DNA damage is a key factor both in the evolution and treatment of cancer. Genomic instability is a common feature of cancer cells, fuelling accumulation of oncogenic mutations, while radiation and diverse genotoxic agents remain important, if imperfect, therapeutic modalities. Cellular responses to DNA damage are coordinated primarily by two distinct kinase signaling cascades, the ATM–Chk2 and ATR–Chk1 pathways, which are activated by DNA double-strand breaks (DSBs) and single-stranded DNA respectively. Historically, these pathways were thought to act in parallel with overlapping functions; however, more recently it has become apparent that their relationship is more complex. In response to DSBs, ATM is required both for ATR–Chk1 activation and to initiate DNA repair via homologous recombination (HRR) by promoting formation of single-stranded DNA at sites of damage through nucleolytic resection. Interestingly, cells and organisms survive with mutations in ATM or other components required for HRR, such as BRCA1 and BRCA2, but at the cost of genomic instability and cancer predisposition. By contrast, the ATR–Chk1 pathway is the principal direct effector of the DNA damage and replication checkpoints and, as such, is essential for the survival of many, although not all, cell types. Remarkably, deficiency for HRR in BRCA1- and BRCA2-deficient tumors confers sensitivity to cisplatin and inhibitors of poly(ADP-ribose) polymerase (PARP), an enzyme required for repair of endogenous DNA damage. In addition, suppressing DNA damage and replication checkpoint responses by inhibiting Chk1 can enhance tumor cell killing by diverse genotoxic agents. Here, we review current understanding of the organization and functions of the ATM–Chk2 and ATR–Chk1 pathways and the prospects for targeting DNA damage signaling processes for therapeutic purposes. © 2010 Elsevier Inc.
I. INTRODUCTION

Cells in multicellular organisms are continuously exposed to DNA damage arising from a variety of endogenous and exogenous sources. These include reactive oxygen species, ultraviolet light, background radiation, and environmental mutagens. To protect their genomes from this assault, cells have evolved complex mechanisms, collectively referred to as DNA damage responses, that act to rectify damage and minimize the probability of lethal or permanent genetic damage. The cellular response to DNA damage encompasses multiple repair mechanisms and checkpoint responses that can delay cell cycle progression or modulate DNA replication. Collectively, these processes are essential to maintain genome stability.

DNA damage responses are orchestrated by multiple signal transduction processes, key among which are the ATM–Chk2 and ATR–Chk1 pathways. Activation of these pathways is crucial for the proper coordination of checkpoint and DNA repair processes; however, they can also modulate other biological outcomes such as apoptosis or cell senescence. In recent years, it has become evident that DNA damage responses are central both for the evolution and therapy of cancer. Inherited defects in DNA damage responses predispose to cancer by enhancing the accumulation of oncogenic mutations, while genome instability is also common in sporadic cancers. More recently, it has become apparent that oncogenic mutations elicit spontaneous DNA damage that can suppress the evolution of incipient cancer cells. Escape from this tumor suppressive barrier may be a major factor in selecting for additional genetic changes during tumor progression such as mutation of the p53 tumor suppressor, the most frequent alteration in human cancer.

Conversely, radiation and genotoxic chemotherapies remain a mainstay of conventional cancer treatment and are likely to remain so for the foreseeable future. Such therapies are, however, imperfect and can incur severe side effects. As a result, much current interest is focused on understanding how normal and tumor cells respond to DNA damage and determining whether DNA damage responses could be exploited or manipulated for therapeutic purposes. Two concepts in particular have attracted attention in recent years. First, inherent defects in genome stability mechanisms, such as homologous recombination, can confer tumor sensitivity to specific genotoxic agents or inhibition of complementary repair pathways. Second, evidence suggests that pharmacological suppression of DNA damage or checkpoint responses can enhance the efficacy of conventional genotoxic agents. Although promising, a full understanding of the biology and functions of the DNA damage signaling pathways will be crucial for the future success of such approaches.
II. ACTIVATION OF THE ATM–CHK2 AND ATR–CHK1 DNA PATHWAYS

DNA damage responses are controlled by biochemical pathways whose principal components and general organization have been conserved from yeasts to humans (Rhind and Russell, 2000). In vertebrates, the two main signaling pathways activated by DNA damage consist of the ATM–Chk2 and ATR–Chk1 protein kinases (Sancar et al., 2004). ATM and ATR are large kinases with sequence similarity to lipid kinases of the phosphatidylinositol-3-kinase (PI3K) family, but which phosphorylate only protein substrates (Abraham, 2001). Key among these substrates are the serine–threonine checkpoint effector kinases, Chk1 and Chk2, which are selectively phosphorylated and activated by ATR and ATM respectively to trigger a wide range of distinct downstream responses (Bartek and Lukas, 2003).

The ATM–Chk2 and ATR–Chk1 pathways respond to different aberrant DNA structures (Fig. 1); ATM is recruited to and activated primarily at DNA double-strand breaks (DSBs) in conjunction with the MRE11:RAD50:NBS1 (MRN) sensor complex (Lee and Paull, 2005; Suzuki et al., 1999), whereas ATR is activated via recruitment to tracts of single-stranded DNA (ssDNA) in association with its partner protein, ATRIP (Dart et al., 2004; Lupardus et al., 2002; Zou and Elledge, 2003).

The basic mechanisms involved in ATM–Chk2 and ATR–Chk1 pathway activation have been elucidated in considerable detail. ATM and Chk2 are activated potently by radiation and genotoxins that induce DSBs, but only weakly, if at all, by agents that block DNA replication without inducing damage (Matsuoka et al., 2000). In undamaged cells, ATM is thought to exist as inactive homodimers. In response to DSBs, inactive ATM homodimers are rapidly induced to autophosphorylate in trans, resulting in dissociation to form partially active monomers (Bakkenist and Kastan, 2003). The exact nature of the primary activating signal that triggers ATM autophosphorylation remains unknown; however, it does not appear to be limited to the immediate vicinity of the damage and may be linked to long-range alterations in chromatin structure (Bakkenist and Kastan, 2003). Serine (S) S1981 was the first autophosphorylation site to be identified; however, this residue is not essential for ATM function, at least in mice (Pellegrini et al., 2006), although its modification is tightly linked to ATM activation under most circumstances (Bakkenist and Kastan, 2003). Subsequent studies have documented additional ATM autophosphorylation sites at S367 and S1893 that may contribute to the activation process, perhaps explaining why S1981 is individually nonessential, while acetylation mediated by the TIP60 acetyl-transferase may also play a role (Lavin and Kozlov, 2007).
ATM monomers are then recruited to DSBs via interactions with the MRN sensor complex (Lee and Paull, 2007), stimulating full activation and providing a platform that enables ATM to act locally on multiple substrates at the site of damage. Local substrates include the variant histone, H2AX, forming the DNA damage-associated γ-H2AX histone mark (Fernandez-Capetillo et al., 2004), the MRN complex itself (discussed in more detail below), the cohesin SMC1 (Kitagawa et al., 2004), and the downstream effector kinase Chk2 (Lukas et al., 2003). ATM phosphorylates Chk2 on a
specific threonine (T) residue, T68, located within an N-terminal serine/threonine-glutamine (SQ/TQ)-rich motif (Ahn et al., 2000). Once phosphorylated, the SQ/TQ motif of one Chk2 molecule is recognized by the phosphopeptide-binding Fork-head associated (FHA) domain of another, leading to transient homodimerization, intermolecular activation loop autophosphorylation, and full activation (Ahn et al., 2002; Cai et al., 2009; Oliver et al., 2006).

Once activated, Chk2 is thought to dissociate from sites of damage and disperse as a monomer throughout the nucleus to act on multiple substrates involved in cell cycle progression, apoptosis, and gene transcription (Lukas et al., 2003). Known substrates of Chk2 include the p53 tumor suppressor protein (Chehab et al., 2000; Shieh et al., 2000) and its regulator MDMX (Chen et al., 2005), Cdc25 family phosphatases (Blasina et al., 1999; Chaturvedi et al., 1999; Matsuoka et al., 1998), the BRCA1 tumor suppressor (Lee et al., 2000), and transcription factors such as FOXM1 (Tan et al., 2007) and E2F1 (Stevens et al., 2003).

It is important to note that ATM also targets other substrates at sites of damage in addition to those mentioned above, including NBS1, BRCA1, MDC1, and p53BP1 among others (Lavin, 2008). In addition, ATM acts on other substrates which do not necessarily concentrate at sites of damage. For example, ATM plays an important role in activating the p53 response to DNA damage both by phosphorylating p53 itself and its stability regulators, MDM2 and MDMX (Chen et al., 2005; Lavin and Kozlov, 2007); however, this is considered to take place in the nucleoplasm rather than specifically at DSBs (Lavin, 2008). In addition, there is increasing evidence that ATM may also have substrates and functions in the cytoplasm (Lavin, 2008).

By contrast, ATR–Chk1 signaling is activated most strongly when DNA replication is impeded, for example as a result of nucleotide depletion or replication-blocking DNA damage lesions such as those inflicted by ultraviolet (UV) light (Abraham, 2001). When replication is blocked, DNA polymerases become uncoupled from the replicative helicase (Byun et al., 2005), generating tracts of ssDNA that rapidly become coated with the trimeric ssDNA-binding protein complex, Replication Protein A (RPA). ATR is recruited to and activated at such tracts in association with its partner protein, ATRIP, which interacts directly with ssDNA complexed with RPA via the 70kD RPA1 subunit (Zou and Elledge, 2003).

Replication fork stalling generates ssDNA directly; however, this structure can also arise through the action of nucleotide excision repair (NER) or at dysfunctional telomeres. In addition, it is important to note that the ATR–Chk1 pathway is also activated in response to DSBs when ssDNA is generated as a result of nucleolytic strand resection (discussed in more detail below). Conversely, replication of damaged DNA can result in DSBs when leading-strand DNA polymerases encounter single-strand nicks or abasic sites. As a result, the ATM–Chk2 and ATR–Chk1 pathways are frequently
activated simultaneously in cells exposed to diverse genotoxic stresses, including ionizing radiation and most or all cytotoxic chemotherapy agents.

Unlike ATM, there is currently no evidence that ATR activation involves autophosphorylation or indeed any other posttranslational modification (Abraham, 2001). Instead, efficient ATR activation and downstream phosphorylation of Chk1 depends on the actions of two mediator proteins, TopBP1 and Claspin. TopBP1, which is recruited to ssDNA-RPA via the PCNA-like RAD9: RAD1: HUS1 checkpoint clamp (Delacroix et al., 2007), contains a domain that stimulates ATR activity, although exactly how this occurs is unclear (Kumagai et al., 2006; Mordes et al., 2008). A second mediator, Claspin, which likely associates with active replication forks during normal replication (Lee et al., 2003), is then subject to ATR-dependent phosphorylation within a short, repeated motif which, once modified, binds Chk1 and serves as a platform for ATR-mediated phosphorylation and activation (Guo et al., 2000). Phosphorylation within the Claspin Chk1-binding motifs depends on ATR kinase activity (Kumagai and Dunphy, 2003); however, the modified residues do not occur within consensus SQ/TQ ATR target sites. So far the kinase directly responsible for this final crucial step in Chk1 activation has not been unambiguously identified; proposed candidates include ATR, Chk1 itself, and Cdc7 (Bennett et al., 2008; Chini and Chen, 2006; Kim et al., 2008).

Interestingly, recent studies have also revealed a requirement for two additional mediators, Timeless and Tipin (Timeless-interacting protein), both for normal replication and for ATR–Chk1 activation in response to replication stress (Kondratov and Antoch, 2007). Timeless binds to both ATR and Chk1 whereas Tipin can interact with Claspin (Kemp et al., 2010). Recent data indicate that like ATRIP, Tipin binds to a specific subunit of the RPA complex (although RPA2 rather than RPA1) and is required for stable association of both Timeless and Claspin with tracts of ssDNA-RPA (Kemp et al., 2010). In addition to checkpoint activation, Timeless and Tipin also seem to be required for replication fork stabilization and restart (Errico et al., 2007). Interestingly, the Drosophila homologue of Timeless is a circadian rhythm regulator, although whether this function is also conserved in mammals is less clear (Kondratov and Antoch, 2007).

Phosphorylated Claspin then recruits Chk1 (Jeong et al., 2003) to ssDNA-RPA complexes, bringing it into close proximity with active ATR (Kumagai and Dunphy, 2003) and enabling ATR to phosphorylate Chk1 directly at multiple S/T-Q sites within the C-terminal regulatory domain, most notably at serines (S) S317 and S345, which are widely monitored as surrogate markers of activation. Phosphorylation of these sites, and in particular serine (S) S345, is essential for Chk1 biological activity, although exactly how these modifications regulate Chk1 catalytic remains poorly understood (Niida et al., 2007; Walker et al., 2009). ATR-mediated phosphorylation is reported to stimulate Chk1 kinase activity by relieving inhibition by the
C-terminal regulatory domain (Oe et al., 2001; Walker et al., 2009); however, it may also promote release of Chk1 from chromatin (Smits et al., 2006). Chk1 also undergoes autophosphorylation during activation (Kumagai et al., 2004); however, this does not occur within the activation loop (Chen et al., 2000), and the exact target sites and functional consequences of this modification have not yet been clearly established.

Once activated, Chk1 is thought to dissociate from Claspin to act on both nuclear and cytoplasmic substrates (Lukas et al., 2003). Important Chk1 substrates involved in cell cycle control include positive and negative regulators of Cdk inhibitory phosphorylation, such as Cdc25A (Falck et al., 2002), Cdc25C (Blasina et al., 1999), and Wee1 (Lee et al., 2001). Chk1-mediated phosphorylation inhibits the activity of both Cdc25A and Cdc25C under conditions of genotoxic stress, although by different mechanisms; phosphorylation of Cdc25A targets the protein for degradation (Falck et al., 2002), while phosphorylated Cdc25C is sequestered in an inactive form through association with 14-3-3 proteins (Peng et al., 1997). Wee1 kinase activity, by contrast, is stimulated by Chk1-mediated phosphorylation (Lee et al., 2001). Chk1 is also thought to modulate recombination by phosphorylating Rad51 (Sorensen et al., 2005) and BRCA2 (Bahassi et al., 2008), and to mediate DNA damage-induced repression of gene transcription through phosphorylation of histone H3 (Shimada et al., 2008). Although predominantly nuclear, a proportion of active Chk1 also localizes at the centrosome, where it is thought to control the timing of activation of the mitotic Cdk1/cyclin B complex, and thus the onset of mitosis, both after damage and during unperturbed cell cycles (Kramer et al., 2004).

As with ATM, ATR is also thought to act on many other substrates in addition to Chk1, including BRCA1, mini-chromosome maintenance (MCM) proteins, and components of the RPA complex (Cimprich and Cortez, 2008). In addition, global proteomic analyses suggest that ATM and ATR probably phosphorylate many other substrates; however, in most cases, the functional significance of these modifications has not yet been established (Matsuoka et al., 2007). In contrast to ATM and Chk2, however, ATR and Chk1 are thought to be active at low levels even during unperturbed cell cycles, particularly during S-phase (Syljuasen et al., 2005), potentially explaining why they are essential in many cell types.

### III. CHECKPOINT FUNCTIONS OF THE ATM–CHK2 AND ATR–CHK1 PATHWAYS

DNA damage or DNA synthesis inhibition in vertebrate cells evokes the activation of multiple, mechanistically distinct checkpoint responses that facilitate repair and promote cell survival (Kastan and Bartek, 2004).
As shown in Fig. 2, DNA damage induces cell cycle delays at the G1/S and G2/M transitions (the G1 and G2 checkpoints), and a transient decrease in the rate of DNA synthesis (the intra-S checkpoint). Of these, the G1 checkpoint is unique in depending primarily on the function of the p53 tumor suppressor protein and its downstream target, the cyclin-dependent kinase inhibitor p21CIP1 (Kastan and Bartek, 2004). G2 arrest, by contrast, is imposed by blocking activation of the mitotic Cdk1-cyclinB complex by preventing removal of the inhibitory threonine 14/tyrosine 15 (T14/ Y15) phosphorylation of Cdk1 (O’Connell et al., 2000). This is achieved, at least in large part, via inhibition of Cdc25 family phosphatases which play an important role in reversing this inhibitory phosphorylation to rapidly activate the Cdk1-cyclin B complex and trigger the onset of mitosis (Boutros et al., 2007). The molecular mechanism of the intra-S checkpoint is less well defined; however, it can involve both active replication fork slowing and suppression of replication origin firing (Grallert and Boye, 2008; Seiler et al., 2007).

When DNA synthesis is blocked, additional replication checkpoint responses are required to stabilize stalled replication forks and prevent the formation of new forks by suppressing late replication origin firing (Branzei...
and Foiani, 2009). Because these functions are essential if cells are to resume replication and complete S-phase when circumstances permit, they are sometimes collectively termed the “replication recovery checkpoint.” In addition, it is essential that cells arrested in S-phase do not attempt mitosis until replication is complete. The mitotic delay triggered by DNA synthesis inhibition, which is also likely mediated through maintenance of inhibitory phosphorylation of Cdk1 (O’Connell et al., 2000), is generally termed the S-M checkpoint to distinguish it from the G2 arrest induced by DNA damage.

Checkpoint mechanisms were first dissected in detail in budding and fission yeast. Each possesses an ortholog of Chk2; Rad53 in budding yeast and Cds1 in fission yeast, while both also express Chk1 homologs (O’Connell et al., 2000). The checkpoint functions of the yeast effector kinases are, however, remarkably variable; in budding yeast Rad53 is the dominant effector of both DNA damage and replication checkpoints, whereas in fission yeast DNA damage responses are assigned to Chk1 while Cds1 regulates replication checkpoint functions (O’Connell et al., 2000). When Chk1 and Chk2 were identified in vertebrates, the obvious question was “what would be the ‘division of labor’ compared to these model organisms?”

Initially it was widely considered that the ATM–Chk2 and ATR–Chk1 pathways acted in parallel, with Chk1 and Chk2 playing overlapping or partially redundant roles in downstream checkpoint responses (as depicted in Fig. 1). Although some early studies suggested that Chk1 and Chk2 shared certain common substrates involved in cell cycle arrest, such as Cdc25 family phosphatases (Chaturvedi et al., 1999; Matsuoka et al., 1998), subsequent genetic and biochemical data have increasingly emphasized ATR–Chk1 as the principal, direct effector of the DNA damage and replication checkpoints, with ATM–Chk2 playing an auxiliary role specifically in the response to DSBs (Bartek and Lukas, 2003; Kastan and Bartek, 2004).

Fundamental differences in the normal physiological functions of the ATM–Chk2 and ATR–Chk1 pathways were initially evident from the phenotypes of mice and cells deficient for each pathway. Thus, germ-line inactivation of ATR or Chk1 results in early embryonic lethality, whereas ATM- and Chk2-knockout mice are viable, and at least in the case of Chk2, remarkably normal (Brown and Baltimore, 2000; Liu et al., 2000; Takai et al., 2000). Similarly, ATM- and Chk2-deficient somatic cells can proliferate successfully in culture (Jallepalli et al., 2003; Lavin and Shiloh, 1997; Xu and Baltimore, 1996), whereas acute genetic inactivation of ATR or Chk1 leads to rapid cell death (Brown and Baltimore, 2003; Liu et al., 2000; Niida et al., 2007). Interestingly, DT40 lymphoma cells represent an exception to this general rule, since they survive genetic inactivation of Chk1 function, albeit with impaired cell growth and survival (Zachos et al., 2003).
ATM-deficient human and mouse cells classically exhibit impaired G1, intra-S, and G2 checkpoint proficiency after DNA damage (Lavin, 2008). As mentioned previously, ATM is an important determinant of p53 stabilization and activation, and evidence suggests that the weakened G1 arrest in ATM mutant cells is attributable to inefficient p53 activation and downstream p21CIP1 induction (Kastan et al., 1992). The mechanism of the intra-S checkpoint is more complex. ATM is thought to suppress DNA replication via two distinct mechanisms; firstly, via direct phosphorylation of NBS1, and secondly through Chk2-mediated degradation of Cdc25A leading to inhibition of Cdk2, which in association with cyclins E or A is required for DNA replication origin firing (Falck et al., 2002).

Interestingly, G2 checkpoint impairment in ATM mutant cells after exposure to ionizing radiation is markedly cell cycle phase-dependent. Thus, ATM-deficient cells in G2 phase at the time of damage are unable to arrest efficiently, whereas cells damaged in G1 and S-phase experience instead a prolonged arrest on reaching G2 compared to genetically normal counterparts (Xu et al., 2002). This has been explained by the existence of two molecularly distinct G2/M checkpoint arrests; “immediate G2 arrest,” which is triggered rapidly in G2 cells, and “G2 accumulation,” which affects cells that reach G2 after traversing S-phase and develops over many hours (Xu et al., 2002). Immediate G2 arrest is ATM-dependent and of relatively short duration, whereas G2 accumulation is ATM-independent (but ATR–Chk1-dependent) and much longer-lasting (Xu et al., 2002). Why this should be is not fully understood; it may reflect a more stringent requirement for ATM-dependent DSB processing for rapid and efficient checkpoint activation in G2 than in S-phase (discussed in more detail below), whereas the prolonged G2 accumulation experienced by ATM mutant cells could reflect a defect in DNA repair (Beucher et al., 2009). As for mechanism, Chk2 was initially invoked as a downstream effector of ATM-dependent G2 arrest via phosphorylation and inhibition of the Cdk1-activating Cdc25C phosphatase (Chehab et al., 2000; Matsuoka et al., 1998); however, as discussed below, Chk2-deficient cells do not exhibit consistent defects in G2 checkpoint proficiency.

As with ATM, Chk2 has been implicated in p53 activation (Chehab et al., 2000), and consistent with this, Chk2-deficient mice show impaired G1 checkpoint arrest and defects in p53-dependent gene transcription after damage (Hirao et al., 2002; Takai et al., 2002). They also show a marked reduction in p53-dependent apoptotic responses in adult and developing tissues and increased organismal survival after whole body irradiation (Hirao et al., 2002; Takai et al., 2002). Perplexingly, however, p53 regulation is not altered in Chk2-deleted HCT116 cells, a human cancer cell line that retains wild-type p53 (Jallepalli et al., 2003). Also puzzling is the fact that G2 checkpoint impairment as a result of Chk2 inactivation is observed
in some, but not all, experimental systems. Thus, MEFs and HCT116 cells deleted for Chk2 retained normal G2 checkpoint proficiency (Jallepalli et al., 2003; Takai et al., 2002), whereas Chk2 knockout DT40 cells exhibited a weakened and delayed G2 arrest at early times after irradiation (Rainey et al., 2008). Interestingly, this defect was most severe in G2 cells, much as described for ATM (Xu et al., 2002), whereas the slower G2 accumulation response was relatively unaffected (Rainey et al., 2008). Variations in the severity of G2 checkpoint deficiency in cells genetically deficient for Chk2 have often been interpreted in terms of compensation by Chk1; however, it is equally possible that the role of Chk2 in this checkpoint simply varies between cell types.

Phenotypic analysis of checkpoint proficiency in ATR- or Chk1-deficient mouse embryos and cells is complicated by loss of viability; however, inactivation of both genes results in profound G2 and S-M checkpoint defects after DNA damage or replication arrest (Brown and Baltimore, 2000; Brown and Baltimore, 2003; Liu et al., 2000; Takai et al., 2000). Consistent with this, Chk1-deficient DT40 cells (which lack functional p53) show complete loss of proficiency for all DNA damage and replication checkpoint responses (Zachos et al., 2003, 2005). Strikingly, these cells show no measurable G2 arrest or intra-S checkpoint activation after irradiation at any dose tested (Zachos et al., 2003), in marked contrast to the partial loss of G2 checkpoint proficiency that results from deletion of Chk2 (Rainey et al., 2008). Loss of G2 checkpoint proficiency was furthermore associated with failure to maintain inhibitory T14/Y15 Cdk1 phosphorylation (Zachos et al., 2003), indicating that Chk1 is essential to restrain the mitosis-promoting activity of Cdc25 family phosphatases after DNA damage. In addition, when DNA polymerase is inhibited Chk1-deficient cells suffer a combination of progressive replication fork collapse and futile origin firing that leads ultimately to S-M checkpoint failure and premature entry to mitosis with unreplicated DNA (Zachos et al., 2005).

Interestingly, Chk1-deficient DT40 cells also exhibit high levels of spontaneous replication fork collapse during unperturbed cell cycles which is compensated by increased replication origin firing (Maya-Mendoza et al., 2007; Petermann et al., 2006). Although this compensation mechanism evidently allows these cells to replicate successfully and to maintain an S-phase of approximately normal length, replication fork collapse as a result of Chk1 inhibition is a potential cause of cell death in other cell types (Syljuasen et al., 2005).

The effects of inhibiting ATR or Chk1 on DNA damage and replication checkpoint proficiency have also been widely explored in cells in culture using various dominant-negative, siRNA depletion, or chemical inhibition approaches. The general consensus that has emerged from such studies [reviewed in (Bartek and Lukas, 2003; Kastan and Bartek, 2004;
Stracker et al., 2009]) is broadly consistent with the genetic analysis in mice and DT40 cells summarized above; namely that ATR and Chk1 are crucial both for the G2 and intra-S checkpoint responses induced by DNA damage and for the S-M and fork stabilization/origin suppression checkpoints triggered by replication arrest. Whether the ATR–Chk1 pathway also contributes to p53-dependent G1 arrest under any circumstances is less clear. Although some early biochemical data implicated ATR and Chk1 as potential regulators of p53 (Shieh et al., 2000; Tibbetts et al., 1999), more recent evidence that ATR–Chk1 activation by DNA damage is largely restricted to the S and G2 phases of the cell cycle makes it seem unlikely to be a major physiological determinant of G1 arrest (Jazayeri et al., 2006; Walker et al., 2009).

IV. THE THREE RS OF DAMAGE SIGNALING: RESECTION, RECOMBINATION, AND REPAIR

In eukaryotes DNA DSBs are repaired via two main mechanisms; nonhomologous end-joining (NHEJ) and homologous recombination repair (HRR). NHEJ occurs throughout the cell cycle; however, because HRR requires a sister chromatid to serve as a template, this mechanism is restricted to the S and G2 phases. Unlike NHEJ, HRR requires extensive DNA damage processing to generate tracts of ssDNA that, once coated with Rad51 recombinase, invade the homologous DNA duplex to initiate repair. Such single-stranded tracts are generated by resection of DSBs in a 3’→5’ direction, a reaction initiated by the endonuclease activity of the MRN complex (Mimitou and Symington, 2009).

Because of its central role in initiating HRR, DNA strand resection at DSBs is regulated during the cell cycle and this regulation is thought to play a major role in restricting HRR to S and G2. In yeasts, Cdk activity controls DNA strand resection via phosphorylation of Sae2 (Wohlbold and Fisher, 2009), a putative nuclease that promotes resection in collaboration with the yeast counterpart of MRN (Huertas et al., 2008). Vertebrate cells express an ortholog of Sae2 in the form of CtIP, which is also required for DSB resection (Sartori et al., 2007). Resection is also cell cycle-regulated in vertebrate cells, and evidence suggests that Cdk5s regulate this process at least in part via direct phosphorylation of CtIP in a manner analogous to yeast (Huertas and Jackson, 2009; Yun and Hiom, 2009). Thus, because Cdk-mediated phosphorylation of CtIP is required for strand resection, increased Cdk activity in S and G2 ensures maximum resection activity in these phases of the cell cycle.
Two other important components required for HRR are the BRCA1 and BRCA2 tumor suppressors. BRCA1 is a multifunctional protein that plays multiple roles in checkpoint activation, DNA repair, and gene transcription in response to DNA damage (Huen et al., 2010). BRCA1 is recruited to sites of ionizing radiation-induced DNA damage and is required for efficient generation of ssDNA at early times after irradiation (Schlegel et al., 2006). Although the mechanism is not fully understood, BRCA1 interacts with MRN and CtIP, suggesting that it too may play a role in strand resection (Chen et al., 2008). BRCA2 by contrast is required for homologous recombination downstream of strand resection through its well-established role in loading the Rad51 recombinase protein (Thorslund and West, 2007).

As shown in Fig. 3, the recent discovery that ATM is required for strand resection and downstream activation of ATR–Chk1 in response to DSBs provides a new framework for understanding the organization and integration of checkpoint and repair pathways (Adams et al., 2006; Cuadrado et al., 2006; Jazayeri et al., 2006; Myers and Cortez, 2006). Although not classically considered a core homologous recombination (HR) factor, ATM is required for efficient HRR of a subset of DSBs specifically in G2 phase (Beucher et al., 2009). Molecular details are still emerging; however, current thinking is that initial recognition of DSBs by the MRN complex leads to recruitment and full activation of ATM. Active ATM then promotes the recruitment of CtIP to sites of damage where it interacts with and stimulates the nuclease activity of MRE11 to initiate strand resection and generate short tracts of ssDNA (You et al., 2009). These may be extended through the actions of other nucleases and helicases, such as Exo1 and BLM, to generate more extensive regions of ssDNA that recruit RPA and form both the initiating substrate for HRR and activating platform for ATR–Chk1 activation (Mimitou and Symington, 2009). Exactly how ATM stimulates resection is not yet known; however, NBS1 and CtIP are both subject to ATM-dependent phosphorylation after damage, and at least in the case of CTIP, this modification appears to be required both for recruitment to DSBs and resection (You et al., 2009).

The ramifications of this model are extensive. Firstly, it explains why ATM is required for rapid activation of ATR–Chk1 in response to DSBs but not DNA polymerase inhibition, since the latter generates extensive tracts of ssDNA directly without the need for DNA damage processing (Byun et al., 2005; Myers and Cortez, 2006). Secondly, it accounts for why ATR–Chk1 activation in response to irradiation-induced DSBs is largely confined to S and G2 phase (Jazayeri et al., 2006; Walker et al., 2009), since this is when levels of Cdk activity become permissive for efficient strand resection (Cerqueira et al., 2009). Thirdly, it explains why other proteins required for ssDNA generation, such as MRE11, NBS1, and BRCA1, are also required both for rapid ATR–Chk1 activation and G2 checkpoint proficiency in response to DSBs (Myers and Cortez, 2006; Yarden et al., 2002).
Fig. 3  ATM is required for DNA strand resection and ATR–Chk1 activation in response to DSBs. In response to DSBs ATM, in conjunction with the MRN complex, CtIP and BRCA1, is required for nucleolytic strand resection to generate tracts of ssDNA. Evidence suggests that ATM stimulates the nuclease activity of MRE11 to initiate resection but that this process may then be extended through the actions of other nucleases and helicases such as Exo1 and BLM. Once complexed with RPA, such tracts form the platform both for ATR-ATRIP recruitment leading to Chk1 activation and also the initiating structure for HRR. BRCA2 promotes loading of Rad51 which displaces RPA leading to strand invasion and subsequent recombination. In contrast, inhibition of DNA synthesis generates tracts of ssDNA-RPA directly without the need for stand resection by stalling replication forks and uncoupling the replicative polymerase and helicase. The various posttranslational modifications involved in ATM–Chk2 and ATR–Chk1 activation shown in Fig. 1 are omitted here for clarity. In contrast to the more conventional scheme depicted in Fig. 1, in this model ATR and Chk1 are the direct effectors of multiple DNA damage and replication checkpoints with ATM acting upstream specifically in the context of DSBs. ATM and Chk2, however, continue to signal DNA damage independently to p53 in a parallel pathway. Please refer to the text for further details and explanation.
Placing ATM upstream of ATR–Chk1 activation in response to DSBs suggests a new interpretation of their overlapping, yet distinct, functions in checkpoint signaling (as depicted in Fig. 3). In this model the ATR–Chk1 pathway is the principal direct effector of the damage and replication checkpoints (apart from p53-dependent G1 arrest), with ATM modulating the response specifically to DSBs indirectly via its role in strand resection. Thus, in ATM mutant cells, rapid activation of ATR and Chk1 in response to DSBs will be impaired as a consequence of inefficient strand resection. This defect is likely to be particularly significant in G2 phase, when resection is the principal mechanism of ssDNA generation. This model predicts therefore that the impact of ATM deficiency on ATR–Chk1 activation and thus G2 checkpoint proficiency in response to DSBs will be greatest in G2 phase, consistent with the immediate G2 arrest defect described in ATM mutant cells (Xu et al., 2002). This model also explains why Chk1-deficient DT40 cells lack any detectable G2 checkpoint after irradiation, despite the continued presence of functional ATM and Chk2 (Zachos et al., 2003), since in this scheme Chk1 is the sole direct downstream effector of G2 arrest. Conversely, because DNA polymerase inhibition generates ssDNA directly without the need for resection, ATR and Chk1 are essential for replication checkpoint responses whereas ATM and Chk2 are not.

Where to place Chk2 in this scheme becomes an interesting question. ATM and Chk2 are activated in response to DSBs at all stages of the cell cycle, including G1, consistent with their established roles in activating p53 and triggering G1 arrest (Lavin and Kozlov, 2007; Takai et al., 2002). Chk2 is, however, variably required for G2 checkpoint proficiency in different cell types, and whether it is a direct effector of this checkpoint has been questioned (Antoni et al., 2007). One intriguing possibility, suggested by the apparent epistatic relationship between Chk1 and Chk2 in DT40 cells (i.e., where G2 checkpoint proficiency is completely abolished in the absence of Chk1 but only impaired in the absence of Chk2), is that Chk2 might also participate in the DNA strand resection process and thus in regulating the efficiency and cell cycle phase-specificity of ATR–Chk1 activation indirectly. Although there is currently no direct evidence for such a role, further investigation seems warranted.

V. ATM–CHK2 AND ATR–CHK1 PATHWAY ALTERATIONS IN CANCER

The importance of genome stability for preventing carcinogenesis is evident both from human cancer predisposition syndromes that result from inherited loss-of-function mutations in DNA damage response genes and
from the occurrence of sporadic mutations affecting such genes in cancers in otherwise genetically normal individuals (Kastan and Bartek, 2004). Examples of both have been found to affect ATM and Chk2 in human cancer, whereas ATR and Chk1 appear to be mutated only rarely. Important insights into the roles of these pathways in tumor formation have also been obtained from experiments using genetically modified mice. In discussing these issues, we also refer in passing to other gene functions, such as the MRN complex and BRCA1/BRCA2, where these are closely linked either to the regulation or downstream functions of the ATM-Chk2 and ATR-Chk1 pathways.

Homozgyous germ-line loss-of-function mutations affecting ATM cause the pleiotropic human disease syndrome Ataxia telangiectasia (AT), characterized by immunodeficiency, neurodegeneration, radiation hypersensitivity, and spontaneous predisposition to lymphoma (Shiloh and Kastan, 2001). Similarly, hypomorphic mutations affecting the genes encoding the functionally related MRE11 and NBS1 proteins give rise to the human conditions Ataxia-like disorder (ATLD), and Nijmegen breakage syndrome (NBS), each of which shares some clinical similarities with AT, although only NBS is clearly associated with cancer predisposition (Stewart et al., 1999; Varon et al., 1998). As with AT humans, ATM knockout mice are predisposed to lymphoma and radiosensitive (Xu et al., 1996), while mice with engineered hypomorphic mutations of NBS1 or RAD50 are also cancer prone (Bender et al., 2002; Kang et al., 2002; Williams et al., 2002). The precise cause of cancer predisposition in humans and mice with inherited defects in ATM or MRN components is not yet known; however, genomic instability and an increased mutation rate resulting from repair and checkpoint defects is presumably an important factor.

Interestingly, although AT is considered to be an autosomal recessive genetic disorder, individuals heterozygous for ATM mutations show an increased incidence of cancer possibly related to medical or occupational radiation exposure (Briani et al., 2006; Swift et al., 1991). In addition, cells from heterozygote individuals show sensitivity to radiation in vitro that is intermediate between those from AT patients and normal individuals (Swift et al., 1991). Taken together, these findings indicate that ATM is a partially penetrant cancer susceptibility gene that might interact with certain environmental predisposing factors. Somatic mutations affecting ATM have also been documented in sporadic lymphoid malignancies and lung adenocarcinomas, although at relatively low incidence (Ding et al., 2008; Gumy-Pause et al., 2004).

In contrast to AT, ATLD, and NBS, human individuals heterozygous for loss-of-function alleles of the BRCA1 and BRCA2 tumor suppressor genes are developmentally normal but suffer from a greatly increased incidence of breast and ovarian cancer (O’Donovan and Livingston, 2010). As mentioned previously, BRCA1 is required for ATR-Chk1 activation, G2 arrest,
and other complex responses to DNA damage (Huen et al., 2010). In general, however, the tumor suppressive functions of BRCA1 and BRCA2 are attributed to their distinct but essential roles in HR-mediated DNA repair (O’Donovan and Livingston, 2010). Because tumorigenesis involves functional inactivation of the remaining functional BRCA1 or BRCA2 allele through loss of heterozygosity or other means (Collins et al., 1995; Neuhausen and Marshall, 1994), the tumors that arise in susceptible individuals consist of cells that are deficient for HRR whereas those in normal tissues remain proficient (Turner et al., 2005). Genomic instability as a result of HRR deficiency is thought to play a key role in the development of such tumors, presumably by accelerating the accumulation of oncogenic mutations (Tutt et al., 2002); however, as discussed below, the presence or absence of DNA repair proficiency in normal and tumor tissue respectively in such individuals also provides an exploitable therapeutic index.

Li Fraumeni syndrome is a multiorgan cancer predisposition condition that is generally, but not exclusively, due to inherited mutations in p53 (Birch, 1994). Heterozygous germ-line mutations in Chk2 were initially reported in a subset of Li-Fraumeni kindreds lacking p53 mutations, consistent with a functional link between Chk2 and p53 (Bell et al., 1999). However, because these mutant alleles were subsequently also found in normal individuals in the general population they are now considered unlikely to be the genetic cause of Li Fraumeni syndrome (Antoni et al., 2007). Nevertheless, studies have established that individuals bearing certain mutant Chk2 alleles do suffer from a statistically significant increase in the incidence of breast, prostate, and other cancers, suggesting that Chk2 is indeed a moderate or low penetrance cancer susceptibility gene in humans (Antoni et al., 2007). Despite this, the tumors that arise in individuals heterozygous for such mutations do not consistently lose the remaining normal Chk2 allele, indicating that Chk2 does not conform to the conventional definition of a tumor suppressor (Antoni et al., 2007). One possibility is that mutant Chk2 proteins exert a dominant-negative effect by inhibiting the endogenous, normal Chk2, to phenocopy loss of function in the heterozygous state. This would be consistent with the occurrence of occasional sporadic Chk2 mutations and rare instances of reduced or absent expression in a variety of different tumor types (Antoni et al., 2007). Alternatively, it is conceivable that Chk2 haploinsufficiency per se synergises with other, as yet unknown, oncogenic events or environmental factors to promote malignant progression.

The impact of Chk2 deficiency on tumorigenesis in mice has also been examined. Although Chk2 knockout mice are developmentally normal and not spontaneously cancer prone (Takai et al., 2002), they are more sensitive to chemical skin carcinogenesis, showing an increase both in overall tumor burden and in the rate at which benign tumors formed after exposure to
chemical carcinogens (Hirao et al., 2002). Whether this increase in tumor sensitivity is attributable to increased genomic instability or perhaps to the relative resistance to stress-induced apoptosis that has been observed in Chk2 knockout mice however remains unclear.

In humans, homozygous hypomorphic mutations affecting ATR give rise to Seckel syndrome. Seckel syndrome is associated with a wide range of deleterious symptoms, including growth retardation and microcephaly; however, such individuals do not suffer from an increased incidence of cancer (Kerzendorfer and O’Driscoll, 2009). Seckel syndrome has also been modeled in mice (Murga et al., 2009), and the consequences of acute conditional genetic inactivation of ATR postdevelopment in adult mice have been investigated (Ruzankina et al., 2007). Degenerative and premature aging-like phenotypes were observed in each case, underscoring the crucial importance of ATR for normal development, stem cell survival, and tissue homeostasis; however, neither showed evidence of cancer predisposition (Murga et al., 2009; Ruzankina et al., 2007). In general therefore it appears that partial or complete inactivation of ATR function, although clearly deleterious in at least some cell types in vivo, does not perturb genome stability in such a way as to promote carcinogenesis. Consistent with this, somatic mutations affecting ATR have not been widely found in cancers (Heikkinen et al., 2005), with the exception of rare sporadic stomach and endometrial tumors with microsatellite instability (MSI) (Menoyo et al., 2001; Vassileva et al., 2002; Zighelboim et al., 2009).

Germ-line mutations in Chk1 have thus far not been implicated in any human disease and, as with ATR, somatic mutations affecting Chk1 appear to be rare in human cancers, although some exceptions have been reported in tumors with MSI (Bertoni et al., 1999; Menoyo et al., 2001). Embryonic lethality in knockout mice precludes direct assessment of the effect of constitutive Chk1 inactivation on spontaneous or induced carcinogenesis; however, some evidence that partial loss of function can promote tumor formation has been reported. Thus, mammary tumors induced by an oncogenic WNT transgene developed more rapidly in Chk1 hemizygous mice than wild-type (Liu et al., 2000). Importantly, however, loss of the remaining functional Chk1 allele was not observed, suggesting that Chk1 continued to be important for the proliferation or survival of the tumor cells (Liu et al., 2000).

More recently, we have examined the effect of experimentally induced hemi- or homozygous conditional deletion of Chk1 in mouse skin on the formation of tumors induced by the chemical carcinogens, DMBA and TPA, using a conditional allele of Chk1 combined with a Keratin14-CreER recombinase transgene (Indra et al., 2000; Lam et al., 2004). This combination allows efficient Chk1 deletion throughout the epidermis in response to systemic treatment with the synthetic estrogen, tamoxifen (LM Tho and DA
Gillespie, unpublished results). We find that homozygous deletion of Chk1 throughout the epidermis is tolerated without acute pathology, presumably because Chk1 is not essential in the postmitotic, terminally differentiated cells that comprise the bulk of the tissue. However, when recombination is induced immediately prior to carcinogen exposure both the number and size of benign papillomas obtained is strongly suppressed (Table 1). Furthermore, the small lesions that form in ablated skin always derive from cells that escape recombination (LM Tho and DA Gillespie, unpublished results). Although the basis of this tumor suppressive effect is not yet fully understood, we hypothesize that Chk1 is probably essential for the proliferation or survival of the epidermal stem cells that are thought to give rise to chemical carcinogen-induced tumors in the skin (Morris, 2004). Consistent with this, developmental or conditional deletion of Chk1 has been shown to lead to rapid cell death in several other tissues including mammary gland, intestine, and lymphocytes (Greenow et al., 2009; Lam et al., 2004; Zaugg et al., 2007).

In marked contrast, Chk1 hemizygous skin supports normal papilloma formation but such hemizygous lesions show an increased probability of progressing to malignant carcinoma (Table 1). We deduce from these experiments that whereas complete loss of Chk1 function is incompatible with skin tumor formation, partial loss of function fosters benign-malignant tumor progression. This conclusion is consistent with the previously described acceleration of WNT-induced tumorigenesis and also evidence that Chk1

<table>
<thead>
<tr>
<th>Cohort</th>
<th>Genotype</th>
<th>Treatment</th>
<th>Mean no. papillomas</th>
<th>% Conversion to carcinoma</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Chk1+/+</td>
<td>K14-CreER</td>
<td>Tamoxifen</td>
<td>17.8 (N = 20)</td>
</tr>
<tr>
<td>2</td>
<td>Chk1Fl/Fl</td>
<td>K14-CreER</td>
<td>Vehicle</td>
<td>15.4 (N = 19)</td>
</tr>
<tr>
<td>3</td>
<td>Chk1Fl/Fl</td>
<td>K14-CreER</td>
<td>Tamoxifen</td>
<td>5.5 (N = 18)</td>
</tr>
<tr>
<td>4</td>
<td>Chk1Fl/+</td>
<td>K14-CreER</td>
<td>Tamoxifen</td>
<td>14.6 (N = 19)</td>
</tr>
</tbody>
</table>

Cohorts of FVB strain mice were bred to express an epithelial-specific K14-CreER transgene (Indra et al., 2000) in combination with either wild-type Chk1 (Chk1+) or a conditional, lox P-modified allele of Chk1 (Chk1Fl) (Lam et al., 2004). At 6 weeks of age mice were treated systemically with Tamoxifen, resulting in efficient Chk1 deletion throughout the epidermis (LM. Tho and D.A. Gillespie, unpublished results), or vehicle control. Mice were then immediately treated with a single dose of the carcinogen, DMBA, followed by twice weekly applications of the tumor promoter TPA for up to 30 weeks (Abel et al., 2009). Total plateau papilloma burden was quantified at that time, or at time of sacrifice if earlier, together with the proportion of papillomas that converted to carcinoma. The reduction in papilloma yield in cohort 3 was highly statistically significant compared to controls (cohorts 1 and 2; Mann Whitney test p < 0.001). Similarly, the rate of conversion to carcinoma was significantly elevated in cohort 4 compared to controls (cohorts 1 and 2; chi-squared test p < 0.025). A more detailed description of these data will be presented elsewhere.
haploinsufficiency can lead to aberrant cell cycle regulation and genomic instability in vivo (Lam et al., 2004; Liu et al., 2000). Conversely, it has been reported that Chk1 hemizygous mice suffer from an increased incidence of anemia associated with increased levels of DNA damage in erythroid progenitors, suggesting that Chk1 haploinsufficiency can also result in degenerative effects in certain cell lineages, perhaps also owing to stem cell death (Boles et al., 2010).

The ability therefore of cells and organisms to tolerate partial or complete loss of function within the ATM–Chk2 and ATR–Chk1 pathways is very different. Impairment or even complete loss of ATM–Chk2 signaling is compatible with cell and organism survival, although frequently at the cost of cancer predisposition, presumably at least in part as a result of genomic instability and more rapid accumulation of oncogenic mutations. In contrast, ATR and Chk1 seem to be essential for the proliferation and survival of many, although not all, cell types, both in vitro and in the developing embryo and adult organism, presumably because they control aspects of DNA metabolism that when dysfunctional lead to cell death rather than survival with mutation. In this scheme Chk1 (and arguably ATR) becomes a logical target for therapeutic strategies based on pharmacological checkpoint suppression (discussed below), although evidence from mouse models that partial loss of Chk1 function (i.e., haploinsufficiency) may promote tumorigenesis, or other undesirable pathologies (Boles et al., 2010), clearly merits careful consideration.

VI. EXPLOITING HOMOLOGOUS RECOMBINATIONAL REPAIR (HRR) DEFECTS FOR CANCER THERAPY

In recent years it has emerged that in addition to predisposing to cancer as a result of increased genomic instability, defective HRR may also render tumor cells inherently vulnerable to specific conventional anticancer agents and also to new strategies based on inhibition of complementary repair pathways. Thus, BRCA1- and BRCA2-deficient tumor cells have been found to be hypersensitive to cross-linking agents such as cisplatin in vitro (Bhattacharyya et al., 2000; Yuan et al., 1999), most probably because HR is the principal mechanism through which replication forks stalled or collapsed by such lesions are repaired or restarted. Importantly, evidence suggests this is also true in vivo and that ovarian cancers arising in BRCA1 and BRCA2 mutation carriers may respond better to platinum-based therapy than similar sporadic tumors (Foulkes, 2006). Remarkably, secondary mutations that restore BRCA1 or BRCA2 function can be a cause both for drug resistance in ovarian cancer cells in culture and treatment failure in
patients, emphasizing the importance of HRR deficiency in determining sensitivity to platinum compounds (Sakai et al., 2008,2009; Swisher et al., 2008).

Poly (ADP-ribose) polymerase 1 (PARP1) is a nuclear enzyme that is activated by DNA single-strand breaks (SSBs) and plays a crucial role in multiple aspects of the DNA damage response (Rouleau et al., 2010). In response to DNA damage active PARP1 binds to SSBs in DNA and catalyzes the synthesis of branched, protein-conjugated poly (ADP-ribose) chains. Many of these chains become linked to PARP1 itself as a result of automodification, although histones, topoisomerases, and many other proteins involved in diverse aspects of DNA metabolism are also substrates for PARP1 (D’Amours et al., 1999). Once formed at sites of damage, such poly (ADP-ribose) chains recruit multiple proteins involved in DNA repair and modulating chromatin structure (Rouleau et al., 2010). In particular, PARP1 both modifies and recruits XRCC1, a key scaffolding factor required for base excision repair (BER). BER excises damaged or mismatched bases from DNA and also repairs single-strand gaps, nicks, and abasic sites, lesions which arise both spontaneously and as a result of oxidative stress or exposure to alkylating agents.

Based on its established role in DNA repair, selective small molecule inhibitors of PARP1 were initially developed with a view to enhancing the potency of DNA damaging chemotherapies (Zaremba and Curtin, 2007). Although PARP1 inhibitors are relatively nontoxic to most cancer cells alone, it was subsequently discovered that tumor cells deficient for BRCA1 or BRCA2 were inherently and exquisitely sensitive to such agents even in the absence of exogenous genotoxic stress (Bryant et al., 2005; Farmer et al., 2005). The basis of this selective sensitization has been explained in terms of synthetic lethality, a term applied to genetic functions or pathways whose individual loss is tolerated but when combined result in lethality (Kaelin, 2005). In this context of course the synthetic lethal interaction occurs between an inherent genetic deficiency for HRR resulting from BRCA1/BRCA2 mutation and a phenocopy of functional BER deficiency that is imposed through pharmacological inhibition of PARP1.

Thus, inhibition of PARP1 leads to accumulation of SSBs and other endogenous DNA damage lesions that would normally be corrected by BER (Jeggo, 1998). Such lesions are particularly problematical in S-phase, since when encountered by replicative polymerases they lead to replication fork collapse and formation of DSBs. HRR is thought to be the main mechanism through which such DSBs can be repaired and replication restarted, enabling HRR proficient cells to tolerate PARP inhibition (Li and Heyer, 2008). However, because BRCA1- and BRCA2-deficient tumor cells are impaired both for HRR and BER under conditions of PARP inhibition, replication-associated DSBs cannot be repaired and thus escalate to lethal proportions (Bryant et al.,
2005; Farmer et al., 2005). As with resistance to platinum compounds, acquired resistance to PARP inhibition can arise through reversion mutations that restore BRCA2 function, at least in cell lines (Edwards et al., 2008).

Importantly, this synthetic lethal principle can be extended to therapy; several recent trials have shown that Olaparib, a potent oral PARP inhibitor, has significant antitumor activity as monotherapy specifically in ovarian cancers arising in BRCA1 and BRCA2 mutation carriers, and remarkably, this activity is achieved without the toxicity associated with conventional genotoxic chemotherapy (Fong et al., 2009, 2010). In addition, much preclinical evidence suggests that PARP inhibition may also synergize with conventional genotoxic chemotherapies, including alkylating agents, platinum compounds, topoisomerase inhibitors, and radiation, both in BRCA1 and BRCA2 mutant and sporadic tumors (Ratnam and Low, 2007). As a result, Olaparib and many other PARP inhibitors are currently undergoing trials both as monotherapies and in combination with conventional agents (Rouleau et al., 2010).

The evident promise of PARP inhibition as a selective therapy against tumors with defects in HRR raises the obvious question of how widespread this phenotype is in sporadic cancers arising in genetically normal individuals. Cell culture studies show that experimental inhibition of many diverse gene functions involved either directly in HRR or in DNA damage signaling more generally (including Rad51, RPA1, NBS1, ATM, ATR, Chk1, Chk2, and certain Fanconi anemia gene products) all result in sensitization to PARP inhibition (McCabe et al., 2006). In addition, human and mouse cells genetically deficient for ATM are also sensitized to PARP inhibition (Loser et al., 2010; Williamson et al., 2010). As already mentioned, inherited mutations in several of these genes result in cancer predisposition; however, there is little evidence that such genes are frequently mutated in sporadic cancers.

The PTEN tumor suppressor, by contrast, is one of the most frequently mutated or inactivated genes in human cancer and leads to upregulation of the PI3-kinase-PKB/ Akt growth and survival signaling pathway (Chalhoub and Baker, 2009). Loss of PTEN has been known for some time to compromise genome stability (Puc et al., 2005); however, recent data have revealed unexpected connections with HR and DNA repair. Remarkably, human HCT116 colon carcinoma cells genetically deleted for PTEN are both deficient for HR and sensitized to PARP inhibitors (Mendes-Pereira et al., 2009). Similar effects have been documented in PTEN-deficient murine astrocytes and human glioblastoma cell line (McEllin et al., 2010). The mechanism through which PTEN affects HR is not yet fully understood; however, two recent studies have shown that Akt, which is upregulated as a result of PTEN loss, can inhibit both strand resection and recruitment of essential HR factors such as BRCA1 and CtIP at radiation-induced DSBs (Tonic et al.,
The prospect that frequently activated oncogenic signaling pathways conventionally linked to cell proliferation and survival may interact with DNA damage response and repair processes suggest that impaired HRR may be widespread in sporadic cancer and that PARP inhibitors could have more generic application than currently thought.

VII. DNA DAMAGE SIGNALING AS A BARRIER TO TUMORIGENESIS

Predisposition syndromes demonstrate that genomic instability can promote cancer; however, in recent years an alternative paradigm has emerged in which DNA damage and the resulting downstream signaling processes act as a tumor suppression mechanism (Halazonetis et al., 2008). This concept originated with the observation that premalignant or early stage lesions in several cancer types, including bladder, breast, lung, and colon, frequently showed evidence of DNA damage as judged by the presence of multiple surrogate or direct markers of damage signaling such as γ-H2AX, and phosphorylated, active forms of ATM, Chk2, and p53 (Bartkova et al., 2005). These markers were not present in proliferating normal tissue, and strikingly, their prevalence diminished again in the more malignant, later stages of disease (Bartkova et al., 2005). Crucially, the signs of DNA damage in early stage lesions preceded the appearance of overt genomic instability or of p53 mutations, both of which are frequent in fully malignant tumors (Bartkova et al., 2005). Based on these observations it was proposed that spontaneous DNA damage occurs early in the evolution of cancer and that the resulting DNA damage signaling processes direct incipient cancer cells to terminal, nonproductive fates such as senescence or apoptosis. It was furthermore postulated that this might provide a selective pressure for specific secondary genetic alterations, such as p53 mutation, that would allow cells to escape these fates and survive (Bartkova et al., 2005).

Although these initial findings were largely correlative, subsequent studies have provided substantial support for this scenario (Bartkova et al., 2006; Di Micco et al., 2006). Thus, while activated oncogenes are known to drive the proliferation of malignant tumor cells, expression of the same oncogenes in naïve, genetically normal cells in culture often results not in cell transformation but instead in growth inhibition through cell senescence or apoptosis (Braig and Schmitt, 2006). Oncogene-induced senescence (OIS) in cultured cells is associated with physical DNA damage and a robust DNA damage response (Di Micco et al., 2006), providing a tractable model to test the role of downstream signaling processes in imposing this phenotype. Remarkably, depletion or inhibition of several key DNA damage signaling components,
including ATM, Chk1, Chk2, and p53, relieved OIS to allow more vigorous cell proliferation and efficient transformation of oncogene-expressing cells (Bartkova et al., 2006; Di Micco et al., 2006). Although cell culture experiments obviously do not accurately recapitulate all features of tumorigenesis in vivo, it is telling that markers of cell senescence and DNA damage signaling are both present in early stage or premalignant lesions and both are lost as tumors progress to more malignant forms (Bartkova et al., 2006).

Such studies have stimulated interest in two questions; firstly, how do oncogenes trigger DNA damage, and secondly, what are the genetic or epigenetic changes which enable tumor cells to survive and proliferate in the face of such damage? With respect to the first question, telomere erosion, reactive oxygen species, or acquired mutations in genes required for genome stability could all plausibly generate DNA damage during tumorigenesis or transformation; however, it has been argued that none of these occur sufficiently frequently to account for OIS either in vitro or in vivo (Negrini et al., 2010). Instead, current evidence favors the view that dominant, growth-promoting oncogenes, such as Ras or EGFR, which are frequently and recurrently mutated in sporadic cancers, disturb the DNA replication process directly in such a way as to generate DNA damage (Halazonetis et al., 2008). Exactly how oncogene-induced replication stress occurs remains unclear; however, CdkS are potential culprits. Ordered cycles of Cdk activation and deactivation are essential for the proper organization and activation of the replication program through the replication origin licensing system (Diffley, 2004). Because Cdk activity is key to uncontrolled proliferation, it is deregulated by oncogene activation and tumor suppressor loss by many different mechanisms. Amplification of Cdk activity as a result of oncogenic signaling therefore may erode these ordered cycles, leading to aberrant over- or under-replication and thus to DNA damage (Blow and Gillespie, 2008). According to this view, uncontrolled proliferation inevitably leads to spontaneous DNA damage and genomic instability (Halazonetis et al., 2008).

With respect to mechanisms that may allow escape from oncogene-induced DNA damage, recent high-throughput sequencing studies have generally failed to find evidence for frequent mutations affecting DNA damage response genes, such as ATM–Chk2 and ATR–Chk1, in sporadic cancers, although clearly this does not rule out functional inactivation by epigenetic or other means (Negrini et al., 2010). By contrast, missense mutations that inactivate the p53 tumor suppressor protein are frequent in sporadic cancer and some genetic evidence is consistent with the idea that these could be selected to permit escape from DNA damage-induced OIS or apoptosis (Negrini et al., 2010). One possible explanation for their prevalence is that mutant p53 proteins can act dominantly, forming complexes with and inhibiting normal p53 or the related p63 and p73 proteins (Brosh and
Rotter, 2009). In contrast, biallelic mutations would presumably be required to inactivate the function of most other DNA damage response genes. At all events, frequent loss of p53 means that many tumor cells lack an effective G1 checkpoint. As discussed below, this defect may render such tumors inherently vulnerable to therapeutic strategies based on checkpoint suppression.

Although inactivation of p53 could plausibly mitigate many of the negative effects of oncogene-induced DNA damage, it does not offer an obvious explanation for why DNA damage signaling per se should be lost in the later stages of tumorigenesis (Bartkova et al., 2005). Also, since malignant tumors commonly exhibit high levels of genomic instability (Negrini et al., 2010), it seems unlikely that the ongoing generation of oncogene-induced DNA damage ceases as tumors progress, but more likely that its presence is simply no longer detected. Why should this be? As previously mentioned, several recent studies have revealed that upregulation of the PI3K-Akt pathway, which is common in cancer as a result of PTEN inactivation and other mechanisms (Chalhoub and Baker, 2009), inhibits both HRR and checkpoint activation by suppressing DNA damage processing (Tonic et al., 2010; Xu et al., 2010). One prediction of these findings is that tumor cells with high PI3K-Akt pathway activity will show a muted response to endogenous DNA damage, potentially providing another mechanism through which oncogene-induced DNA damage could be tolerated. However, a second prediction is that this would inevitably be associated with genomic instability and an increased mutation rate, since ongoing oncogene-induced DNA damage would continue undetected but unabated in cells with high PI3K-Akt activity. Finally, it seems possible that tumors which have evolved through such a route might show an altered response to conventional genotoxic therapies, since presumably the recognition and signaling of therapeutic DNA damage would also be suppressed.

VIII. CHECKPOINT SUPPRESSION AS A THERAPEUTIC PRINCIPLE

Radiation and genotoxic chemotherapies remain the mainstays of cancer treatment. Although new, molecularly targeted, drugs like imatinib have revolutionized treatment of rare cancers such as chronic myeloid leukemia (Agrawal et al., 2010), there seems little prospect that conventional therapies will be replaced in the treatment of other, more common malignancies in the foreseeable future. Such treatments are, however, of limited efficacy and toxic not only to tumor cells but also to normal tissues, leading to severe side-effects. The realization that radiation and essentially all genotoxic
anticancer agents potently activate the ATM–Chk2 and ATR–Chk1 signaling pathways has stimulated much interest in whether these could be manipulated pharmacologically to enhance the efficacy of conventional therapies.

Several considerations argue that this may be the case. Firstly, defects in DNA damage responses, whether inherent or experimentally imposed, can result in sensitization to genotoxic stress. Examples already mentioned include the acute radiosensitivity of ATM-deficient cells, the inherent vulnerability of BRCA1- and BRCA2-deficient cells to cross-linking agents, and the synthetic lethality that results when cells with HRR deficiency are subject to PARP inhibition. Secondly, enhanced DNA damage signaling has been linked to radio- and chemo-resistance in leukemia and gliomas (Bao et al., 2006; Nieborowska-Skorska et al., 2006), raising the possibility that this might be reversed. Finally, it has been argued that the frequent functional inactivation of p53 in human cancer creates a generic and exploitable distinction between tumor and normal cells in terms of checkpoint proficiency. In essence this hypothesis holds that loss of p53-mediated G1 arrest under conditions of genotoxic therapy will render tumor cells more dependent on checkpoints activated in S and G2 phases than their genetically normal counterparts. Obviously this strategy is predicated on the assumption that in tumor cells the primary function of these residual p53-independent checkpoints is protective; that is, that their inhibition will either escalate damage, promote the formation of more lethal lesions, or trigger some mechanism of cell death that would not otherwise occur in the absence of checkpoint suppression.

Over the past decade considerable evidence has accumulated to support this concept. Thus, inhibition of Chk1 using either siRNA depletion or the selective chemical inhibitor, UCN-01, has been shown to potentiate cell killing by a wide range of genotoxic agents, including ionizing radiation, alkylating agents, nucleoside analogs, cisplatin, and topoisomerase inhibitors (Carrassa et al., 2004; Cho et al., 2005; Ganzinelli et al., 2008; Hirose et al., 2001; Karnitz et al., 2005; Koniaras et al., 2001; Wang et al., 1996; Yu et al., 2002). In many, although not all, of these studies Chk1 inhibition resulted in a greater degree of sensitization in tumor cells that were deficient for p53 than in their proficient counterparts, consistent with the idea that loss of G1 arrest indeed creates a therapeutic index. Sensitization has also been observed as a consequence of inhibiting ATM, ATR, and other downstream components involved checkpoint regulation such as the Cdk1-regulating kinases, Wee1 and Myt1 (Karnitz et al., 2005; Mukhopadhyay et al., 2005). In comparison, where tested, inhibition of Chk2 has in general been found to have little or no effect on cell survival under conditions of damage, emphasizing the fundamental functional distinction between Chk1 and Chk2 (Carrassa et al., 2004; Karnitz et al., 2005; Pan et al., 2009).
Although these studies support the general principle that checkpoints are protective and help tumor cells to survive exposure to genotoxic stress, exactly how checkpoint suppression leads to cell death remains poorly understood. Evidence for amplified levels of damage, mitotic catastrophe with damaged or incompletely replicated DNA, and increased levels of apoptosis have all been reported (Carrassa et al., 2004; Cho et al., 2005; Ganzinelli et al., 2008; Hirose et al., 2001; Karnitz et al., 2005; Koniaras et al., 2001; Yu et al., 2002). This is likely to be a complex issue; however, since genotoxic agents with distinct mechanisms of action activate different combinations of DNA damage and replication checkpoint responses, while the effects of some genotoxins may also depend on cell cycle phase and thus vary among individual cells in a population. The cellular consequences of suppressing checkpoint responses and the mechanisms of induced cell death that contribute to sensitization are therefore likely to be equally variable and complex.

Some of this complexity is illustrated by studies in Chk1 knockout DT40 lymphoma cells examining the relationship between checkpoint deficiency and cell survival under different conditions of genotoxic stress. Compared to wild-type, Chk1-deficient DT40 cells are markedly sensitive to ionizing radiation, the DNA polymerase inhibitor aphidicolin, and the nucleoside analog 5-fluorouracil (5-FU); however, the mechanism of cell death arising from checkpoint deficiency is different in each case. Thus, abrogation of the Chk1-dependent G2 checkpoint leads to division with lethal damage after irradiation, presumably because G2 arrest would normally provide a vital opportunity to repair such damage prior to mitosis (Zachos et al., 2003). By contrast, when DNA polymerase is blocked with aphidicolin, deficient fork stabilization/origin suppression and S-M checkpoints result in massive replication fork collapse and lethal premature entry to mitosis with unreplicated DNA (Zachos et al., 2003, 2005). 5-FU also inhibits DNA replication through its inhibitory action on thymidylate synthase (TS) leading to nucleotide pool depletion (Longley et al., 2003); however, sensitization in this case proved to stem from uncontrolled replication in the presence of drug due to loss of Chk1-mediated slowing of DNA synthesis, rather than fork collapse followed by premature entry to mitosis (Robinson et al., 2006). Failure to slow replication resulted in enhanced incorporation of 5-FU into cellular genomic DNA and a large increase in DSBs compared to Chk1 proficient cells (Robinson et al., 2006). Thus, the consequences of replication checkpoint suppression vary according to the nature of the genotoxic agent and the mechanism of DNA synthesis inhibition.

The many preclinical “proof-of-principle” studies over the past decade or so have encouraged efforts within the pharmaceutical industry to develop drugs targeting the ATM–Chk2 and ATR–Chk1 pathways, some of which have already entered clinical trials. Thus far these efforts have concentrated
mainly on Chk1, presumably in recognition of its role as the ultimate effector of both DNA damage and replication checkpoints and the fact that it is thought to be expressed and active in virtually all tumors (Dai and Grant, 2010). Drugs specifically targeting ATM and Chk2 are, however, also under development, although at an earlier stage, and also other checkpoint-regulating kinases such as Wee1 and Myt1 (Antoni et al., 2007; Ashwell et al., 2008; Dai and Grant, 2010). Thus far no selective inhibitors of ATR have been reported.

UCN-01 was the first Chk1 inhibitor approved for clinical trials; however, undesirable pharmacological characteristics combined with lack of selectivity limited its utility and led to the development of several new and more selective agents (Ashwell et al., 2008; Dai and Grant, 2010; Fuse et al., 2005). Several of these have reached clinical trials while others remain under preclinical development. Current Chk1 inhibitors in Phase I clinical trials include AZD7762 (AstraZeneca), PF-00477736 (Pfizer), XL844 (Exelixis), and SCH 900766 (Schering-Plough). It is important to note that although each of these drugs was developed to inhibit Chk1, several also have significant, and in one case even greater, activity against Chk2. Whether the potential for dual inhibition of both Chk1 and Chk2 is relevant to the biological effects of these agents as reviewed below is currently unclear.

Each of these agents has shown promising preclinical activities that recapitulate many of the effects of experimental Chk1 inhibition on DNA damage and replication checkpoint responses described above (Ashwell et al., 2008; Dai and Grant, 2010). In addition to monitoring effects on cell survival, several of these studies have sought evidence of drug efficacy in terms of Chk1 inhibition and to gain insight into potential mechanisms of synergistic cell killing. Biochemical readouts of Chk1 activity include turnover of Cdc25A phosphatase, phosphorylation of Cdc25C on serine 216 (S216), and increased levels of inhibitory T14/Y15 phosphorylation of Cdk1. To document the biological consequences of Chk1 inhibition, nuclear foci of γ-H2AX and Rad51 provide a means of quantifying DNA damage and proficiency for HRR respectively, while premature entry to mitosis can be detected by flow cytometry when cells with incompletely replicated DNA become positive for histone H3 phosphorylated on serine 10 (pS10) (Zachos et al., 2005).

AZD7762 is a potent ATP-competitive inhibitor of Chk1 and Chk2 that is currently in clinical trials in combination with gemcitabine and irinotecan for solid malignancies (Morgan et al., 2010; Zabludoff et al., 2008). In preclinical studies AZD7762 has been shown to synergize with ionizing radiation, irinotecan, and gemcitabine in a variety of tumor cell lines and xenografts with evidence of greater potency in p53-deficient cells (Morgan et al., 2010; Zabludoff et al., 2008). In each case AZD7762 treatment resulted in stabilization of Cdc25A, decreased levels of T14/Y15 phosphorylated Cdk1, and an increase in mitotic entry compared to DNA damaging
agent alone, indicating efficient Chk1 inhibition and checkpoint override (Morgan et al., 2010; Zabludoff et al., 2008). Enhanced cell killing by AZD7762 in the context of ionizing radiation was also associated with increased and persistent γ-H2AX staining and a marked suppression of Rad51 focus formation, indicating that drug treatment resulted in higher levels of DNA damage and also likely resulted in suppression of HRR (Morgan et al., 2010; Zabludoff et al., 2008).

Gemcitabine is an antimetabolite and DNA strand-terminator that inhibits DNA replication via inhibition of both ribonucleotide reductase and DNA polymerase, stalling replication forks and arresting cells in S-phase (Ewald et al., 2008). A more detailed analysis of gemcitabine chemosensitization revealed that AZD7762 treatment caused collapse of stalled replication forks, ectopic replication origin firing, and ultimately premature entry to mitosis with unreplicated DNA and apoptosis (McNeely et al., 2010). Interestingly, siRNA depletion of Cdk1 mitigated cell death under conditions of AZD7762 and gemcitabine treatment, suggesting that premature entry to mitosis as a result of S-M checkpoint failure was a direct cause of cell death (McNeely et al., 2010). Replication fork collapse also resulted in high levels of DSBs and ATM activation, and consistent with this, AZD7762 enhanced gemcitabine cytotoxicity particularly effectively in cells with DSB repair defects (McNeely et al., 2010).

PF-00477736 is a potent ATP-competitive inhibitor of Chk1 (Ki 0.49 nM) with more modest activity towards Chk2 (Ki 47 nM) in vitro. It is in combination trials with gemcitabine for the treatment of advanced solid tumors (Ashwell et al., 2008; Dai and Grant, 2010). In preclinical studies PF-00477736 has been shown to abrogate both the G2 and intra-S-phase checkpoints in cells treated with camptothecin and gemcitabine respectively, and to enhance the cytotoxicity of gemcitabine and carboplatin in cell and xenograft assays (Blasina et al., 2008). Unexpectedly, PF-00477736 also synergizes strongly with docetaxel in cells and xenografts, releasing cells from mitotic arrest and enhancing apoptosis (Zhang et al., 2009). The mechanism of this synergy is not yet fully understood. High concentrations of docetaxel can induce DNA damage which could be a factor; however, other findings have shown that Chk1 is required for spindle checkpoint function and mitotic progression, raising the possibility that Chk1 inhibitors might enhance the effects of antimitotic drugs more generally (Carrassa et al., 2009; Peddibhotla et al., 2009; Zachos et al., 2007).

XL-844 is a more potent inhibitor of Chk2 (Ki 0.07 nM) than Chk1 (Ki 2.2 nM). XL-844 is in combination trials with gemcitabine for the treatment of advanced solid tumors and lymphoma (Ashwell et al., 2008; Dai and Grant, 2010). In PANC-1 cells treated with gemcitabine XL-844 has been shown to override the S-phase checkpoint leading to increased levels of Chk1 phosphorylation (S317) and γ-H2AX, indicative of increased
levels of damage, followed by subsequent premature entry into mitosis and decreased clonogenic survival (Matthews et al., 2007). Similarly, SCH-900766 is a selective Chk1 inhibitor that is in combination trials with gemcitabine for the treatment of solid tumor and lymphoma and cytarabine for the treatment of acute leukemia. SCH-900766 has been shown to abrogate both the intra-S and G2 checkpoints resulting in sensitization of tumor cells to IR and alkylating agents, although full details of these effects are not yet in the public domain (Dai and Grant, 2010).

Taken together, these studies validate Chk1 as a target whose pharmacological inhibition can potentiate tumor cell killing by a wide range of genotoxic agents in vitro. As depicted in Fig. 4, much remains to be learned about the detailed mechanisms involved in chemosensitization, however, Chk1 inhibition can clearly both amplify the extent of damage inflicted by a given agent and promote the formation of more lethal lesions, for example by triggering stalled replication fork collapse to form DSBs. In addition, evidence suggests that damage escalation as a result of Chk1 inhibition can enhance tumor cell killing both by conventional routes, for example by increasing apoptosis, but also by triggering novel mechanisms such as premature entry to mitosis with unreplicated DNA. Clearly, the challenge now will be to determine whether these preclinical principles established in the laboratory using novel Chk1 inhibitor drugs will have utility in the clinic.

IX. FUTURE PERSPECTIVES

Genomic instability has long been recognized as a cardinal feature, and arguably an important cause of, cancer, however, in recent years it has also emerged as a potential Achilles heel that offers new therapeutic opportunities. Thus far this prospect has been most evident in cancers that arise in predisposed individuals, for example in BRCA1 and BRCA2 mutation carriers, where impairment of one particular form of DNA repair specifically in tumor cells creates sensitivity to both existing and novel treatments. Although similar loss-of-function mutations do not appear to be common in sporadic cancers, genomic instability is, perhaps because DNA damage response genes are inactivated epigenetically, or the proteins they encode are inhibited by oncogenic signaling processes. This raises the possibility that individual sporadic cancers might also be selectively targeted if the functional basis and consequences of genomic instability in individual tumors could be understood. Alternatively, it may be possible to improve the efficacy, or mitigate the undesirable side-effects, of existing genotoxic therapies by developing drugs that inhibit DNA damage responses controlled by the ATM–Chk2 and ATR–Chk1 pathways. Evidence suggests that inhibition of the Chk1 protein
kinase in particular may have therapeutic potential, particularly in tumors that have suffered loss of p53 function during their evolution. Such efforts are, however, in their infancy, and as understanding of the biological and molecular functions of these pathways deepens, additional rational therapeutic strategies based on genome stability defects will likely emerge.

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