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SGM SPECIAL LECTURE

Analysis of fission yeast DNA structure checkpoints

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Overview

Work in *Schizosaccharomyces pombe* and *Saccharomyces cerevisiae* has identified many of the elements which define the pathways that monitor DNA status within the cell and delay the cell cycle when this is appropriate. Checkpoints include a detection mechanism, a signal transduction process and an effector that interacts with the cell cycle machinery. Two distinct but overlapping checkpoint pathways can be delineated in *Schiz. pombe* which monitor DNA metabolism and send signals to the mitotic apparatus: the DNA damage checkpoint, which arrests mitosis when DNA is damaged, and the DNA replication checkpoint, which prevents mitosis during ongoing or stalled DNA replication. The DNA structure checkpoint pathways also co-ordinate other functions, including transcriptional activation and the reversible arrest of DNA replication after DNA damage or S phase arrest. Many of the proteins required for the checkpoint pathways have been identified. Our current model suggests that a core complex of at least six or more proteins is required to sense DNA structure changes and to activate two downstream kinases, Chk1 and Cds1, which signal to mitotic control and replication proteins. In this way, checkpoints are able to co-ordinate repair, mitosis and replication. Chk1 is phosphorylated in response to DNA damage but not in response to DNA replication arrest. Cds1 is activated by DNA damage only during S phase and is also activated in response to DNA replication arrest with hydroxyurea. This demonstrates that the biochemical outputs of the checkpoint pathway are dependent on the initial signalling event and confirms our genetic conclusions. The DNA structure checkpoint proteins have been conserved throughout eukaryotes, and work in mammalian cells suggests a role in both the DNA damage response and in controlling progress through meiosis, possibly by monitoring the status of recombination events.

Background

The first description of the checkpoint concept was published by Weinert & Hartwell (1988). This work centred around the *Sacch. cerevisiae rad9* mutant, which is sensitive to DNA damage. The authors linked this sensitivity to an inability to arrest mitosis following irradiation, and genetically defined the checkpoint pathways. Two years later Enoch & Nurse (1990) described the first characterization of a fission yeast checkpoint mutant. In this case the defect was in responding to the replicative status of the genome. Interestingly, this mutation was an allele of the *cdc2* gene, and suggested that the checkpoint pathway that monitored the status of DNA replication directly affected the activity of the Cdk complex.

My interest in fission yeast checkpoints arose from my postdoctoral work at the Cell Mutation Unit, University of Sussex, where we began a molecular characterization of the DNA repair genes of *Schiz. pombe* (Lehmann, 1996). After reading the work of Weinert & Hartwell (1988) concerning *RAD9* in *Sacch. cerevisiae*, I started to look at the phenotypes of the DNA damage-sensitive mutants of *Schiz. pombe* more closely. I also instigated a screen for checkpoint mutants using various phenotypes as end-points, the most successful of which turned out to be looking for co-sensitivity to both DNA damage and to the replication inhibitor hydroxyurea. From amongst the previously described DNA-damage-sensitive (*rad*) mutants, *rad1*, *rad3*, *rad9* and *rad17* were identified as defective in all the DNA-structure-dependent checkpoints including pre-mitotic arrest following DNA damage and the prevention of mitosis during DNA synthesis arrest by hydroxyurea (Al-Khodairy & Carr, 1992). The mutants screen, in addition to throwing up over 60 alleles of these four genes, also identified two more loci with the same phenotype and a couple of loci with distinct phenotypes (Al-Khodairy *et al.*, 1994). These included the *chk1* gene (Walworth *et al.*, 1993) and the *rad24* gene (Ford *et al.*, 1994), which appeared to be specific for the DNA damage checkpoint pathway,

but did not affect the DNA replication checkpoint pathway.

Several other groups were also working in the same area: Rowley *et al*. (1992) identified the same four known *rad* mutants as defective in the mitotic checkpoints, Tamar Enoch identified the same loci looking for checkpoint mutants that could not respond correctly to DNA replication arrest (Enoch *et al*., 1992) and Nancy Walworth in David Beach's laboratory identified Chk1 through a genetic interaction with a specific $p34^{cdc2}$ allele (Walworth *et al*., 1993). When put together, we were able to use all this data to construct a simple model of the *Schiz. pombe* checkpoint pathways (Sheldrick & Carr, 1993). This model proposed that two separate checkpoints were operating in *Schiz. pombe*, with distinct inputs (DNA damage or stalled DNA replication) that resulted in different outputs (defined by either the *cdc2.3w* mutant in the case of S phase arrest or *chk1* in the case of DNA damage). While it is perhaps not unexpected that two different pathways would be operating in these different circumstances, it was surprising that both pathways absolutely required the function of the same six or more proteins (the checkpoint Rad proteins). Furthermore, at this point, in *Sacch. cerevisiae*, a linear pathway was being proposed where two different branches of sensing (DNA damage and DNA replication) converged to activate a single signal transduction pathway to arrest mitosis (Weinert *et al*., 1994).

Characterization of checkpoint genes and mutants

To understand the function of the checkpoint pathways, and to begin to dissect the mechanisms by which these sense and signal the presence of specific DNA or DNA–protein structures, we began a systematic molecular and phenotypic characterization of the checkpoint genes. Between our own work and that of Tamar Enoch, Nancy Walworth and Suresh Subramani, the six checkpoint *rad* genes, along with *chk1*, *rad24* and *hus5*, were sequenced and their associated mutants characterized (Al-Khodairy *et al*., 1994, 1995; Bentley *et al*., 1996; Ford *et al*., 1994; Griffiths *et al*., 1995; Kostrub *et al*., 1997; Murray *et al*., 1991; Sunnerhagen *et al*., 1990; Walworth *et al*., 1993). This molecular and phenotypic characterization revealed a number of interesting points. Perhaps the most provocative was the observation that the checkpoint *rad* gene products in some way protected the cells from the consequences of insults during S phase (Enoch *et al*., 1992). This was at the time unexpected, since it had previously been assumed that cells were dying during the abortive mitosis. In fact, Tamar Enoch clearly showed that checkpoint *rad* mutant cells died when exposed to hydroxyurea as a result of being unable to reverse the arrest of S phase after a brief treatment with hydroxyurea.

Similarly, we were able to demonstrate that a specific checkpoint *rad* mutant allele resulted specifically in sensitivity to DNA damage during the G1/S phase of the cell cycle, but not during G2 (Al-Khodairy *et al*., 1994). This mutant, *rad26.T12* continues to be of particular interest to us. Complete deletion of the *rad26* gene results in loss of all the checkpoints, as is typical of the checkpoint *rad* mutants. However, the *rad26.T12* mutation [which is an arginine to tryptophan change at residue 541 (Lindsay *et al*., 1998)] has a completely normal mitotic arrest after DNA damage, but still remains sensitive to DNA damage. This formally separated an element of the radiation sensitivity for the checkpoint pathway away from the inability to arrest mitosis. Using genetic analysis, we were able to separate the sensitivity to radiation associated with loss of the checkpoint pathway into at least two elements, that caused by loss of mitotic arrest (best defined by the *chk1* mutant) and that which is specific to G1/S phase and which was at this point defined by the *rad26.T12* mutation. Combining both these mutations in one cell led to a phenotype not dissimilar to deletion of the entire *rad26* gene (Al-Khodairy *et al*., 1994).

Mutational analysis of the *rad17* gene also identified a role for checkpoint proteins in an aspect of DNA repair (Griffiths *et al*., 1995). Rad17 contains several domains with similarity to the subunits of RFC (replication factor C), suggesting that Rad17 functions to bind specific DNA structures. Mutagenesis of several of these conserved residues revealed a phenotype that made cells specifically sensitive to ionizing radiation (but not to UV or hydroxyurea exposure) but which did not affect the ability of cells to arrest mitosis. Furthermore, the Rad1 protein was found to be homologous to the *Ustilago maydis* Rec1 gene product (Carr, 1994; Long *et al*., 1994), which has been characterized as a nuclease (Thelen *et al*., 1994). Using the two hybrid system and co-immunoprecipitation we have shown that Rad1 and Rad17 associate together. It is intriguing to speculate that this association of a potential nuclease with a potential DNA-binding protein might be important for specific aspects of DNA repair or checkpoint signalling, although this has not yet been demonstrated.

Signalling in checkpoint pathways

Several protein kinases have been identified in the checkpoint pathways. One of the checkpoint Rad proteins, Rad3, is a large protein with a C-terminal domain which is a member of a subfamily of kinases with homology to PI-3 kinase (Bentley *et al*., 1996). The most characterized protein kinase in this family is the DNA-PK catalytic subunit (Jeggo *et al*., 1995). DNA-PKcs is activated by association with the Ku70/80 heterodimer when the latter binds to DNA. An assay for Rad3 activation has not yet been identified, but it is likely that Rad3 exists in a complex with other members of the checkpoint Rad group of proteins, and we have proposed that these may act as specific activating subunits of the Rad3 kinase in a manner analogous to the Ku–DNA-PKcs interactions (Carr, 1997). Although such a model is yet to be proven, genetic and immuno-

precipitation data are consistent with the existence of a checkpoint protein complex.

Two additional kinases have been shown to act downstream of Rad3. The Chk1 protein kinase is phosphorylated in response to DNA damage (Walworth & Bernards, 1996). This phosphorylation event correlates to biological activity (although it has not been possible to see Chk1 kinase activity increase after DNA damage) and is dependent on the integrity of the checkpoint Rad proteins. Similarly, the Cds1 kinase (Murakami & Okayama, 1995) acts downstream of the checkpoint Rad proteins (Lindsay *et al*., 1998). Cds1 is also phosphorylated by DNA damage and its activity against MBP (myelin basic protein) in immunoprecipitates is increased after DNA damage or after exposure of cells to hydroxyurea. Analysis of Cds1 activation and phosphorylation through the cell cycle demonstrates that Cds1 can only be activated by DNA damage or hydroxyurea when cells are in S phase, thus Cds1 defines an S-phase-specific subpathway of the checkpoint response (Lindsay *et al*., 1998).

Interestingly, Cds1 null mutant cells remain able to arrest mitosis during hydroxyurea exposure, even though they are sensitive to this treatment. Combining a Cds1 mutant with the Chk1 null mutant resulted in a phenotype which is similar to deletion of the *rad3* gene. This implied that the mitotic arrest seen in Cds1 null mutants after hydroxyurea exposure was dependent on Chk1. It is formally possible that Chk1 and Cds1 have overlapping redundant roles in hydroxyurea-imposed mitotic arrest. However, Nancy Walworth had demonstrated that Chk1 does not become phosphorylated during hydroxyurea exposure (Walworth & Bernards, 1996), suggesting it is not normally involved in this response. We looked at the phosphorylation of Chk1 in *cds1* null cells and found that Chk1 is phosphorylated in response to hydroxyurea when Cds1 function is missing (Lindsay *et al*., 1998). This strongly implies that Cds1 normally acts in a way that prevents the activation of Chk1 when cells are blocked in S phase. Our interpretation of this is that Cds1 protects cells from catastrophic and lethal DNA lesions arising during S phase arrest, possibly by stabilizing stalled replication structures. In the absence of Cds1, replication structures may be prone to disassociate from the DNA, leaving the possibility that DNA lesions are created that are both lethal, and potential activators of the DNA damage checkpoint and therefore of Chk1.

Checkpoint pathway(s) co-ordinate several different DNA damage responses

It has become clear that the checkpoint proteins co-ordinate a large number of DNA damage and S phase arrest responses. In *Sacch. cerevisiae* the checkpoint proteins are required for the induction of a range of gene products after DNA damage and treatment with hydroxyurea, as well as being required to prevent progress into S phase and through S phase in the presence of DNA damage (Aboussekhra *et al*., 1996; Allen *et al*., 1994; Kiser & Weinert, 1996). In *Schiz. pombe*, the *rad1* gene is required to induce the transcription of *sup22*, an RNR (ribonucleotide reductase) subunit, after DNA damage and S phase arrest by hydroxyurea (Taylor *et al*., 1996). Noel Lowndes has recently demonstrated that this is true for all the checkpoint *rad* genes, and that mutations in the downstream kinases *cds1* and *chk1* do not completely abolish this induction (personal communication). It is also clear from our recent work that in *Schiz. pombe* the checkpoint which slows down S phase after DNA damage is dependent on the Rad3 protein. Interestingly, Cds1 activity is required for survival after DNA damage during S phase. The work of Murray *et al*. (1997) has demonstrated that the *rqh1* gene product, the *Schiz. pombe* RecQ homologue (Stewart *et al*., 1997), acts in a DNA damage response which is dependent on Cds1 and the checkpoint proteins, and which acts in association with recombination proteins such as Rhp51, the *Schiz. pombe* RecA homologue (Muris *et al*., 1996). This damage response is required for cells to survive unrepaired DNA damage during S phase, and may be formally analogous to post-replication repair in bacteria, where polymerases stall at DNA lesions and the resulting gap is repaired by recombination with the daughter strand. In human cells, several RecQ homologues have been identified. The *BLM* gene is defective in Bloom's syndrome patients (Ellis *et al*., 1995) and the *WRN* gene is defective in Werner's syndrome patients (Yu *et al*., 1996). While cells derived from these two syndromes are not specifically sensitive to DNA damage, both syndromes are characterized by elevated genomic instability and cancer proneness (Ellis, 1997). It is thus possible that, like the situation with loss of the p53-dependent G1 arrest checkpoint, loss of S-phase-specific repair functions results in genomic instability as opposed to decreasing survival in clonogenic assays (Hartwell & Kastan, 1994).

Models for DNA structure checkpoints

Early work by Rao & Johnson (1970) fusing mammalian cells in different stages of the cell cycle demonstrated that an intrinsic signal is present during S phase that is able to prevent mitosis. In the light of the formulation of the checkpoint concept, it is suggested that this work reveals the operation of the DNA structure checkpoint pathway during normal DNA synthesis. We have looked for the activation of Cds1 during S phase, but have not found evidence that Cds1 is activated when S phase proceeds unperturbed. Similar studies suggest the same is true for the *Sacch. cerevisiae* homologue Rad53 (Sanchez *et al*., 1996; Sun *et al*., 1996). This could suggest one of two things, either an intrinsic checkpoint does not operate during normal S phase, or an intrinsic checkpoint links unperturbed S phase and mitosis, but it is independent of Cds1. We have proposed two models to account for all the data which surround the DNA replication checkpoint responses in *Schiz. pombe*, one of which is consistent with the lack of an intrinsic checkpoint during S phase, and a second which allows

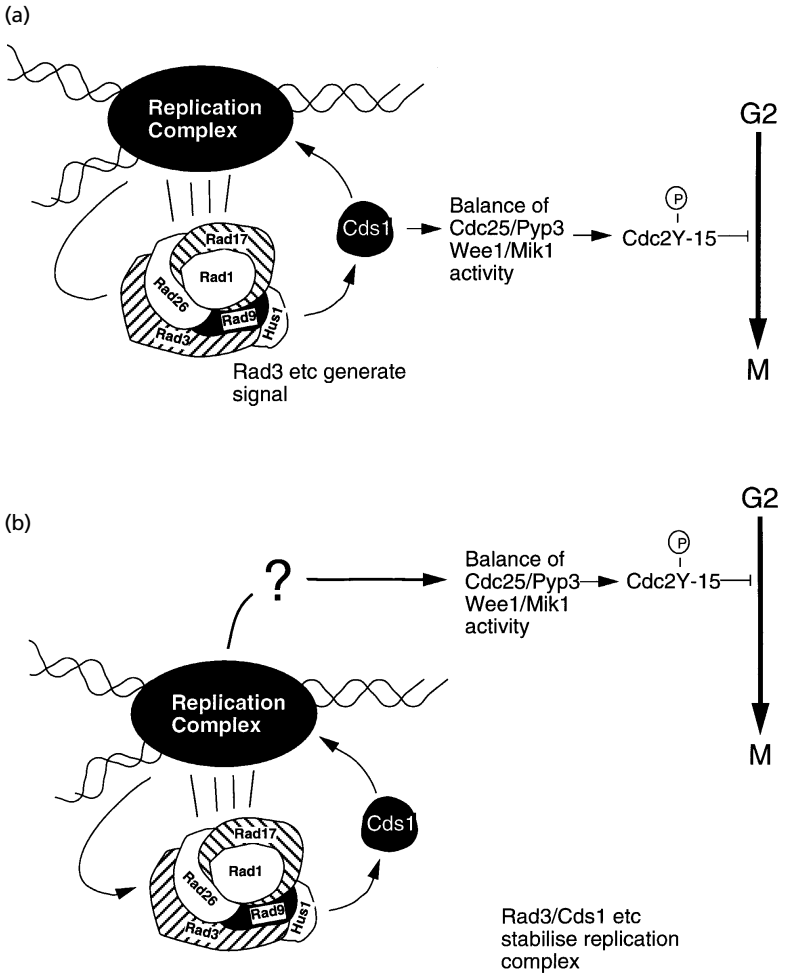

Fig. 1. Models for the checkpoint function during S phase. (a) Cds1 is activated by perturbations to S phase in a manner dependent on the checkpoint Rad proteins. Cds1 mediates S phase arrest by phosphorylating components of the replication complex and mediates mitotic arrest by phosphorylating a substrate that leads to inactivation of $p34^{cdc2}$. (b) Cds1 only acts to phosphorylate the replication machinery. This would prevent inappropriate DNA damage and maintain the structure(s) that are activating a Cds1-independent mitotic arrest signal. Such a signal would also be independent of the checkpoint Rad proteins, until replication was perturbed, when maintenance of the appropriate replication structures becomes pre-requisite.

for the operation of an intrinsic link between S phase and mitosis in the absence of perturbation (Fig. 1).

In both models the DNA status is monitored by Rad3 and its subunits (the remaining checkpoint Rad proteins), which are proposed to form a guardian complex that can interact with either specific DNA structures or DNA–protein complexes at specific points in the cell cycle. In this way the guardian complex would either directly activate or allow the indirect activation of the appropriate signalling kinases. In the case of DNA damage outside S phase, the signalling kinase would be the Chk1 kinase, which then effects mitotic arrest by phosphorylating the regulators of $p34^{cdc2}$ [either Wee1, Cdc25 or both Wee1 and Cdc25 (Furnari *et al*., 1997; O'Connell *et al*., 1997)]. In the case of DNA damage during S phase, or in response to hydroxyurea treatment and stalled DNA replication, the signalling kinase would be Cds1. In our first model, Cds1 would be responsible for inactivating $p34^{cdc2}$, possibly by affecting its regulators, and for stabilizing DNA replication (and possibly co-ordinating replication and repair). In our second model, Cds1 only acts on replication/repair, and it is the act of stabilizing or retaining replication structures in the appropriate state that allows the continued signalling of an intrinsic checkpoint. In this model, Cds1 and the guardian complex are not directly functioning as checkpoint proteins, since the intrinsic checkpoint functions in their absence, but are specialized replication proteins which are required to retain the conformation of the DNA replication apparatus which becomes a pre-requisite for the continuation of the checkpoint signal.

By extension of this model, it is also possible to propose two models for the DNA damage checkpoint response (Fig. 2). In the first, the pathway operates in a linear manner: DNA damage is detected by a guardian complex containing Rad3, which results in the phosphorylation of Chk1, which itself phosphorylates Cdc25 and Wee1 to prevent the activation of $p34^{cdc2}$ by tyrosine dephosphorylation. In the second model, Chk1 (and possibly other associated proteins) may be able to directly detect DNA damage (or a by-product of DNA damage such as protein/DNA structures or single-stranded DNA). The function of the checkpoint Rad complex would be to facilitate access of Chk1 to such structures. While the current data do not distinguish between these possibilities, one attraction of the second model is that it would help explain how checkpoints can

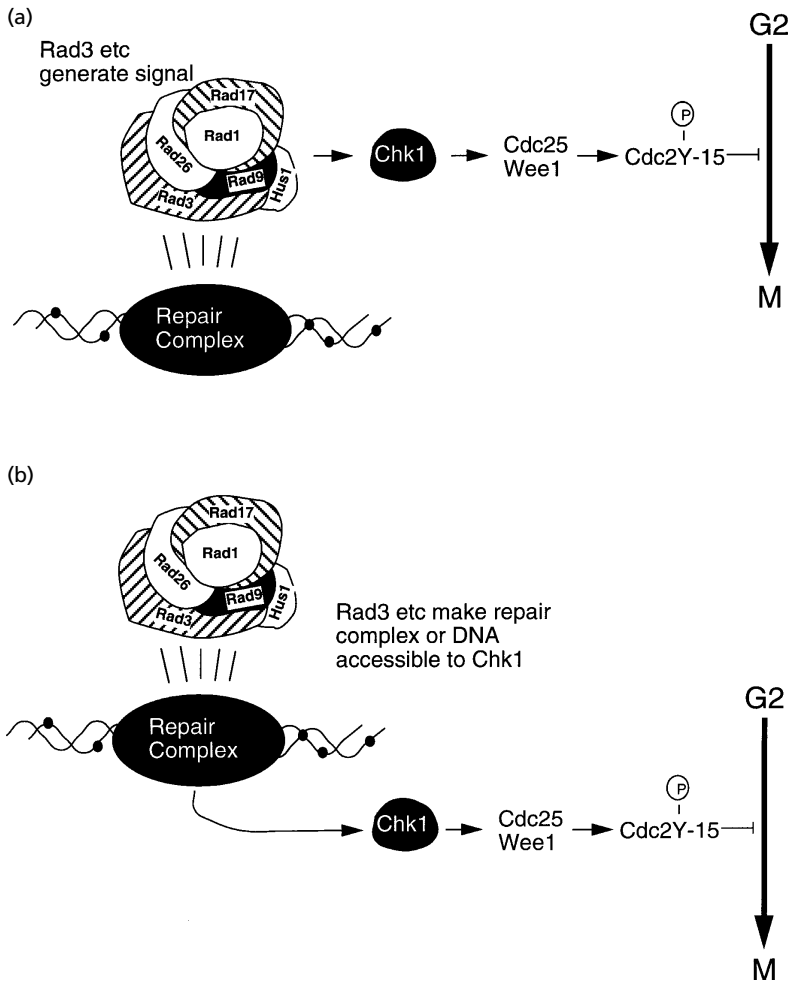

Fig. 2. Models for the checkpoint function after DNA damage. (a) Chk1 is activated by DNA damage or its repair in a manner dependent on the checkpoint Rad proteins. Chk1 mediates mitotic arrest by phosphorylating Wee1 (activation) and/or Cdc25 (inactivation) leading to inactivation of $p34^{cdc2}$. (b) Chk1 can access DNA damage or DNA repair structures independently of the Rad3 complex, but requires the Rad3 complex to make this an efficient process.

be reinstated in *Sacch. cerevisiae* by overexpression of an unrelated human protein when key checkpoint proteins are mutated or entirely absent (Pati *et al.*, 1997).

The DNA structure checkpoint proteins are conserved in human cells

In mammalian cells three DNA damage checkpoints have been identified. The G1 checkpoint prevents progress into the cell cycle after DNA damage. The intra-S phase checkpoint slows down S phase in the presence of DNA damage (presumably to allow co-ordination between DNA replication and DNA repair). The G2 checkpoint delays the onset of mitosis when the DNA is damaged in G2 (Hartwell & Kastan, 1994). The *ATM* gene (*A*taxia *T*elangiectasia *M*utated) is the only human checkpoint gene which has been identified by function and which is related to a yeast checkpoint gene (Savitsky *et al.*, 1995). *ATM* is homologous to *Schiz. pombe rad3* and *Sacch. cerevisiae TEL1* and *MEC1*, and is required for the correct functioning of the DNA structure checkpoints in response to ionizing radiation. A second related gene, *ATR* (*AT*axia- and *R*ad-related) has been identified by sequence similarity to *Schiz. pombe rad3* (Bentley *et al.*, 1996; Cimprich *et al.*, 1996). *ATR* is more closely related to *rad3/MEC1* and is able to complement some phenotypes associated with mutations of *Sacch. cerevisiae MEC1*. *ATR* did not complement mutations in *Schiz. pombe rad3*, but is able to physically associate with *rad3* to form heteromers (Bentley *et al.*, 1996).

More recently a number of human genes have been identified from Est libraries which are clearly structural homologues of the *Schiz. pombe* and *Sacch. cerevisiae* checkpoint proteins (Table 1). In particular, a human homologue of Chk1 has been identified which is modified in response to DNA damage and is able to phosphorylate a specific and biologically relevant residue of human Cdc25 proteins (Peng *et al.*, 1997; Sanchez *et al.*, 1997). This phosphorylation event creates a 14-3-3-protein-binding site, and the binding of a 14-3-3 protein to this site is proposed to inactivate the Cdc25 phosphatase. Such an interpretation is consistent with the work in *Schiz. pombe*, where Rad24 function has been placed downstream of Chk1 and there is evidence that Cdc25 has a role during mitotic arrest after DNA damage (Barbet & Carr, 1993; Ford *et al.*, 1994; Furnari *et al.*, 1997). Taking all the data together it would seem that a conserved pathway exists between yeast and human cells for the DNA damage checkpoint at mitosis: DNA structure changes are monitored by complex(es)

Table 1. Gene products involved in the DNA structure checkpoints

Schiz. pombe	*Sacch. cerevisiae*	Human	Comment
Rad1	Rad17	Est	Possible nuclease
Rad3	Mec1	ATM, ATR	Lipid kinase motif
Rad9	–	hRad9	None
Rad17	Rad24	Est	Replication factor C homology
Rad24/25	Bmh1/2	14-3-3	14-3-3
Rad26	–	–	None
Hus1	–	Est	None
Hus5	Ubc9	hsUbc9	Ubiquitin-conjugating
Chk1	Chk1	hChk1	Cdc25 kinase
Cds1	Rad53	–	S-phase kinase
Rqh1	Sgs1	Blm, Wrn, RecQL	Helicase
Rhp9	Rad9	–	BRA1 C-terminal domains

of proteins centred around the large lipid kinase motif proteins (Rad3/Atm/Atr). These complex(es), directly or indirectly, activate the Chk1 kinase, which in turn phosphorylates the regulators of $p34^{cdc2}$. This ensures that $p34^{cdc2}$ itself is not activated when DNA damage is present.

The mouse ATM and ATR proteins and the mouse Chk1 kinase homologue, have all been shown to associate with meiotic chromosomes in patterns that suggest participation in the monitoring of recombination events and/or the signalling of this information to co-ordinate the onset of the meiotic divisions (Flaggs *et al*., 1997; Keegan *et al*., 1996). This would be consistent with recent work in *Sacch. cerevisiae* which has demonstrated a requirement for the DNA structure checkpoint proteins to monitor double-strand breaks associated with meiotic recombination (Lydall *et al*., 1996). It is thus apparent that the DNA structure checkpoint proteins are conserved both structurally and functionally across eukaryotes.

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