DNA REPLICATION CHECKPOINT CONTROL

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1. ABSTRACT

The eukaryotic cell cycle comprises two critical phases, DNA replication (S phase) and the subsequent distribution of an equivalent genome to each of two daughter cells at mitosis (M phase). A signal transduction cascade called the replication checkpoint has evolved to ensure that M phase does not occur prior to the completion of S phase. The mitotic regulators targeted by this checkpoint have recently been identified in the fission yeast *Schizosaccharomyces pombe*. As was the case for the DNA damage checkpoint, studies on the replication checkpoint in fission yeast promise to provide an excellent framework for analogous studies in mammalian cells.

2. INTRODUCTION

Successful cellular proliferation requires the temporal coordination of a number of critical events. Most importantly, the integrity of the genome must be ensured before it is passed to daughter cells. In unicellular organisms such as yeast, loss of genetic material may lead to cell death. However, in higher eukaryotes genomic instability is causally related with tumorigenesis. To combat this instability cells have evolved 'checkpoints' that constantly monitor the genome and prevent the irreversible step of mitosis until DNA aberrations have been resolved. Weinert and Hartwell coined the term 'cell cycle checkpoint' to define a mechanism that maintained the observed order of events each cell cycle, for example, prevented the onset of mitosis during S phase or in the presence of DNA damage. They envisaged two possible ways of achieving this dependency: either the product of an earlier cell cycle phase was required for a later event or, an active mechanism restrained cell cycle progression until each phase was complete. The latter mechanism proved to be true as it was possible to isolate a mutation in yeast *rad9* that uncoupled the normal dependency of mitosis on the completion of DNA repair (1). The observations of Hartwell and Weinert (2) prompted a search for further 'relief of dependency mutations'. Many such mutations were found both in budding and fission yeasts, defining a number of checkpoints, including the replication or S-M checkpoint (3).

Budding and fission yeasts have played a major role in defining much of what we understand about checkpoints to date. Analysis of mammalian checkpoints has been greatly facilitated by isolating homologues of checkpoint genes previously characterized in yeast. The checkpoint organization in fission yeast appears to bare the most in common with mammalian systems and thus will be the focus of this review.

3. THE TARGET OF CHECKPOINT CONTROL

The pivotal step in mitotic induction is the activation of the cyclin-dependent kinase Cdc2 and it is therefore the logical target of the checkpoint machinery. This is true in most species (4-8) with the exception of budding yeast in which the Cdc2 homologue, Cdc28, is not targeted. In fission yeast, Cdc2 is held at interphase levels of activity by phosphorylation on tyrosine 15 (Y15)(9). This phosphorylation is performed by the mitotic inhibitory kinases Wee1 and Mik1 and removed prior to mitosis by the phosphatase Cdc25. Rhind and co-workers showed that inhibitory Y15 phosphorylation of Cdc2 was maintained during both DNA damage and replication checkpoint arrests in fission yeast (7, 10). These were important observations but still left the question of how the Y15 phosphorylation was maintained. The Cdc2 regulators Wee1, Mik1 and Cdc25 were the logical targets through which the checkpoints could impinge on the mitotic machinery and will be discussed later ('Effectors').

4. COMPONENTS OF THE REPLICATION CHECKPOINT

Checkpoint mechanisms consist of three essential parts: a way of sensing that a cell cycle event is aberrant or
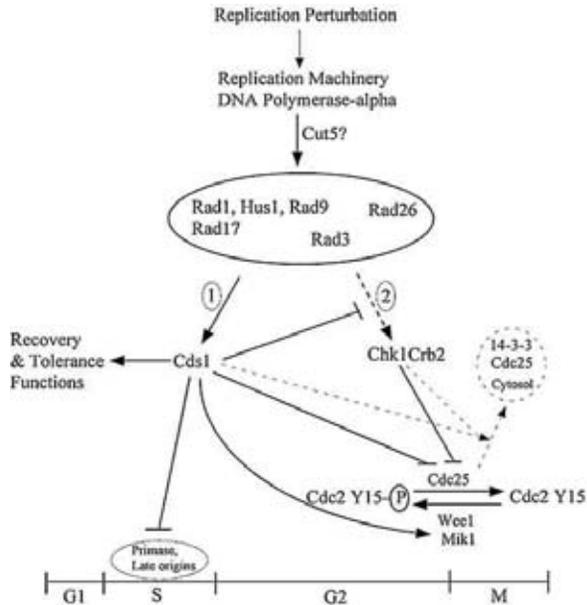
Figure 1. A model of replication checkpoint control in *S. pombe*. Many of the components shown have homologues in humans and the mechanism of action is likely to be largely conserved. In response to replication perturbation, a signal is received by Rad3and the other essential Rad proteins, probably from the replication machinery. Cds1 becomes phosphorylated and activated in a checkpoint Rad dependent manner (1). Activated Cds1 prevents mitosis by inhibiting Cdc25 and positively regulating the Cdc2 Y15 kinases. In addition to targeting the mitotic machinery, Cds1 activates a sub-pathway for replication arrest tolerance, and prevents the activation of Chk1 during S phase. If the Cds1 pathway is compromised, Chk1 may be activated resulting in mitotic arrest (2). See text for detailed description.

incomplete, a signal transduction cascade and finally, effectors that arrest cell cycle progression until the problem is resolved. The replication checkpoint prevents mitosis in the presence of incompletely replicated DNA. This checkpoint has been studied primarily using hydroxyurea (HU), an inhibitor of ribonucleotide reductase, that blocks replication through nucleotide deprivation. It has been suggested that early replication origins in budding yeast fire in the presence of HU but stall within ~10 kilobases of the origin (11). This response to HU is likely to be conserved in other eukaryotes. Many other replication perturbations can activate the replication checkpoint, including thermosensitive alleles of DNA polymerases alpha and epsilon (12). These will be discussed below under 'sensors'.

4.1. Sensors

How partially replicated DNA is detected is still unknown but there are a number of observations that suggest the signal arises from the replication machinery itself. Studies on proteins that are part of the replication apparatus are complicated by the fact that they are usually essential. However, spore germination studies have been performed with fission yeast diploids, heterozygous for the initiating DNA polymerase Pol-alpha (13). Pol-alpha deleted cells partially replicated their DNA, probably using residual maternal protein and proceeded to inappropriate mitosis without checkpoint arrest. These data suggest that Pol-alpha is required for the replication checkpoint even after the initiation of bulk DNA synthesis. Similar studies have been carried out recently using spores germinated from diploids that have one thermosensitive and one deleted allele of Pol-alpha (14). In this background, at the Pol-alpha restrictive temperature, there is no functional maternal protein to initiate replication in Pol-alpha deleted cells. As previously observed by D'Urso and co-workers these cells proceeded to catastrophic haploid mitosis. Data in the latter paper also suggested that it was the catalytic activity of Pol-alpha and not the physical presence of the protein that was required to activate the replication checkpoint. In budding yeast an essential DNA polymerase, epsilon (Pol-epsilon) has been suggested to be a sensor for the replication checkpoint (15). C-terminal truncations of Pol-epsilon do not affect its essential function but abrogate the replication checkpoint. This may be a result of disrupting the single strand 'sensing' ability of a zinc finger found at the C-terminus of Pol-epsilon or, loss of a protein-protein interface important for checkpoint function. This checkpoint role for Pol-epsilon is not conserved in fission yeast (16).

A number of other proteins required for replication in fission yeast are also required for activating the replication checkpoint. Deletions or mutations of Cdc18, Cdt1 and Cut5 uncouple or do not allow the establishment of the replication checkpoint (17-19). Cut5 is notable in that cells carrying thermosensitive alleles of Cut5 enter aberrant mitosis at restrictive temperature without completing replication (19). In addition, Cut5 mutants inactivate an already established replication checkpoint. That is, if Cut5 mutant cells are first treated with HU to activate the replication checkpoint and then shifted to restrictive temperature, cells enter aberrant mitosis due to loss of the checkpoint. It is interesting to note that although Pol-alpha is required to initiate and maintain the replication checkpoint, thermosensitive Pol-alpha alleles arrest in a replication checkpoint dependent manner at the restrictive temperature (13, 14). Therefore, although Cut5 and Pol-alpha deletion mutants display the same phenotype, thermosensitive alleles give distinct effects. The difference may be explained by the extent of allele inactivation at restrictive temperature. Cut5 was reported to be highly susceptible to proteolytic cleavage (20) and hence, the thermosensitive allele may become rapidly degraded as its conformation is compromised at higher temperatures, mimicking a deletion.

In conclusion, it appears that replication must be initiated to establish the replication checkpoint and prevent lethal haploid mitosis. After initiation, proteins involved in ongoing replication are required to maintain the replication checkpoint. At present, Cut5 is a possible candidate for a protein that is at the interface between the replication machinery and the checkpoint signal transduction cascade (21). The Saccharomyces cerevisae homologue of Cut5,

Dbp11, interacts with Pol-epsilon and is required for the replication checkpoint (22). The signal that is detected by the checkpoint sensors during replication is not known but has been suggested to be single stranded DNA that accumulates when replication is blocked (15).

4.2. Signal Transducers

The fission yeast replication checkpoint shares a number of upstream components with the DNA damage checkpoint (figure 1). These include Rad3, Rad1, Rad9, Rad26, Rad17, Hus1 and Cut5. A central element of damage and replication checkpoints in metazoans and yeasts is a PI-3 like kinase, Rad3 in fission yeast, ATM and ATR in mammalians and Mec1 in budding yeast (23-25). Loss of function of these kinases in different species abrogates DNA structure checkpoints. Rad3 shares homology with DNA dependent protein kinase (DNA-PK) that is activated by association with proteins that flag sites of DNA strand breakage. This suggests that Rad3 may be intimately involved in the sensing of aberrant DNA structures and initiating a kinase cascade. In fission yeast, Rad1 and Hus1 have been shown to form a complex in a Rad9 dependent manner (26). Hus1 appears to be phosphorylated in response to DNA damage and to a lesser extent by HU treatment. An analogous complex has also been observed in mammalian cells, between the human homologues of Hus1, Rad1 and Rad9, highlighting the conservation of these checkpoint rad genes from fission yeast to humans (27). It should be noted that in *S. pombe*, Rad1 and Hus1 associate in a Rad9 dependent manner. However, in mammalian cells Rad9, Rad1 and Hus1 have all been shown to physically interact.

Functional assignment of the central checkpoint rad genes from homology searches is for the most part uninformative. However, Rad1 has recently been suggested to share structural similarity with proliferating cell nuclear antigen (PCNA) and to possess exonuclease activity (28, 29). PCNA forms a homotrimer that encircles DNA and 'slides' along the genome during replication keeping the polymerase complex on its substrate. The loading of PCNA onto DNA requires several factors, including a protein called replication factor C (RFC). Interestingly, Rad17 shares homology with RFC and the human Rad17 homologue interacts with human Rad1 (29). Therefore, Rad1 as an exonuclease may be involved in processing sites of damaged DNA in a mechanism that requires it to encircle DNA in a Rad17 dependent fashion. The Rad17 and Rad1 homologues in S. cerevisiae are also suggested to be involved in DNA damage processing (30). Clearly, more biochemical characterization will be required to determine the precise roles of these rad proteins in checkpoint signaling.

The rad genes described above are, from a number of genetic and biochemical arguments, upstream components in the checkpoint signal cascade. There are two downstream kinases in this cascade that are activated differentially by the Rad proteins depending on the stimulus. The G2 DNA damage response is mediated by the serine/threonine kinase Chk1 (31). Chk1 becomes phosphorylated following DNA damage in a checkpoint rad dependent manner and, Chk1 mutants are defective in G2 arrest (31). In wild type fission yeast Chk1 is not phosphorylated (activated) in response to HU treatment or certain thermosensitive polymerase alleles that arrest cells in early S phase. However, Chk1 is activated by HU in cells that are deleted for Cds1, suggesting a role for Chk1 in the replication checkpoint (12, 32-34). That Chk1 is downstream of the six Rad proteins was suggested by overexpression studies (35). Chk1 overproduction causes cell cycle arrest in G2 that is dependent on Cdc2 Y15 phosphorylation but independent of the checkpoint Rads (7, 35). In addition, Chk1 has been shown to associate with and phosphorylate Cdc25, a regulator of Cdc2 (see 'Effectors') (36). Chk1 may also target Wee1, an inhibitor of Cdc2 (6). Recent studies suggest that Rad3 may bind and phosphorylate Chk1 directly (37).

Another serine/threonine kinase, Cds1, mediates the cell cycle response to replication perturbations. This kinase was first described as a suppressor of a thermosensitive allele of DNA polymerase alpha (38). S phase arrest or perturbation results in Cds1 activation in a checkpoint Rad dependent manner (12, 32). The activation of Cds1 is apparently restricted to S phase in response to DNA damage, nucleotide depletion or certain thermosensitive polymerase alleles (12, 32). There is a suggestion that Rad3 binds and phosphorylates Cds1, although no activation of Cds1 was observed (37). Overproduction of Cds1 results in a Rad independent and Cdc2 Y15 dependent G2 arrest, suggesting it is in proximity to the mitotic machinery (32). Furthermore, Cds1 has been shown to bind and phosphorylate the N-terminus of Wee1, phosphorylate and inactivate Cdc25 and cause accumulation of Mik1, all in a checkpoint dependent manner (see 'Effectors') (32, 34). In addition to eliciting cell cycle arrest during replication blocks, Cds1 has functions in maintaining cell viability following various forms of S phase insult. This role of Cds1 will be discussed under 'Intra S phase checkpoint and Cds1 mediated recovery function'.

The above observations raise an interesting question, that is, how do the same checkpoint Rad proteins mediate activation of Cds1 or Chk1, depending on the stimulus and cell cycle position. A simple explanation would be that Chk1 and Cds1 are reciprocally localized to the nucleus. In this situation, Cds1 would be nuclear during S phase and cytoplasmic in G2. However, Cds1 accumulates in the nuclei of cells during S phase and is never excluded completely from the nucleus [Brondello, 1999 #1337]. Experiments using a constitutive nuclear form of Cds1 showed that exclusion of Cds1 from the nucleus is not an essential regulatory step [Brondello, 1999 #1337]. In addition, Chk1 appears to be nuclear at all cell cycle positions (39). These observations argue against a simple model where access to the checkpoint Rads is regulated by compartmentalization. Another possibility is that there are specific 'adaptor proteins' that recruit Chk1 or Cds1 to the Rad complex depending on the stimulus. Some evidence exists to support this idea. The Crb2 protein associates with Chk1 in the yeast two-hybrid system and Crb2 delete cells are defective in Chk1

activation (21). Crb2 also interacts with Cut5 (Rad4), an essential protein that is required for both the replication checkpoint and the UV induced G2 damage checkpoint (21). Therefore, Crb2 may act to bring Chk1 to Cut5 and the activated Rad complex during a G2 damage response. Another report demonstrated that Chk1 activity is suppressed in the presence of activated Cds1 [Brondello, 1999 #1337]. Therefore, the existence of an S phase specific adaptor for the activation of Cds1 could also explain how Chk1 and Cds1 are active in different phases of the cell cycle. These models predict that it should be possible to isolate mutations in some of the checkpoint Rad proteins, which abrogate one checkpoint response and not the other. Indeed, an allele of Rad26 was isolated that was defective in monitoring S phase progression, but left the damage checkpoint intact (40). This is compelling evidence for the adaptor model; however, direct interaction of Cds1 and Chk1 with unique sites on the Rad proteins cannot be excluded.

Using overproduction assays Cds1 has been shown to co-immunoprecipitate with Rad26 in fission yeast (12). This interaction did not occur *in vitro* and therefore may be dependent either on an adaptor protein or on a post-translational modification. The latter would be an interesting possibility because Cds1 contains a motif called the forkhead associated domain (FHA). This domain appears to bind phospho-peptides and is found in numerous proteins involved in signal transduction pathways (41, 42). Rad53, the *S. cerevisiae* homologue of Cds1, contains two FHA domains that are required for its checkpoint functions. Following DNA damage, the C-terminal FHA domain of Rad53 binds hyper-phosphorylated Rad9, and this binding is required for the phosphorylation of Rad53 (42). Mutations in the C-terminal Rad53 FHA domain abrogate the G2/M cell cycle arrest following DNA damage, but leave the replication checkpoint intact. The FHA domain in Cds1 is near the amino terminus and is required for the *S. pombe* replication checkpoint (43). It is possible that Rad26 is modified in a replication checkpoint specific manner and recruits Cds1 to the Rad complex for activation.

4.3. Effectors

In fission yeast, Cdc2 is the ultimate target of the checkpoint signals that arrest cell cycle progression (4-8). As described above, the Cds1 and Chk1 kinases are at the interface between the checkpoint pathways and the mitotic regulators. The regulators of Cdc2 Y15 phosphorylation, the tyrosine kinases Wee1 and Mik1 and the phosphatase Cdc25, were the most obvious targets for Cds1 and Chk1. Although Chk1 was initially described to act solely in the G2 damage checkpoint, it has more recently been implicated in the replication checkpoint (12, 32-34). Therefore, the discussion on how the replication checkpoint acts on the mitotic machinery will include the effects of Chk1. It is important to note that these kinases are not completely redundant in checkpoint function. In this case, 'checkpoint function' does not refer solely to cell cycle arrest. Cds1 and Chk1 are equally capable of preventing mitosis during an HU induced replication block. However, Cds1 is necessary and sufficient for the replication checkpoint, but Chk1 is neither necessary nor sufficient for the normal response to replication arrest (12, 32-34). Chk1 is not normally activated by HU, but in the absence of Cds1, Chk1 is activated and can arrest cells, preventing premature mitosis (12, 32). Despite this arrest, Cds1 null cells rapidly lose viability during HU treatment (32). This highlights what is termed the recovery or tolerance function of Cds1 and will be described later (12, 44).

4.3.1. Dropping the Anchor, the Role of 14-3-3 Proteins

Chk1 was first described to bind and phosphorylate Cdc25 in both fission yeast and human cells, following DNA damage (36, 45, 46). Recently, Cds1 has also been shown to phosphorylate Cdc25 on the same sites as Chk1 (33, 34, 47). This phosphorylation regulates the activity of Cdc25 in at least two ways. Firstly, several sites on Cdc25 are phosphorylated by Cds1/Chk1 and this inhibits Cdc25 phosphatase activity *in vitro* (34, 48). Secondly, the phosphorylation of human Cdc25C on serine 216 creates a binding site for 14-3-3 proteins (46). 14-3-3 proteins are phosphoserine-binding proteins that are involved in many signaling pathways and have been implicated in the regulation of normal cellular proliferation and checkpoint function (49-51). The binding of 14-3-3 proteins to Cdc25 *in vitro* has little or no effect on the phosphatase activity of Cdc25, suggesting that *in vivo* 14-3-3 proteins somehow limit access of Cdc25 to Cdc2 (52). Elegant immunolocalization studies in fission yeast have now shown how this is likely to be accomplished (39). Just before mitosis, the Cdc2/cyclin B complex is localized in the nucleus; thus, Cdc25 must be nuclear in late G2 to effect Cdc2 activation. Indeed, Cdc25 localization changes throughout the normal cell cycle, accumulating in the nucleus in late G2 and being largely cytosolic in the other phases (39). Deletion of Rad24, a fission yeast 14-3-3 homologue, results in constitutive nuclear localization of Cdc25 and a concomitant advancement of mitosis. This result suggests that Rad24 plays a role in the regulation of Cdc25 activity during the normal cell cycle. Rad24 contains a nuclear export sequence and is apparently confined to the cytosol (39). These facts are consistent with a situation in which Cdc25 is exported from the nucleus, and held in the cytosol, by associating with Rad24. When cells reach the size for mitosis, Cdc25 is released into the nucleus (driven by an NLS in Cdc25) following de-phosphorylation of its Rad24 binding sites. This system would require a checkpoint independent phosphorylation of Cdc25 to drive Rad24 association (Chk1 or Cds1 delete cells have no obvious cell cycle defect). In support of this is the existence of a Cdc25C Serine 216 kinase in mammalian cells, called C-TAK1 (51). During a replication or DNA damage checkpoint arrest, Cdc25 remains cytosolic and this helps prevent mitosis. In the damage checkpoint this is dependent on Chk1 (39) and in the replication checkpoint may be dependent on Cds1. Therefore, Chk1 and Cds1 may act to maintain the 14-3-3 binding sites in Cdc25 during prolonged cell cycle arrests, in which cells have surpassed the size control requirement for mitosis.

4.3.2. Wee1 and Mik1

The first putative connection between the replication checkpoint and the mitotic machinery was the

observation that Cds1 binds to and phosphorylates the N-terminus of Wee1 in a checkpoint dependent manner (32). The *in vivo* significance of this phosphorylation is still unknown and is complicated by the redundancy of checkpoint targets (see Cdc25 and Mik1). However, it is interesting to note that the N-terminus of Wee1 is auto-inhibitory (53) and therefore, Cds1 may act to relieve this inhibitory effect. It has recently been shown that in *Xenopus* egg extracts, exogenously added Wee1 protein is stabilized by the replication checkpoint(54).

The replication checkpoint is intact in cells deleted for both Wee1 and Cdc25 (55). This fact suggested that Mik1 may also be a target of the replication checkpoint. Indeed, Mik1 accumulates during an HU induced arrest and this increase in Mik1 abundance is dependent on the presence of both Cds1 and Rad3 (32). Therefore, activation of the replication checkpoint causes the accumulation of a dosage dependent inhibitor of Cdc2. The accumulation of Mik1 is a result of both positive transcriptional regulation and protein stabilization (56). Mik1 is not phosphorylated *in vitro* by Cds1 and therefore its stabilization is unlikely to be a direct effect of Cds1 (43).

5. THE INTRA S PHASE CHECKPOINT AND, 'TOLERANCE' OF REPLICATION BLOCKS

As described earlier ('Effectors'), Cds1 plays additional roles in the response to replication perturbations, beyond effecting mitotic delay. That is, in cells deleted for Cds1 that retain a mitotic delay following HU treatment, viability is rapidly lost (32, 38). In addition, UV irradiation of cells in S phase results in a Cds1 dependent slowing of replication (12, 57). Rad53, the *S. cerevisiae* Cds1 homologue is also required for the analogous function in budding yeast. Interestingly, this Cds1 dependent slowing of replication does not appear to enhance survival of cells that are irradiated in G1 or S phase (57). However, Chk1 deleted cells that lack a G2 damage checkpoint are less sensitive to UV damage than cells deleted for both Chk1 and Cds1 (or one of the essential Rad proteins) (43, 44). This clearly demonstrates a role for Cds1 in UV damage tolerance. What does Cds1 do during HU treatment or following UV irradiation to maintain cell viability? The first response may be to stop or pause replication forks (depending on the severity of replication perturbation) and stabilize these to prevent the formation of aberrant DNA structures. To achieve this, Cds1 may target components of the replication machinery such as the DNA polymerase-alpha primase, as proposed for Rad53 in *S. cerevisiae* (58). In addition to stalling active replication forks, Cds1 may prevent initiation at late-firing origins of replication. Rad53 has been shown to perform this function in *S. cerevisiae* (59). To maintain reversibility of stalled replication, Cds1 may control a subgroup of proteins including Rqh1 (44, 60). Rqh1 is a homologue of the prokaryotic RecQ helicase and loss of its function results in irreversible S phase arrest (60). The role of Rqh1 appears to be the suppression of hyper-recombination that would otherwise occur following replication arrest (60).

6. MODEL OF THE REPLICATION CHECKPOINT AND CONCLUSIONS

A number of important questions on how the replication checkpoint functions have been answered in recent literature. This allows us to 'flesh out' the existing model of the replication checkpoint and highlights its complexity compared with that of the G2 damage checkpoint in fission yeast (figure 1). This model encompasses the current results of both genetic and biochemical experiments on the replication checkpoint. However, an alternate model has been proposed in which Cds1 functions solely to stabilize stalled replication forks (12). In this model, an intrinsic checkpoint coupling S phase and mitosis operates during a replication arrest, possibly by sequestration of Cdc2. The fact that the replication checkpoint is dependent on the maintenance of Cdc2 Y15 phosphorylation, argues that Cdc2 sequestration is not the sole mechanism preventing mitosis. In addition, a preponderance of evidence now exists showing that Cds1 and Chk1 directly regulate the modulators of Cdc2 Y15 phosphorylation (Wee1, Mik1 and Cdc25).

As described earlier there are many similarities between the mechanisms of checkpoint arrest in fission yeast and humans. These include a central PI-3 like kinase, Rad3 in fission yeast and ATM in humans. Rad3 and ATM are both required for the activation of the downstream kinases Cds1 and Chk1. In addition, Rad1, Hus1 and Rad9 form a complex in fission yeast and this complex is conserved in humans. Conservation that is even more striking is observed at the level of Cdc25 regulation. Human and fission yeast Chk1 proteins phosphorylate the same sites on human Cdc25C. Since Cds1 and Chk1 phosphorylate the same sites in fission yeast Cdc25, Cds1 would also be likely to phosphorylate human Cdc25C on serine 216.

The role of Cds1 and Rqh1 in damage tolerance and HU resistance may also be conserved to humans. Rqh1 has several homologues in humans including the Bloom's helicase (61). Mutations in the Bloom's gene (*BLM*) results in genomic instability and cancer predisposition (62). This is in part a result of hyper-recombination, as observed in the fission yeast *rqh1* mutants.

The parallels between human and fission yeast checkpoint organization highlighted above, suggest that studies on fission yeast will continue to pave the way for analogous studies in humans.

7. ACKNOWLEDGEMENTS

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