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The multiple checkpoint functions of CHK1 and CHK2 in maintenance of genome stability

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1. ABSTRACT

Cell cycle checkpoints are pivotal mechanisms safeguarding genome stability. Cells that harbor defects in checkpoints are predisposed to genome instability and neoplastic transformation. Two structurally-unrelated protein kinases, CHK1 and CHK2, are implicated in several major checkpoints of the cell cycle, providing a crucial linkage between the upstream sensors of the checkpoints and the cell cycle engine. Variations of the ATM/ATR-CHK1/CHK2-CDC25-CDK axis underlie the molecular basis of the replication checkpoint, the intra-S phase checkpoint, and the G_2 DNA damage checkpoint. Although some aspects of the pathway remain contentious, the ATM/ATR-CHK1/CHK2-p53-$p21^{CIP1/WAF1}$-CDK axis is believed to play an important role in the G_1 DNA damage checkpoint. Recent data also reveal that CHK1 may play a role in the spindle-assembly checkpoint. Finally, CHK1 and CHK2 are implicated in linking the cell cycle to diverse processes such as senescence and the circadian cycle. In this review article, we provide an overview of how the multi-tasking nature of CHK1 and CHK2 is achieved in vertebrate cells.

2. INTRODUCTION

Checkpoint mechanisms operate throughout the cell cycle to safeguard genome stability. For example, the DNA damage checkpoints ensure that damaged DNA is neither replicated nor segregated to daughter cells until repaired. Similarly, the replication checkpoint ensures that cells do not enter mitosis until DNA replication is completed. The spindle-assembly checkpoint is another major checkpoint that inhibits mitotic exit until all the chromosomes have achieved bipolar spindle attachment. Cells that harbor defects in these pathways are prone to genome instability and neoplastic transformation. Two structurally-unrelated protein kinases, CHK1 and CHK2, are implicated in all these major checkpoints of the cell cycle. These two protein kinases are activated by upstream sensors of the checkpoints and in turn phosphorylate targets of the cell cycle engine. In this review article, we summarize how CHK1 and CHK2 function in multiple checkpoints to ensure the genomic integrity in vertebrate cells. Emerging checkpoint-independent functions of CHK1/CHK2, such as in the circadian cycle, are also discussed.

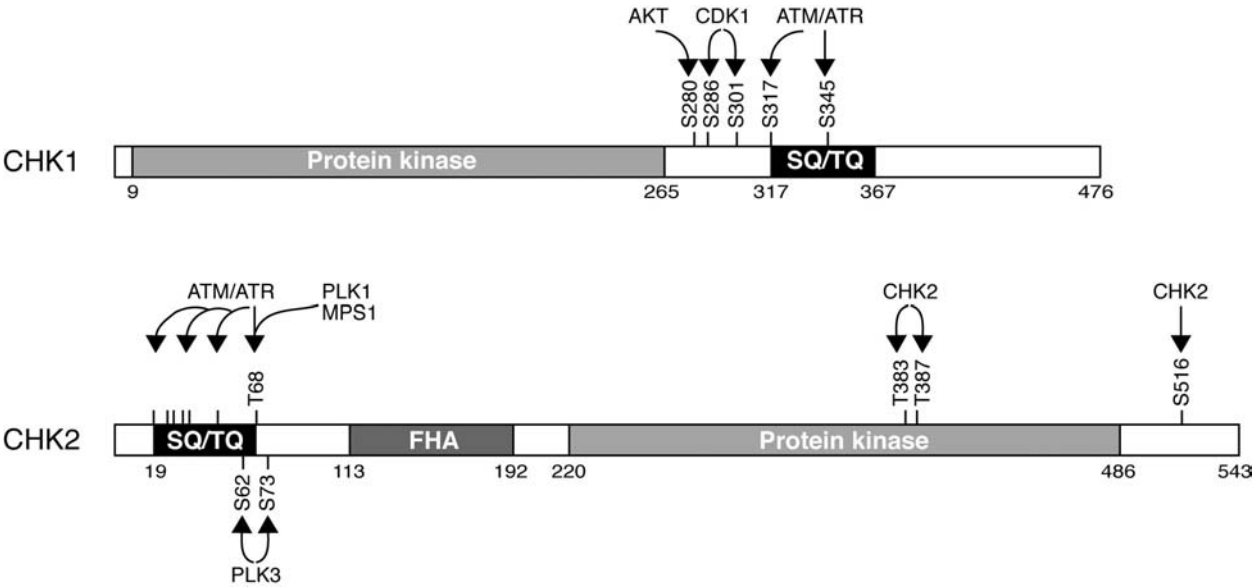

Figure 1. Schematic diagram of CHK1 and CHK2. Elements in human CHK1 and CHK2, including the protein kinase domain, the SQ/TQ domain, and the FHA domain are shown to scale. Phosphorylation sites by various protein kinases are shown. The potential SQ/TQ sites in CHK2 are Ser19, Thr26, Ser28, Ser33, Ser35, Ser50, and Thr68.

3. STRUCTURE AND REGULATION OF CHK1 AND CHK2

Although CHK1 and CHK2 display many similarities in functions, these two protein kinases share no significant sequence homology outside the protein kinase domain. CHK1 is comprised of a kinase domain at the NH_2-terminal half and a regulatory domain at the COOH-terminal half of the protein (Figure 1). Several sites within the SQ/TQ domain (including Ser317 and Ser345) are phosphorylated by ATM and ATR in response to replication stress or DNA damage (1-5). ATM (ataxia-telangiectasia mutated) is a PI-3 (phosphoinositide 3-kinase)-related protein kinase encoded by a gene mutated in the cancer-prone disorder ataxia telangiectasia. Following exposure to ionizing radiation (IR) or other genotoxic insults that elicit DNA double-strand breaks, ATM is autophosphorylated, leading to dimer dissociation and activation of the kinase (6). An ATM-related protein kinase, ATR (ATM and Rad3-related), is activated by a broader spectrum of stress including ultraviolet irradiation, hypoxia, and replication stress. ATM and ATR specifically phosphorylate Ser-Gln (SQ) and Thr-Gln (TQ) motifs such as those present in the SQ/TQ domain of CHK1/CHK2 (7).

The upstream sensors that initiate the activation of ATM/ATR consist of an intricate network of large protein complexes, of which many components contain the BRCT domain. These include the RAD9-HUS1-RAD1 (9-1-1) clamp and the RAD17-containing clamp loader that facilitate ATR-mediated activation of CHK1 (8). Another large complex that participates in ATM/ATR activation is composed of BRCA1, BLM, and MRN (MRE11-RAD50-NBS1) (termed the BRCA1-associated genome surveillance complex) (9). A detailed discussion of these upstream activators that initiate the signaling cascade is beyond the scope of this review.

Early studies conferred the idea that ATM and ATR activate CHK2 and CHK1 respectively, and that the two pathways are largely linear and non-overlapping (1, 2, 4). More recent observations, however, indicate that these pathways are not mutually exclusive. Depending on the particular stress signal, there is a high degree of redundancies in the system. For example, although ATR regulates a majority of the late responses after IR, both ATM and ATR contribute to early delay in M phase entry (10, 11). Only double deletion of ATR and ATM completely eliminates the IR-induced G_2 DNA damage checkpoint. Similarly, both CHK1 and CHK2 are believed to participate in the G_2 DNA damage checkpoint (see below).

A hint of the molecular regulation of CHK1 was obtained by the fact that deletion of the COOH-terminus activates the kinase even in the absence of stress (12-14). These results argue for the existence of an autoinhibitory domain. Indeed, the COOH-terminal region of rat CHK1 was shown to interact with its kinase domain (15). Likewise, the observations that the COOH-terminal region of *Xenopus* CHK1 can interact with and inhibit the kinase domain, but not full-length CHK1, suggest an intramolecular interaction in the CHK1 molecule (16). Phosphorylation of the SQ/TQ motifs by ATR or mutation of the SQ/TQ motifs to phospho-mimic residues abolishes the kinase domain-inhibitory domain interaction. Conversely, the interaction is maintained even after stress if the SQ/TQ motifs are rendered nonphosphorylatable (16). A possible model is that in the absence of stress, CHK1 is kept inactive by intramolecular interaction between the COOH-terminal domain and the kinase domain. This interaction is disrupted following ATM/ATR-mediated phosphorylation of the SQ/TQ domain, freeing the kinase domain to phosphorylate its substrates. In this connection, while two SQ sites (Ser317 and Ser345) have been

extensively investigated and shown to be important for CHK1 regulation, the significance of the other SQ sites (Ser357 and Ser366) is not known. Another complication is that phosphorylation of $CHK1^{Ser345}$ creates a 14-3-3 binding site that is required for nuclear retention of CHK1 during checkpoint activation (17).

The functional domains of CHK2 are arranged very differently from those of CHK1. Compelling evidence suggests that the activation of CHK2 involves intermolecular interaction and autophosphorylation. In comparison to CHK1, the kinase domain of CHK2 is located at the COOH-terminal half of the protein, and the NH_2-terminal regulatory region contains a SQ/TQ domain and a FHA domain (Figure 1). It is well established that a major ATM phosphorylation site is Thr68 in the SQ/TQ domain (18-21). However, other sites in the SQ/TQ domain are also targeted by ATM, because only mutation of all seven SQ/TQ sites (Ser19, Thr26, Ser28, Ser33, Ser35, Ser50, and Thr68) abolishes ATM phosphorylation (19). The FHA domain is dispensable for Thr68 phosphorylation but is necessary for efficient autophosphorylation in response to IR (22-24). After DNA damage, phosphorylation of Thr68 precipitates the autophosphorylation of Thr383 and Thr387 (25, 26). These two residues are located within the activation loop of the kinase domain and are essential for CHK2 activity. Another autophosphorylation site of CHK2, Ser516, is phosphorylated after DNA damage and mutation to a non-phosphorylatable residue impairs the activity and proper checkpoint function of CHK2 (27). Interestingly, while autophosphorylation of Ser516 appears to be carried out by cis, Thr383 and Thr387 can be phosphorylated in trans by another molecular of CHK2 (26). The FHA domain also mediates CHK2 oligomerization via direct interaction with phosphorylated Thr68 on another CHK2 molecule (22, 24). Although Thr68 phosphorylation is required for the initial oligomerization and activation of CHK2, it is not essential for the maintenance of oligomerization or kinase activity (28). Collectively, the existing data indicate that although both CHK1 and CHK2 are transducers of the ATM/ATR-mediated signaling pathway, the two kinases are regulated very differently.

4. THE CHK1/CHK2-CDC25-CDK AXIS AND THE G_2 DNA DAMAGE CHECKPOINT

The best understood functions of CHK1 and CHK2 are their actions on members of the CDC25 phosphatase family (CDC25A, CDC25B, and CDC25C). CDC25s are dual-specific phosphatases that dephosphorylate two residues within the catalytic site of cyclin-dependent kinases (CDKs) (Thr14 and Tyr15 in the archetypical member CDK1). Each member of the CDC25 family appears to possess unique functions during the cell cycle: CDC25C functions mainly in activating CDK1 during mitosis; CDC25B is believed to activate CDK1 specifically at the centrosome; CDC25A acts on both CDK1 and CDK2, regulating multiple points at G_1/S transition, S phase, and mitosis. Progress in the past several years has unraveled very similar underlying principles involving CDC25s in the replication checkpoint, the intra-S DNA damage checkpoint, and the G_2 DNA damage checkpoint. In essence, DNA damage or replication stress activates CHK1 and CHK2 through ATM/ATR-mediated mechanisms, resulting in the inactivation of CDC25s (and in some cases, also the activation of WEE1), thereby promoting CDK inhibition and cell cycle arrest.

In the case of the G_2 DNA damage checkpoint, the ultimate target of the CHK1/CHK2 pathway is cyclin B1-CDK1 (CDC2). In the unperturbed cell cycle, cyclin B1 starts to accumulate from S phase and forms a complex with CDK1. The complex is kept inactive through MYT1- and WEE1-dependent phosphorylation of $CDK1^{Thr14/Tyr15}$. During mitosis, the stockpile of Thr14/Tyr15-phosphorylated CDK1 is abruptly activated by CDC25-mediated dephosphorylation (reviewed in (29)). Once a portion of cyclin B1-CDK1 is activated, it catalyzes its own activation by an intricate network of feedback loops. WEE1 is phosphorylated by CDKs, facilitating its degradation by the ubiquitin ligases $SCF^{\beta\text{-}TrCP}$ and SCF^{TOME1} (30-33). Conversely, CDC25C is activated by multiple CDK phosphorylations (reviewed in (34)). These feedback loops allow cyclin B1-CDK1 to behave as a bistable system (35), converting graded inputs into switch-like, irreversible responses once a critical portion is activated. Our knowledge of this bistable system is certainly not complete as the initial trigger of the system still remains elusive, though cyclin A2-CDK may be a candidate (36).

Based on these premises, the G_2 DNA damage checkpoint delays mitotic entry by exploiting the normal control of cyclin B1-CDK1. The current paradigm states that the checkpoint exerts its effects mainly through a signaling cascade involving CHK1 and CHK2, thereby maintaining CDK1 in the Thr14/Tyr15-phosphorylated state. In support of this, gene disruption or siRNA downregulation of CHK1 results in a defective G_2 DNA damage checkpoint (1, 37-39). Similarly, deletion of *CHK2* in mouse cells disrupts G_2 DNA damage checkpoint maintenance (40), although very similar studies using an independent knockout model did not reach the same conclusion (41).

ATM/ATR phosphorylates and activates CHK1 and CHK2 (see above), which in turn phosphorylate and inactivate members of CDC25 phosphatase family. All three isoforms of CDC25 are likely to collaborate in the timely activation of cyclin B1-CDK1 during mitosis. Nevertheless, mouse cells lacking both CDC25B and CDC25C display minimum defects in cell cycle progression (42). In contrast, CDC25A may play a nonredundant role in cyclin B1-CDK1 activation in mouse cells (43). Underscoring the importance of CHK1 and CHK2, all three isoforms of CDC25 can be phosphorylated by the two protein kinases (44, 45).

Phosphorylation of $CDC25C^{Ser216}$ by CHK1/CHK2 inactivates its phosphatase activity either directly (46, 47) or indirectly through the creation of a 14-3-3 binding site (44, 48). Binding of 14-3-3 masks a

proximal nuclear localization sequence and anchors CDC25C in the cytoplasm, preventing efficient access of CDC25C to the nuclear cyclin B1-CDK1 complexes. Interestingly, phosphorylation of a proximal site (Ser214) by cyclin B1-CDK1 inhibits further phosphorylation of $CDC25C^{Ser216}$ (49). This provides an elegant mechanistic explanation for the suppression of DNA damage-mediated CDC25C inactivation during mitosis.

CDC25A, arguably the most important member of the CDC25 family, is also inactivated by CHK1 and CHK2. Unlike CDC25C, CDC25A is targeted to rapid degradation through a ubiquitin-mediated mechanism by CHK1/CHK2 (50, 51). During mitotic exit and early G_1, CDC25A stability is controlled by the APC/C^{CDH1} complex (52). In contrast, CDC25A degradation is carried out by the $SCF^{\beta\text{-}TrCP}$ complexes during interphase (53, 54). The canonical SCF ubiquitin ligase complexes consist of a SKP1 and a cullin subunit, together with a variable F-box-containing protein that is responsible for recognizing specific substrates. β-TrCP is a WD40 repeat-containing F-box protein that recognizes a doubly phosphorylated phosphodegron (also called DSG motif) found in diverse targets like I□B, β-catenin, and EMI1. Importantly, $SCF^{\beta\text{-}TrCP}$-dependent turnover of CDC25A is enhanced in response to DNA damage. Phosphorylation of $CDC25A^{Ser76}$ by CHK1 is required for the phosphorylation of a phosphodegron centered at Ser82 (by an as-yet-unidentified kinase), creating a binding site for β-TrCP. Interestingly, β-TrCP also binds to a separate nonphosphorylated sequence in CDC25A (the DDG motif) and plays a role in CHK1-induced ubiquitination and degradation of CDC25A. This mechanism presumably also applies to CDC25B, as CDC25B also contains a DDG motif and binds to β-TrCP (55).

The least studied member of the CDC25 family, CDC25B, is believed to possess a unique role in activating cyclin B1-CDK1 at the centrosome (56). A growing body of evidence indicates that CHK1 may shield centrosomal cyclin B1-CDK1 from unscheduled activation by CDC25B during normal G_2 phase and presumably also during the G_2 DNA damage checkpoint. The molecular basis of this activity may be due to CHK1-dependent phosphorylation of $CDC25B^{Ser323}$, creating a docking site for 14-3-3 that prevents access of substrates to the catalytic site (57-61). Dissociation of CHK1 from the centrosomes at the end of G_2 phase, together with positive regulatory phosphorylation of $CDC25B^{Ser353}$ by Aurora-A (62), enables CDC25B to activate the centrosomal cyclin B1-CDK1 and initiate mitosis.

Finally, there is evidence that WEE1, the kinase that opposes the action of CDC25s, is also regulated by CHK1. Phosphorylation of the COOH-terminal tail of WEE1 by CHK1 promotes 14-3-3 binding and increases the kinase activity of WEE1 (63, 64). However, it is curious that inactivation of CHK1 with the inhibitor UCN-01 decreases WEE1 activity but does not appear to affect the interaction between WEE1 and 14-3-3 (64).

5. THE CHK1/CHK2-CDC25-CDK AXIS AND THE S PHASE CHECKPOINTS

It is generally accepted that similar mechanism as the G_2 DNA damage checkpoint operates for the intra-S DNA damage checkpoint, which halts DNA replication in response to DNA damage. A key player that governs both the initiation and completion of DNA replication is CDK2, which is able to associate with both cyclin A and cyclin E. Functions attributed to CDK2 include the loading of CDC45 onto replication origins and the degradation of the licensing factor CDC6 (reviewed in (65)). A major determinant of the intra-S phase checkpoint is believed to be CDC25A, which is eliminated upon phosphorylation by CHK1 and CHK2 (see above). Removal of CDC25A triggers the accumulation of Thr14/Tyr15-phosphorylated CDK2, thereby stalling S phase progression (50, 51). Cells that are defective in the intra-S phase checkpoint, such as those derived from ataxia-telangiectasia, often exhibit radio-resistant DNA synthesis.

Many studies have also detailed the role of CHK1 in the replication checkpoint, which stabilizes stalled replication forks and prevents late origin firing when DNA synthesis is inhibited (2, 66). There is a consensus in the field that stalled replication forks mainly activates the ATR-CHK1-CDC25A pathway, with very little contribution from ATM and CHK2. A partner for ATR, called ATRIP (ATR-interacting protein), appears to be required for recruiting ATR to single-stranded DNA present at stalled replication forks. Depletion of ATRIP in human cells by siRNA (67) and immunodepletion of ATRIP from *Xenopus* egg extracts (68) indicate that ATRIP is critical for ATR function. Furthermore, phosphorylation of CHK1 by ATR in *Xenopus* egg extracts requires binding of ATR to ATRIP (69), TopBP1 (70), as well as another CHK1-associated protein called Claspin (68). Phosphorylated Claspin docks with a phosphate-binding site in the kinase domain of CHK1, which may mimic activating phosphorylation (71).

Relatively little is known about how the checkpoint is inactivated to allow the cell cycle to continue. One such mechanism of checkpoint recovery is probably achieved by degradation of Claspin, which is mediated by a PLK1- (72, 73) and $SCF^{\beta\text{-}TrCP}$-dependent ubiquitination process (74, 75). CHK1 itself has also been reported to be degraded by the ubiquitin-proteasome pathway following genotoxic stress. The degradation is triggered by phosphorylation of $CHK1^{Ser345}$ and is mediated by SCF complexes containing CUL1 or CUL4A (76). Apart from protein degradation, dephosphorylation by protein phosphatases may also play an important role in checkpoint recovery. The p53-induced type 2C serine/threonine phosphatase PPM1D/Wip1 can reverse ATM/ATR-mediated phosphorylation of CHK1, CHK2, and p53 (77-79). However, another study indicates that PP2A is the main phosphatase that dephosphorylates CHK1 (80).

Similarly to the mechanism of the intra-S DNA damage checkpoint, activated CHK1 targets CDC25A for degradation after replication block. This accounts for the inhibition of CDK2 and the interruption of S phase

progression. Conceptually, it is vital to also prevent the precarious activation of cyclin B1-CDK1 and mitotic entry as long as replication remains incomplete. This is sometimes referred to as the S-M checkpoint. By dual inhibition of both CDK1 and CDK2 using the same principle, the ATR-CHK1-CDC25 pathway is able to prevent both replication and mitosis concurrently (37, 81). In this connection, agents that uncouple the checkpoint can induce premature entry into mitosis. This is exemplified by caffeine, which is an inhibitor of ATM and ATR (82, 83). On entering mitosis prematurely in the presence of replication block or DNA damage, many cells undergo a poorly defined type of cell death termed mitotic catastrophe (84, 85). At least under some conditions, mitotic catastrophe has been shown to be negatively regulated by CHK1 (86) or CHK2 (87). Mitotic catastrophe can also occur after checkpoint adaptation (88, 89). Thus mitotic catastrophe may be a final mechanism to safeguard genome instability and transformation in cells that have defective checkpoints.

6. THE CHK1/CHK2-P53-P21$^{CIP1/WAF1}$-CDK AXIS

When cells suffer DNA damage during G_1 phase, it is critical for the cell to halt the entry into S phase until the DNA is repaired. Failure to establish this checkpoint allows the damaged DNA to be replicated, which may predispose cells to genome instability. It is well established that the G_1 DNA damage checkpoint involves the stabilization and activation of the tumor suppressor p53, which in turns transcriptionally activates the CDK inhibitor p21$^{CIP1/WAF1}$, leading to the inhibition of cyclin E-CDK2 complexes and G_1 arrest. Since, in addition to its role in the G_1 checkpoint, p53 has also been implicated in the G_2 checkpoint (90, 91), it is conceivable that some of the G_2 functions involving CHK1/CHK2 may be due to actions through p53 instead of the CDC25 pathway.

The activity of p53 is highly regulated by a remarkable web of mechanisms including protein-protein interaction, acetylation, neddylation, phosphorylation, sumoylation, and ubiquitination (reviewed in (92)). In unstressed cells, p53 is restrained by binding to MDM2, itself a transcriptional target of p53, in a negative feedback loop. MDM2 binds to the NH_2-terminal transactivation domain of p53 and inhibits its transcriptional activity directly. MDM2 also shuttles p53 out of the nucleus by the virtue of its nuclear exporting signal. Finally, MDM2 is a ubiquitin ligase that targets p53 for ubiquitin-mediated proteolysis. Upon DNA damage, p53 is phosphorylated at several sites in its NH_2-terminal transactivation domain, which inhibits the interaction of p53 with MDM2, resulting in p53 stabilization (93, 94).

The crucial event in p53 activation is thus the phosphorylation of the NH_2-terminal residues by checkpoint-stimulated protein kinases. Following DNA damage, ATM and ATR are activated and phosphorylate Ser15 at the NH_2-terminal region of p53 (95-98). ATM also activates p53 indirectly by phosphorylating the COOH-terminal region of MDM2 (Ser395 in human MDM2), which reduces the p53-inhibitory potential of MDM2 by disrupting the nucleo-cytoplasmic shuttling of p53 (99). A different residue (Ser407) has been reported to be phosphorylated by ATR in response to DNA damage or replication stress, which interferes with the MDM2-dependent nuclear export of p53 (100).

A MDM2-related protein, MDMX, collaborate with MDM2 in inhibiting p53. MDMX binds and inhibits the transcriptional activity of p53 without targeting p53 for ubiquitination (101). In the absence of stress, MDMX binds and stabilizes MDM2 by preventing its self-ubiquitination (102, 103). After DNA damage, MDMX is phosphorylated at Ser403 by ATM and at Ser342 and Ser367 in a CHK2-dependent manner (104). Phosphorylation of MDMX^{Ser367} stimulates 14-3-3 binding and nuclear import (105). Together, these phosphorylations enhance the degradation of MDMX by MDM2. Removal of MDMX in turns destabilizes MDM2 and promotes the accumulation and activation of p53.

Apart from directly phosphorylating p53, ATM also induces p53 phosphorylation indirectly via CHK1 and CHK2. CHK2 was first shown to be able to interact with p53 and phosphorylate p53^{Ser20} *in vitro* (40, 106, 107). Notably, CHK2-p53 complex formation is abrogated by cancer-associated mutations in the FHA domain of CHK2, or by mutations in the tetramerization domain of p53 (108).

If CHK2 is indeed an upstream activator of p53, animals that lack CHK2 should share at least some features of animals with p53 deletion. Indeed, although *CHK2*$^{-/-}$ mice are normal and fertile, they are more resistant than wild type mice to sublethal doses of IR as a result of ineffective apoptosis (41). In support of a role of CHK2 in the G_1 DNA damage checkpoint, the IR-induced G_1 arrest is impaired in *CHK2*$^{-/-}$ mouse embryonic fibroblasts (41). The stabilization of p53 after DNA damage is also reduced in *CHK2*$^{-/-}$ mouse embryonic cells (40), leaning support of a role of CHK2 in p53 regulation. Other studies indicated that although protein stabilization and phosphorylation of Ser23 (equivalent to Ser20 in human p53) are apparently normal in *CHK2*$^{-/-}$ mouse embryonic fibroblasts, p53-dependent transcription of target genes such as *CIP1/WAF1* is defective (41).

Despite the strong link between CHK2 and p53^{Ser20} phosphorylation in the G_1 DNA damage checkpoint, several lines of evidence have challenged the biological significance of this pathway. These contentions mainly center around the questions whether CHK2 is indeed a major kinase for p53^{Ser20}, and whether Ser20 phosphorylation itself is critical for the DNA damage checkpoint.

Although CHK2 can phosphorylate p53^{Ser20} *in vitro*, a paradoxical point is that Ser20 does not fit the consensus CHK2 phosphorylation site found in other CHK2 substrates (7). Furthermore, p53-derived peptide containing Ser20 turns out to be a poor substrate for CHK2 (109, 110). However, the relatively poor phosphorylation of Ser20-containing peptides can be reconciled by the discovery of a distinct CHK2 docking site in the DNA binding domain of

p53 (110). Binding of CHK2 to this docking site may change the conformation of p53 and juxtapose Ser20 for more efficient phosphorylation.

A much weaker than expected contribution of CHK2 in p53 regulation is also revealed by addressing the functions of CHK2 using reverse genetic approaches. Depletion of CHK2 with various techniques, including gene knockout in mouse embryonic fibroblasts (41), homologous recombination in human cancer cells (111), or RNAi (109) resulted in only partial or no defects in the DNA damage-induced phosphorylation and stabilization of p53. One possible explanation is that in the absence of CHK2, the checkpoint function could be compensated by CHK1. CHK1 is evidently capable of phosphorylating $p53^{Ser20}$ *in vitro*, with a preference for tetrameric to monomeric p53 (107). In addition to Ser20, CHK1 also phosphorylates sites in the COOH-terminal domain of p53 (107). Phosphorylation of these sites affects the COOH-terminal acetylation and the activation of p53-targeted promoters (112). Although it has been demonstrated that expression of an antisense CHK1 construct reduces the abundance of p53 protein (107), knockdown of CHK1 with siRNA in another study reveals that CHK1 does not play a role in p53 stabilization after DNA damage (109).

In the connection between CHK1 and p53 activation, there is also evidence of feedback controls. Transcription of *CHK1* is downregulated by activated p53 through a $p21^{CIP1/WAF1}$- and pRb-dependent mechanism (113, 114). Recruitment of other proteins such as the p52 NF-kB subunit by p53 to the promoter region represses *CHK1* transcription (115). The expression of *CHK1* is affected by p53 in a tissue-specific manner, as *CHK1* mRNA is induced in spleen, thymus, and dermal fibroblasts, but reduced in lung and testis in the absence of p53 (116). Similarly, there is evidence that *CHK2* transcription can be downregulated by p53 (117). A CCAAT box in the *CHK2* promoter, which can bind the transcription factor NF-Y, is responsible for the repression by p53.

Even if CHK1 or CHK2 are important in regulating $p53^{Ser20}$ phosphorylation, there is also the issue of which of the many phosphorylation sites in p53 are in fact important for regulating its activity. The NH_2-terminal transactivation domain that is responsible for binding to MDM2 (residue 18-26) is predominantly unstructured in solution, and only acquires its a-helical conformation when in complex with MDM2 (118, 119). The prevailing view is that phosphorylation of specific residues within this stretch of amino acids disrupts MDM2 docking, relieving the inhibition of p53 by MDM2. However, it is clear that multiple sites in the p53 transactivation domain can be phosphorylated and the consequences of phosphorylation appear to be site- or even cell-specific. Phosphorylation of $p53^{Ser15}$ (carried out by ATM, ATR, or DNA-PK) was initially believed to be important in regulating interaction with MDM2 (93). However, more recent data suggest that Ser15 phosphorylation does not directly affect the binding to MDM2, but promotes the interaction with CBP/p300 and is subsequently involved in transcriptional activation (120-122). The CHK1/CHK2 phosphorylation site, Ser20, appears to be important in the regulation of the turnover rate of p53 after DNA damage by disrupting MAD2-p53 interaction (123, 124). However, recent studies suggest that phosphorylation of Ser15 and Ser20 alone may not be necessary for p53 regulation. Introduction of Ala-substituted mutants of Ser18 or Ser23 (the mouse equivalents of Ser15 and Ser20, respectively) into the endogenous p53 gene of mouse embryonic fibroblasts showed no effect on p53 accumulation upon genotoxic stress (125, 126).

Another phosphorylation site in the transactivation domain of p53 is Thr18. Compare to Ser15 and Ser20, phosphorylation of Thr18 significantly attenuates the interaction between p53 and MDM2 (120, 127-129). Several protein kinases are known to be able to phosphorylate Thr18, including DNA-PK (130), VRK1 (human vaccinia-related kinase 1) (131, 132), and CK1 (casein kinase) (129). Interestingly, phosphorylation of Thr18 may require the prior phosphorylation of Ser15, suggesting cooperation of phosphorylation of the p53 NH_2-terminal residues. In this connection, it is likely that multiple cooperative phosphorylation of the NH_2-terminal residues, rather than of a single residue, determines the activation of p53.

7. CHK1/CHK2 AND THE SPINDLE-ASSEMBLY CHECKPOINT

During mitosis, microtubules are radiated from two opposite spindle poles and capture chromosomes by attaching to their kinetochores. Sister chromatids segregation is only initiated when all the chromosomes have achieved bipolar attachment to the spindles and have aligned at the metaphase plate. The current paradigm states that unattached kinetochores or the absence of tension between the paired kinetochores activates the spindle-assembly checkpoint. This checkpoint maintains high level of active cyclin B1-CDK1 by inhibiting APC/C^{CDC20} (reviewed in (133)). The underlying mechanism remains ill defined, but a growing body of evidence indicates that unattached kinetochores attract the components of the checkpoint machinery (including BUB1, BUB3, BUBR1, MAD1, MAD2, MAD3, MPS1, and CENP-E) to the kinetochores. This catalyzes the formation of diffusible mitotic checkpoint complexes (components include MAD2, BUBR1, and BUB3) which in turn inhibit the APC/C^{CDC20} complexes (134). Cells that harbor defects in the spindle-assembly checkpoint may exit mitosis prematurely and are prone to chromosomal instability and neoplastic transformation (reviewed in (135)).

Only very recently has CHK1 been implicated in the regulation of mitosis. Depletion of CHK1 in HeLa cells by RNAi leads to a metaphase block, accompanying with chromosome misalignment during metaphase and chromosome lagging during anaphase (136). By disrupting *CHK1* in the chicken B cell line DT40, Zachos *et al.* (2007) found that CHK1 is required for the sustained anaphase delay when the spindle function is disrupted by Taxol (137). Since CHK1 can directly phosphorylate Aurora-B *in*

vitro, it is possible that the proper regulation of Aurora-B kinase activity may underlie the spindle-assembly checkpoint function of CHK1. Among other functions, Aurora-B targets BUBR1 to kinetochores (138). Accordingly, the kinetochore localization of BUBR1 is lost in CHK1-disrupted cells (137).

There is evidence that the spindle-assembly checkpoint or mitosis may regulate CHK1 reciprocally. CHK1 is phosphorylated during mitosis at Ser286 and Ser301, most likely by cyclin B1-CDK1 (139). The phosphorylation of CHK1 during the activation of the spindle-assembly checkpoint correlates with the inhibition of the kinase (12). In contrast, IR-mediated DNA damage during mitosis induces $CHK1^{Ser345}$ phosphorylation. Activation of CHK1 during mitosis may be involved in delaying mitotic exit and preventing cytokinesis (140).

Another protein kinase that may regulate CHK1 during mitosis is AKT (protein kinase B), a central effector of the PI-3 kinase signaling pathway. In HeLa and MDCK cells, the activity of AKT is low during S phase but rises in G_2/M, coinciding with the decrease in CHK1 kinase activity (141). Importantly, AKT can phosphorylate $CHK1^{Ser280}$ and prevent the ATM/ATR-dependent activation of CHK1 (142). Phosphorylation of Ser280 also triggers ubiquitination and cytoplasmic retention of CHK1 (143). Since the activity of AKT is antagonized by the potential tumor suppressor PTEN, a possible mechanism that the loss of PTEN may promote genome instability may be through the AKT-CHK1 pathway (143).

On the other hand, there is evidence suggesting crosstalk between CHK2 and the spindle-assembly checkpoint. MPS1 (TTK) is a dual specificity protein kinase that is localized to the kinetochores and centrosomes. Studies from several organisms indicate that MPS1 is required for the spindle-assembly checkpoint and centrosome duplication. Interestingly, MPS1 can bind and phosphorylate $CHK2^{Thr68}$ *in vitro* (144). Moreover, depletion of MPS1 with siRNA interferes with $CHK2^{Thr68}$ phosphorylation and CHK2 function in the G_2 DNA damage checkpoint (144).

Yet another link between mitosis and CHK2 may be coming from the polo-like kinases. PLK1 is a crucial effector for several mitotic events, including cyclin B1-CDK1 activation, chromosome condensation, chromosome segregation, and mitotic exit. It has been shown that PLK1 co-localizes with CHK2 to centrosomes and the midbody (145). Interaction between PLK1 and CHK2 peaks in mitosis and involves the polo box domain of PLK1 and phosphorylated $CHK2^{Thr68}$ (146). The mutual regulation of the two proteins is highlighted by the fact that PLK1 is able to phosphorylates $CHK2^{Thr68}$, and their interaction stimulates the activity of PLK1. In marked contrast, experiments using RNAi against CHK1 suggest that CHK1 is a negative regulator of PLK1, although it is not clear whether this effect is direct or not (136). Finally, it has been shown that the polo-like kinase PLK3 interacts with and phosphorylates CHK2 at Ser62 and Ser73, which facilitates subsequent phosphorylation of $CHK2^{Thr68}$ by ATM (147, 148).

8. SENESCENCE, STEM CELLS, AND CIRCADIAN CLOCK

Telomere shortening in normal human fibroblasts causes replicative senescence. It has been shown that telomere shortening activates a DNA damage-like response, with DNA damage foci components forming at the eroded telomeres. Importantly, both ATM/ATR and CHK1/CHK2 are activated during this process, eventually causing a p53-dependent growth arrest (149, 150). Furthermore, inactivation of CHK1/CHK2 in senescent cells can restore cell cycle progression. These data are consistent with the model that replicative senescence may represent a special case of permanently activated DNA damage checkpoint (151).

In contrast to somatic cells, embryonic stem (ES) cells undergo neither telomere shortening nor replicative senescence. ES cells are pluripotent, retaining the capacity to differentiate into any cell type in the organism. ES cells must have robust mechanisms to maintain the stability of their genomes in order to transmit faithful copies of undamaged DNA to progeny. Interestingly, ES cells lack a functional G_1 DNA damage checkpoint. Both the ATM-CHK2-CDC25A-CDK2 and the ATM-CHK2-p53-$p21^{CIP1/WAF1}$-CDK2 pathways appear to be compromised (152, 153). The lack of cell cycle arrest after DNA damage in ES cells may underlie the high level of apoptosis, and consequently may give rise to a relatively low mutation rate. Although ATM appears to be activated normally after DNA damage in ES cells, CHK2 function may be impaired due to sequestration at centrosomes (154). Indeed, ectopic expression of CHK2 is able to restore the CDC25A-mediated DNA damage checkpoint (but not the p53-dependent pathway).

In mammals, a master circadian clock resides in the suprachiasmatic nuclei of the hypothalamus. In the absence of external cues (such as light), this circadian cycle continues to operate with a cycle time of approximately 24 hours. A connection between the circadian cycle and the cell cycle has long being recognized, as exemplified by the direct regulation of *WEE1* transcription by circadian clock components (155). It is likely that restricting the cell cycle to nighttime, when there is less likelihood of DNA damage due to UV radiation is advantageous during evolution. Recent data also reveal a link between the circadian cycle and the cell cycle through CHK1 and CHK2. The human homologue of the *Drosophila* Timeless (TIM1) protein interacts with CHK1 and the ATR-ATRIP complex and plays an important role in the replication checkpoint and the intra-S phase checkpoint (156). Similarly, the human homologue of the clock protein CLK-2 (HCLK2) associates with CHK1, ATR, ATRIP, and Claspin, and may promote the checkpoint function by preventing unscheduled CHK1 degradation (157). In support of this, downregulation of HCLK2 compromises both the replication checkpoint and the intra-S phase checkpoint (157).

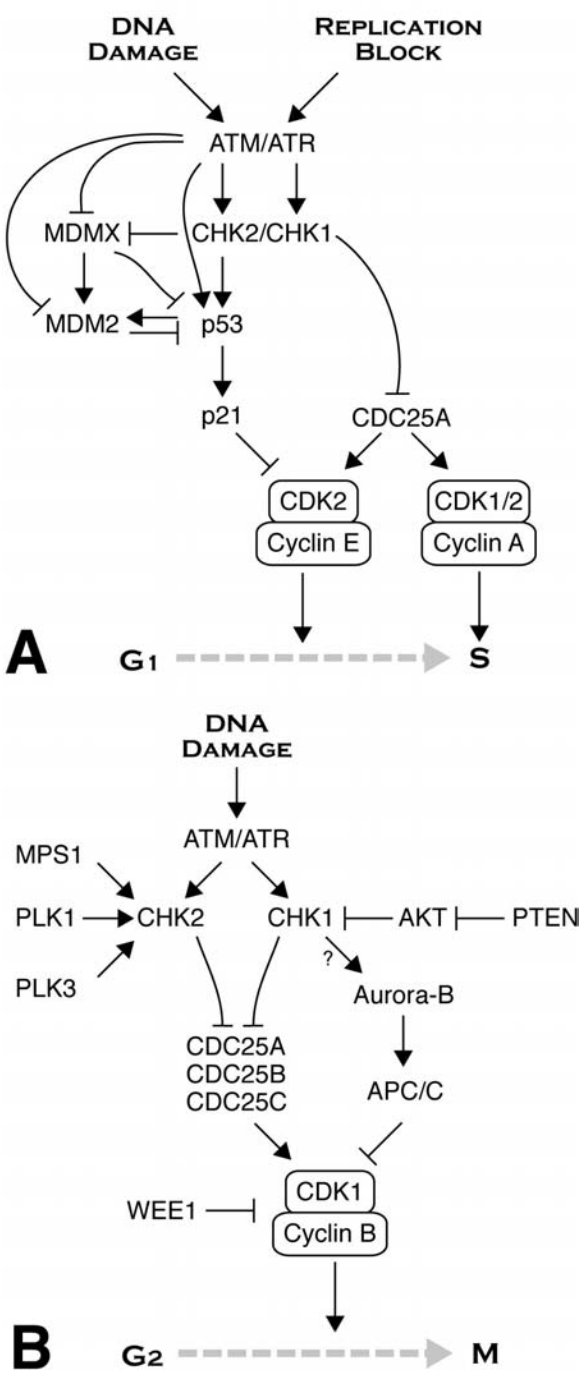

Figure 2. Checkpoint pathways mediated by CHK1 and CHK2. Selected functions of CHK1 and CHK2 during G_1 and S (A) and during G_2 and mitosis (B). See text for details.

Another core clock protein, PER1, is also found to interact with CHK2 and ATM (158). A role of PER1 in regulating the DNA damage responses is further verified by the result that overexpression of PER1 sensitizes cells to DNA damage-induced apoptosis. Conversely, downregulation of PER1 reduces DNA damage-induced apoptosis. In further support of a link between CHK2 and the circadian clock, the *Neurospora* clock protein PRD-4 is found to be an ortholog of mammalian CHK2 (159).

9. CONCLUSIONS AND PERSPECTIVES

CHK1 and CHK2 are mediators that link upstream activators, such as ATM and ATR, to cell cycle responses. Malfunctioning of CHK1/CHK2-dependent checkpoints can predispose cells to genomic instability. It is remarkable that CHK1 and CHK2 appear to play pivotal roles in a number of important checkpoints that monitor DNA damage, completion of replication, as well as mitotic spindle assembly (Figure 2). Why are the CHK1/CHK2 pathways so robust in response to different types of stress? It will be important to figure out which of the many functions of CHK1 and CHK2 are more critical under different conditions and in different cell types.

In carrying out their checkpoint functions, CHK1 and CHK2 phosphorylate a number of targets. While a number of proteins have been shown to be substrates of CHK1 and CHK2, it is expected that many others await to be described. Whole genome screens of CHK1/CHK2 phosphorylation sites in response to different types of stress will be informative. The effects on the targets are diverse, some of which can be classified into those that promote protein-protein interaction, which frequently involving 14-3-3 (CDC25B, CDC25C, and WEE1), hindering protein-protein interaction (p53-MDM2/MDMX), affecting the enzymatic activity (CDC25C and Aurora-B), and targeting for ubiquitin-mediated degradation (CDC25A and possibly MDMX). It is likely that new principles will be uncovered after a fuller spectrum of targets are discovered.

Relatively little is known about the inactivation of CHK1/CHK2, and for that matter, of the checkpoints in general, when the defects are mended. Are the phosphatases that reverse the activating phosphorylation in CHK1/CHK2 actively regulated by the checkpoint? Another exciting area of research will be the precise connection between CHK1/CHK2 and the circadian cycle. Given the current interest in stem cell research, the functions of CHK1/CHK2 in normal stem cells and cancer stem cells will also be an active area to explore.

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