DNA damage checkpoint maintenance through sustained Chk1 activity

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Summary

The G2 DNA damage checkpoint prevents mitotic entry in the presence of DNA damage. This requires the activation of the phosphoinositide-3-kinase-related protein kinases ATR and ATM in human cells and the ATR homologue Rad3 in the fission yeast *Schizosaccharomyces pombe*. Rad3 activates the effector protein kinase Chk1 by phosphorylation. However, in fission yeast, inactivation of Rad3 following checkpoint activation has no impact on checkpoint duration. This demonstrates that Rad3 is not required for checkpoint maintenance and that the processes of checkpoint initiation and maintenance are distinct. Chk1 is required for checkpoint initiation but its role in checkpoint maintenance has not been investigated. We show here that Chk1 kinase activity is rapidly induced following irradiation and is maintained for the duration of a checkpoint arrest. On entry to mitosis, there is a transient decrease in Chk1 activity and phosphorylation, but Chk1 activity remains higher than that observed in unirradiated cells. We have generated temperature-sensitive alleles of *chk1*, which phenocopy *chk1* deletion at the non-permissive temperature. Using these alleles, we have shown that inactivation of Chk1 during a checkpoint arrest leads to premature checkpoint termination, resulting in catastrophic mitoses that are a hallmark of checkpoint failure. Therefore, unlike Rad3, Chk1 is an important determinant of both checkpoint initiation and maintenance.

Key words: Checkpoint, Chk1, DNA damage, Protein kinase

Introduction

The cellular response to DNA damage involves both the repair of lesions and the activation of signalling pathways, known as checkpoints, that delay cell cycle progression until the completion of repair (Hartwell and Weinert, 1989). DNA damage checkpoints share the common architecture of detectors, signal transducers and effectors (O'Connell et al., 2000). Conceptually, the checkpoint delay can be divided into three phases: initiation; maintenance during repair; and termination once repair is completed to allow cell cycle progression.

The G2 DNA damage checkpoint acts to prevent mitotic entry in the presence of DNA damage and is highly conserved from fission yeast to humans. Initiation of this checkpoint in mammalian cells involves activation of the ATM and ATR protein kinases. These serine/threonine kinases share sequence homology with the phosphoinositide-3 lipid kinases (PI-3Ks) (O'Connell et al., 2000) and are therefore termed PI-3K-related kinases (PIKKs). In fission yeast, the earliest known biochemical marker of checkpoint initiation is the activation of the ATR homologue Rad3 (Edwards et al., 1999). In addition, PIKK family members phosphorylate several checkpoint proteins, including the ATR-interacting protein ATRIP (Cortez et al., 2001), Rad26 in *Schizosaccharomyces pombe* and members of RFC- and PCNA-related complexes, which are required for transduction of the checkpoint signal (O'Connell et al., 2000).

PIKK family members ultimately inhibit entry into mitosis by activation of the effector kinase Chk1, an event that is dependent on all known upstream checkpoint proteins (Martinho et al., 1998; Walworth et al., 1993; Walworth and Bernards, 1996). This activation is achieved via the Rad3/ATR-dependent phosphorylation of key serine residues located in the C-terminal regulatory domain, specifically S345 in *S. pombe* and S317 and S345 in human cells (Capasso et al., 2002; Guo et al., 2000; Liu et al., 2000; Lopez-Girona et al., 2001; Zhao and Piwnica-Worms, 2001). In *S. pombe*, Chk1 activation also requires the BRCT-domain protein Crb2 (Esashi and Yanagida, 1999; Saka et al., 1997), which transiently binds to phosphorylated Chk1 during a checkpoint arrest (Mochida et al., 2004). In both humans and *S. pombe*, Chk1 phosphorylation is also required for its interaction with 14-3-3 proteins, which are essential for checkpoint function (Capasso et al., 2002; Chen et al., 1999; Jiang et al., 2003).

Chk1 elicits a G2 arrest through regulation of the Wee1 tyrosine kinases and Cdc25 tyrosine phosphatases. Together, these proteins control the activity of the cyclin-dependent kinase Cdc2 (Furnari et al., 1997; O'Connell et al., 1997; Raleigh and O'Connell, 2000), which is universally required for mitotic entry (Nurse, 1990). Deletion of *chk1* results in checkpoint failure, whereas Chk1 overexpression elicits a sustained G2 arrest that is independent of its phosphorylation or the presence of upstream checkpoint proteins (Capasso et

al., 2002; Ford et al., 1994; Lopez-Girona et al., 2001; O'Connell et al., 1997; Walworth et al., 1993).

A genetic analysis of *S. pombe* Rad3 has shown that this protein is required only for the initiation and not the maintenance of the G2 DNA damage checkpoint. If a temperature-sensitive (ts) allele of Rad3 is inactivated before irradiation, checkpoint initiation fails and cells prematurely enter mitosis, leading to the accumulation of mitotic abnormalities and a reduction in cell viability. However, if Rad3 is inactivated following irradiation, the checkpoint response is unaffected (Martinho et al., 1998). These experiments have established that the initiation and maintenance of the checkpoint are distinct events. Although the mechanism(s) by which the checkpoint is maintained is currently unclear, there are several plausible means by which this could occur. One possibility is that sustained Chk1 activity ensures maintenance of Cdc2 inhibition. Alternatively, Chk1 might be dispensable following the phosphorylation of Cdc25 and Wee1, and checkpoint maintenance might be imposed at this level of the cascade, via dephosphorylation, subcellular localization or interaction with regulatory proteins such as 14-3-3. To date, there is no evidence to support or refute any mechanism of checkpoint maintenance

We have developed an in vitro kinase activity assay for *S. pombe* Chk1 and shown that Chk1 is activated following irradiation in a manner dependent on Rad3-mediated phosphorylation of serine-345 (Capasso et al., 2002). Using this assay, we now show that Chk1 is rapidly activated following irradiation, and that this activity is maintained for the duration of a checkpoint arrest. Coincident with mitotic entry, there is a moderate but highly reproducible reduction of Chk1 activity and phosphorylation. These findings indicate that Chk1 function extends beyond initiation of the checkpoint. Furthermore, using ts alleles of *chk1*, we show that inactivation of Chk1 at any point after checkpoint initiation leads to lethal mitotic entry. This establishes the requirement for Chk1 for checkpoint initiation and maintenance in *S. pombe*. Given the highly conserved nature of the G2 DNA damage checkpoint pathway, we predict that human cells will be regulated in a similar fashion.

Materials and Methods

Fission yeast methods

All strains are derivatives of wild-type *S. pombe 972 h*$^-$ and *975 h*$^+$. Standard procedures and media were used for culture growth, transformation, microscopy and genetic analysis (Moreno et al., 1991).

Checkpoint analyses

Synchronous cultures were prepared by centrifugal elutriation and irradiated as described previously (O'Connell et al., 1997; Verkade et al., 1999) or by a *cdc10-M17* block-release-block protocol (Verkade and O'Connell, 1998). Cell cycle progression was followed by septation, binucleate or mitotic indices, averaged from three counts of at least 100 4′,6-diamino-2-phenylindole (DAPI) stained cells. Samples for estimation of cell number were fixed in 3.7% formaldehyde and counted as described previously (O'Connell et al., 1997). For 150 Gy ionizing radiation, cells at a density of 4×10^6 cells ml^{-1} were irradiated using a Varian Linear Accelerator with a 6 MeV electron beam at a dose rate of 38 Gy minute^{-1}. The source was set at 80 cm above the flasks that were covered with a sheet of Perspex. Alternatively, cells were irradiated using a ^{137}Cs source at a dose rate of 0.7 Gy minute^{-1}.

DNA-damage survival assays

For ultraviolet-C (UV-C) assays, cells were plated at various densities on YES agar [yeast extract plus supplements, described in (Moreno et al., 1991)] and irradiated in a Stratalinker (Stratagene). For ionizing radiation assays, cells were irradiated using the Linear Accelerator as described above. For methyl methanesulfonate (MMS) sensitivity assays, cultures were diluted to 4×10^6 cells ml^{-1} and 5 μl of tenfold dilutions were spotted onto YES agar containing the indicated concentration of MMS. For all survival assays, temperature was maintained during damage treatment, sample collection, plating and incubation. Colonies were counted and percentage survival was expressed as a proportion of unirradiated controls.

Chk1 immunoprecipitation and kinase assays

Cells were disrupted in immunoprecipitation (IP) buffer (10 mM $NaPO_4$ pH 7.0, 0.15 M NaCl, 1% NP-40, 10 mM EDTA, 50 mM NaF, 2 mM DTT, 1 mM PMSF, 3 mg ml^{-1} N-p-Tosyl-L-phenylalanine chloromethyl ketone (TPCK), 10 mg ml^{-1} E64, 100 mM benzamidine, 2 μg ml^{-1} aprotinin, 2 μg ml^{-1} leupeptin, 2 μg ml^{-1} pepstatin) using acid-washed glass beads (Sigma) and a cell homogenizer. Five μg anti-haemagglutinin (anti-HA) 12CA5 monoclonal antibody (Roche) was added per milligram of protein in a total volume of 1 ml (with IP buffer). Samples were incubated at 4°C on a rotator for 1-2 hours, then 20 μl of protein-A/Sepharose (50% v/v slurry in IP buffer) was added and samples incubated for a further 30-60 minutes at 4°C on a rotator. Protein-A/Sepharose beads were washed five times with IP buffer and three times with 1× kinase buffer (50 mM Tris-HCl pH 7.0, 1 mM DTT, 5 mM $MgCl_2$, 0.4 mM $MnCl_2$, 25% glycerol (G5516; Sigma), 0.1% Triton X-100, 100 μM ATP). The washed Sepharose was resuspended in 24 μl kinase reaction buffer [1× kinase buffer, 12 μg peptide (RIARAASMAALARK), 10 μCi [γ-^{32}P]ATP (3000 Ci mmole^{-1})] and incubated at 30°C for 15 minutes in an Eppendorf thermomixer. The Sepharose was pelleted and 20 μl of the supernatant was spotted onto P81 paper (Whatmann), which was then washed three to five times in ~200 ml 0.5% orthophosphoric acid, rinsed with ethanol and dried. The dried p81 papers were then mixed with 5 ml Ready-safe scintillant (Beckman) and counted in a liquid scintillation counter. For synchronous cell populations, Chk1 activity was expressed as activity detected above background, where background was measured as activity in kinase assays using an isotype control IgG. For assays performed on asynchronous cell populations, Chk1 activity was expressed as activity detected above background, where background was measured as the activity detected for *chk1* kinase dead cells. Each assessment of non-specific background produced essentially the same value.

Lysate preparation and western blots

For detection of Chk1-HA, extracts were prepared using acid-washed glass beads and cell homogenization. For asynchronous cells, extracts were prepared in 8 M urea, 100 mM Na_2HPO_4, 10 mM Tris-HCl, pH 8.0. For synchronous cells, extracts were prepared for immunoprecipitation and assay of Chk1 activity (as above) and aliquots retained and used for immunoblotting. A total of 20-50 μg protein was separated by sodium-dodecyl-sulfate polyacrylamide-gel electrophoresis (SDS-PAGE) on a 7% gel run at 200 V for 45 minutes and transferred to nitrocellulose in 25 mM Tris, 192 mM glycine, 20% methanol at 60 V for 45 minutes. The HA tag was detected with 12CA5 monoclonal antibody and a horseradish-peroxidase-coupled secondary antibody (Amersham) and proteins

were detected using enhanced chemiluminescence (ECL) reagent (Roche).

Results

Regulation of Chk1 throughout a checkpoint arrest

In response to irradiation, Rad3-mediated phosphorylation of Chk1 at serine-345 leads to induction of Chk1 kinase activity (Capasso et al., 2002). Using a HA-tagged allele of *chk1* (*chk1:ep*), the hyperphosphorylated form of the protein can be detected as a slower migrating species by SDS-PAGE (Walworth and Bernards, 1996). Rad3 is required only to initiate the G2 DNA damage checkpoint, demonstrating that checkpoint initiation and maintenance are separable processes (Martinho et al., 1998). Checkpoint maintenance could operate through Chk1 and/or further downstream, through its substrates Cdc25 and Wee1. Therefore, we asked whether Chk1 was required simply as a checkpoint initiator or for checkpoint initiation and maintenance.

If Chk1 were required for maintenance of the G2 DNA damage checkpoint, one would expect its kinase activity to remain elevated for the duration of a checkpoint-induced arrest. Previously, Chk1 phosphorylation has been used as a marker of checkpoint activation (Walworth and Bernards, 1996). However, it has not been shown conclusively that Chk1 phosphorylation strictly correlates with Chk1 activity. Here, we have used a recently developed Chk1 kinase assay (Capasso et al., 2002) to determine whether Chk1 is active for the duration of a checkpoint arrest and to assess the relationship between Chk1 phosphorylation and kinase activity throughout a checkpoint delay.

We prepared synchronous cultures of G2 cells by centrifugal elutriation, which were then irradiated with 150 J m^{-2} UV-C or left untreated. In these experiments, in which a large population of cells was required for biochemical analysis, synchrony was somewhat compromised. Thus, the duration of the checkpoint arrest was approximately 60-75 minutes, which is slightly longer than previously described for smaller, more synchronous cultures (O'Connell et al., 1997; Raleigh and O'Connell, 2000) (Fig. 1A). Most of both unirradiated and irradiated populations completed mitosis, as evidenced by a doubling in cell number. No significant change in Chk1 activity was observed in unirradiated cultures for the duration of the experiment (data not shown). In the irradiated population, both the phosphorylation and induction of Chk1 kinase activity occurred rapidly and were maximal by ~30 minutes after irradiation (Fig. 1A,B). As cells entered mitosis, Chk1 activity was reduced by ~40% but it remained significantly higher than the basal level as the population passed into the next cell cycle. This was coupled with a reduction in Chk1 phosphorylation, although hyperphosphorylated Chk1 remained visible in all time points after irradiation and levels increased as cells passed into the next cell cycle. Owing to diminished synchrony in the larger cell populations used, it is likely that the magnitude of the changes in Chk1 activity and phosphorylation as cells pass mitosis have been underestimated. The induction of Chk1 activity and phosphorylation was not significantly increased with doses of UV-C radiation above 50 J m^{-2} (Fig. 1C) (Walworth and Bernards, 1996), despite the substantially longer cell cycle delay observed (Al-Khodairy and Carr, 1992).

We conclude that Chk1 phosphorylation and activity are induced by irradiation and that this induction is saturated at relatively low experimental doses of UV-C. Furthermore, both Chk1 activity and Chk1 phosphorylation remain high for the duration of the checkpoint, suggesting a requirement for Chk1 function beyond checkpoint initiation.

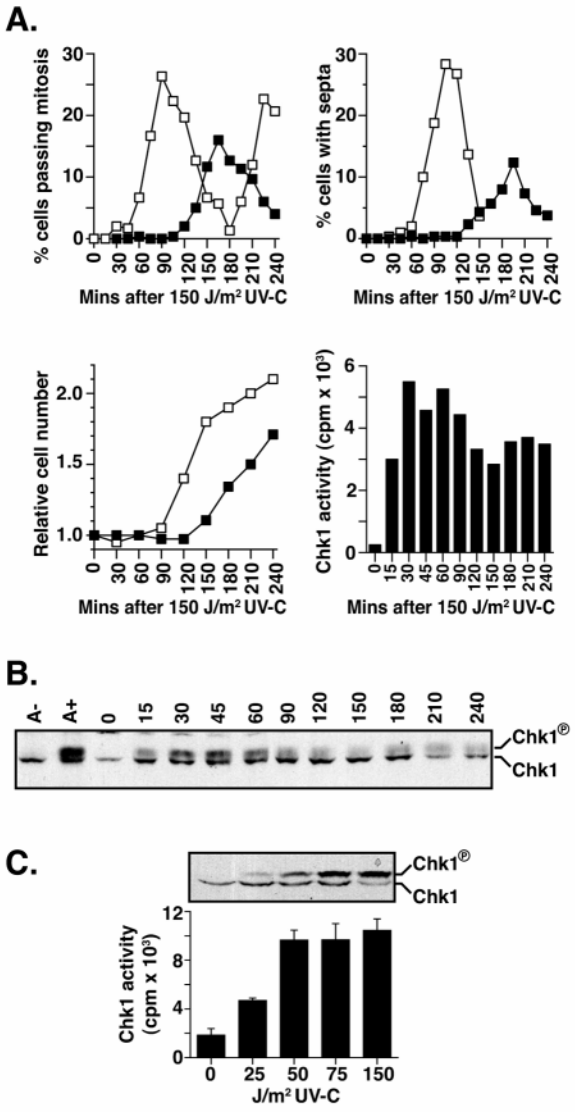

Fig. 1. Chk1 kinase activity is rapidly induced following UV irradiation and remains elevated for the duration of the checkpoint. (A) Checkpoint response of wild-type (*chk1:ep*) G2 cells to 150 J m^{-2} UV-C. Cells were synchronized by centrifugal elutriation and then irradiated (■) or left untreated (□) (*t*=0). The proportion of cells either passing mitosis or with septa and cell number was determined. Chk1 kinase activity was assayed at the time points indicated. Data shown is representative of three independent elutriations. (B) Phosphorylated Chk1 (Chk1$^{(P)}$) was detected by western blotting. (C) Asynchronous wild-type cells (*chk1:ep*) were irradiated with 0 J m^{-2}, 25 J m^{-2}, 50 J m^{-2}, 75 J m^{-2} or 150 J m^{-2} UV-C, and extracts were prepared 30 minutes after irradiation. The average Chk1 kinase activity from three concurrent assays is shown and error bars represent standard error. Chk1 and hyperphosphorylated Chk1 (Chk1$^{(P)}$) was detected by immunoblotting with anti-HA antibodies

Isolation of ts *chk1* alleles

To address directly the requirement for Chk1 function for G2 DNA damage checkpoint maintenance, we sought to inactivate Chk1 conditionally following the establishment of a checkpoint arrest. To this end, we performed two screens to isolate ts alleles of *chk1*. The first screen involved the site-specific mutagenesis of the non-catalytic domain of HA-tagged Chk1 (J. Ross and M. O'Connell, unpublished), followed by integration of the mutants into the endogenous *chk1* locus. These integrants were then screened for hypersensitivity to the DNA-damaging drug MMS at 36°C, but not at 25°C. This screen resulted in the isolation of *chk1-ts1* (E472D). The second screen involved the mutagenesis of *pREP1::chk1* in XL-1Red *Escherichia coli* (Stratagene), which caused the accumulation of point mutations in this plasmid. The resultant plasmids were then transformed into *chk1Δ* cells and transformants were incubated at 25°C in the presence of thiamine to repress the *nmt1* promoter and prevent Chk1 expression. Colonies were then replica plated to 25°C and 36°C in the absence of thiamine, to allow promoter derepression. Those colonies that failed to grow at 25°C in the absence of thiamine (owing to the induction of a cell cycle arrest by *chk1* overexpression) but grew at 36°C (owing to the inactivation of the mutant *chk1* at this temperature) were retained. The resultant ts alleles were sequenced and the mutations identified were reintroduced into the endogenous *chk1* locus. From this screen, the alleles *chk1-ts2* (E111R), *chk1-ts3* (L151S), *chk1-ts4* (I389S) and *chk1-ts5* (ΔI484) were isolated.

To determine whether these mutants were suitable for experiments that examine the role of *chk1* in checkpoint maintenance, we analysed the function of these *chk1-ts* alleles in response to DNA damage induced by ionizing radiation, UV-C radiation and MMS at both 25°C and 36°C. In all cases, *chk1-ts1* cells were found to be hypersensitive to DNA damage at 36°C, and to be slightly more sensitive than wild-type cells at 25°C (Fig. 2A,B). Therefore, the *chk1-ts1* allele essentially phenocopies *chk1Δ* at 36°C and wild-type *chk1* at 25°C. The remaining four alleles (*chk1-ts2* to *chk1-ts5*) behaved similarly, but only when expressed from the endogenous *chk1* promoter carried on a multicopy plasmid (data not shown).

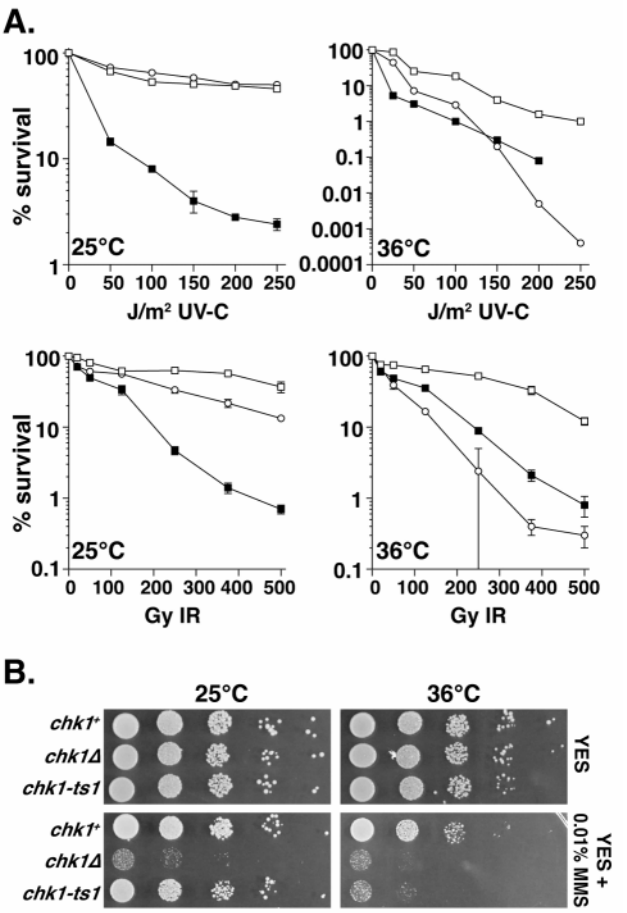

Fig. 2. *chk1-ts1* is a conditional allele of *chk1* that is active at 25°C but not at 36°C. (A) UV-C and ionizing radiation survival curve for wild-type (*chk1:ep*) (□), *chk1Δ* (■) and *chk1-ts1* (○) at 25°C and 36°C. Strains were grown at the temperature shown and irradiated with the indicated dose of UV-C (J m^{-2}). Data are normalized against unirradiated controls, and error bars represent s.e.m. (*n*=3-9). (B) MMS sensitivity assay for *chk1:ep*, *chk1Δ* and *chk1-ts1*. Strains were grown at the temperatures indicated then serial dilutions of culture inoculated onto YES agar plates containing 0.01% MMS. Plates were incubated at the specified temperature to allow colony formation.

Chk1-ts1 is conditionally checkpoint defective

To determine whether the increased sensitivity of *chk1-ts1* to DNA-damaging agents at 36°C was caused by checkpoint failure, the DNA-damage response of the *chk1-ts1* cells was examined within a single cell cycle. Despite many attempts, we were unable to synchronize this strain by centrifugal elutriation, despite obtaining populations of cells of equal size. We do not know the reason for this effect, although backcrossing did not alleviate the problem. We were unable to use *cdc25-22* block and release to generate synchrony, given the temperature-sensitive nature of *chk1-ts1* and the possibility that this protocol might inappropriately induce synthetic checkpoint defects (Harvey et al., 2004). Therefore, asynchronously growing cultures were treated with 150 J m^{-2} UV-C radiation to determine the checkpoint proficiency at 25°C and 36°C. Mitotic progression in these experiments was measured by septation (that is, the presence of a medial septum, which appears transiently ~20 minutes after mitosis). The mitotic delay induced by a checkpoint arrest is accompanied by a transient loss of septated cells.

At 25°C, there was a clear cell cycle delay and decrease in septation in both wild-type (*chk1:ep*) and *chk1-ts1* cells ~45 minutes after irradiation (Fig. 3A). The arrest was shorter in *chk1-ts1* than in the wild type, suggesting a weak checkpoint defect. In both strains, septation returned to basal levels from ~90 minutes onwards, indicating the resumption of cell cycle progression. There was no decrease in septation observed in *chk1Δ* cells, which fail to arrest in response to DNA damage. At 36°C, wild-type cells arrested normally and remained arrested for ~60 minutes before re-entering the cell cycle (Fig. 3A), whereas *chk1Δ* cells and cells carrying the *chk1-ts1* allele failed to arrest in response to irradiation. When wild-type *S. pombe* cells arrest in response to DNA damage, cells continue to grow but fail to divide, resulting in cell elongation. Therefore, we examined the length of *chk1-ts1* cells in

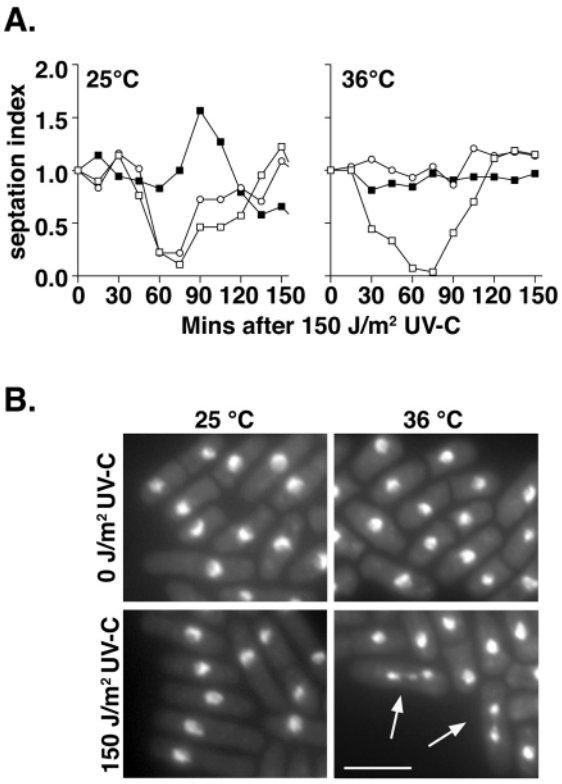

Fig. 3. *chk1-ts1* is conditionally checkpoint defective. (A) Checkpoint assays for asynchronously growing wild-type (*chk1:ep*) (□), *chk1Δ* (■) and *chk1-ts1* (○) at 25°C and 36°C. Cells were irradiated with 150 J m^{-2} UV-C and septation indices counted at each time point. Data are normalized against the time of irradiation (*t*=0). (D) DAPI-stained *chk1-ts1* cells from both the 25°C and 36°C asynchronous time courses, with or without UV-C irradiation. Cells showing aberrant mitotic figures are indicated by the arrows. Scale bar, 10 μm.

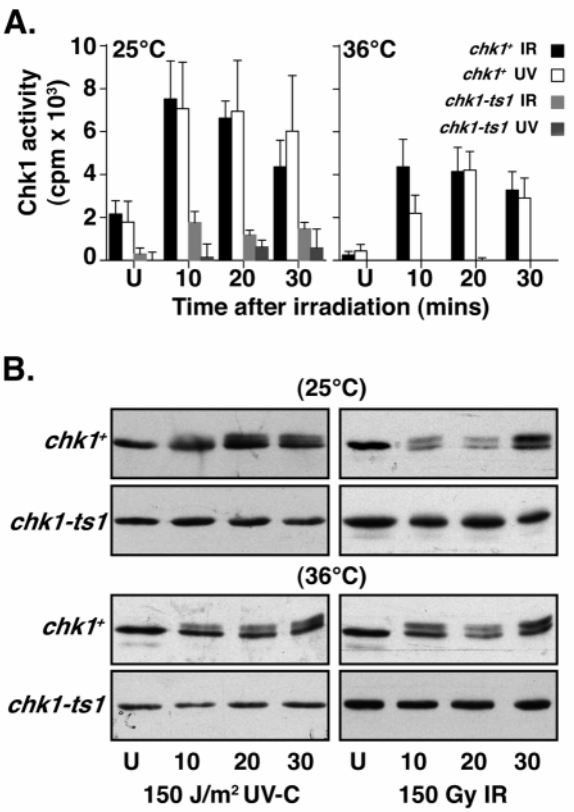

Fig. 4. *chk1-ts1* kinase activity is induced following irradiation at 25°C but not at 36°C. (A) Comparison of kinase activity of asynchronous wild-type (*chk1:ep*) and *chk1-ts1* cells grown at 25°C or 36°C and irradiated with 150 J m^{-2} UV-C or 150 Gy ionizing radiation. Samples were taken 10 minutes, 20 minutes and 30 minutes after irradiation. Error bars represent standard errors. (B) Chk1 and hyperphosphorylated Chk1 (top) was detected by immunoblotting with anti-HA antibodies.

response to UV-C radiation at 25°C and 36°C. In contrast to cells treated at 25°C, UV-C irradiated *chk1-ts1* cells at 36°C were not elongated and binucleate cells were evident (Fig. 3B). This is consistent with a lack of checkpoint function in *chk1-ts1* cells at 36°C. In addition, there were mitotic abnormalities observed in UV-C-irradiated *chk1-ts1* cells at 36°C, indicative of checkpoint failure. We conclude that *chk1-ts1* is conditionally checkpoint defective at 36°C.

Chk1-ts1 is conditional for kinase activity

Considering the position of the *chk1-ts1* mutation in the non-catalytic domain (E472D), we wished to determine whether Chk1 activity was altered in this strain. Therefore, we performed Chk1 kinase assays on wild-type (*chk1:ep*) and *chk1-ts1* cells irradiated with 150 J m^{-2} UV-C radiation or 150 Gy ionizing radiation. Chk1-ts1 was found to be induced following irradiation at 25°C, although much less than wild-type Chk1 (Fig. 4A), consistent with its modest checkpoint defect at this temperature. At 36°C, although there was a reduced induction of in vitro kinase activity detected in irradiated wild-type cells compared with that at 25°C, activity above basal levels was readily detectable. By contrast, there was no detectable Chk1 activity in irradiated *chk1-ts1* cells grown and irradiated at 36°C. It is notable that the higher temperature reduced both viability and Chk1 activation of both strains following UV-C irradiation, although to different extents (Fig. 2A, Fig. 4A). Because 36°C is the highest tolerable temperature for *S. pombe*, it is probable that there are multiple causes for the observed reduction in the viability and it cannot be attributed solely to decreased Chk1 kinase activity, especially given that these viability effects extend to *chk1Δ* cells.

To determine whether Chk1-ts1 was phosphorylated similarly to wild-type Chk1 in response to DNA damage, we also analysed Chk1 phosphorylation by western analysis in these experiments. For wild-type cells, the hyperphosphorylated form of Chk1 was detected in irradiated samples at both 25°C and 36°C (Fig. 4B). However, we were unable to detect any phosphorylated species of the Chk1-ts1 mutant protein following irradiation at 36°C, although there was a very slight mobility shift of Chk1-ts1 at 25°C after ionizing radiation.

Role of Chk1 in checkpoint maintenance

If Chk1 were required for checkpoint maintenance then one

would expect that inactivation of Chk1 after the initiation of the G2 DNA damage checkpoint would result in premature mitotic entry. Therefore, we determined the effect of inactivation of Chk1 during a checkpoint that had been initiated at 25°C using the *chk1-ts* mutants (Fig. 5A). First, we used MMS or ionizing radiation to inflict DNA damage in these experiments. We pre-established a robust checkpoint arrest at 25°C with either a 16 hour exposure to 0.0075% MMS or treatment with a cumulative 250 Gy dose of ionizing radiation delivered over 6 hours at 0.7 Gy minute^{-1}. During these treatments at 25°C, in which there is a 4 hour cell cycle, checkpoint induction in wild-type (*chk1:ep*) cells led to significant cellular elongation owing to continued growth during the imposed cell cycle delay (Fig. 5B). A similar elongation was observed for *chk-ts1*, but not for *chk1Δ*, because these cells continued into mitosis. The cultures of checkpoint-arrested cells were then shifted to 36°C and the proportion of cells with aberrant mitoses was determined every 15 minutes for 1 hour. In treated samples, t_0 indicates the effect of the DNA damage induced at 25°C. No aberrant mitoses were observed in wild-type cells over the time course for ionizing-radiation-treated cells, although a background of ~6% aberrant mitoses were seen in MMS-treated cells (Fig. 5A). The *chk1Δ* cells treated with MMS or ionizing radiation showed many aberrant mitotic figures at t_0 owing to the absence of a checkpoint, and this increased only slightly over the hour at

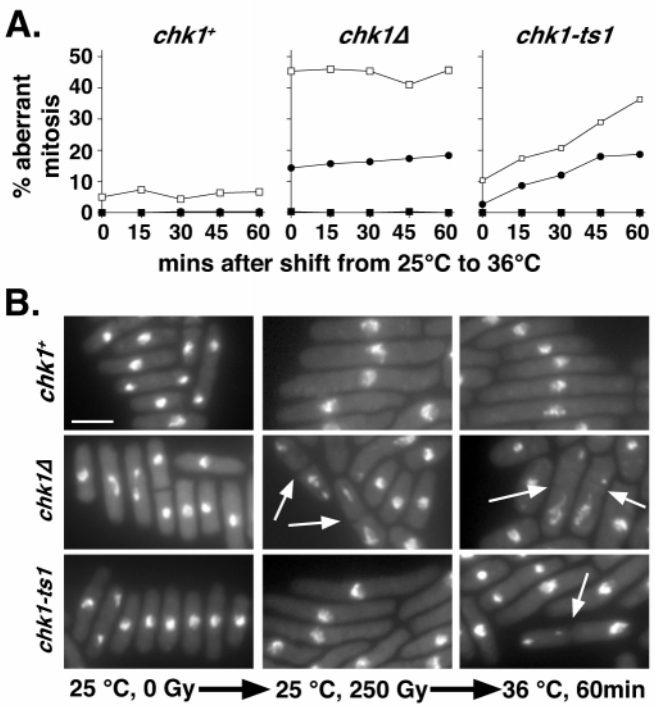

Fig. 5. Inactivation of *chk1-ts1* following the accumulation of DNA damage results in a rapid increase in aberrant mitoses. (A) Asynchronous wild-type (*chk1:ep*), *chk1Δ* and *chk1-ts1* cells were treated with either 0 Gy or 250 Gy ionizing radiation (0.7 Gy minute^{-1}, 6 hour exposure) or 0.0075% MMS (16 hour exposure) at 25°C. Cultures were then shifted to 36°C to inactivate the *chk1-ts1* allele. For each strain, the number of aberrant mitoses was counted every 15 minutes for 1 hour after temperature shift for irradiated (●), MMS-treated (□) and untreated (■) cells. (B) DAPI-stained wild-type (*chk1:ep*), *chk1Δ* and *chk1-ts1* cells are shown at 25°C, at 25°C following 250 Gy irradiation and following a temperature shift after irradiation to 36°C for 60 minutes. Cells showing aberrant mitotic figures are indicated by the arrows.

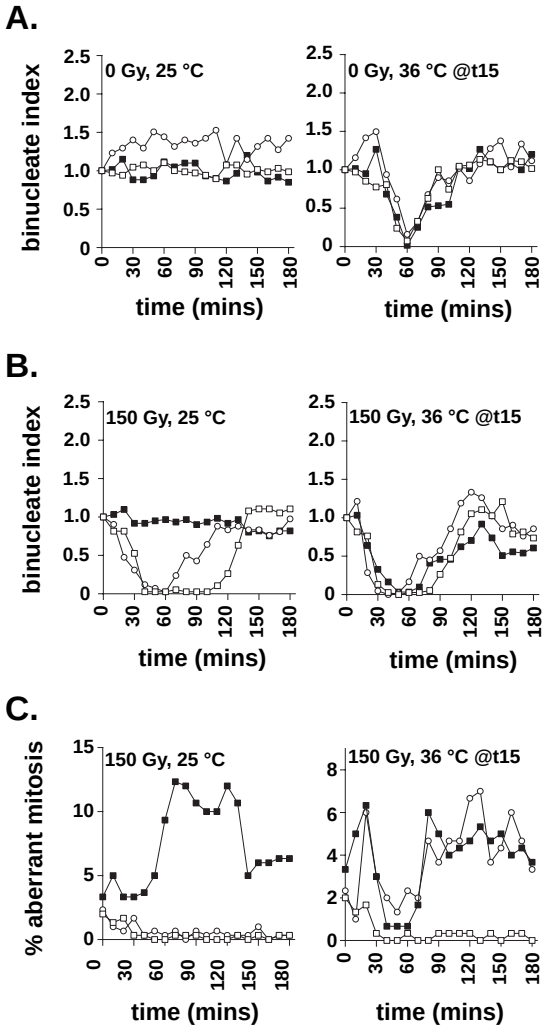

Fig. 6. Chk1 is required for ionizing-radiation-induced checkpoint maintenance. (A) Asynchronously growing wild-type (*chk1:ep*) (□), *chk1Δ* (■) and *chk1-ts1* (○) cells were incubated at 25°C for the duration of the experiment or shifted to 36°C at 15 minutes after irradiation. The proportion of binucleate cells was counted at the time points indicated by DAPI and calcafluor staining, and normalized to that of t_0 (binucleate index). In each case, shifting the culture to 36°C led to a *chk1*-independent decrease in the number of binucleate cells as a result the temperature-shift-induced heat shock. (B) The checkpoint response of asynchronously growing wild type (*chk1:ep*) (□), *chk1Δ* (■) and *chk1-ts1* (○) to 150 Gy ionizing radiation. Cultures were incubated at 25°C for the duration of the experiment or shifted to 36°C at 15 minutes after irradiation. The percentage of binucleate cells was counted at the indicated timepoints. In the absence of Chk1 activity (*chk1-ts1* and *chk1*Δ), the percentage of binucleate cells increased more rapidly than wildtype (*chk1:ep*) controls. (C) Checkpoint proficiency of asynchronously growing wild-type (*chk1:ep*) (□), *chk1* (■) and *chk1-ts1* (○) to 150 Gy ionising radiation. Cultures were incubated at 25°C for the duration of the experiment, or shifted to 36°C at 15 minutes post irradiation. The proportion of aberrant mitoses was determined at the indicated time points. The proportion of aberrant mitoses is higher in the absence of Chk1 activity (*chk1-ts1* and *chk1Δ*).

36°C. However, in *chk1-ts1* cells, there was a rapid accumulation of aberrant mitoses for both MMS- and ionizing-radiation-treated cells following Chk1-ts1 inactivation at 36°C. The alleles *chk1-ts2* to *chk1-ts5* behaved in a similar manner when expressed from a multicopy plasmid (data not shown). We conclude that inactivation of Chk1 leads to a loss of checkpoint maintenance.

To corroborate these findings, we undertook a second series of experiments. In this case, wild-type (*chk1:ep*), *chk1-ts1* and *chk1Δ* cells were mock irradiated or exposed to 150 Gy ionizing radiation, delivered rapidly at 38 Gy minute^{-1} at 25°C (total 3.95 minutes). The cultures were left at 25°C or shifted to 36°C at 15 minutes (t_{15}) after irradiation. As a measure of cells progression into mitosis, the number of binucleate cells and the number of aberrant mitoses were monitored for each strain and untreated controls.

In untreated cells incubated at 25°C, there was no evidence of a decrease in the number of binucleate cells for any strain (Fig. 6A). However, the shift to 36°C led to a decrease in the proportion of binucleate cells in all strains regardless of radiation dose. This decrease can be attributed to a heat shock, which causes a cell cycle delay in *S. pombe* (Polanshek, 1977). Therefore, the response to the temperature shift in irradiated populations included a component of heat-shock delay. It should be realized that such a heat-shock effect is not evident in cells that have been previously arrested in the cell cycle (Verkade and O'Connell, 1998) (Fig. 5).

In cells irradiated at 25°C, a cell cycle delay resulting from initiation of the G2 DNA-damage checkpoint was observed for both wild-type (*chk1:ep*) and *chk1-ts1* cells, albeit that *chk1-ts1* cells had a shortened delay compared to the wild type (Fig. 6B). Consistent with the presence of a functional checkpoint in wild-type and *chk1-ts1* cells at 25°C, there were few aberrant mitoses (<2%) in these strains (Fig. 6C). In *chk1Δ* cells, no delay was observed and there was a considerable number of aberrant mitoses in irradiated cultures at 25°C. Therefore, as expected from the previous experiments, the *chk1-ts1* allele is also checkpoint proficient at 25°C by this protocol.

In *chk1-ts1* cultures shifted to 36°C following irradiation, the proportion of binucleate cells decreased in a manner similar to that of wild-type cells (Fig. 6B). However, *chk1-ts1* cells consistently re-entered the cell cycle earlier than wild-type cells at this temperature, indicating that this lack of Chk1 function caused cells to enter mitosis prematurely, following a *chk1*-independent heat-shock delay. This was accompanied by an increased number of aberrant mitoses in *chk-ts1* cells, to a level similar to that observed for *chk1Δ* cells, accounting for approximately 50% of mitoses observed (Fig. 6C). In wild-type cells, the number of aberrant mitotic figures remained low at 36°C (Fig. 6C). In *chk1Δ* cells, the number of aberrant mitoses following ionizing radiation was reduced in cultures shifted to 36°C compared with those incubated at 25°C. This was due to the cell cycle delay imposed by the heat shock in these cultures, which, like a G2 delay enforced by inactivation of Cdc25, rescues the mitotic defects of irradiated *chk1Δ* cells (Walworth et al., 1993). Therefore, *chk1-ts1* initiates a checkpoint response at 25°C, allowing time for DNA repair and resulting in a low number of aberrant mitoses (Fig. 6C). When shifted to 36°C, *chk1-ts1* is rapidly inactivated, causing premature resumption of the cell cycle.

In a third series of experiments, a *cdc10-M17* block-release-block protocol was used to synchronize cells in G2 (Verkade and O'Connell, 1998). Cdc10 is a transcription factor required for passage of the G1-S transition (Simanis and Nurse, 1989) and thus *cdc10-M17* cells arrest in G1 at the non-permissive temperature of 36°C. However, these cells need only to be shifted down to 25°C for approximately 30 minutes for Cdc10-M17 to allow passage of G1 and S phase into G2 (Verkade and O'Connell, 1998; Verkade et al., 2001). Owing to time and temperature restraints for synchrony, it was only feasible to use UV-C irradiation in these experiments. Under this protocol, *chk1-ts1* cells had a clear UV-C-induced checkpoint delay at 25°C, as observed previously, which was shorter than that of wild-type cells (Fig. 7). In asynchronously growing cells, the survival of this strain under UV-C irradiation mirrors that of wild-type cells (Fig. 4), suggesting that this shorter delay is

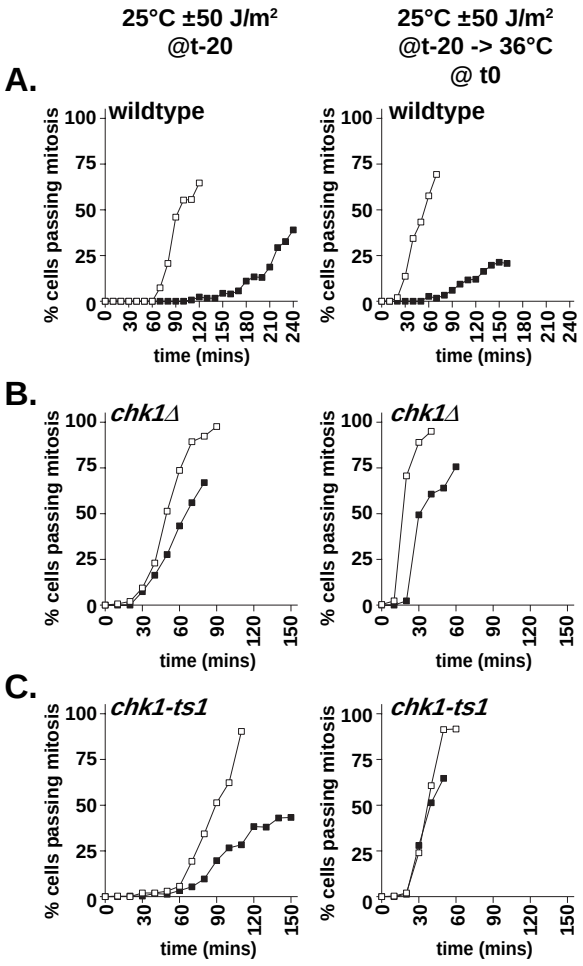

Fig. 7. Chk1 is required for UV-C-induced checkpoint maintenance. (A) Wild-type (*cdc10-M17chk1:ep*) cells were grown to mid-logarithmic phase at 25°C and then shifted to 36°C for 4.75 hours, after which the cultures were shifted to 25°C. Half of the culture was irradiated with 50 J m^{-2} UV-C (■) and the other half mock irradiated (□). After an additional 20 minutes at 25°C, one irradiated and one mock-irradiated culture were shifted to 36°C, with duplicate cultures left at 25°C. Samples were taken at the indicated times and cells passing mitosis assayed by DAPI staining. (B) Repeat of the experiment in (A) but using *cdc10-M17chk1Δ* cells. (C) Repeat of the experiment in (A) but using *cdc10-M17chk1-ts1* cells.

sufficient to maintain viability or that the synchronization protocol is affecting the duration of the delay. When cells were shifted to 36°C 20 minutes after irradiation, there was no evidence of a checkpoint arrest for *chk1-ts1* and there was a concomitant increase in aberrant mitoses (data not shown). An arrest was retained in wild-type controls under the same conditions. Under both conditions, either no delay or a delay of one time point was observed in *chk1Δ* cells (Fig. 7), which, in synchronous cultures, exhibit a short but reproducible delay only to UV-C irradiation (Al-Khodairy et al., 1994; Capasso et al., 2002).

Together, these experiments demonstrate a role for *chk1* not only in checkpoint initiation but also in checkpoint maintenance. Furthermore, they demonstrate that failure to maintain the G2 DNA-damage checkpoint following initiation of a checkpoint response and temporary cell cycle delay leads to a loss of mitotic fidelity.

Discussion

A physiological response to DNA damage detected in G2 phase involves the initiation of a checkpoint arrest and the maintenance of this cell cycle delay until the completion of DNA repair. Failure to do so results in mitotic entry with unrepaired or partially repaired DNA, leading to defects in chromosome segregation and a consequent reduction in cell viability. In recent years, a sophisticated model has emerged of how the many checkpoint proteins co-operate to ensure the activation of Chk1 (O'Connell et al., 2000), the effector kinase for the G2 DNA-damage checkpoint and thus initiation of the checkpoint signalling cascade. However, what role, if any, Chk1 might play in maintenance of the checkpoint has not been investigated.

We have shown here that Chk1 activity is dynamically regulated following DNA damage and that this activity is required for the duration of a G2 checkpoint. In synchronous cultures, Chk1 kinase activity and phosphorylation were rapidly induced following DNA damage, and remained high for the duration of a checkpoint arrest. Chk1 activity was reduced as cells recover from the checkpoint arrest, although to levels significantly higher than basal, and there was a concomitant reduction in Chk1 phosphorylation as cells re-enter the cell cycle. We further demonstrated that the inactivation of Chk1 activity during an already-established DNA damage checkpoint leads to checkpoint failure, resulting in premature mitotic entry and mitotic abnormalities. Chk1 is therefore required for not only the initiation of the G2 DNA-damage checkpoint but also checkpoint maintenance, during which we propose that Chk1 is responsive to the detection of ongoing DNA repair.

It is unclear how Chk1 activity is maintained to ensure that cells remain checkpoint arrested until the completion of DNA repair. Chk1 activation requires the phosphorylation of its C-terminal domain by Rad3 (Capasso et al., 2002; Lopez-Girona et al., 2001; Martinho et al., 1998). However, Rad3 is only required for checkpoint initiation and its inactivation following DNA damage does not affect the ability of a cell to maintain a checkpoint arrest (Martinho et al., 1998). Thus, it must be possible to maintain Chk1 activity independent of Rad3 function. Perhaps the phosphorylated epitope of Chk1 is protected or Chk1 dephosphorylation is prohibited for the duration of a checkpoint arrest. Alternatively, another kinase might phosphorylate Chk1. Three proteins have been reported to interact with Chk1. The 14-3-3 proteins Rad24 and Rad25 interact specifically with the S345 phosphorylated form of Chk1 (Capasso et al., 2002; Chen et al., 1999). The BRCT-protein Crb2 has been found to interact with Chk1 in the yeast two-hybrid system, is required for Chk1 phosphorylation and associates with phosphorylated Chk1 (Esashi et al., 2000; Mochida et al., 2004; Saka et al., 1997). Therefore, maintaining a phosphorylation-dependent interaction between Chk1 and either 14-3-3 or Crb2 might contribute to checkpoint maintenance. In vivo, this might include the interaction of the C-terminal regulatory domain of Chk1 with these or other proteins. In this regard, it is notable that three of the five *chk1-ts* alleles we identified were mutations in this domain.

Having demonstrated a role for Chk1 in checkpoint maintenance, it follows that Chk1 is an obvious target for signalling pathways involved in checkpoint release and resumption of the cell cycle. We show here that a decrease in Chk1 phosphorylation and activity occurs coincident with cell cycle re-entry, yet both remain above basal. This might suggest that only a proportion of Chk1 within the cell, for example that which is in active complexes, need be inactivated for cell cycle resumption. Alternatively, Chk1 regulation might be more complicated than simple phosphorylation events.

The *chk1-ts1* mutant we have isolated has substantially reduced kinase activity compared with the wild type at 25°C, yet is checkpoint proficient at this temperature. This implies that more Chk1 is activated in the cell than is required for a checkpoint arrest. Moreover, the observed SDS-PAGE mobility shift of Chk1-ts1 in response to DNA damage is minimal compared with that of the wild type. This might indicate that activation of the Chk1-ts1 allele requires a reduced threshold of Rad3-dependent phosphorylation. However, a number of mutations in Chk1 can affect the magnitude of this mobility shift (Capasso et al., 2002) and so these data must be interpreted carefully. We also find that maximal Chk1-ts1 activity is significantly lower than that at which wild-type cells re-enter the cell cycle. This suggests that Chk1 regulation in vivo is more complicated than currently thought. However, by necessity, we have used in vitro assays of Chk1 activity and this might only partially reflect the activity in vivo. Further to this, it has been shown that S345 phosphorylation is not required for the activity of recombinant Chk1 (O'Connell et al., 1997), nor for the G2 arrest elicited by Chk1 overexpression (Lopez-Girona et al., 2001) or the basal activity observed in unirradiated cells (Capasso et al., 2002). Chk1 regulation might involve mechanisms other than S345 phosphorylation, such as localization and interaction with substrates or regulatory proteins.

We have shown here that regulation of Chk1 activity is important for checkpoint maintenance, but is it the duration of Chk1 activity or the level of Chk1 activity that is crucial in the checkpoint signalling cascade? We show that the level of Chk1 activation is saturated at relatively low doses of UV-C and did not thereafter increase with increasing doses of UV-C radiation. Therefore, rather than the absolute level of Chk1 activity determining the duration of a checkpoint delay, it appears that Chk1 activity must reach a critical threshold for checkpoint maintenance. This, together with our finding that Chk1 activity is transiently reduced upon mitotic entry,

indicates that the duration of Chk1 activity is the crucial determinant of checkpoint duration.

In addition to finding a role for Chk1 activity in checkpoint maintenance, we were surprised that Chk1 phosphorylation and activity increases following a brief decrease observed upon checkpoint release. This might indicate that a low level of unrepaired lesions persists in the cell. The signal generated from these lesions must be insufficient to block mitosis but might be more potent in the subsequent G1 and S phases, when cells are particularly sensitive to DNA damage (Al-Khodairy et al., 1994; Verkade et al., 2001). Consistent with this notion, we have only observed the transient loss of hyperphosphorylated Chk1 in synchronous cultures as they pass through mitosis, not in asynchronous cultures. In irradiated asynchronous cultures at any time after irradiation, there are a significant number of interphase cells with activated Chk1. This would mask the modest reduction in Chk1 phosphorylation and activity that we observe at mitotic entry in synchronous cultures and might explain why this change in Chk1 activity and phosphorylation has not been reported previously.

Our finding that Chk1 activity is required throughout the DNA-damage checkpoint has important consequences for anticancer therapeutics. Most tumour cells have lost G1 checkpoints and resort only to a G2 arrest in response to genotoxic stress. Abolishing their G2 checkpoint might selectively sensitize such tumour cells to treatment, because normal cycling cells will continue to use their G1 checkpoint (Koniaras et al., 2001). There is emerging evidence that Chk1 inhibition is a selective way to fulfil this objective (Jackson et al., 2000; Zachos et al., 2003; Zhao et al., 2002). Our finding that Chk1 is required for both checkpoint initiation and maintenance provides a broad window during which the specific inactivation of Chk1 by inhibitory drugs might be of benefit as an anticancer regime.

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