by repeated phosphopeptide motifs that are phosphorylated by Chk1 and perhaps by other kinases as well (Kumagai and Dunphy 2003; Chini and Chen 2006). Thus, the Chk1–Claspin interaction is promoted by stalled replication forks and by the basal activity of Chk1 itself. Brought into proximity of the Chk1–Claspin complex, ATR phosphorylates Chk1 at two serine residues (S317 and S345) in a regulatory domain located in the c-terminus (Zhao and Piwnica-Worms 2001). While the Chk1 regulatory domain is evolutionarily divergent, the serine residues required for human Chk1 activation are within short regions of homology that are conserved in yeast.

Detailed analyses of the functions of the individual Chk1 phosphorylation sites have provided insights into the functions of Chk1 and the distinct roles played by Chk1 in unperturbed and damaged cells. Genetic model systems in mouse and human cells have demonstrated Chk1 to be essential for cellular viability, as is ATR (Liu et al. 2000). Phosphorylation of Chk1 on its S345 site appears to be intrinsic to the essential function of Chk1; *CHK1* alleles with mutations (S to A) that disrupt the S345 site do not support cellular viability (Niida et al. 2007; Wilsker et al. 2008). In contrast, mutations of the S317 are tolerated. However, cells harboring S317 mutant Chk1 proteins lose the ability to mediate the G<sub>2</sub>/M checkpoint in response to DNA damage and exhibit defects in DNA replication, including decreased replication fork velocity and increased fork stalling in unperturbed cells (Wilsker et al. 2008). Studies of Chk1 mutants have therefore revealed that Chk1 plays an essential role during normal cell growth and also has a nonessential role in the DNA damage response. These distinct roles are genetically separable by mutation of a single ATR phosphorylation site at S317 (Niida et al. 2007; Wilsker et al. 2008).

Phosphorylation of Chk1 plays a prominent role in its localization. In unstressed cells, a pool of Chk1 protein remains stably bound to chromatin. Phosphorylation of Chk1 on S317 and S345 by ATR results in the rapid dissociation of this bound fraction (Smits et al. 2006). Following DNA damage or replicative stress, the majority of Chk1 in the cell becomes rapidly phosphorylated, suggesting that both chromatin-bound and unbound fractions of Chk1 are ultimately modified by ATR. In the prevailing model, unphosphorylated Chk1 in effect circulates to sites of

activated ATR (Smits 2006). Once phosphorylated by ATR at regions of RPA-coated single-stranded DNA, Chk1 is released from chromatin to phosphorylate its downstream substrates.

Chk1 localization is also controlled by cellular pathways generally unrelated to DNA damage signaling. The AKT kinase, which is negatively regulated by the PTEN tumor suppressor, phosphorylates Chk1 on S280 (Puc et al. 2005). The phosphorylation of Chk1 at this site promotes its monoubiquitination and promotes its sequestration in the cytoplasm. It is believed that the enhanced phosphorylation of Chk1 by AKT contributes to the checkpoint deficiencies observed in PTEN-deficient cancers (Puc and Parsons 2005; Puc et al. 2005).

Chk1 controls the intra-S and the G<sub>2</sub>/M checkpoints, DNA damage-responsive pathways by which damaged cells transiently halt DNA replication and are prevented from entering mitosis, respectively (Stracker et al. 2009). Key components of the checkpoint pathways are the Cdc25 family of phosphatases. The three Cdc25 proteins Cdc25A, Cdc25B, and Cdc25C remove inhibitory phosphate moieties from the cyclin-dependent kinases (CDKs), and thereby promote cell cycle transitions (Boutros et al. 2006; Karlsson-Rosenthal and Millar 2006). The effect of Chk1 on the Cdc25 proteins is to inhibit their activity by triggering their degradation (in the case of Cdc25A) or causing their sequestration in the cytoplasm (Cdc25B and Cdc25C). By inhibiting the Cdc25 phosphatases required for CDK activity, activated Chk1 is required for the checkpoint pathways that block cell cycle transitions.

Other functions of Chk1 include the activation of DNA repair by the Fanconi Anemia pathway. Required for the repair of interstrand DNA cross-links, the Fanconi Anemia genes encode a multisubunit protein complex that is initially activated by ATR (Gurtan and D'Andrea 2006). Chk1 phosphorylates the FancE subunit on two residues (T346 and S374) that promote its degradation (Wang et al. 2007). The Chk1 sites on FancE are required for cell survival after treatment with DNA cross-linking agents.

### 2.3.2 *Chk2*

As Chk1 is directly phosphorylated by ATR in response to RPA-coated single-stranded DNA, so is Chk2 is phosphorylated by ATM in response to DSBs (Bartek et al. 2001; Ahn et al. 2004; Stracker et al. 2009). The kinase domains of Chk2 and Chk1 are highly related, and pharmacological inhibitors of these kinases typically exhibit cross-specificity. Beyond their kinase domains, Chk2 and Chk1 are structurally and functionally distinct (McGowan 2002).

In striking contrast with the ATR–Chk1 pathway, the ATM–Chk2 pathway is nonessential. The Chk2 gene can be homozygously disrupted in human and mouse cells (Hirao et al. 2000; Jallepalli et al. 2003), and therefore does not play a critical role in cell proliferation. Chk2 mutations occur at a significant frequency, but the role of Chk2 in cancer has been controversial. The original identification of Chk2

mutations in patients with a variant of Li Fraumeni syndrome known as Li Fraumeni-like syndrome suggested that Chk2 mutations might phenocopy highly penetrant p53 mutations (Bell et al. 1999). In accordance with this hypothesis, studies of Chk2 activity in vitro demonstrated that Chk2 can phosphorylate p53 on a site (S20) involved in its activation (Hirao et al. 2000; Shieh et al. 2000). However, the most prevalent Chk2 alteration is a truncating mutation (designated *1100delC*) that is found in some populations at a frequency as high as 1% (Sodha et al. 2000). The discordance between the high frequency of the *CHK2*<sup>1100delC</sup> allele and the low incidence of Li Fraumeni-like syndrome suggests that Chk2 mutations are not functionally equivalent to p53 mutations (Sodha et al. 2002). Studies in human cells demonstrate that p53 can be activated in the absence of Chk2 (Jallepalli et al. 2003), while analyses of knockout mice suggest that Chk2 is required for some p53-dependent functions in some cells types (Jack et al. 2002). It thus would appear that Chk2 can promote p53 activation in some tissues, perhaps in response to tissue-specific stimuli. Large-scale population-based studies have demonstrated that carriers of the *CHK2*<sup>1100delC</sup> allele have three- to fivefold increased risk of developing breast cancer (Meijers-Heijboer et al. 2002; Weischer et al. 2008). *CHK2* is therefore a tumor suppressor gene with incomplete penetrance, similar to ATM.

Chk2 has an N-terminal cluster of ATM/ATR recognition sites known as the SCD, a Forkhead-associated domain (FHA) that is involved in phosphorylation-dependent protein–protein interactions and a kinase catalytic domain toward the c-terminus (Ahn et al. 2004; Stracker et al. 2009). More than 25 distinct Chk2 phosphorylation sites have been identified. Activated ATM phosphorylates Chk2 on many of the recognition sites in the c-terminal SCD, including residue T68. Once phosphorylated, the SCD becomes a docking site for a second Chk2 molecule. The multimerization of Chk2 brings the activation domains of the kinase loops into close proximity, thus promoting the autophosphorylation of multiple residues in the catalytic site. The autophosphorylation of the N-terminal sites increases enzymatic activity. This two-stage activation mechanism is common to the homologs of Chk2 in yeast (Oliver et al. 2007).

The substrate specificity of Chk2 closely resembles that of Chk1 (Stracker et al. 2009). Accordingly, Chk2 has been shown to phosphorylate key cell cycle regulators such as p53 and the Cdc25 protein phosphatases. The phenotypes of Chk2 knockout human cells and mice are generally mild and do not phenocopy mutations in ATM. Chk2 knockout cells retain their ability to upregulate p53 and activate checkpoints following DNA damage, and apoptotic pathways appear to be defective in only a subset of tissues (Jack et al. 2002; Jallepalli et al. 2003).

### 2.3.3 *p38MAPK/MK2*

The mitogen-activated protein kinase-activated protein kinase-2 (MK2) is potently stimulated by the various activators of the p38 family, which response to diverse forms of cell stress (Roux and Blenis 2004). Loss of function of MK2 in knockout



mice leads most apparently to deficiencies in immune responses. A role for the p38MAPK/MK2 in checkpoint control was suggested by the finding that MK2 can function downstream of ATR and phosphorylate the same sites on Cdc25 proteins as Chk1 and Chk2, following exposure to UV radiation (Manke et al. 2005). Cdc25B and Cdc25C are sequestered in the cytoplasm following MK2-dependent phosphorylation.

## 2.4 Cooperation Between ATM and ATR

ATM and ATR are both activated by DSBs (Abraham 2001; Osborn et al. 2002). The activation of ATM occurs within minutes of a DSB, in cells that are in any phase of the cell cycle. In contrast, activation of ATR by DSBs is delayed and restricted to cells that are in S and G<sub>2</sub> (Jazayeri et al. 2006). Cells from ataxia-telangiectasia patients deficient in ATM function exhibit markedly impaired phosphorylation of downstream substrates and diminished DSB responses, despite the presence of wild-type ATR. Such observations underscore the primary importance of ATM in the DSB response. Recent studies have demonstrated that ATM is in fact required for activation of ATR by DSBs (Adams et al. 2006; Cuadrado et al. 2006; Jazayeri et al. 2006; Myers and Cortez 2006). Unlike other downstream components of the DSB response, ATR is not known to be an ATM substrate. Rather, ATR is activated indirectly as a result of ATM-initiated processing of DSBs that involves the components of the MRN complex. The DNA end processing by the MRN complex in effect causes DSBs that efficiently activate ATM to be converted to regions of RPA-coated single-stranded DNA that efficiently activate ATR. This DSB conversion is CDK-dependent, which causes ATR activation to be cell cycle phase-specific (Jazayeri et al. 2006). In the absence of ATM, the conversion of DSBs to RPA-coated single-stranded DNA is significantly slower and less robust, and the activation of ATR is similarly decreased.

Following its indirect activation by ATM, ATR plays a critical role in the overall DSB response. Most notably, cells engineered to be completely or even partially deficient in ATR function exhibit clear defects in the G<sub>2</sub>/M checkpoint triggered by ionizing radiation (Hurley et al. 2007). Additionally, ATR-deficient cells exposed to ionizing radiation fail to enter S-phase and complete DNA replication, suggesting a failure to stabilize early replication complexes in the presence of DSBs (Hurley et al. 2007).

ATM has been observed to be detectably activated by agents that primarily inhibit DNA replication (Dodson and Tibbetts 2006; Stiff et al. 2006); this cross-activation has been attributed to phosphorylation by ATR of the ATM S1981 auto-phosphorylation site (Stiff et al. 2006). The ATR-dependent phosphorylation of this site is independent of the MRN complex, suggesting that ATM activation by this pathway is mechanistically distinct from its autoactivation after ionizing radiation. Cumulatively, these data show that the cooperation between ATM and ATR appears

to work in both directions. While ATM and ATR control parallel pathways, these apical sensor kinases clearly work together to respond to structurally diverse DNA lesions (Hurley and Bunz 2007).

Is there crosstalk between the ATM and ATR pathways? The shared substrate specificity of ATM and ATR presents the possibility that downstream substrates could be activated by either kinase. Activation of the ATM substrate Chk2 has been shown to result from treatment with DNA replication stressors known to activate ATR (Stiff et al. 2006). Chk1, known to be strongly phosphorylated by ATR, is phosphorylated in an ATM-dependent manner after exposure to ionizing radiation (Gatei et al. 2003). Each of these observations could be interpreted as evidence of crosstalk. However, recent insights into the cooperation between ATM and ATR have cast these observations in a new light. It is important to consider that DNA lesions are not static structures, but rather are rapidly processed and metabolized (Jazayeri et al. 2006). DSBs can be converted to regions of single-stranded DNA by the processing of broken ends that is ATM-dependent and MRN-mediated. DSB can alternatively arise from stalled replication forks that expose single-stranded DNA to the effects of nucleases. It now appears that relationships that on the surface appear to be crosstalk between upstream kinases are in fact the result of inter-conversion between DSBs and RPA-coated single-strand DNA. It is now generally believed that the respective activation of Chk1 and Chk2 by ATR and ATM is highly specific.

## **2.5 Mediators of the DNA Damage Response: BRCT-Containing Proteins**

Downstream of ATM and ATR are a complex family of proteins that share a common, highly conserved motif first identified in the breast cancer susceptibility gene 1 (BRCA1). The BRCA1 c-terminal (BRCT) motif is a phosphoprotein-binding domain involved in protein–protein interactions and oligomerization (Yu et al. 2003). Proteins containing tandem BRCT domains at their C-termini function to facilitate the interaction between signaling (PIKK) and transduction (checkpoint kinase) molecules, and thus act as mediators of the signaling cascade activated by DNA damage or DNA replication stress (Mohammad and Yaffe 2009). Among the BRCT-containing proteins recently found to function in DNA damage signaling are BRCA1, 53BP1, MDC1/NFBD1, and MCPH1/BRIT1. Together, these proteins represent the functional homologs of the first checkpoint protein ever described, budding yeast Rad9 (which is unrelated to the human Rad9 component of the 9-1-1 complex). Importantly, while yeast Rad9 functions to unidirectionally mediate signals from the PIKKs to the checkpoint kinases, several of the human homologs appear to additionally affect the upstream activation of ATM (Mochan et al. 2003; Aglipay et al. 2006; Wilson and Stern 2008). Both the diversity of mediators and the bidirectionality of the signaling pathways suggest a higher order of complexity in mammalian cells as compared with yeasts.

### 2.5.1 *BRCA1*

Many of the genes involved in the DNA damage repair pathways function as tumor suppressors. Prominent among these is *BRCA1*, which mutated in about one-half of inherited breast and ovarian cancers. Many of the cancer predisposing mutations in *BRCA1* affect the BRCT domain, suggesting that the role of *BRCA1* in the DNA damage response is intrinsic to its role in tumor suppression. *BRCA1* is phosphorylated after DNA damage on multiple sites by ATM (Cortez et al. 1999) and Chk2 (Zhang et al. 2004) and relocates rapidly to sites of damage and stalled DNA replication forks (Okada and Ouchi 2003; Ouchi 2006). Cells containing *BRCA1* mutant proteins display defective intra-S and G<sub>2</sub>/M checkpoints and hypersensitivity to ionizing radiation (Deng 2006). The individual phosphorylation sites are differentially required for the G<sub>2</sub>/M and the intra-S checkpoints, demonstrating the independent regulation of these pathways and the overall complexity of the relationship between *BRCA1* and checkpoints (Xu et al. 2001). The phosphorylation of *BRCA1* by Chk2, also encoded by a tumor suppressor gene involved in breast cancer, is required for efficient repair of DSBs (Lee et al. 2000; Zhang et al. 2004).

### 2.5.2 *53BP1*

53BP1 is a component of the DNA damage response that was originally identified by virtue of its physical interaction with the tumor suppressor protein p53 (Iwabuchi et al. 1994). Genetic analyses have demonstrated the importance of 53BP1 for the stabilization of p53, the phosphorylation of Chk2 and the activation of intra-S and G<sub>2</sub>/M checkpoints after DNA damage (DiTullio et al. 2002; Wang et al. 2002; Ward et al. 2003). After exposure to ionizing radiation, 53BP1 relocates rapidly to nuclear foci that also contain ATM, which correspond to sites of DSBs. Cells deficient in ATM exhibit defects in the relocation of 53BP1, indicating that 53BP1 functions in ATM-dependent pathways (DiTullio et al. 2002). 53BP1-knockout mice are cancer prone, radiosensitive, and develop similar cancers as ATM-knockout mice (Ward et al. 2004). Interestingly, loss of one or both copies of *53BP1* in p53-null mice significantly accelerated the rate of cancer development, demonstrating that 53BP1 and p53 proteins function together to suppress tumors (Ward et al. 2005).

While a significant body of data suggests that 53BP1 mediates the phosphorylation of ATM substrates, the exact function of 53BP1 remains unclear. 53BP1 contains tandem BRCT motifs, but these are not required for its oligomerization or for downstream DNA repair functions (Ward et al. 2006). Localization of 53BP1 to damage sites has been shown to require a tandem Tudor domain, a methyl-protein-binding motif required for the initial recruitment of 53BP1 to chromatin (Huyen et al. 2004). 53BP1 contains 15 identified ATM/ATR phosphorylation sites (Matsuoka et al. 2007). These sites are phosphorylated after ionizing radiation as well as UV radiation, suggesting that ATR as well as ATM can play an upstream role in its activation (Jowsey et al. 2007).

### 2.5.3 *MDC1/NFBD1*

Mediator of DNA damage checkpoint protein 1 (MDC1/NFBD1) is a BRCT-domain-containing protein that is phosphorylated in response to DNA damage (Stucki and Jackson 2004). After its phosphorylation, MDC1/NFBD1 relocalizes to sites of DNA lesions and functions to facilitate the recruitment and accumulation of cell cycle checkpoint and DNA repair factors to sites of DNA damage (Lukas et al. 2004; Xu et al. 2008), a characteristic shared with the other BRCT-containing proteins. Originally identified in a random screen of large cDNAs, MDC1/NFBD1 was predicted to function in the DNA damage response on the basis of its domain structure (Stucki and Jackson 2004). Depletion of MDC1/NFBD1 causes defects in checkpoint activation and apoptosis and sensitizes cells to DNA-damaging agents (Goldberg et al. 2003; Stewart et al. 2003).

### 2.5.4 *MCPH1/BRIT1*

MCPH1/BRIT1 was identified independently as a disease gene that encodes microcephalin and as a BRCT domain-containing inhibitor of the catalytic component of telomerase (hTERT). Like the more extensively characterized BRCT-domain protein 53BP1, MCPH1/BRIT1 regulates the intra-S and G<sub>2</sub>/M checkpoints, and localizes to  $\gamma$ H2AX foci after irradiation (Xu et al. 2004; Lin et al. 2005; Alderton et al. 2006). Depletion of MCPH1/BRIT1 leads to decreased expression of BRCA1 and Chk1, and causes radiosensitivity. The clinical similarity of primary microcephaly, caused by MCPH1/BRIT1 mutations, and ATR–Seckel syndrome, a recessive disease caused by hypomorphic mutations in ATR (O’Driscoll et al. 2004), suggest the importance of an ATR–BRCA1–Chk1 signaling pathway in brain development (Lin et al. 2005; Alderton et al. 2006).

MCPH1/BRIT1 contains three BRCT domains that facilitate its interactions with the chromatin-remodeling complex SWI/SNF. By recruiting this complex to the sites of DNA lesions, MCPH1/BRIT1 promotes chromatin relaxation which is thought to facilitate the access to the lesions by DNA repair proteins (Peng et al. 2009).

## 2.6 Activation of p53 by Upstream Kinases

Among the first human proteins found to contribute to the DNA damage response was the tumor suppressor p53. The p53 gene is among the most widely mutated of cancer genes, and contributes to the development of approximately one-half of all cancers. Upon its activation by upstream signaling pathways, p53 contributes significantly to numerous growth inhibitory pathways, including cell cycle checkpoints, apoptosis and senescence (Vogelstein et al. 2000). The p53 protein is modified by a broad array of posttranslational modifications, many of which are believed to

contribute to its activation (Horn and Vousden 2007; Kruse and Gu 2008). The best characterized among these are the phosphorylation moieties that are added by the kinases of the DNA damage signaling network (Appella and Anderson 2001).

p53 normally has a very short half-life, due to its association with Hdm2, a ubiquitin E3-ligase that targets p53 for proteasomal degradation (Kubbutat et al. 1997). In unperturbed cells, p53 is therefore maintained at low levels. After DNA damage, p53 is phosphorylated on multiple sites. These phosphorylation events are concurrent with the dissociation of p53 from Hdm2 and its stabilization. Thus activated by DNA damage, p53 transactivates the transcription of a large number of genes that contribute to the diverse outcomes of the DNA damage response (Vogelstein et al. 2000).

While p53 is known to be strongly activated by DNA damage and replication stress, the exact mechanism of its activation remains unclear (Kruse and Gu 2009). ATM-null cells from patients with ataxia-telangiectasia exhibit a markedly diminished p53 response to ionizing radiation, that is both less robust and temporally delayed compared with cells with wild-type ATM (Canman et al. 1994; Kastan and Lim 2000). ATM, ATR, Chk1, and Chk2 have all been reported to directly phosphorylate p53 on several N-terminal sites (Canman et al. 1998; Tibbetts et al. 1999; Chehab et al. 2000; Hirao et al. 2000), most prominently S15 and S20, that appear to play a role in protein stabilization *in vitro*. It has been unclear whether the direct phosphorylation of p53 by any one of the upstream kinases is primarily important, or to what extent the p53 response is due to indirect effects of DNA damage signaling. In recent years, studies of knock-in mouse models have called the role of the N-terminal p53 phosphorylation sites into question. Mutations of the mouse equivalents of human S15 and S20 (S18 and S23 in mouse) notably fail to eliminate the responsiveness of p53 levels to DNA damage and other stimuli (Wu et al. 2002; Saito et al. 2003; MacPherson et al. 2004; Sluss et al. 2004), although double mutants in both of these residues do exhibit significant apoptotic defects in some tissues (Chao et al. 2006). These results suggest that p53 stabilization may be a combination of direct and indirect effects of the DNA damage signaling pathways, including the stimulation of other types of posttranslational modifications (Kruse and Gu 2009).

## 2.7 Diverse Substrates of the Human PIKKs

The two-tiered structure of the yeast DNA damage and replication stress pathways (Fig. 2.1) has guided the identification of homologous proteins that function in the human DNA damage response. The checkpoint and mediator proteins that play critical roles in the growth arrest and survival following DNA damage remain the most highly characterized substrates of ATM and ATR. Recently, unbiased proteomic approaches have significantly broadened our view of the extent and scope of the DNA damage responses. Over 700 proteins are robustly phosphorylated at more than 900 sites by the combined activation of ATM and ATR in response to the

DSBs caused by ionizing radiation (Matsuoka et al. 2007). Similarly, UV radiation, a potent inhibitor of DNA replication, was found to induce the phosphorylation of nearly 500 proteins (Stokes et al. 2007; Stokes and Comb 2008). Within these two unexpectedly large sets of proteins are many that are commonly activated by both stimuli and therefore probably represent the combined effects of ATM and ATR activation. A significant number of substrates appear to be exclusively dependent on either ATM or ATR (Stokes and Comb 2008).

A common theme among the downstream targets of ATM/ATR is the phosphorylation of multiple targets within individual pathways, suggesting many potential nodes of regulation (Matsuoka et al. 2007). For example, many substrates participate in the regulation of the successive stages of DNA replication. Prevalent among the ATM/ATR target proteins are those that are involved in each step of DNA replication, including origin recognition (ORC proteins), replication complex assembly (MCM proteins), clamp loading (RFC1 and RFC3), and DNA synthesis (DNA polymerase epsilon, GINS). Identification of these substrates promises mechanistic insights into the genetically observed roles of ATM and ATR in controlling DNA replication after DNA damage. Other functional modules impacted by the combined functions of ATM and ATR are the Fanconi anemia pathway and the nucleotide excision repair pathway, and combined pathways required for DNA repair by homologous recombination (Matsuoka et al. 2007).

The functional diversity of the ATM/ATR phosphoproteome is striking. Many of the recently identified ATM/ATR substrates implicate pathways with no prior relationship to DNA damage signaling. For example, multiple proteins in the PTEN/AKT pathway, which responds to growth stimuli such as insulin, were found to be robust ATM/ATR substrates, including AKT, its adaptors, regulators, and downstream effectors (Matsuoka et al. 2007). The elucidation of these pathways, and the ways they are functionally integrated will provide experimental challenges for years to come.

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