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Sensing, Signaling, and Responding to DNA Damage: Organization of the Checkpoint Pathways in Mammalian Cells

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Abstract The DNA damage and replication checkpoints are signaling mechanisms that regulate and coordinate cellular responses to genotoxic conditions. Unlike typical signal transduction mechanisms that respond to one or a few stimuli, checkpoints can be activated by a broad spectrum of extrinsically or intrinsically derived DNA damage or replication interference. Recent investigations have shed light on how the damage and replication checkpoints are able to respond to such diverse stimuli. The activation of checkpoints not only attenuates cell cycle progression but also facilitates DNA repair and recovery of faltered replication forks, thereby preventing DNA lesions from being converted to inheritable mutations. Recently, more checkpoint targets from the cell cycle and DNA replication apparatus have been identified, revealing the increasing complexity of the checkpoint control of the cell cycle. In this article, we discuss current models of the DNA damage and replication checkpoints and highlight recent advances in the field. J. Cell. Biochem. 94: 298–306, 2005.

Key words: DNA damage; replication interference; cell cycle checkpoints; cell cycle arrest; genomic instability; DNA damage recognition

Fidelity of the eukaryotic genome is maintained by coordinated actions of cellular pathways, including DNA repair, chromatin remodeling, apoptosis, and cell cycle checkpoints. The DNA damage checkpoint surveys the structural integrity of genomic DNA, while the DNA replication checkpoint monitors various aspects of DNA synthesis. Albeit seemingly distinctive in functions, the DNA damage checkpoint and the replication checkpoint share many critical components (Fig. 1) and are therefore functionally linked. Under genotoxic conditions, the sensors of DNA damage or replication interference generate distinct checkpoint signals, primarily in the form of kinase-mediated protein phosphorylations. Such signals, often bearing lesion specificity, are processed to effect downstream targets. The end targets of the checkpoints include components of the cell cycle, DNA replication, and DNA repair machinery. By attenuating cell cycle progression and/or DNA synthesis, checkpoints afford repair mechanisms extra time to remove DNA lesions and allow disrupted replication forks to recover rather than giving rise to strand breaks. Furthermore, checkpoints may also play active roles in stimulating and coordinating DNA repair and replication fork recovery. Thus, checkpoints are specialized signal transduction mechanisms responsible for maintaining genetic integrity by controlling both "gatekeepers" and "caretakers" [Kinzler and Vogelstein, 1997]. Collectively, these damage-control mechanisms minimize the risk of DNA lesions being converted into inheritable mutations. Deficiencies in the damage and replication checkpoints have a profound impact on

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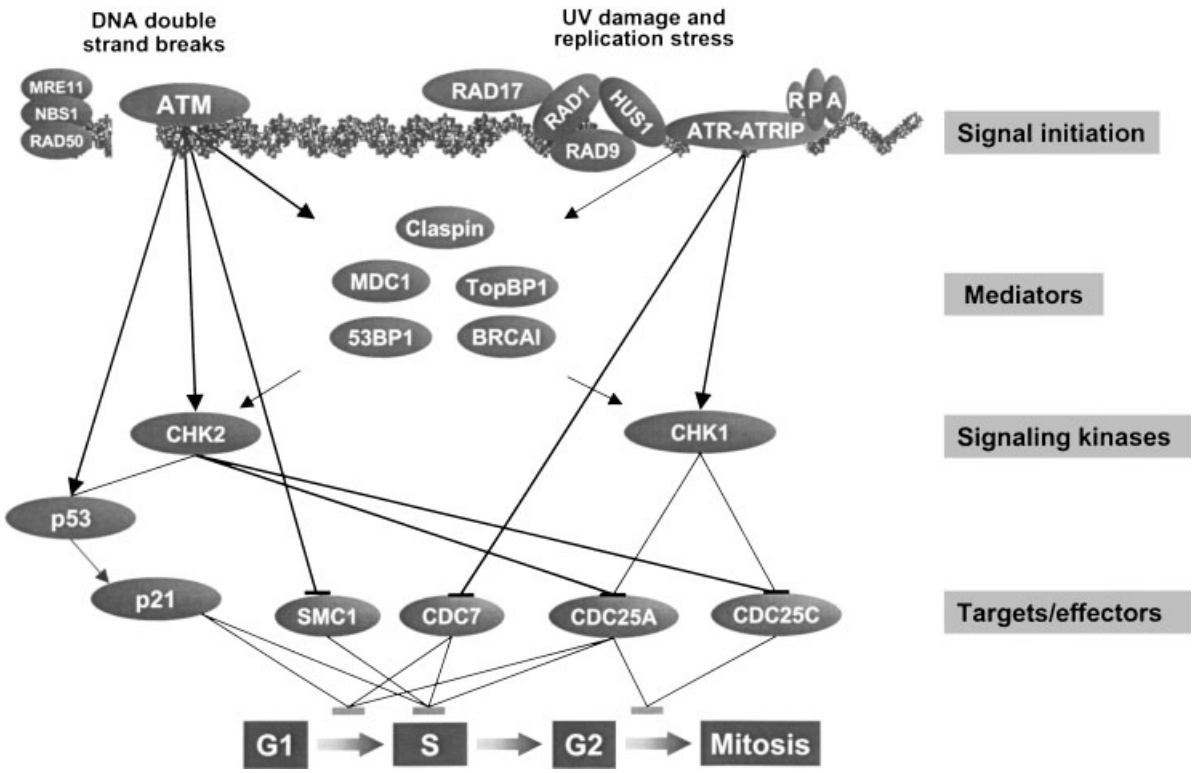

Fig. 1. Current model of the mammalian DNA damage and replication checkpoints. A line ending with an arrowhead indicates activation. A line ending with a bar indicates inhibition.

genomic stability. The biological functions of many important tumor suppressor genes, such as ATM, p53, and Brca1, are recognized in the context of the damage and replication checkpoints. Moreover, genomic instability arisen from compromised checkpoint mechanisms has been firmly linked to cancer development [Hartwell and Kastan, 1994; Kinzler and Vogelstein, 1997].

Studies from the past decade have identified many gene products participating in the damage and replication checkpoints in eukaryotes. Genetic analyses, particularly those carried out in budding and fission yeast systems, have established the overall framework of the checkpoints. However, many important questions remain unanswered, and new components of the mammalian checkpoints continue to emerge. Here, we focus on the molecular organization of the damage and replication checkpoints and provide an updated view of several critical aspects. Like many other signaling pathways, the damage and replication checkpoints can be divided into three sequential elements: damage recognition and signal initiation, signal mediation and processing, and targeting of effector processes [Elledge, 1996; Zhou and Elledge, 2000]. The contents of our discussion are organized accordingly.

DAMAGE RECOGNITION AND SIGNAL INITIATION

As cellular DNA is a highly reactive molecule, its structural integrity is constantly threatened by endogenous and exogenous genotoxic agents. When a threshold is reached, presumably when the extent of damage is beyond the steady-state capacity of DNA repair pathways, a checkpoint signal is produced. Two protein kinases of the PI-3-kinase-like kinase family, ATM and ATR, are central to the initiation of the damage and replication checkpoints in response to various genotoxic agents. These two kinases are the signal initiation point of two major branches of the damage and replication checkpoints [Abraham, 2001]. The kinase activity of ATM is activated when DNA double-strand breaks (DSBs) occur [Canman et al., 1998], whereas ATR responds broadly to DNA damage and replication interference such as that caused by hydroxyurea treatment [Abraham, 2001]. Activation of the ATR kinase, as assessed by its primary target, Chk1, requires its associated

protein ATRIP [Cortez et al., 2001] and two additional protein complexes, the Rad17 and 9-1-1 complexes. The 9-1-1 complex, containing Rad9, Rad1, and Hus1, structurally resembles the trimeric proliferating cell nuclear antigen (PCNA) clamp that functions in DNA replication and repair [Venclovas and Thelen, 2000]. The Rad17 protein, structurally similar to subunits of the replication factor C (RFC) [Griffiths et al., 1995], forms a RFC-like complex with the four small RFC subunits that acts as a DNA damage-activated loader of the 9-1-1 clamp [Bermudez et al., 2003; Ellison and Stillman, 2003; Zou et al., 2003; Majka et al., 2004]. Loss of Rad17 or Rad1 have a major impact on ATR-dependent checkpoint signaling but not on ATM-dependent Chk2 activation [Weiss et al., 2002; Wang et al., 2003; Zou and Elledge, 2003; Bao et al., 2004]. The interactions among ATR-ATRIP, Rad17, and Rad9 complexes might be critical for initiating the checkpoint signals in response to DNA damage and replication disruption.

From a molecular perspective, DNA lesions can be grouped according to the chemical nature of base modifications. Major categories of DNA lesions include dimers, mismatches, base modifications (oxidative damage, aberrant methylation, depurination, etc.), bulky adducts, intrastrand and interstrand cross-links, and single-strand breaks and DSBs [Friedberg et al., 1995]. Each type of lesion is detected by damage recognition proteins from specialized repair pathways, such as base excision repair, nucleotide excision repair, nonhomologous-end-joining, and recombinational repair.

In contrast to these lesion-specific recognition mechanisms, DNA damage checkpoints have to respond virtually to all types of lesions as well as disruption of DNA synthesis. So far, there is little evidence that the damage checkpoints possess lesion-specific damage recognition proteins. Therefore, a number of potential damage recognition mechanisms have been examined. The prevailing model at present is that a sustained presence of single-strand DNA (ssDNA) is the triggering structure for the ATR-dependent damage and replication checkpoint, because ssDNA is generated during repair of virtually all types of DNA lesions or when replication forks are stalled [Sogo et al., 2002]. Recently, biochemical analyses have provided firm evidence in support of this model. Depletion of replication protein A, an ssDNA-binding protein complex from *Xenopus* extracts, prevents DNA replication and the binding of ATR to chromatin [You et al., 2002]. Furthermore, in a purified in vitro system, ATRIP, the regulatory partner of ATR, binds to replication protein A-coated ssDNA, revealing a direct connection between ssDNA and checkpoint signal activation [Zou and Elledge, 2003]. Additional evidence supporting this model came from budding yeast. A recent study indicates that damage processing of UV lesions, which usually produces 28–31 base long ssDNA gaps for each lesion, is required in checkpoint activation during G_1/G_0 [Giannattasio et al., 2004].

Alternative mechanisms may act in parallel or in concert with the ssDNA-based damage recognition mechanism. Physical interactions with repair proteins may also direct checkpoint signal initiation kinases to the site of damage. In human cells, ATR associates with Msh2, a protein involved in mismatch repair [Wang and Qin, 2003]. Ddc1/Rad9, a subunit of the 9-1-1 clamp, interacts with nuclear excision repair factor Rad14 during UV-induced checkpoint activation [Giannattasio et al., 2004]. Taken together, processing of DNA lesions into ssDNA-containing intermediates, accumulation of ssDNA during replication fork blockage, and interactions between repair and checkpoint sensor proteins appear to be fundamental steps in triggering activation of the damage and replication checkpoints.

Unlike ATR, ATM primarily responds to DSBs, and its activation seems to be faster than that of ATR. The Mre11-Nbs1-Rad50 (MNR) complex may be a crucial sensor for the ATM pathway. In human cells, MNR is required for the damage-induced chromatin association of ATM [Andegeko et al., 2001; Carson et al., 2003] and for efficient ATM autophosphorylation after damage [Uziel et al., 2003]. In budding yeast, Xrs2, the homolog of Nbs1, is required for localizing Tel1, the ATM homolog, to DSBs [Nakada et al., 2003]. However, a recent study reported that low levels of ATM autophosphorylation could occur in Nbs1 mutant cells, suggesting the existence of other sensing mechanisms [Kitagawa et al., 2004]. It has been proposed that ATM might sense and respond to changes of chromatin structures. However, the mechanisms of such sensing processes remain unclear. It should be noted that although MNR may function upstream of

ATM in signaling, Nbs1 itself is a substrate of ATM, and phosphorylation of Nbs1 might play a signaling role downstream of ATM activation [Gatei et al., 2000; Lim et al., 2000; Zhao et al., 2000] (see below).

SIGNAL MEDIATION AND PROCESSING

In addition to the 9-1-1 complex and Rad17, efficient activation of the ATR-dependent pathway also requires the function of several additional proteins including Brca1, Claspin, TopBP1, and Mdc1. In the case of ATM, 53BP1 and Mdc1 also appear to be critical for the phosphorylation of many ATM substrates. These proteins were proposed to function as "mediators" of checkpoint signaling because they are clearly important for the phosphorylation of specific subsets of ATR or ATM substrates in response to specific types of DNA damage or replication interference. For example, Claspin is required for ATR-dependent phosphorylation of Chk1 and Brca1. It binds to chromatin structure and physically interacts with ATR, Brca1, and Chk1 during damage and replication stress [Kumagai and Dunphy, 2000; Chini and Chen, 2003; Lin et al., 2004]. Similarly, 53BP1 and Mdc1 are important in DNA damage-induced foci formation and phosphorylation of ATM targets [Wang et al., 2002; Goldberg et al., 2003; Lou et al., 2003; Stewart et al., 2003]. Although there is no clearly defined enzymatic activity associated with these mediator proteins, most of the mediators (TopBP1, 53BP1, Mdc1, and Brca1) carry BRCA1 C-terminal repeat (BRCT) domains. Tandem BRCT domains have recently been identified as phosphoserine- or phosphothreonine-specific binding modules [Manke et al., 2003; Yu et al., 2003]. Furthermore, all of the mediators are themselves substrates of ATR and/or ATM. The phosphorylation of certain mediators might generate docking sites for other mediators or downstream effectors with phosphoserine- or phosphothreonine-binding motifs (i.e., Chk1 and Chk2). Therefore, the following possible role for mediators can be envisioned: Initial checkpoint signals, such as phosphorylation on Rad17 and Rad9 and autophosphorylation on ATM or ATR, are recognized by mediator proteins through their BRCT motifs. Binding of mediators to the upstream signaling molecules facilitates the recruitment of downstream targets such Chk1 and Chk2.

In addition to recruiting and presenting substrates to ATR and ATM, some of the mediators might also regulate the kinases themselves. For example, Brca1, Mdc1, and 53BP1 are important for efficient ATM autophosphorylation after damage [Mochan et al., 2003; Kitagawa et al., 2004 #40]. It is possible that these mediators might function to further recruit or stimulate the kinases after the initial activation by the sensors. Alternatively, these mediators might facilitate the sensing of specific types of DNA damage.

With the help of mediators, checkpoint signals originating from either DNA damage or replication interference are transmitted, in the form of protein phosphorylation, to two major signal-transducing kinases—Chk1 and Chk2. These two kinases in turn regulate downstream targets, such as Cdc25A, Cdc25C, and p53, to control cell cycle progression and DNA synthesis. The Chk1 kinase is highly conserved among eukaryotes. Chk1 contains multiple serine glutamine/threonine glutamine motifs that are consensus binding sites for ATM and ATR. Activation of Chk1, as indicated by Ser345 phosphorylation, is predominantly ATR-dependent [Liu et al., 2000]. Homozygous deletion of Chk1 leads to embryonic lethality in mice. At the cellular level, loss of Chk1 results in viable but proliferation-retarded cells [Liu et al., 2000; Zachos et al., 2003b]. Noticeably, $Chk1^{-/-}$ DT40 cells exhibit major defects in S phase control in response to DNA damage and replication interference. The impact on the G2/M checkpoint is relatively small [Zachos et al., 2003b]. The Chk2 kinase is the main target of ATM, responding primarily to formation of DSBs [Matsuoka et al., 2000]. A recent study showed that Chk2 homozygous deletion produced viable mice with radiation resistance and moderate defects in the G_1/S checkpoint [Takai et al., 2002], suggesting a more important role of Chk2 in the regulation of apoptosis. Such a notion is supported by the finding that loss of Chk2 could rescue Brca1 deletion-induced proliferation deficiency [McPherson et al., 2004]. Therefore, the function of mammalian Chk2 appears to be considerably different from that of its budding yeast homolog Rad53, which performs vital checkpoint functions. Since Chk2-dependent p53 phosphorylation and transcriptional activation have been observed, p53 was considered a major effector of Chk2 [Takai et al., 2002]. However, the role of Chk2 in p53 regulation was

recently questioned by two studies using a human Chk2 knockout cell line and small interfering RNA against human Chk2 [Ahn et al., 2003; Jallepalli et al., 2003]. Thus, how Chk2 regulates apoptosis in human cells remains to be determined.

TARGETING OF EFFECTOR PROCESSES

The primary targets of the damage and replication checkpoints are components of the cell cycle machinery and DNA replication factors. At present, several mechanisms involved in establishment of the G_1/S, S, and G2/M checkpoints have been revealed.

Control of G_1/S Entry

Cells received DNA damage during the G_1 phase of the cell cycle are delayed from entering S phase by the G_1/S checkpoint. This is a crucial step for the prevention of mutagenesis, because once a cell enters S phase, unrepaired lesions have the potential to be converted into inheritable mutations through DNA synthesis and therefore directly compromise genetic integrity. The p53 tumor suppressor plays an important role in the DNA damage induced G_1/S checkpoint. p53 is phosphorylated by ATM, ATR, and Chk2 depending on the nature of the DNA damage incurred. Phosphorylation allows p53 to dissociate from Mdm2, a p53 E3 ubiquitin ligase that restricts p53 activity through targeted protein degradation [Maya et al., 2001]. A major consequence of p53 activation is transcriptional upregulation of the cyclin-dependent kinase (CDK) inhibitor p21/CIP1/WAF1. Through direct binding of p21 to G_1 CDK-cyclin complex, the G_1/S transition is suppressed. (Another major effect of p53 activation is apoptosis, which is not a topic of this article.)

Tyrosine phosphorylation of CDKs provides temporal restriction of their function during the orderly execution of cell cycle stages. When CDK activity is needed, such restriction is reversed by a group of tyrosine phosphatases known as Cdc25A, Cdc25B, and Cdc25C. Cdc25A is capable of removing inhibitory tyrosine phosphorylation from both Cdk1- and Cdk2 kinases to promote entry into and progression through S phase and mitosis. Therefore, inhibition of Cdc25A activity can arrest cell cycle progression at various stages, including the G_1/S transition. Recently, Cdc25A was identified as a Chk1 target when DNA damage occurs [Jin et al., 2003]. Phosphorylated Cdc25A is subject to the Skp1/Cul1/F-box protein ubiquitin ligase-mediated protein turnover, contributing to DNA damage-induced G_1 block.

Cdt1 is a licensing factor required for the formation of the prereplication complex. Together with Cdc6, Cdt1 promotes loading of the MCM2-7 proteins onto chromatin. Cdt1 is therefore essential for replication initiation. Upon DNA damage, Cdt1 was found to undergo Cul4-Roc1-dependent ubiquitination and proteolysis [Higa et al., 2003]. Such loss of Cdt1 would effectively block the onset of S phase due to ablation of licensed replication origins. This could be another mechanism by which cells with DNA damage exhibit G_1 delay. Since this pathway appears to be independent of the ATM-Chk2 branch, ATR or other signaling pathway may be involved in conditioning Cdt1 for Cul4-Roc1-mediated ubiquitination.

Control of DNA Replication

Cells in S phase respond to both DNA damage and disruption of DNA synthesis. The initiation of an S-phase checkpoint signal can be DNA lesions or replication forks stalled by DNA lesions. The S-phase checkpoint has profound importance in the maintenance of genomic stability. First, S phase offers a last line of defense against DNA lesions before they are converted into inheritable mutations. Second, S phase presents the best opportunity for revealing DNA lesions since each lesion will inevitably be encountered by the replication machinery and may stall DNA replication forks. Several mechanisms have been identified for the attenuation of the S phase in response to DNA damage and replication stress.

Radioresistant DNA synthesis is a hallmark of cells with a failed S-phase checkpoint. In normal cells, DNA damage or depletion of nucleotide substrate triggers immediate slowing down of DNA synthesis. In cells with a defective S-phase checkpoint, DNA synthesis persists in the presence of such conditions. A number of mechanisms have been identified for the attenuation of the S phase. As mentioned earlier, the Cdc25A phosphatase is required for Cdk activity during S-phase progression [Vigo et al., 1999]. Cdc25A is a target for both Chk1 and Chk2 [Falck et al., 2001; Jin et al., 2003], depending on the nature of DNA damage or replication stress. In human cells, the

phosphorylation of Cdc25A by Chk1 is clearly linked to its degradation [Jin et al., 2003]. In *Xenopus*, the phosphorylation of Cdc25s by Chk1 blocks their interactions with CDK-cyclin complexes [Uto et al., 2004]. Furthermore, it has been shown in *Xenopus* that the activity of Cdk2-cyclin E, a target of Cdc25A, is inhibited by the DSB-activated ATM pathway [Costanzo et al., 2000]. The downregulation of Cdk2-cyclin E prevents Cdc45 from binding to replication origins, thereby repressing the initiation of DNA replication. In addition to CDK-cyclins, Cdc7, another protein kinase required for the initiation of DNA replication, is also regulated by the S-phase checkpoint. It was shown that etoposide, an inhibitor of topoisomerase II, activates the ATR checkpoint pathway and diminishes the activity of Cdc7 in *Xenopus* extracts [Costanzo et al., 2003].

Defects in NBS1 and SMC1 also give rise to a radioresistant DNA synthesis phenotype. Mutations in the NBS1 gene cause the human chromosomal instability disorder Nijmegen breakage syndrome, and NBS1 mutant cells are similar to that of ataxia-telangiectasia cells [Carney et al., 1998]. The checkpoint function of Nbs1 was revealed when it was identified as a direct substrate of ATM upon radiation treatment, placing a function of Nbs1 downstream of ATM [Lim et al., 2000]. The cohesin protein, Smc1, also plays an important role in the S-phase checkpoint as an ATM substrate [Kim et al., 2002; Yazdi et al., 2002]. More recently, it was demonstrated that Nbs1 is required in the Smc1-dependent S-phase checkpoint [Kitagawa et al., 2004]. Therefore, an ATM-Nbs1-Smc1 pathway appears to be another major mechanism for the control during S phase. However, it remains unclear what direct impact Smc1 may have during S-phase progression.

The complete S-phase checkpoint involves several distinct elements. The observed immediate decline in the DNA synthesis rate is mediated by suppression of late-firing origins and perhaps slowing down of replication fork progression. Replication forks stalled by encountering lesions then require proper stabilization. Moreover, stalled replication forks need to be restarted. In budding yeast, Mec1-dependent checkpoint signaling is essential in suppression of late-firing origins as well as in recovery of stalled or collapsed replication forks [Desany et al., 1998; Lopes et al., 2001; Tercero and Diffley, 2001]. $ATR^{-/-}$ mouse cells accumulate DSBs when released from aphidicolin-induced replication block [Brown and Baltimore, 2000]. $Chk1^{-/-}$ DT40 cells showed multiple S-phase checkpoint defects, including a prolonged S phase and inability to resume DNA synthesis upon release from replication blockade [Zachos et al., 2003a]. Thus, it appears that the function of the S-phase checkpoint may extend beyond simply arresting S-phase progression to include facilitating recovery of DNA replication after damage repair.

Control of Mitotic Entry

The mitotic entry checkpoint (G2/M) prevents the onset of mitosis when DNA damage occurs. Delaying entry into mitosis affords repair mechanisms extra time to remove DNA lesions before they are passed on to daughter cells. Recombination repair mechanisms, which provide the highest fidelity of lesion removal, are also believed to operate more efficiently during late S and G2 phase due to the presence of sister chromatids and chromosomes. DNA lesions that impede the equal segregation and distribution of chromosomes, such as DSBs and DNA interstrand crosslinks, are expected to elicit a more profound response. In contrast, lesions such as UV-induced dimers and base modifications may elude the G2/M checkpoint as they can still be repaired in the succeeding G_1 and S phases.

The mitotic entry requires dephosphorylation of Cdc2 on Tyr14 and Tyr15. This process depends on the Cdc25C phosphatase in mammalian cells. Chk1 phosphorylates Cdc25C on Ser216, which results in sequestration of Cdc25C activity from the nucleus by 14-3-3 [Lopez-Girona et al., 1998]. Therefore, the ATR-Chk1-Cdc25C pathway is recognized as one of the mechanisms mediating G2/M arrest. In fact, this mechanism was the first elucidated pathway of DNA damage leading to negative control of cell cycle components [Peng et al., 1997; Sanchez et al., 1997]. Similarly, an ATM-Chk2-Cdc25C pathway may also function in parallel with the ATR-Chk1-Cdc25C pathway [Matsuoka et al., 2000]. Inhibition of Cdc25A may also contribute to the delay of mitotic entry upon DNA damage. Interestingly, $ATR^{-/-}$ $ATM^{-/-}$ mouse cells are able to arrest before mitosis in response to hydroxyurea or aphidicolin treatments [Brown and Baltimore, 2003], indicating the existence of unidentified mechanisms for G2/M arrest.

BEYOND BUYING TIME FOR DNA REPAIR

The original concept of the cell cycle checkpoints held that these mechanisms are in place to ensure the orderly onset of cell cycle stages and the delay of cell cycle progression in the presence of DNA damage or replication interference. However, recent investigations, particularly studies in mammalian genetic models, indicate that the checkpoint pathways carry out functions much beyond simply delaying cell cycle progression. These are reflected by the essentiality of certain checkpoint genes, such as ATR, CHK1, RAD17, and RAD9, in embryonic development and cell proliferation. These findings suggest that certain checkpoint components are involved in fundamental processes such as proper maintenance of DNA replication forks. It was recently suggested that the checkpoint signal-initiating kinases ATM and ATR may even be involved in control of the overall timing of replication in *Xenopus* extract under unperturbed conditions [Shechter et al., 2004]. It is expected that more essential functions of checkpoint factors in normal cell growth will be identified in future studies. Segregation of function mutations would be particularly important in revealing such functions, which may lead to identification of additional tumor development mechanisms. Of course, many questions remain. What are the roles of ATR and the 9-1-1 complex during normal cell growth since these proteins appear essential for sustaining cell proliferation? How is the damage checkpoint activated when processing of lesions does not lead to substantial amounts of single-strand breaks (i.e., short patch base excision repair has only a single base gap intermediate). How do other Cdc25 phosphates participate in G2/M control (Cdc25C knockout has no apparent phenotype [Chen et al., 2001])? How are the checkpoint signals reversed so that cells can resume the cell cycle progression? These and many other unknowns require identification of additional checkpoint factors and mechanisms. Understanding of the DNA damage and replication checkpoint pathway has and will continue to advance our understanding of the maintenance of genomic stability and tumorigenesis.

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