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TOWARD MAINTAINING THE GENOME: DNA Damage and Replication Checkpoints

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■ **Abstract** DNA checkpoints play a significant role in cancer pathology, perhaps most notably in maintaining genome stability. This review summarizes the genetic and molecular mechanisms of checkpoint activation in response to DNA damage. The major checkpoint proteins common to all eukaryotes are identified and discussed, together with how the checkpoint proteins interact to induce arrest within each cell cycle phase. Also discussed are the molecular signals that activate checkpoint responses, including single-strand DNA, double-strand breaks, and aberrant replication forks. We address the connection between checkpoint proteins and damage repair mechanisms, how cells recover from an arrest response, and additional roles that checkpoint proteins play in DNA metabolism. Finally, the connection between checkpoint gene mutation and genomic instability is considered.

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INTRODUCTION

Growth and division of a single cell to yield two daughter cells requires the coordination of numerous events, in particular the faithful replication and partitioning of the cell's genetic material to each daughter cell. At the extreme, errors in this process could mean death for a unicellular organism, and for multicellular organisms, faulty cell division may ultimately culminate in developmental defects or oncogenesis.

To ensure the fidelity of division, cells have evolved general mechanisms called checkpoints that monitor the successful completion of cell cycle events. Checkpoints are typically not essential for cell cycle events per se, but rather they make certain that events are completed correctly and in the proper order. When one cell cycle event has not been successfully completed, checkpoints will delay progression until the step is correctly accomplished, and only then will they relieve the arrest to allow the cell to move to the next phase.

Given that cells are constantly under the assault of endogenous and exogenous forms of damage, maintaining a complete, undamaged genome is a continual challenge and of vital importance to the cell and future cellular generations. All eukaryotic cells, except certain embryonic cells, possess checkpoints to monitor virtually every cell cycle event involving DNA metabolism (86). Cells not only supervise the process of DNA replication during S-phase to ensure correct completion of this event, but they also monitor the state of DNA throughout the entire cell cycle to minimize the accruement of damage. In addition to arresting cells with compromised DNA, these checkpoints also mediate repair of the damage, a role still being elucidated. Therefore, these DNA checkpoints—or, perhaps more specifically, DNA maintenance checkpoints—include what may have traditionally been thought of as two classes: (*a*) the DNA damage checkpoints that recognize and respond to DNA damage, and (*b*) the replication checkpoints that monitor the fidelity of copying DNA.

The importance of DNA checkpoints in human pathology, especially cancer, is now well established. The p53 tumor suppressor, the most infamous of checkpoint genes, is the only one routinely mutated in sporadic human cancers [reviewed in (11)]. Although p53's role in G1 arrest was first described 11 years ago (98), our understanding of its function is incomplete. As for many checkpoint genes, p53 is not essential for cell viability yet plays key roles in at least two response pathways after DNA damage: G1 arrest and apoptosis [reviewed in (186)]. Early hypotheses that arrest provides time for DNA repair whereas apoptosis eliminates cells with

irreparable DNA damage from cycling proves insufficient. In addition, how each p53 function—G1 arrest, G2 arrest, and apoptosis—relates to human cancer awaits the description of molecular mechanisms in specific cellular contexts.

Other human disease genes have links to cancer pathology, although again those links are unresolved. ATM (ataxia telangiectasia mutated) is a protein kinase central to all DNA maintenance responses that, when absent, results in the disease ataxia telangiectasia (AT). AT is characterized at the cellular level by gross chromosomal rearrangements and radioresistant DNA synthesis, and at the organismal level by immune deficiency, cerebellar degeneration, and an increased predisposition to cancer (107, 164). Not known is whether the disease is due to defects in DNA damage responses or to defects in some other cellular activity regulated by ATM. Humans harboring mutations in proteins that have recently been shown to interact with ATM in DNA damage recognition, Mre11 and Nbs1, also present with AT-like symptoms, thereby suggesting a connection to checkpoint and DNA repair defects (26, 180). Additional links include mutation of either Chk2 or p53, both of which act downstream of ATM, resulting in a genetic disease called Li Fraumeni syndrome that dramatically predisposes patients to cancer development (14, 125). Mutations in the checkpoint protein BRCA1, another substrate of ATM, result in defects in S-phase and G2 arrest (220) and also cause defects in DNA repair (1, 15, 135). Notably, an estimated 50% of inherited cases of breast cancer are likely due to mutations in the *BRCA1* gene, and *BRCA1* mutation is implicated in almost all families with histories of both ovarian and breast cancer (73). Such results clearly demonstrate that DNA checkpoints play a central role in human pathology, and we now must face the challenge of understanding the molecular intricacies as to how and why.

In the past few years, studies in organisms from budding and fission yeasts, the fungus Aspergillus, nematodes, Drosophila, mouse, to mammalian systems have combined to enhance our understanding of these DNA maintenance checkpoints in cell cycle biology. The recurrent theme is the importance of these checkpoints in maintaining genome stability and the correlation of loss of this function with the human pathology of cancer. Cells lacking functional checkpoints display genomic instability due to a failure to properly respond to DNA damage, faulty DNA replication, or aberrant chromosome segregation, resulting in an accelerated mutator phenotype (116). Recent understanding of molecular mechanisms summarized in this review may provide some insight into the causes of genomic instability and suggest strategies for the development of therapeutics. Of promise, researchers hope to exploit checkpoint defects in cancer cells to selectively kill them.

This review describes our current understanding of the molecular and genetic pathways of the DNA damage and replication checkpoints. We present working models based on available data from studies of various organisms, most notably those performed in mammalian cells and in budding and fission yeast, and point out that many molecular and biochemical details needed to support some of the proposed models are lacking. Checkpoint controls in different organisms indicate a high degree of conservation, although occasional divergences do occur.

THE CHECKPOINT PROTEINS

The checkpoint pathways involve three major groups of proteins that act in concert to translate the signal of damaged DNA into the response of cell cycle arrest and repair. These groups include (*a*) sensor proteins that recognize damaged DNA directly or indirectly and function to signal the presence of abnormalities, initiating a biochemical cascade of activity; (*b*) transducer proteins, typically protein kinases, that relay and amplify the damage signal from the sensors by phosphorylating other kinases or downstream target proteins; and (*c*) effector proteins, which include the most downstream targets of the transducer protein kinases, and are thus regulated, usually by phosphorylation, to prevent cell cycle progression. Table 1 summarizes the principal orthologous checkpoint proteins identified thus far in mammals and budding and fission yeasts. Within the text, the mammalian nomenclature is used

TABLE 1 Orthologous checkpoint proteins

Protein function	**Mammals**	***S. pombe***	***S. cerevisiae***
Sensors			
RFC1-like	Rad17	Rad17	Rad24
PCNA-like	Rad9	Rad9	Ddc1
	Rad1	Rad1	Rad17
	Hus1	Hus1	Mec3
BRCT-containing	BRCA1	Crb2/Rph9	Rad9
	TopBP1	Cut5	Dpb11
DSB recognition/repair	Mre11	Rad32	Mre11
	Rad50	Rad50	Rad50
	Nbs1		Xrs2
Replication proteins			
recruits polymerases	TopBP1	Cut5	Dpb11
needed for replication		Drc1	Drc1
DNA polymerase	Pol2	Cdc20	Pol2
DNA helicase	BLM, WRN*	Rhq1/Rad12	Sgs1
Topoisomerase	Top3	Top3	Top3
clamp loader	Rfc2–5	Rfc2–5	Rfc2–5
binds ssDNA	Rpa2		Rfa2
Transducers			
PI3-kinases (PIKK)	ATR	Rad3	Mec1
	ATM	Tel1	Tel1
PIKK binding partner	ATRIP	Rad26	Ddc2/Lcd1
Effector Kinases	Chk1	Chk1	Chk1
	Chk2	Cds1	Rad53
Replication fork	—	—	Tof1
stabilizers	Claspin	Mrc1	Mrc1

*WRN—mutated in Werner syndrome.

unless otherwise stated. Below, we name and briefly discuss the primary proteins involved in DNA checkpoint responses. More detailed discussion of their roles follows in subsequent sections.

Sensors

Proteins have been placed in the sensor class based on genetic and biochemical findings or inference, as little direct biochemical evidence yet exists. Some sensors are thought to directly associate with damaged DNA; others are most likely indirectly associated by interactions with the aforementioned sensors. The types of sensor proteins that associate with a particular DNA lesion may serve as recognition complexes to modulate, recruit, and localize specific target proteins for that lesion type.

Rad17-RFC AND 9-1-1 These two complexes of proteins are presumed to act in concert with one another and to have functional analogs in DNA replication. As first shown in budding and fission yeasts, Rad17 interacts with four replication factor C subunits (Rfc2, Rfc3, Rfc4, Rfc5) to form a pentameric structure, referred to as the Rad17- RFC complex (79, 81, 114). Another complex is a heterotrimeric ring composed of Rad9, Hus1, and Rad1, termed the 9-1-1 complex (23, 28, 84, 179, 198, 201). The RFC subunits found in Rad17-RFC are more commonly recognized for their association with Rfc1 during replication to form what is termed the "clamp loader." The replication RFC clamp loader complex recognizes single-strand/double-strand DNA junctions and loads the homotrimeric PCNA "sliding clamp" complex, which encircles the DNA to act as a general scaffold upon which DNA polymerases and other DNA replication proteins are assembled (193). One infers from sequence and structural similarities that during a checkpoint response the Rad17-RFC complex may recognize damage and load the 9-1-1 sliding clamp onto DNA. Indeed, association of Rad17-RFC and the 9-1-1 heterotrimer has been identified in vitro, and each is inferred to bind to sites of DNA damage in vivo as the Rad17 -RFC complex binds preferentially to DNA with primer-template-like structures (114). The 9-1-1 complex may have an additional role of processing DNA damage to generate more single-stranded DNA, perhaps required to enhance damage signaling to other checkpoint components (122, 146). Although the two human Rad17-RFC and 9-1-1 complexes have been reconstituted individually in vitro from recombinant proteins, loading of the 9-1-1 complex onto DNA by Rad17-RFC has not yet been shown. A recent report, however, indicates that mammalian Rad17 recruits the 9-1-1 complex to sites of damage in vivo as in yeast, although the consequences of this association have yet to be elucidated (235).

BRCA1 BRCA1 appears to function as an adaptor of checkpoint initiation by localizing additional substrates for transducer kinase phosphorylation and by perhaps linking checkpoint arrest to DNA damage repair. BRCA1 colocalizes with

a number of proteins involved in DNA repair and/or replication, including Rad51, PCNA, Mre11-Rad50-Nbs1, histone deacetylases, the DNA helicase BLM (mutated in Bloom's Syndrome), and mismatch repair proteins (167, 207, 225, 232). Additionally, BRCA1 directly binds specific DNA structures that are sequence independent in vitro (147). Consistent with these associations, BRCA1 affects homologous recombination, mismatch repair, and transcription-coupled repair (1, 77, 135, 178, 206, 207).

Based on functional and sequence similarities, we suggest that the fission yeast Crb2 and the budding yeast Rad9 proteins should be considered BRCA1 orthologs. All three proteins share limited sequence similarity in that each contains two BRCT (Brca1 Carboxy Terminal) domains, which are motifs most likely involved in dimerization [reviewed in (92)]. In addition, their regulation is similar: BRCA1, Crb2, and Rad9 are all phosphorylated by ATR and ATM orthologs after damage (39, 56, 92, 158, 190, 200), and each is required to activate downstream protein kinases (76, 80, 109, 158, 159, 226). Furthermore, each acts to induce a G2/M cell cycle arrest to allow time for DNA repair (158, 211, 215, 220). Disparities in other protein functions are expected, but the functional core similarities listed here warrant their being termed orthologs.

Mre11-Rad50-Nbs1 The Mre11-Rad50-Nbs1 (MRN) complex localizes to sites of double-strand breaks (DSBs) in vivo and plays vital roles in DNA metabolism, including DSB repair, meiotic recombination, and telomere maintenance (32, 50, 128, 234). Cells deficient for Mre11 or Nbs1 continue DNA replication after X-ray damage, known as radioresistant DNA synthesis (RDS), indicating defective checkpoint signaling during S-phase (170). Additionally, such mutations affect checkpoints at all phases of the cell cycle (24, 223).

REPLICATION PROTEINS We have grouped these proteins because they are all localized at sites of replication forks, play roles in DNA replication, and have checkpoint defects within S-phase. This group includes budding yeast topoisomerase III [Top3 (31)], the Pol2 subunit of the DNA polymerase from budding yeast (138), the budding and fission yeast Drc1 protein required for DNA replication (138a, 203), the mammalian DNA helicase BLM and orthologous proteins (45, 52, 65, 66), the mammalian polymerase recruiter TopBP1 and orthologous proteins (8, 124, 199, 222), eukaryotic members of the RFC clamp loader complex (102, 137, 139, 172), and the Rpa heterotrimer that binds single-strand DNA in mammals and budding yeast (163, 205).

Transducers

Transducers include the protein kinases that, when activated by the presence of DNA damage, initiate a signal transduction cascade that propagates and amplifies the damage signal to ultimately cause cell cycle arrest.

ATM AND ATR Both ATM and ATR (ATM and Rad3-related) are members of the phosphoinositide 3-kinase related kinases (PIKKs), which are large proteins ranging between 275-500 kDa that possess a unique protein kinase domain at their C termini with little sequence conservation outside this region (2).

Following observations in yeast, ATR has recently been shown to form a heterodimer with the associated protein ATRIP, which is required for the checkpoint signaling pathway (38, 54, 142, 157, 202, 216). The mechanism of ATR-ATRIP activation remains elusive. As ATR is a member of the PIKK family that includes DNA-PK, it is interesting to note that in vitro DNA-PK kinase activity is mostly activated upon association with the Ku heterodimer that binds double-stranded DNA ends (176). Whether ATRIP possesses detectable DNA binding activity or is required for protein kinase activity of ATR is not yet known, although ATR and ATRIP colocalize to intranuclear foci after DNA damage or inhibition of replication (38). In a recent budding yeast study, Rouse & Jackson show that the ATRIP ortholog, Ddc2 (also known as Lcd1/Pie1), can bind DNA independently of Mec1, the ATR ortholog, and that Ddc2 is required for the recruitment of Mec1 to sites of DNA damage (157a).

ATR in mammalian cells and Mec1 in budding yeast are essential for cell viability, whereas Rad3 in fission yeast is not. In *Saccharomyces cerevisiae*, this essentiality is due to Mec1's role in regulating dNTP levels (229, 231). As ATR's essential function has yet to be clearly defined, regulation of dNTP pools in mammalian cells is an obvious place to look. Alternately, because mammalian genomes are so large, the DNA damage sustained on a continuous basis may require the constant monitoring via ATR to ensure viability. For instance, the Rad51 recombination protein is not essential in yeast but it is in mammalian cells (194); perhaps mammalian and yeast genomes incur the same number of breaks per unit of DNA, but the mere fact that the mammalian genome is 250 times larger than the yeast genome makes repair proteins essential.

Chk1 AND Chk2 Chk1 and Chk2 are classic serine-threonine kinases that are required for cell cycle arrest in response to DNA damage. As downstream kinases, they are phosphorylated by ATM/ATR-dependent processes, may additionally undergo autophosphorylation [demonstrated for *S. cerevisiae* Rad53 (76) and Chk1 (202a)], and potentiate phosphorylation of downstream targets.

Effectors

Many targets of transducer phosphorylation have yet to be identified, and known targets are typically not well conserved across species. We discuss the effects of effector phosphorylation throughout the remaining sections only where appropriate.

THE MOLECULAR SIGNALS THAT ACTIVATE CHECKPOINT RESPONSES

The nature of DNA structures that are recognized by the sensor proteins to activate checkpoint responses remains obscure. Even in the well-defined bacterial SOS response system that leads to activation of the RecA protein, defining the DNA damage signal in vivo has been a formidable task, though it is likely single-strand DNA (53). In eukaryotic cells, even the simplest hypothesis becomes complex when accounting for the type of damage, the quantity of lesions, and the various protein kinases and substrates involved. Results from in vivo studies that utilize irradiation or alkylating agents to induce DNA damage—both of which cause a vigorous checkpoint response—are ambiguous owing to the myriad of DNA lesions generated (209). Furthermore, there are no defined biochemical systems that can be used to determine mechanisms of activation in vitro.

Despite these difficulties, a prevailing view for eukaryotic cells is that different kinds of lesions are converted to single-strand DNA (ssDNA) and double-strand DNA breaks (DSBs), two common structures that then signal checkpoints. Moreover, the weight of evidence suggests that the two signals activate different arms of the checkpoint pathways: DSBs activate a pathway containing the ATM kinase family, whereas ssDNA activates a checkpoint pathway containing the ATR kinase family (Figure 1).

Activation of the ATR checkpoint pathway by ssDNA rests on extrapolation from three key observations in budding yeast. In yeast, a single defined DSB generated by the HO endonuclease is rapidly converted to long tracts (>5 kb) of ssDNA by degradation of the 5′ strand (181). This ssDNA causes an acute arrest of chromosome segregation that requires the ATR homolog, Mec1, and presumably not the ATM homolog, Tel1 (161). In the second study, inactivation of Cdc13, a protein normally associated with telomeres that aids in their protection, leads to extensive ssDNA near telomere ends. This defect also elicits a profound Mec1-dependent arrest that is Tel1 independent (67, 69). Third, unrepaired DSBs (due to mutation of the RecA homolog Dmc1) generated during meiosis undergo 5′ end resection to create ssDNA and require Mec1 for meiotic arrest (121). The length or quantity of ssDNA required to activate arrest is not known, nor are the proteins needed to associate with ssDNA, though RPA is a plausible candidate (117, 163).

In budding yeast, evidence regarding the activation of Tel1 by DSBs is less extensive, as these breaks are typically converted rapidly to ssDNA. However, a recent study shows activation of Tel1 using cells where DSB resection is inferred to be blocked in meiosis by *rad50S* or *sae2* mutation (195). Meiotic cells with a resected DSB require Mec1 for arrest (121), whereas meiotic cells with an unresected DSB require Tel1 and, enigmatically, Mec1 for arrest (195). This study also shows that mitotic cells with a *mec1* mutation show extreme sensitivity to the damaging agent methylmethane sulfonate (MMS) (195). *mec1 sae2* or *mec1 rad50S* mutants, however, show partial suppression of sensitivity, re-establishment of downstream Rad53 phosphorylation, and restoration of cell cycle arrest, and this suppression

requires an intact TEL1 gene. These data support a model whereby suppression of sensitivity in *mec1 sae2* or *mec1 rad50S* mutants is achieved because of enhanced activation of the parallel checkpoint pathway mediated by Tel1, which may specifically respond to DSBs. Elimination of both Mec1- and Tel1-dependent pathways results in enhanced lethality when DNA damage is incurred (195), presumably because both branches of the DNA damage response have been abrogated.

Many other indirect observations support the view that ssDNA activates ATR homologs and that DSBs activate ATM-like kinases. γ-irradiation of mammalian cells, which is thought to induce DSBs, causes rapid accumulation of ATM kinase activity, whereas ATR activity increases only at a later time, perhaps in response to DSB processing (217, 221). Moreover, immunoprecipitated ATM is more enzymatically active after extraction from cells treated with IR or radiomimetic agents (10, 25). In contrast, disruption of DNA replication in budding and fission yeasts and mammalian cells, thought to generate extensive ssDNA regions, results in responses that require activated ATR but not ATM (4, 83, 88, 149).

One structure critical for activating checkpoint controls is hypothesized to arise from aberrant replication forks. Of principal importance may be the structure and quantity of stalled forks required to signal; whether ssDNA, DSBs, or other structures specific to faulty forks are needed to signal is unknown. The structures of replication forks from wild-type and checkpoint-mutated cells have recently been reported in an electron micrograph study of replication forks isolated from budding yeast (178a). These results suggest that stalled forks contain more ssDNA than replicating forks, and that this is part of the signal for arrest. Just as one DSB is sufficient to cause arrest in yeast cells (161), we envision that one stalled fork is likewise sufficient to signal. Whether a single unperturbed DNA replication fork may also be sufficient to form a signal is unclear [see (196)].

THE MOLECULAR PATHWAYS ASSOCIATED WITH DNA DAMAGE

Numerous genes and proteins that act in checkpoint pathways have now been identified. We present the general mechanisms for pathways responding to ssDNA and DSBs (Figure 1), recognizing that most molecular aspects rely on inference and are based, in part, on the expectation of functional conservation of orthologous proteins.

ATR and Single-Strand DNA Response Complexes

The ATR-ssDNA checkpoint pathway likely involves four sets of protein complexes that assemble directly or indirectly on DNA to initiate and propagate a signal for arrest and to recruit damage repair proteins. How these four protein complexes interact in concert to mediate a DNA damage response is largely speculative. Like the replicative RFC complex with PCNA, the Rad17-RFC pentamer

may bind ssDNA and facilitate the binding of the 9-1-1 heterotrimer to damage; the ATR-ATRIP heterodimer may bind damage independently, as suggested by yeast studies (106, 132, 157). The Rad17-RFC complex together with the 9-1-1 complex may then recruit substrates, including BRCA1 and its associated proteins, for phosphorylation by ATR. Indeed, in fission and budding yeast, the ATR-like kinase requires components of both the yeast Rad17-RFC complex and the yeast 9-1-1 complex to phosphorylate Crb2 and Rad9 (56, 158). Once phosphorylated, BRCA1 may activate downstream protein kinase cascades, aiding in amplification of the damage signal by recruiting additional phosphorylation substrates. ATR-like protein kinases can phosphorylate some substrates, like the H2AX component of nucleosomes, independent of sensor proteins (208). Phosphorylation of other substrates by ATR is dependent on Rad17-RFC, 9-1-1, and BRCA1 complexes, which suggests that these complexes serve to localize some but not all substrates.

How ATR becomes activated during a checkpoint response is uncertain, although *Xenopus* studies may provide some indication. Guo et al. (83) purified ATR from egg extracts using DNA-cellulose and found enhanced ATR activity, 10- to 20-fold above that of ATR immunoprecipitated directly from egg extracts. This suggests that binding to DNA activates ATR activity or selectively purifies an activated form of ATR (2).

ATM and Double-Strand Break Response Complexes

The ATM protein kinase, orthologous to Tel1 in both budding and fission yeast, has been studied more extensively than ATR for two reasons: (*a*) in 1995, ATM was identified as the gene defective in the syndrome AT, whereas no association between ATR deficiency and a disease yet exists, and (*b*) unlike ATR, ATM is not essential for cell viability, (18, 46, 164). Cells lacking ATM display chromosomal instability, RDS, and extreme sensitivity to ionizing radiation (IR) and radiomimetic drugs. AT cells are defective for cell cycle checkpoints at G1/S, S, and G2/M phase transitions in response to radiation-induced damage [reviewed in (2)].

In accord with the view that ATM recognizes dsDNA ends, purified ATM associates with nonspecific DNA fragments in vitro, demonstrating a modest affinity for linear DNA over supercoiled molecules, and often localizing at DNA ends (40, 175). At DSBs, ATM may associate with the MRN complex of proteins, which could then serve to recruit ATM substrates for phosphorylation, much like the 9-1-1 and Rad17-RFC complexes do for ATR. That ATM and MRN act together at DSBs is inferred from the fact that cells from patients with mutations in *NBS1*, presenting as Nijmegan breakage syndrome, also exhibit radiosensitivity and chromosomal fragility like AT cells (17), and patients with mutations in *MRE11* present with clinical symptoms and cellular defects much like those seen in AT, thus giving rise to the name "AT-like disorder" (180). Budding yeast studies additionally support the model that ATM and MRN act coordinately at DSBs. Usui et al. (195) determined that unresected DSBs created during meiosis activate Tel1

in vivo through the MRX complex, where checkpoint activation is dependent on Mre11 acting as a damage sensor. The MRX complex consists of Mre11, Rad50, and the budding yeast protein, Xrs2, which is replaced by Nbs1 in mammals.

Identifying the targets of ATM phosphorylation will likely provide insight into the mechanism of checkpoint activation in response to DSBs [reviewed in (97)]. What can be gleaned at this point, however, is that, at least for two cases, ATM regulates several proteins within the same pathway to ensure the control of a key target. In regulating p53, ATM employs three different means to stabilize the protein. ATM, or a closely associated kinase, phosphorylates p53 on Ser^{15} in vitro, and most likely also in vivo, as AT cells show much reduced levels of p53 phosphorylated at the Ser^{15} residue in response to IR (25). This phosphorylation by ATM likely causes p53 transcriptional activation (51), while phosphorylation of p53 at Ser^{20} by Chk2 acting downstream of ATM, reduces the ability of ubiquitin ligase Mdm2 to bind p53, thus promoting its stabilization (34, 87, 90, 169). ATM further ensures p53 stabilization by phosphorylating Mdm2, thereby preventing export of p53 to the cytoplasm where it is degraded (101). Another "tri-strategy" approach involves regulation of BRCA1. ATM directly phosphorylates BRCA1 after activation by DSBs caused by ionizing radiation, although the consequence of this phosphorylation is unknown (39, 71). Moreover, activated Chk2, downstream of ATM, additionally phosphorylates BRCA1 on another site that is required for the dissociation of BRCA1 and Chk2 (109). ATM also phosphorylates CtIP, forcing dissociation of this inhibitor from BRCA1 (111).

THE MOLECULAR PATHWAYS ASSOCIATED WITH REPLICATION FORKS

The regulation of DNA replication by checkpoint controls may be most important in both genome stability and potentially in cancer therapy. Indeed, a major role of checkpoint proteins may be to stabilize stalled replication forks, since fork collapse can lead to chromosome rearrangements and, thus, genome instability or cell death. Genome instability can activate oncogenes leading to unregulated, abnormal cell growth. Further destabilizing replication forks in cancer cells might provide an avenue for therapy. Much of the current interest in replication fork biology has been motivated by findings in bacteria where replication fork collapse is thought to be a common feature (occurring about once in every two cell divisions), with RecA acting to restore failed forks by homologous recombination (41).

Replication forks slow or stall when they encounter DNA adducts, creating a physical impediment, or when dNTP pools are limiting. Replication disruption elicits four cellular responses: a block to initiation of replication (origin firing), slowing of elongation, maintenance of slowed or stalled replication forks, and a block to mitosis (Figure 2). In budding yeast, checkpoint proteins regulate three of these four responses. After DNA damage, if the key Mec1 and Rad53 regulators are mutated, late origins of replication continue to fire (162), stalled replication forks are not stabilized (48, 119, 188), and cells enter mitosis (213).

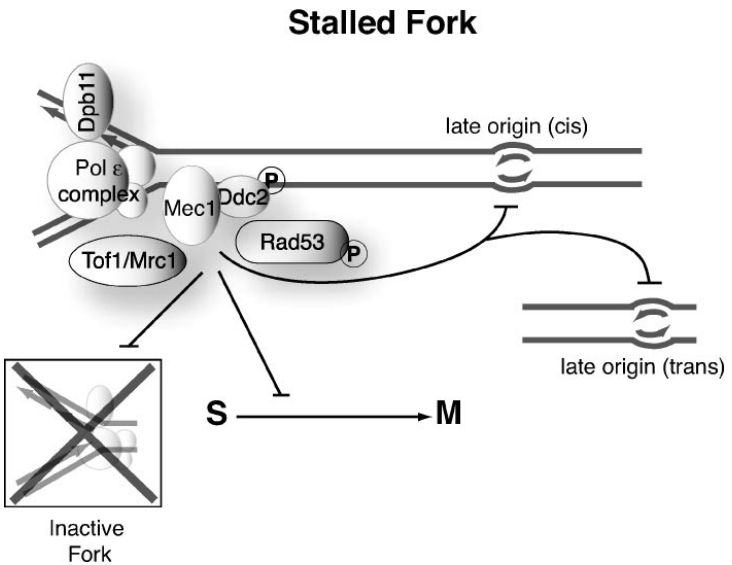

Figure 2 A stalled replication fork activates three common checkpoint mechanisms: (*a*) stabilization of the fork by blocking fork collapse, (*b*) a block to mitosis, and (*c*) blockage of late origin firing both in *cis* and in *trans*.

Many of the mechanisms controlling these responses are at least partially understood. Surprisingly, slowing of elongation does not require the yeast checkpoint genes; the replication apparatus inherently slows upon encountering damage, likely due to a physical block in fork progression. The Mec1 and Rad53 checkpoint proteins in *S. cerevisiae* are, however, required for the other three responses. Blocking of late origin firing in budding yeast likely occurs though Rad53 phosphorylation of Dbf4-Cdc7, a protein kinase required for the firing of all origins (214), and in higher cells through inhibition of Cdc25A and Orc function (59). Inhibition of mitosis in budding yeast involves regulation of Esp1 and Pds1, which in turn regulate cleavage of cohesins between sister chromatids required for chromosome segregation (191). Inhibition of mitosis in higher eukaryotes involves inhibitory phosphorylation of Cdc2 by blocking Cdc25C activity (160).

The roles of Mec1 and Rad53 proteins in preventing catastrophic termination of forks are likely crucial (48, 119, 188). That checkpoint proteins might regulate stability of replication forks was first suggested by Enoch et al. (57), who found two mutants, *rad3* and *cdc2-3w*, defective in blocking mitosis of S-phase-arrested cells in *Schizosaccharomyces pombe*. The *rad3* mutant dies in S-phase, whereas the *cdc2-3w* mutant dies only upon entry into mitosis. The *S. pombe* Rad3 (ATR-like) protein kinase was thereby considered to be required for recovery from replication stress. This recovery function probably entails stabilizing slowed or stalled replication forks, now shown most convincingly in budding yeast studies.

The Diffley and Foiani groups independently showed in *S. cerevisiae* that wild-type cells slow the rate of replication when treated either with MMS to induce damage or with hydroxyurea (HU) to deplete dNTP pools. In wild-type cells fork stability is maintained such that replication by the stalled or slowed fork can resume when cellular conditions permit (119, 188). In Mec1- and Rad53-deficient cells, however, replication forks collapse and fail to resume replication. One can now infer that stability of forks is a primary determinant for damage-sensitivity in checkpoint mutants from the following observations. Hypomorphic *mec1* mutants and *mrc1* null mutants are relatively resistant to MMS, yet fail to block late origin firing or entry into mitosis (5, 143). Hence, it is inferred that *mec1* and *mrc1* mutants are damage sensitive owing to their inability to stabilize replication forks and not owing to defects in blocking replication from late origins or preventing mitotic entry. An additional implication is that checkpoint proteins may act efficiently in *cis* to retain a fork, but require more activity to act in *trans* to block late origin firing and mitotic progression. For these latter purposes, Mrc1 (and perhaps Tof1 and Rad9) may be a potentiator or amplifier of the damage signal in S-phase.

Interestingly, one can make a similar inference for mammalian cells in that fork stability, and not cell cycle progression per se, may be a key determinant of damage sensitivity. Mutants for ATM are defective for inhibition of origin firing or RDS. Suppressors of $ATM^{-/-}$ mutations have been identified that restore inhibition of late replication origins, yet the strains remain damage sensitive (95).

The mechanism of fork stabilization is not known [although it may involve regulation of DNA primase (126)], nor is the exact fate of stalled, intact replication forks known. Recently, an interaction was reported between a Holliday resolving protein and the Cds1 checkpoint protein kinase in fission yeast (16). Checkpoint proteins may well stabilize forks by preventing cleavage of key intermediates. Thus, in a larger scheme, checkpoint proteins may prevent repair of stalled forks by recombination pathways.

THE G1 CHECKPOINT

Most eukaryotic cells damaged in G1 exhibit a pronounced delay prior to S-phase. This arrest in G1 allows vital time for repair and prevents replication of a damaged template. Otherwise, uninhibited DNA replication would convert one gapped chromosome into two sister chromosomes, one of which contains a DSB. Although its role in cell physiology seems clear, the role of the G1 checkpoint is often the subject of debate because it is very different in higher compared to in lower eukaryotes.

In budding yeast, the G1 checkpoint exists but is very weak; damage induces a delay that lasts far less than an hour, and most damage remains unrepaired (61, 174). For example, a double-strand break induces no detectable delay in G1 but leads to a prolonged arrest in G2 (213). This may reflect the ability of budding yeast to efficiently repair DSBs by homologous recombination using the intact sister homolog. What little delay is detected in G1 involves Rad53-dependent

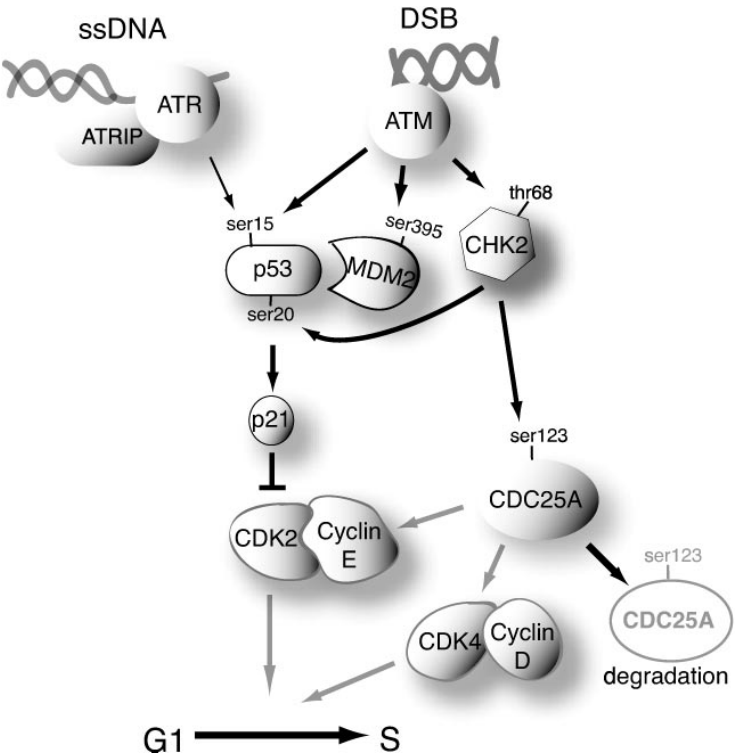

Figure 3 The G1 checkpoint in mammalian cells primarily functions to block Cdk2-cyclin E activity. This is achieved by stabilizing p53 and degrading Cdc25A to maintain Cdk2 inhibitory phosphorylation. Gray arrows denote functions that are lost upon activation of the checkpoint cascade, and labeled amino acids on proteins indicate sites of phosphorylation.

phosphorylation of the Swi4/6 transcription factors. This phosphorylation inhibits transcription of G1 cyclins, thereby slowing entry into S-phase (173).

In contrast to the weak G1 arrest in yeast, DNA damage induces a very robust G1 arrest via action of p53 in higher eukaryotes from *Xenopus* to mammals. This G1 arrest now appears to have two waves of action (Figure 3). The first immediate delay provides time for repair but lasts only several hours, whereas a slower and sometimes irreversible delay may function to remove cells from the cell cycle (12, 123).

The first delay occurs within minutes after damage, employs posttranslational modification of proteins, and is p53-independent. ATM-dependent activation of Chk2 leads to phosphorylation of Cdc25A, thereby priming it for ubiquitination and proteasome destruction (123). Loss of Cdc25A consequently regulates Cdk2 by maintaining its inhibitory phosphorylation on Tyr^{15} and inhibiting its

association with cyclin E, a necessary step for progression into S-phase. An elegant set of experiments using a cell-free system derived from *Xenopus* eggs showed that Cdk2-cyclin E inhibition also prevents complete formation of prereplicative complexes on DNA to inhibit the start of replication (40). All prereplication complex components are present—ORC, Cdc6, CDC7, and MCM proteins—except Cdc45, which requires Cdk2-cyclin E activity for assembly at origins where it then attracts DNA polymerases. Cdc25A also regulates Cdk4 activity, and, as indicated by a much earlier study performed with UV treatment, destruction of Cdc25A results in maintenance of the Tyr^{17} inhibitory phosphorylation on Cdk4 that also prevents S-phase entry (187).

A second G1 delay mechanism also involves ATM/ATR and Chk2, but takes several hours to initiate because regulation for this block to S-phase entry occurs at the transcriptional level. Here, ATM/ATR and Chk1/Chk2 activate and stabilize p53, as discussed earlier, causing transcriptional induction of p21, which then inhibits the Cdk2-cyclin E complex (55, 168). The p53-dependent block in G1 maintains the arrest initiated by the Cdc25A pathway to allow sufficient time for repair of DNA damage. How recovery from arrest is achieved or whether, in fact, the p53-dependent delay is reversible in all cell types is poorly understood (27, 49). Also undetermined is whether these two checkpoint cascades can occur independently; that is, can limited damage that requires little time for repair induce only the posttranslational cascade for a brief delay, or are both cascades always activated together, regardless of the extent of damage (12)?

Whether G1 arrest or apoptosis is more important in mammalian cells in preventing cancer is as yet unresolved. If both mechanisms function to remove damaged cells from cycling, then both may play prominent roles in cancer evasion.

THE S-PHASE CHECKPOINT

Description of S-phase checkpoint pathways is under way, driven predominantly by studies of budding and fission yeast. Based on details inferred from genetic observations, we present models of each organism in an attempt at deriving unifying mechanisms. Details of the fates of forks in the two yeasts are similar, but not identical, and the possibility that mammalian cells exhibit similar features is addressed.

A traditional view held by many is that cells in S-phase activate one of two checkpoint pathways to either signal DNA damage through the intra-S checkpoint or aberrant replication forks through the replication checkpoint. Due to key features shared by these two pathways, we believe that these two responses can be integrated into one pathway, termed simply the S-phase checkpoint. Much of this model is based on key observations of alternative pathways in S-phase control both in budding yeast (Figure 4) and fission yeast (Figure 5) (5, 64, 184).

The first step in S-phase checkpoint activation is stalling of replication forks owing either to the depletion of dNTPs or to an encounter with DNA damage. We consider these two forms of damage similar because they both slow replication fork

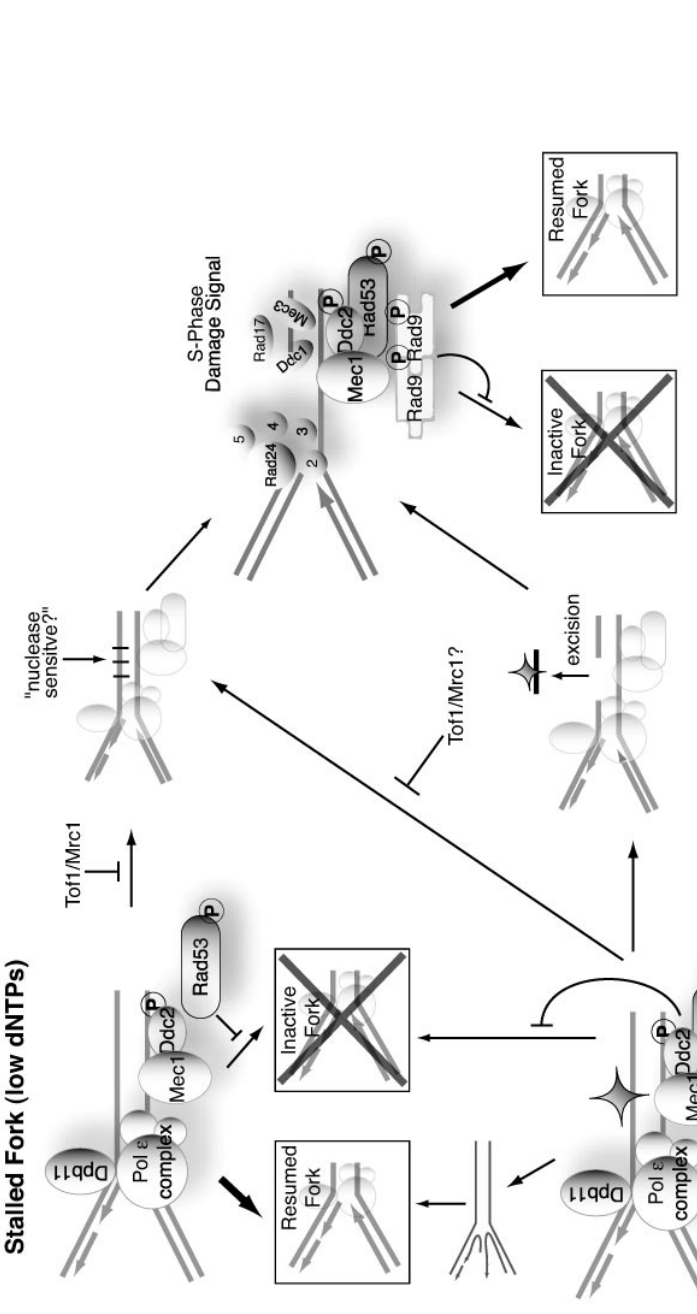

Figure 4 In budding yeast, replication forks stall upon encountering DNA lesions or low dNTP pools, signaled by proteins localized at sites of replication. Tof1 and Mrc1 block the conversion of stalled forks prone to form alternative structures. Potential attack by nucleases or lesion processing induces activation of the checkpoint sensors that specifically recognize DNA damage to further maintain fork stability.

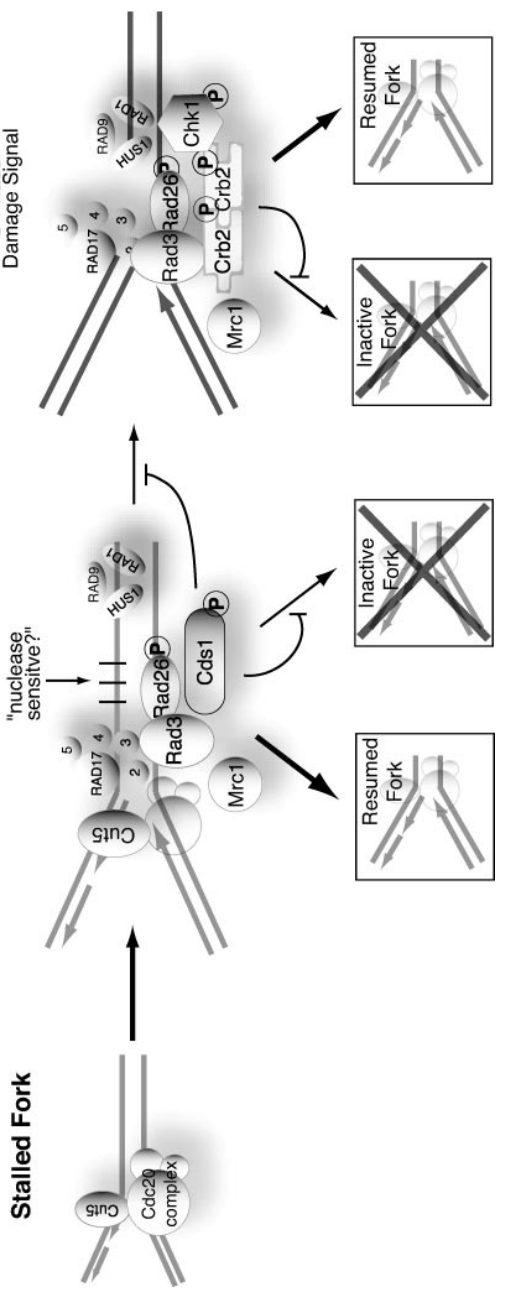

Figure 5 In fission yeast, stalled replication forks are immediately converted to alternative structures that are blocked from possible nuclease attack and collapse. Mrc1 additionally functions to block possible nuclease attack. Those forks that undergo nuclease processing activate the checkpoint sensors that respond to DNA damage to maintain fork stability.

progression and either insult can culminate in fork collapse in checkpoint mutants. Both mechanisms of stalling replication forks activate the S-phase checkpoint pathway by proteins that are localized at the fork sites. The proteins associated with replication forks that display checkpoint activity include the DNA helicases Sgs1 and Top3 (31, 66); Pol2, a subunit of DNA pol [essential for DNA replication and implicated in DNA repair (138)]; Dpb11, a protein that interacts with and recruits Pol2 to replication origins (204); Drc1, an essential protein for DNA replication that interacts with Dpb11 (203); and RFC subunits 2, 3, 4, and 5 (102, 137, 139, 172). These proteins, along with contributing functions from Mrc1 and Tof1, somehow activate Mec1 and Rad53 to carry out the common S-phase responses detailed in Figure 4 (5, 64).

To explain the role of budding yeast Rad9 within S-phase, we propose that a stalled fork can be converted to a different fork form that activates other checkpoint proteins, like Rad9, to somehow maintain or restore fork stability (Figure 4). Based on genetic analysis, we suggest that the primary stalled fork is prevented from conversion to this other form by Tof1 and Mrc1, because in the absence of Tof1 or Mrc1, S-phase checkpoints become dependent on Rad9 (5, 64). These "converted" forks can resume replication provided that they activate Mec1 and Rad53 for fork stabilization.

We suggest that a stalled fork at a lesion is subject to an additional fate. Lesion processing followed by replication generates a fork with a DSB. In this situation, the DNA damage created is sufficient to signal the damage checkpoint proteins, such as Rad9, that then prevent late origin firing and spindle elongation via Mec1 and Rad53. Therefore, the damage checkpoint serves as a backup to the replication checkpoint. This model explains why damage induces a weak Rad9-dependent S-phase response that becomes greatly enhanced in cells lacking either Tof1 or Mrc1 (5, 64).

We propose that in fission yeast, and therefore perhaps in mammalian cells, a similar, though not identical, progression of replication fork conversions may occur (Figure 5). First, replication forks blocked in fission yeast always activate Rad3 using the damage sensor proteins and Mrc1 (184). We speculate that a stalled replication fork may constitutively convert to an altered form that then signals using damage sensor proteins. Next, this fork intermediate may convert to another form, a process actively prevented by Cds1/Chk2 (113). This is supported by the key observation that *cds1* mutants are not appreciably sensitive to hydroxyurea, and when HU-treated require Chk1 activity for viability (113, 127). We suggest that Cds1 prevents the conversion of the first broken fork structure, while Chk1 acts as a failsafe mechanism, like budding yeast Rad9, to rescue any forks that have converted to the second altered fork form. Tests of these models linking DNA fork structures to protein functions are close at hand. Foiani and colleagues have shown that replication forks formed in yeast can be isolated and their structures determined by electron microscopy (178a).

In mammalian cells, the genetic basis of S-phase checkpoints has also been well documented (144), though the underlying mechanisms remain obscure. BRCA1,

ATM, and MRN complexes are involved after γ-irradiation, and ATR is likely involved after UV damage (59, 112, 208, 220, 227, 228). Cdc25A is also implicated as a downstream effector since it appears to regulate ORC complexes at initiation (59). It is unknown whether mammalian replication forks experience similar options of conversion as we propose in yeast. Differences between lower and higher eukaryotes include much longer tracts of DNA between origins in higher eukaryotes, suggesting that processivity might be more critical in mammals and may require other or additional forms of regulation by checkpoints. Whatever mechanisms operate in eukaryotic cells, fork stability mediated by checkpoint proteins is likely to be critical for genome stability. Furthermore, if replication defects are ongoing and persistent in cancer cells, then the potential for developing therapeutic strategies based on defects in replication fork regulation are attractive.

THE G2/M CHECKPOINT

Though well characterized in both yeast systems, the details of G2/M arrest in higher eukaryotic cells are still being determined. The picture thus far is that fission yeast and higher eukaryotes, both target Cdc2 to maintain its inhibitory phosphorylations as a principal means to block the G2/M transition. This is achieved by targeting various parallel pathways, including the phosphatases that promote mitosis, the kinases that block Cdc2 function, and other proteins that regulate these regulators, which all converge to regulate Cdc2 activity (Figure 6).

DNA damage in mammalian cells causes the activation of four different downstream kinases that independently block the activity of Cdc25C, the phosphatase that promotes mitosis by removing phosphates at Cdc2 inhibitory sites. First, activation of ATR by damage leads to Chk1 phosphorylation (83). Chk1 then negatively regulates Cdc25C by phosphorylating it at Ser^{216} (151, 160). This phosphorylation of Cdc25C creates a binding site for 14-3-3 proteins, and in the bound state renders Cdc25C either catalytically less inactive, sequestered in the cytoplasm, or both (78, 120). In addition to activation by ATR, Chk1 may be activated by BRCA1 to induce a G2/M arrest, although this has yet to be confirmed by in vitro analysis (226). By a second mechanism, ATM activation by DNA damage in G2 leads to Chk2 activation that also phosphorylates Cdc25C at Ser^{216} to block its function (129, 233). And now two new regulators of Cdc25C are emerging, both members of the Polo-like kinase (Plk) family. Plk3 resembles Chk1 and Chk2, as it is activated in an ATM-dependent manner, physically interacts with Cdc25C, and phosphorylates this protein at Ser^{216} to inhibit its activity (177, 218). Unlike the kinases that inhibit Cdc25C activity by Ser^{216} phosphorylation, Plk1 is recognized for its role in promoting mitotic entry by phosphorylating Cdc25C to activate it. Both ATR and ATM are thought to phosphorylate Plk1, perhaps via Chk1 and Chk2 activation, to block Plk1 activation of Cdc25C (177, 197). Plk1 inhibition correlates with a decrease in Cdc2-cyclin B kinase activity, and this inhibition is completely

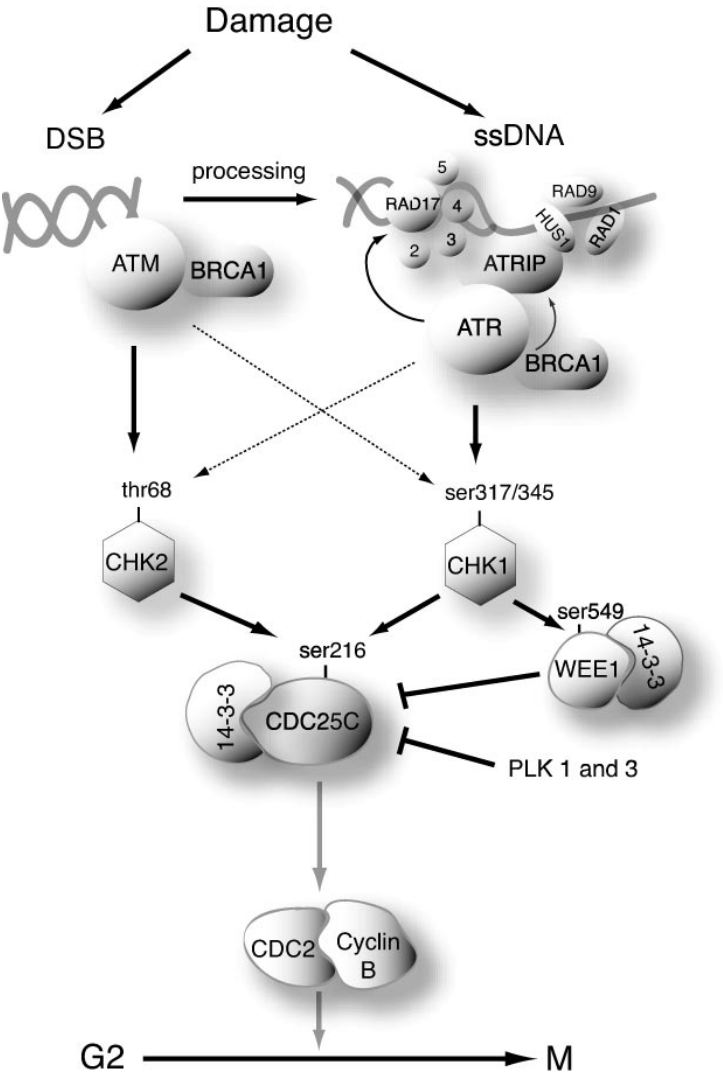

Figure 6 The G2 checkpoint in mammalian cells primarily functions to block Cdc2-cyclin B activity. The common means of maintaining Cdc2 inhibitory phosphorylation is by blocking Cdc25C phosphatase activity, namely by promoting its association with 14-3-3-proteins. Gray arrows denote functions that are lost upon activation of the checkpoint cascade, and labeled amino acids on proteins indicate sites of phosphorylation.

blocked by caffeine, an inhibitor of ATM/ATR. Additionally, expression of an unphosphorylatable Plk1 cannot be inhibited in the presence of DNA damage and does not initiate a G2/M arrest, further implicating Plk1 as another regulator of Cdc25C activity (177). Adding an additional level of G2/M control, Plk1 protein stability now appears to be regulated by the checkpoint protein Chfr, which delays entry into mitosis when cells are under mitotic stress (96, 166). Using *Xenopus* cell-free extracts, Kang et al. showed that Chfr ubiquitinates the human Plk1 to target it for degradation, thereby inhibiting Cdc25C activation, resulting in a delay in Cdc2 activation (96). The mechanism of Chfr activation has yet to be determined.

While the majority of 14-3-3-bound Cdc25C localizes to the cytoplasm, some Cdc25C remains in the nucleus (233), implying that other mechanisms are required for inhibition of Cdc25C phosphatase activity. In mammalian cells, inhibition may be accomplished by the Cdk inhibitor p21 by the following mechanism (7). p21, Cdc25C, and Cdc2-cyclinB can all bind PCNA, but not all at the same time (85, 99, 219). PCNA may act as a platform to mediate protein interactions, and binding of p21 and Cdc25C to a PCNA-Cdc2-cyclin B complex may be a mutually exclusive event (7). Therefore, p21 interaction with PCNA-Cdc2-cyclin B may exclude Cdc25C from interacting with Cdc2 to dephosphorylate it for mitotic progression. By an additional method, p21 may act directly on Cdc2. Although p21 does not affect the inhibitory phosphorylation sites (Thr^{14} and Tyr^{15}) on Cdc2, p21 does block the activating phosphorylation of Cdc2 on Thr^{161}, mediated by the Cdk-activating kinase, CAK (177).

Another major player in the G2/M arrest is the 14-3-3 protein. 14-3-3 not only regulates Cdc25C as discussed previously, but it also regulates Wee1. Experiments using *Xenopus* extracts demonstrated that 14-3-3 binds to Wee1 when phosphorylated at Ser^{549} and that this site on Wee1 is phosphorylated by Chk1 (108). By both recombinant protein and immunodepletion analysis, Chk1 phosphorylation of Wee1 promotes 14-3-3 binding, which significantly enhances the inhibitory kinase activity of Wee1 toward Cdc2. By another mechanism identified in colorectal cell lines, p53 mediates 14-3-3σ induction after damage; σ expression is needed for G2/M arrest and to prevent "mitotic catastrophe" (33, 89). Based on Western analysis of immunoprecipitated complexes, 14-3-3σ appears to bind to the Cdc2-cyclin B complex to sequester it in the cytoplasm to maintain G2 arrest (33). Whether the same mechanism functions in non-colorectal cells is unknown.

Myt1 is yet another possible target of checkpoint mechanisms in mammalian cells. The Myt1 fission yeast ortholog, Mik1, is regulated by checkpoint components. After DNA damage or replication defects Mik1 protein accumulates in a Chk1-dependent manner that is required for both initiation and maintenance of a G2/M arrest (9, 154). Checkpoint regulation of Myt1 in mammalian cells has not yet been demonstrated, however overexpression of Myt1 induces a G2 delay by retaining Cdc2-cyclin B in the cytoplasm (115). Additionally, Myt1 is down-regulated in a cell-cycle-dependent manner through phosphorylation by Akt, leading to the meiotic G2/M transition (141).

Regulation of cyclin B may also contribute to the G2/M arrest (21). Studies from two laboratories demonstrated that induction of DNA damage leads to retention of cyclin B in the cytoplasm and that forcing nuclear localization of cyclin B partially abrogated the G2/M damage-induced arrest (94, 192). Moreover, cyclin B and Cdc2 are both transcriptionally down-regulated upon checkpoint induction, correlating with a reduction in protein, which further reinforces the G2 arrest (43, 185).

In response to UV damage only the mitogen-activated protein kinase p38 is rapidly activated to induce a G2/M delay. Active p38 phosphorylates Cdc25B at Ser^{309}, demonstrated both in vitro and in vivo, to induce 14-3-3 binding, which then leads to the arrest response (22). 14-3-3 binding to Cdc25B blocks access of substrates to the catalytic site to inhibit Cdc25B activity (63). Notably, p38 apparently plays no role in affecting 14-3-3 binding to Cdc25C in vivo (22).

The budding yeast G2/M checkpoint arrests cells principally at the metaphase-anaphase transition, and the effectors targeted to cause arrest differ from those in fission yeast and higher eukaryotes. This checkpoint primarily halts arrest by two mechanisms that converge on the regulation of degradation of cohesins [(191, 224; reviewed in (6)]. The anaphase promoting complex (APC) plays a major role in regulating G2/M in budding yeast. If future work demonstrates that the APC in higher eukaryotes is subject to checkpoint regulation in response to DNA damage, with the polo-like kinases likely targets (159, 177), then insights from budding yeasts mechanisms can be drawn to direct experimentation in mammalian cells or *Xenopus* egg extracts.

DAMAGE REPAIR AND RECOVERY

DNA Checkpoints and Their Link to Repair

The checkpoint proteins arrest the cell cycle in the presence of DNA damage, presumably to allow the cell time for repair. The checkpoints are proposed to act not simply as a switch for arrest, but also to directly recruit DNA repair machinery or perhaps even play direct roles in repairing DNA. A role in repair is borne out by the fact that checkpoint-deficient cells temporarily arrested with drugs after infliction of DNA damage are not as viable as similarly treated wild-type cells [reviewed in (156)]. Thus, checkpoint proteins clearly have roles presumably in DNA repair above and beyond that of inducing cell cycle arrest and fork stability. Here we present some of the indirect links and the few direct links between checkpoint machinery and DNA repair mechanisms uncovered thus far.

Yeast and mammalian cells indirectly contribute to repair responses by inducing transcription of a number of genes encoding proteins involved in repair (12, 62, 70, 111, 153, 165). Checkpoint proteins also regulate dNTP pools, perhaps a common feature involved in DNA repair, since limiting dNTP pools enhances damage sensitivity (183, 210, 231).

Hard evidence supporting the notion that checkpoint and repair proteins interact to enhance cell survival is scarce, whereas soft evidence abounds. ATM is linked to the repair of DNA damage by homologous recombination (HR) based on the fact that AT cells defective in HR components (*rad54* mutation) are no more sick than AT cells, whereas AT cells defective for non-homologous end-joining (NHEJ; *ku70* mutation) show drastic increases in chromosomal instability (134). The inference is that $ATM^{-/-}$cells are already defective for HR, resulting in no increased defect upon *rad54* mutation, while they are proficient for NHEJ. BRCA1, also implicated in HR repair (135) as well as transcription-coupled repair (1, 77), is thought to be involved in various repair processes. BRCA1 transiently associates with a number of repair proteins, including mismatch repair proteins, Nbs1, and the BLM helicase, in the BRCA1-associated genome surveillance complex (BASC) after DNA damage treatment (207). Colocalization after damage induction has also been demonstrated for BRCA1 and Rad51 (15), BRCA1 and PCNA (167), and BRCA1 and the MRN complex (232). Based on the DNA damage-sensitivity of Mre11-Rad50-Nbs1 mutants, their ability to process DSBs, and their genetic grouping with the Rad52 epistasis group, it was assumed that the MRN complex also plays a role in HR. However, numerous studies suggest that the roles of the MRN complex in arrest and in repair (either by HR or by NHEJ) are distinct [reviewed in (44)].

One protein likely to be involved in both signaling and repairing damage is RPA, the heterotrimeric ssDNA binding protein. RPA contributes essential functions to DNA replication, repair, and recombination [reviewed in (93)]. Studies in both mammals and yeast indicate that the middle RPA subunit, Rpa2, is phosphorylated by ATM (DNA-PK phosphorylation has also been demonstrated) following fork stalling and DNA damage induction, and this phosphorylation abrogates DNA replication as measured by in vitro SV40 replication (20, 72, 140, 205). Since Rpa2 phosphorylation is likely ATM-dependent and, at least in budding yeast, downstream factors (Rad9 and Rad53) are not required for this event, Rpa2 phosphorylation may be necessary to stabilize stalled replication forks or to halt replication until DNA damage is repaired. The involvement of Rpa2 phosphorylation in mediating DNA repair is currently unclear. Interestingly, in budding yeast the large RPA subunit (Rpa1) is additionally phosphorylated by Mec1, and this event requires Rad9 and the downstream kinase Rad53, placing the event farther down the checkpoint cascade (19). Whereas Rpa2 is also normally phosphorylated during the cell cycle, as well as in response to damage, Rpa1 is only phosphorylated upon genotoxic and HU insult, and the extent of phosphorylation correlates with the amount of damage induced. Given the late timing and modulation by levels of DNA damage within the cell, Rpa1 phosphorylation mediated by Mec1 may be involved in some aspect of DNA repair (117).

The only conclusive evidence tying the checkpoint response directly to DNA repair is that of Rad55 activation in budding yeast and Crb2 regulation in fission yeast. In budding yeast, Rad55, a recombinational repair protein, undergoes specific phosphorylation mediated by Mec1 following treatment by numerous damaging

agents (13). Mutant *rad55* cells are proficient for arrest and induced gene expression in response to DNA damage, but a *mec1* mutant that cannot phosphorylate Rad55 shows an 88-fold reduction in the amount of damage-induced recombination. That Rad55 is a terminal substrate of the checkpoint cascade and that a major repair defect is evident when Mec1 fails to phosphorylate Rad55 suggests a link between checkpoints and repair processes. In fission yeast, the checkpoint protein Crb2 has a role in regulating topoisomerase III (Top3; 29). Crb2 hyperphosphorylation in response to DNA damage not only initiates a checkpoint arrest response, but Crb2's hyperphosphorylated state is needed to regulate Top3 activity to prevent aberrant hyperrecombination mediated by the RecQ-like helicase, additionally linking checkpoints and repair mechanisms.

Recovery, Adaptation, and Apoptosis

Upon repair of damage, cells resume cycling. But is the completion of repair per se sufficient to turn off the checkpoint arrest? Indeed, data indicate that the checkpoint cascade must be actively down-regulated before the cell cycle can resume. In budding yeast, overexpression of Ddc2 (the ATRIP ortholog) results in an irreversible G2/M arrest after DNA damage (36). One interpretation of this result is that the ability to shut off the checkpoint response is abrogated in Ddc2-overexpressing cells. Additional evidence that recovery from damage is an active process is supported by the fact that yeast undergo adaptation, a process by which cells resume cell cycle progression despite the presence of unrepaired DNA (161). The activity of Rad53 and Chk1 must be down-regulated to permit adaptation, indicating that the kinase cascade must be shut off (150). Adaptation involves Cdc5, which may operate in a feedback loop to inactivate the checkpoint cascade (159). Additionally, the inactivation event may be a regulatory phosphorylation on Rad9, a necessary protein for Rad53 activity. Fission yeast employ such a mechanism of terminating arrest whereby they shut off the activity of Crb2, the Rad9 ortholog, via inhibitory phosphorylation carried out by Cdc2 (58). The clearest example of checkpoint inactivation in mammalian cells involves the p53-inducible protein Wip1. Genotoxic stress leads to p53 activation by p38 phosphorylation, consequently inducing transcription of a number of genes, including Wip1 (182). Accumulation of the Wip1 phosphatase then inactivates p38, demonstrated both in vivo and in vitro, leading to p53 transcriptional down-regulation and a reduced apoptotic response. Attenuation of cell cycle arrest was not specifically examined, but it, too, is predicted to be affected.

In many higher eukaryotes, extensive DNA damage that cannot be repaired during a checkpoint-controlled cell cycle arrest is thought to initiate cell death via the apoptotic pathway. That p53 is a key effector of ATM/ATR protein kinases during checkpoint responses strongly suggests that checkpoint controls and apoptotic controls are coordinated processes. For example, mice harboring mutations in p53 or ATM are defective in response to DNA damage for both checkpoint activation and apoptosis [reviewed in (100)]. Also, studies in *Caenorhabditis elegans* suggest

a prominent role in arrest and apoptosis for an orthologous Rad1 sensor protein in germ cells (68). (Curiously, somatic cells in *C. elegans* do not undergo either fate, only their germ cells do.) Whether checkpoint protein interaction with damage is somehow central to the switch between arrest and apoptosis or whether the decision lies elsewhere in signal transduction is unknown. Association between checkpoint pathways and apoptosis is suggested by the interaction between the human Rad9 damage sensor protein and BCL apoptotic proteins (105).

ADDITIONAL ROLES OF CHECKPOINT PROTEINS IN DNA METABOLISM

The checkpoint proteins initially identified as controlling the cell cycle have many additional roles in DNA metabolism. These include roles in transcription of damage-responsive genes and roles in DNA repair (discussed in the "Repair" section), telomere biology, meiotic functions different from those operating in mitotic cells, and control of dNTP pools. Furthermore, proteins that act together in one pathway or response may not act together in a second response, indicating that "complexes" do not necessarily function constitutively.

Prominent among the additional roles of checkpoint proteins is their part in telomere biosynthesis. In mammals, ATM mutants have shorter telomeres, as do budding yeast *tel1* mutants. These initial observations led the way to the remarkable finding that the fission yeast Rad3 and Tel1 proteins are required for telomere addition (130), followed by similar findings for budding yeast Mec1 and Tel1 proteins (155). Tel1 appears to recruit telomerase to sites of novel telomere addition, although exactly how Tel1 does so and whether Mec1 does so as well is unknown. In budding yeast, Mec1 and Dun1 also affect gene expression of telomere-proximal genes, suggesting a role in chromatin structure (42, 47). An effect on telomere chromatin is also indicated by the finding that in budding yeast some repair and chromatin proteins are relocalized from the telomeres to sites of DNA damage—recruitment that requires checkpoint protein functions [Mec1, Rad9, and Rad53 (118, 131, 133)]. An additional and opposite effect on telomeres is mediated by budding yeast Mec3 and Rad17 proteins; inexplicably, *mec3* mutants have shorter telomeres and *rad17* mutants have longer telomeres (37, 118). Recall that Mec3 and Rad17 both act coordinately in cell cycle arrest in what is the equivalent of the 9-1-1 mammalian complex. *rad1* mutants in *C. elegans* also have shorter telomeres (3), implying a role for this class of proteins in telomere biosynthesis in many organisms. That checkpoint proteins might interact with telomeres is not unexpected, given that telomeres, by their nature, might resemble a DSB. Hence, checkpoint proteins might be expected to interact with telomeres because they may resemble damage and/or to perhaps aid in their protection

Study of meiosis has provided yet another fertile ground for discovery of checkpoint protein functions. Checkpoint proteins play many roles in meiosis, including imparting two cell cycle delays and regulating partner choice in recombination (82, 121, 189). Checkpoint protein functions in meiosis are sometimes

divergent from their roles in mitosis. For example, Rad9 is not required for early meiotic arrest (121), and members of the 9-1-1 complex act at independent steps during meiotic arrest (91, 189). Meiotic studies also reveal the diversity of arrest mechanisms involving Cdc2 in different organisms. The budding yeast meiotic arrest (called the pachytene arrest) and the fission yeast mitotic G2/M arrest after DNA damage involves inhibitory phosphorylation of Cdc2, yet the budding yeast mitotic G2/M arrest and the fission yeast meiotic arrest enigmatically do not. The mechanisms of meiotic arrest in fission yeast remain unclear.

Finally, budding yeast checkpoint proteins have other important roles in the regulation of DNA replication independent of cell cycle progression and replication fork stability. Mec1 and Rad53 regulate the synthesis of dNTPs by blocking activity of Sml1, an inhibitor of ribonucleotide reductase (30). Mec1 and Rad53 inhibit Sml1, illustrated by the fact that *mec1* and *rad53* mutants are inviable due to Sml1 inhibition of the ribonucleotide reductase protein (Rnr1) and that *mec1* and *rad53* mutants become viable when *sml1* is deleted (230). Mec1 also appears to regulate replication fork movement in specific "replication slow zones" within the yeast chromosome, and slow replication is again relieved by mutation of *sml1* or up-regulation of *RNR1* (48; R. Cha & N. Kleckner, manuscript submitted). To this end, Mec1 may somehow regulate replication fork progression and/or more simply regulate the level of dNTPs.

CONCLUDING REMARKS REGARDING GENOME STABILITY

The importance of checkpoints in biology, and especially in human cancer, is likely due to their preeminent roles in maintaining genomic stability. Multiple significant links between checkpoint proteins and genome stability have been identified in model organisms, as well as in mammalian cells. In mammals, ATM, ATR, BRCA1, Chk2, and p53 mutants all show instability. For ATM, some of this instability may be due to shortening of telomeres, leading to telomere fusions and breakage-fusion-bridge events [reviewed in (145)]. However, like most other checkpoints proteins, ATM is likely involved in preserving stability by several mechanisms independent of telomere biology. For example, $ATM^{-/-}$ mutants exhibit elevated mitotic recombination (104). In another example, BRCA1 mutants have partial defects in homologous recombination and transcription-coupled repair (1, 135). As better systems to study instability in mammalian cells are developed, our understanding of these processes should improve.

In budding yeast, the roles of checkpoint proteins in genome stability are being examined. General mechanisms linking checkpoint protein function and genome stability are illustrated by four examples discussed below. In the first example, Paulovich et al. (148) showed that after UV irradiation, checkpoint

proteins promote error-prone repair carried out by Rev3/Rev7, which form a polymerase complex that replicates over DNA damage by inserting noncognate nucleotides. Whether a cell cycle delay is required for Rev3/7 function or whether checkpoint proteins act more directly, perhaps by recruiting Rev3/7 to UV lesions, is unknown. Second, checkpoint proteins seem to promote allelic recombination (13). Defects in this process lead to an increased frequency of ectopic recombination in meiotic (82) and mitotic cells (60), as well as defects in intergenic mitotic recombination (13). Third, *rad9* and other checkpoint mutants spontaneously lose chromosomes (103, 212). One mechanism of loss was, until recently, thought to involve mitosis of a cell with a chromosome that has an unrepaired DSB. Surprisingly, a recent study indicates that a cell completely defective for the G2/M checkpoint (a *dun1 chk1* double mutant) undergoes no detectable spontaneous chromosome loss (103). Therefore, chromosome loss in *rad9* mutants is likely due to a defect in some other cellular activity.

Finally, and in our view most significantly, checkpoint proteins may play key roles in maintaining stalled replication forks, because when defective the collapsed forks may lead either to cell death or to genomic rearrangements. Myung & Kolodner (136) analyzed the effects of checkpoint mutations on a spectrum of chromosomal arrangements that they call gross chromosome rearrangements, or GCR. They suggest that GCRs in yeast structurally resemble those rearrangements seen in cancer cells. By surveying checkpoint mutants for GCR they found that the proteins regulating S-phase responses generally had significant roles in maintaining stability. Defects in both Mec1 and Tel1 lead to a 10,000-fold increase in instability. But which of their many functions contributes to stability remains uncertain; they mediate essentially all responses to DNA damage, are required for telomere synthesis, and play roles in DNA replication. Defects in Pds1 also strongly affect stability, probably due to a role in sister chromatin cohesion (rather than to a defect in its role in G2/M arrest, since *chk1* mutants having a similar arrest defect demonstrate no instability). Linking complex replication fork biology, checkpoint protein function, and genome stability remains a major challenge, but one we anticipate is fundamental to understanding cancer etiology and treatment.

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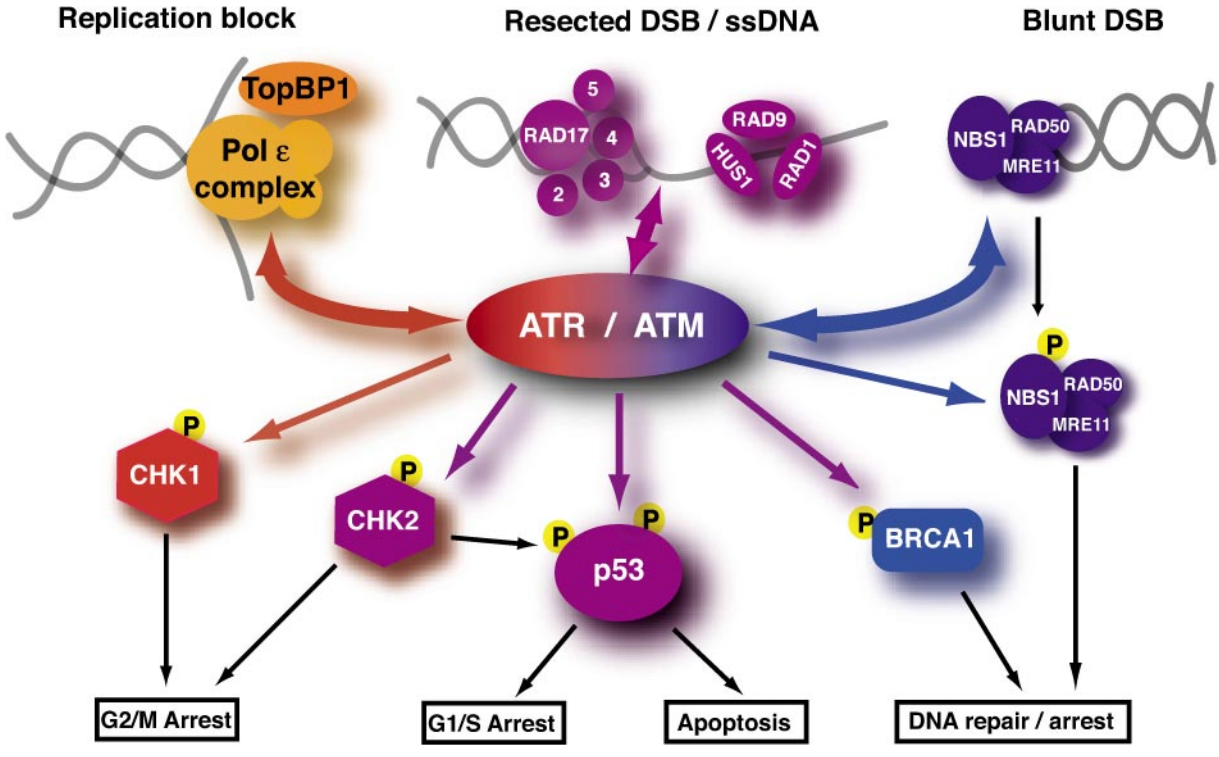

Figure 1 ATR and ATM are the central proteins regulating checkpoint responses to various forms of DNA damage. Activation of ATR and its downstream effects are indicated in red, whereas activation and downstream effects of ATM are indicated in blue (not absolute; some exceptions of overlapping function do occur). Signals and downstream effects common to both kinases are designated in purple.