Mrc1 transduces signals of DNA replication stress to activate Rad53

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Cells experiencing DNA replication stress activate a response pathway that delays entry into mitosis and promotes DNA repair and completion of DNA replication. The protein kinases ScRad53 and SpCds1 (in baker's and fission yeast, respectively) are central to this pathway. We describe a conserved protein Mrc1, mediator of the replication checkpoint, required for activation of ScRad53 and SpCds1 during replication stress. *mrc1* mutants are sensitive to hydroxyurea and have a checkpoint defect similar to *rad53* and *cds1* mutants. Mrc1 may be the replicative counterpart of Rad9 and Crb2, which are required for activating ScRad53 and Chk1 in response to DNA damage.

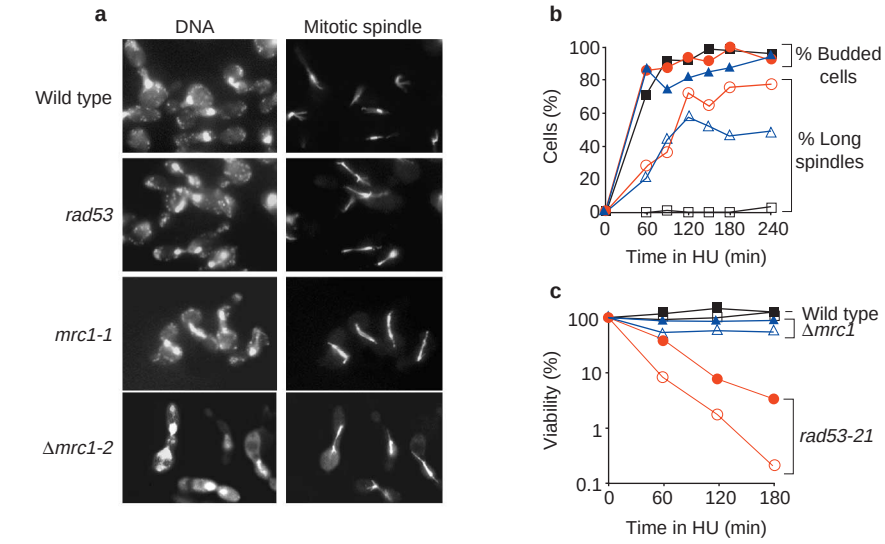
In response to DNA damage and DNA replication stress, cells activate a stress response pathway that ultimately activates two families of checkpoint kinases, Chk1 and Chk2[1]. Human Chk2 is a tumour suppressor required for activation of p53 in response to ionizing radiation[2–5]. In response to DNA damage, activation of the yeast checkpoint kinase homologues, *Saccharomyces cerevisiae* (Sc)Rad53, ScChk1 or *Schizosaccharomyces pombe* (Sp)Chk1, is controlled by the ataxia-telangiectasia-related (ATR) kinase homologues ScMec1 or SpRad3 and mediated by the BRCT-repeat (domain present in the tumour suppressor BRCA1 carboxy terminus) proteins ScRad9 or SpCrb2 (refs 6–11). Activation of the Chk2 homologues ScRad53 or SpCds1 in response to DNA replication blocks also requires ScMec1 and SpRad3, but not ScRad9 or SpCrb2, suggesting there is a specific mediator of the replication stress response that works in place of ScRad9 or SpCrb2 to transduce stress information. In a screen for new replication checkpoint mutants in *S. cerevisiae* we identified the conserved protein Mrc1, which is required for full activation of ScRad53 and SpCds1 during replication stress. ScMrc1 is phosphorylated in a *MEC1*- and *RAD53*-dependent manner and is required to prevent mitotic entry in the presence of hydroxyurea (HU). Mrc1 is partially redundant with respect to ScRad9 and SpCrb2 in transducing information about DNA replication stress in budding and fission yeast. Mrc1 shares sequence similarity with Crb2, supporting the notion that Mrc1 may be the replicative counterpart of the Rad9 and Crb2 proteins.

Results

***MRC1* is a nonessential gene that functions in the S-phase checkpoint response and during DNA replication.** We carried out a genetic screen to identify *S. cerevisiae* mutants that fail to grow in the presence of 100 mM HU. We further required that mutants elongate their spindles, and die when treated with HU for short periods. We isolated one allele of *MRC1* and several alleles of *MEC1* and *RAD53*. *MRC1* (YCL061C) was cloned by complementing the HU-sensitive phenotype of the *mrc1-1* mutant. *MRC1* is a nonessential gene, and a Δ*mrc1-2::HIS3* strain was constructed by replacing the majority of the reading frame with *HIS3*. A Δ*mrc1-3::his5*$^{+}$ strain was also created by replacing the entire *MRC1* coding region with *S. pombe his5*$^{+}$; this strain behaves identically to Δ*mrc1-2*. The deletion mutants and *mrc1-1* mutants are equally sensitive to HU, failing to grow on plates containing 120 mM HU (data not shown).

To examine the DNA replication checkpoint, G1-arrested cells were released into media containing 200 mM HU, and monitored for the presence of elongated spindles and divided nuclei, which signifies entry into anaphase. Wild-type cells arrested with short pre-anaphase spindles, whereas *rad53-21* and *mrc1-1* mutants failed to arrest (Fig. 1a). Spindle elongation in Δ*mrc1-2* mutants occurs to a significant extent (~50%), with timing identical to that of a *rad53-21* mutant (Fig. 1b). Δ*mrc1-2* mutants enter anaphase within 60–120 min of HU treatment, when DNA replication is still blocked (data not shown). *mrc1* mutants are tenfold more resistant to killing by HU than *rad53* mutants (Fig. 1c), indicating that factors other than spindle elongation contribute to lethality in *rad53* mutants[12].

MRC1 encodes a highly charged 1096-amino-acid protein (36% of residues are charged) that has several regions clustered with positive or negative charges. Mrc1 also contains an SQ cluster domain between amino acids 90 and 202 (nine SQ and TQ pairs). These domains are often phosphorylation sites for ATM-like kinases such as Mec1 (ref. 1). We searched databases and found candidate homologues in both *Candida albicans* (data not shown) and *S. pombe* (Fig. 1d). The similarity between these proteins is highest along their C-terminal halves. In addition to these very similar homologues, we found a significant similarity with the *S. pombe* Crb2/Rhp9 protein ($P = 0.00033$). The highest degree of similarity occurs in the central region of Mrc1. Crb2 is considered to be the *S. pombe* analogue of the *S. cerevisiae* Rad9 protein, as both bind to and are required for phosphorylation of the Chk1 protein kinase in response to DNA damage[11,13,14]. Rad9 and Crb2 both contain a C-terminal BRCT repeat domain that is not conserved with Mrc1. Crb2 is not required for activation of Cds1 in response to DNA replication stress[8]. Similarly, Rad9 is not required for activation of Rad53 in response to DNA replication blocks[9,10]. We also found similarity between *S. pombe* Mrc1 and the human and *Xenopus* (X) Claspin proteins (Fig. 1d). XClaspin is required for



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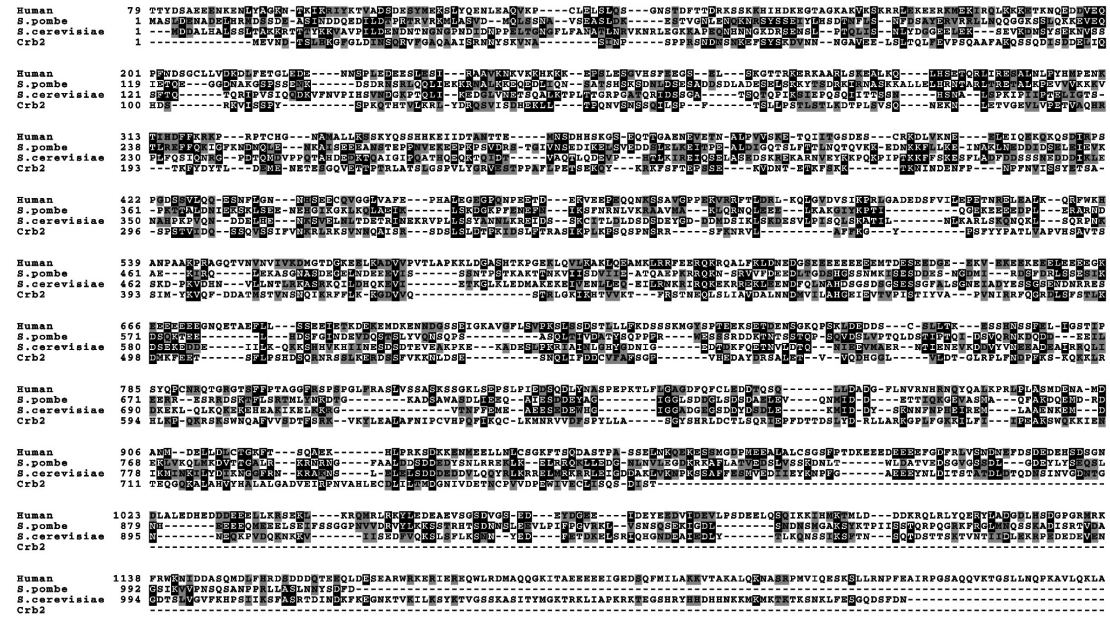

Figure 1 **MRC1 is required for cell-cycle arrest in response to replication blocks.** Wild-type (Y300), *rad53-21* (Y301), *mrc1-1* (Y1121) and Δ*mrc1-2::HIS3* (Y1122) cells were arrested in G1 with α-factor and then released at 30 °C into YPD containing HU as indicated. **a**, DNA and mitotic-spindle staining of cells at 120 min after release into 200 mM HU. Wild-type cells maintain short spindles, whereas *rad53* and *mrc1* mutants enter anaphase and show separated DNA. **b**, Percentage of budded cells (filled symbols), and percentage of anaphase spindles (open symbols) were quantified for 240 min after release into 200 mM HU. Wild type is represented by black squares, Δ*mrc1-2* by blue triangles, and *rad53-21* by red circles. **c**, Survival (per cent viability) of wild-type (black squares), Δ*mrc1-2* (blue triangles), and *rad53-21* (red circles) cells treated with 20 mM HU (filled symbols) or 100 mM HU (open symbols). **d**, Alignment of the *S. cerevisiae* Mrc1 protein with *S. pombe* Mrc1, human Claspin and *S. pombe* Crb2/Rhp9. Identities (shaded black) and similarities (shaded grey) are shown.

replication-checkpoint-induced XChk1 phosphorylation[15].

***MRC1* is not required for the DNA damage checkpoint.** We determined whether Mrc1, like Rad53, is involved in the G2/M DNA damage checkpoint by constructing *cdc13-1* Δ*mrc1-2* double mutants. Cells bearing the temperature-sensitive *cdc13-1* mutation accumulate single-stranded DNA at 32 °C and arrest as large-budded cells when the G2–M DNA damage checkpoint is activated. Cells defective in this checkpoint continue to divide and eventually die as microcolonies[16]. Log-phase cultures grown at 24 °C were plated on YPD medium and incubated at 32 °C. After 16 h, nearly 90% of *cdc13-1* and *cdc13-1 mrc1* mutant cells were arrested in G2, having two or four cell bodies, whereas *cdc13-1 rad53-21* cells formed microcolonies, indicating a checkpoint defect (Fig. 2a). These results demonstrate that the checkpoint function of Mrc1 is required only in response to replication stress.

***MRC1* is involved in the intra-S-phase checkpoint.** *rad53* mutants fail to slow down DNA replication in the presence of continual DNA damage from methylmethanesulphonate (MMS), a response also known as the intra-S-phase checkpoint[17]. We examined this response in Δ*mrc1* mutants and compared it with that of wild-type cells (Fig. 2b). We arrested strains in G1 and released them into YPD containing 0.033% MMS. *mrc1* mutants progress

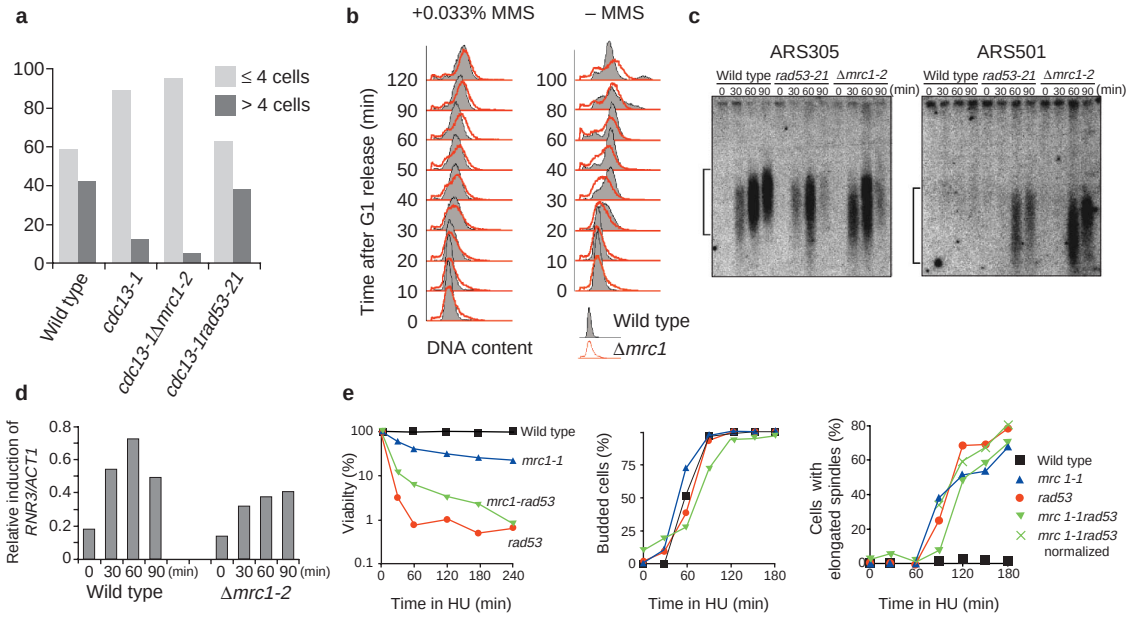

Figure 2 **Mrc1 functions in S phase and is required for responses to replication stress and DNA damage. a**, The DNA damage checkpoint response of *mrc1* mutants is intact. Wild-type (Y300), *cdc13-1* (Y809), Δ*mrc1-2cdc13-1* (Y1124) and *rad53-21 cdc13-1* (Y810) cells were grown to log phase at 24 °C, sonicated and plated onto pre-warmed YPD at 32 °C. Sixteen hours later, arrested cells (≤4 cell bodies; grey bars) and microcolonies (>4 cell bodies; black bars) were scored. **b**, Analysis of the intra-S-phase checkpoint in *mrc1* mutants. G1-arrested cultures of wild-type (Y1130) and Δ*mrc1* (Y1132) cells were released into media with or without 0.033% MMS. Samples were processed at the indicated times for FACS analysis. The FACS profiles for wild-type (shaded) and *mrc1* (red lines) cells are overlaid for comparison. **c**, Southern analysis of replication origins ARS305 and ARS501 in HU-treated wild-type (Y300), *rad53-21* (Y301) and Δ*mrc1-2* (Y1122) cells. G1-arrested cells were released into YPD containing 200 mM HU and collected for DNA extraction at the indicated times. Short single-stranded DNAs synthesized from fired origins are visualized as smears and are indicated by brackets. **d**, Transcriptional induction of the *RNR3* gene in HU-treated cultures of wild-type (Y300) and Δ*mrc1-2* (Y1122) strains. G1-arrested cells were released into YPD containing 200 mM HU. Cells were collected at the indicated times for RNA extraction. After northern analysis and quantification using a phosphorimager, the amount of *RNR3* was normalized to *ACT1* mRNA. **e**, Wild-type (Y300; black squares), *rad53-21* (Y301; red circles), *mrc1-1* (Y1121; blue triangles) and *mrc1-1rad53-21* (Y1125; green inverted triangles) were arrested in G1 and then released into YPD containing 200 mM HU at 24 °C. Viability, per cent budded cells and per cent anaphase spindles were measured. When the number of anaphase spindles in the *mrc1-1rad53-21* double mutant is normalized to the amount of large-budded cells present (green crosses), the extent of its checkpoint defect is very similar to that of *rad53* mutants.

through S phase slightly faster than the wild type in the presence of MMS. The peaks representing DNA content for MMS-treated *mrc1* mutants at the 50, 60 and 90 min time points have advanced further than the peaks representing the DNA content of wild-type cells. Mrc1 is therefore involved in the intra-S-phase checkpoint.

We also observed that, in the absence of treatment to induce DNA damage, Δ*mrc1* strains replicate more slowly than do wild-type strains. DNA replication is completed 10–20 min later in Δ*mrc1* cells than in wild-type cells at 30 °C (Fig. 2b) and 24 °C (data not shown) indicating a role for *MRC1* in S phase. A function for Mrc1 in DNA replication is consistent with the fact that *MRC1* transcription is regulated over the cell cycle with messenger RNA levels peaking at the start of S phase[18]. Because *mrc1* mutants replicate more slowly than wild-type cells, it is possible that a much more severe defect in the intra-S-phase checkpoint occurs in *mrc1* strains, but is partially masked by their slower rate of DNA replication.

Mrc1 is required for delaying late origin firing in response to replication blocks. In addition to cell-cycle arrest, HU induces a *RAD53*-dependent inhibition of late origin firing[19], as well as the transcription of DNA damage-inducible genes, including *RNR3* (ref. 20). To determine whether *MRC1* is required to block late origin firing in the presence of HU, we used an assay that detects replication intermediates as short single-stranded DNAs that can be probed with sequences surrounding replication origins[19]. ARS305 is an early firing origin of DNA replication whose activation is not affected by HU treatment, whereas ARS501 typically fires late in S phase and is suppressed in the presence of HU. We measured activation of ARS305 and ARS501 in the presence of 200 mM HU in wild-type, *rad53* and *mrc1* cells released from a G1 block. As expected, ARS305 firing was detected in all three strains. In wild-type cells, replication initiation at ARS501 was not observed for at least 90 min. In contrast, both Δ*mrc1-2* and *rad53-21* mutants failed to delay ARS501 firing, as replication intermediates were observed 60 min after release (Fig. 2c). Mrc1 is therefore required to prevent late origin firing in response to replication stress. In similar experiments, we found that *mrc1* mutants show a slightly reduced transcriptional response relative to wild-type cells (Fig. 2d).

Mrc1 is part of the S phase checkpoint signal transduction pathway. Although *rad53* mutants are far more sensitive to HU than Δ*mrc1-2* mutants, Δ*mrc1-2* and *rad53-21* mutants exhibit similar defects in DNA replication checkpoint as measured by spindle elongation during HU exposure. To determine whether Mrc1 and Rad53 act in the same replication checkpoint pathway or function independently, we analysed double mutant phenotypes. We tested whether the replication checkpoint defect of the *mrc1-1 rad53-21* strain was more severe than those of the *rad53-21* and *mrc1-1* single mutants. Cells were arrested in G1 with α-factor then released into media containing 200 mM HU. Samples were monitored for budding, viability and inappropriate entry into anaphase. At the start of the experiment, 10% of the double-mutant population was resistant to α-factor arrest (large budded cells at 0 min) and may represent dead or arrested cells. Three hours after release, 10% of the population remained unbudded (Fig. 2e, budded cells) which rendered

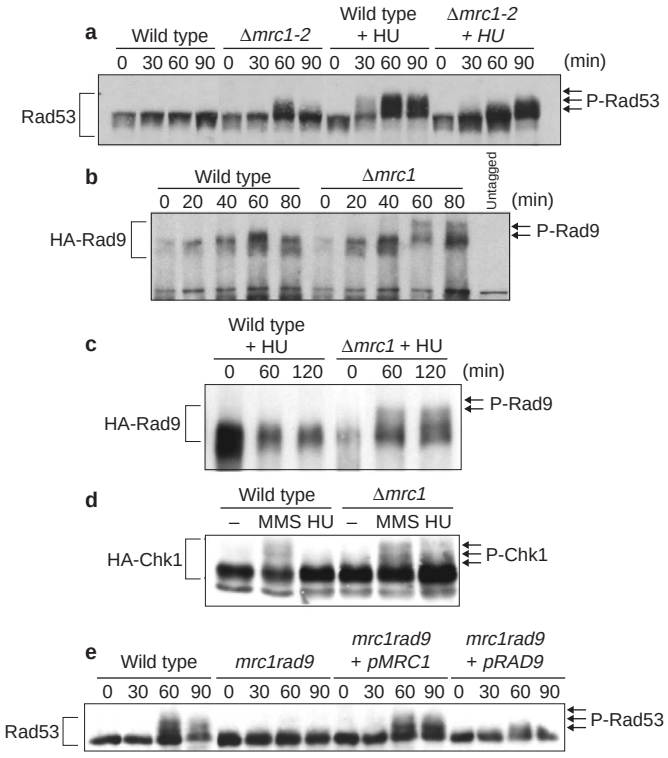

Figure 3. Mrc1 is required for Rad53 activation during replication stress. **a**, G1-arrested wild-type (Y300) and Δ*mrc1-2* (Y1122) cells were released into YPD with or without 200 mM HU. Samples were collected every 30 min for protein extraction. We carried out western analysis using anti-Rad53 antibodies. The arrows at right indicate hyperphosphorylated Rad53. **b**, Strains containing HA-tagged Rad9, wild type (Y1126) and Δ*mrc1-3* (Y1127), were G1-arrested and released into YPD. Protein samples were collected every 20 min for western analysis of HA-Rad9. The arrows at the right indicate the position of hyperphosphorylated Rad9 observed in the presence of DNA damage. **c**, Asynchronous cultures of wild-type (Y1126) and Δ*mrc1-3* (Y1127) cells were treated with 200 mM HU. Protein samples were taken at 0, 60 and 120 min for western analysis of HA-Rad9. The time 0 lane for wild type is overloaded relative to the other lanes. **d**, Log phase cells containing a HA-Chk1 plasmid — wild type (Y1128) and Δ*mrc1-3* (Y1129)— were treated with 200 mM HU or 0.1% MMS for 1 h. Protein samples were taken before and after treatment for western analysis of HA-Chk1. The staining above the main band (indicated by arrows) represents phosphorylated Chk1. **e**, Strains over-expressing *RNR1* — wild type (Y1130), Δ*mrc1* Δ*rad9* (Y1131), Δ*mrc1* Δ*rad9* + *pRAD9* (Y1132) and Δ*mrc1* Δ*rad9* + *pMRC1* (Y1133) — were synchronized in G1 and released into YPD containing 200 mM HU. Samples were collected every 30 min for protein extraction and western analysis with anti-Rad53 antibodies.

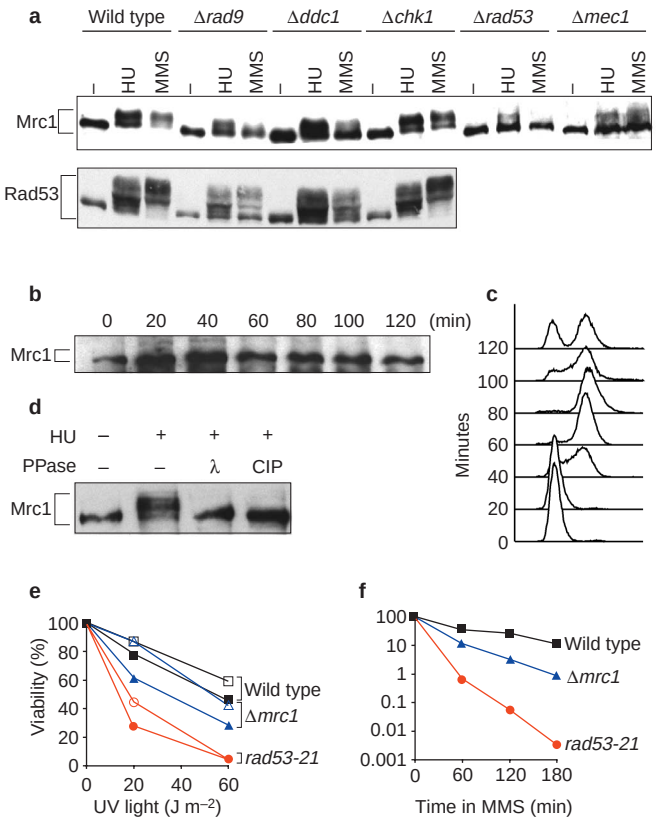

Figure 4 **Mrc1 is phosphorylated in response to HU.** **a**, Checkpoint-induced modification of Mrc1. Asynchronous cultures of wild-type (Y300), Δ*rad9* (Y438), Δ*ddc1* (Y1135), Δ*chk1* (Y801), Δ*rad53* (Y606) and Δ*mec1* (Y581) strains were treated with 200 mM HU for 1.5 h or 0.1% MMS for 1 h. Protein extracts were collected after precipitating the culture with TCA, subjected to SDS–PAGE and immunoblotted using anti-Mrc1 antibodies. Rad53 western blots were also performed on extracts from wild-type, Δ*rad9*, Δ*ddc1* and Δ*chk1* cells. **b**, Mrc1 is not modified during a normal cell cycle. A wild-type culture of G1-arrested cells was released into YPD. Samples were taken every 20 min for protein extraction and western analysis with anti-Mrc1 antibodies. **c**, A FACS profile that represents the culture in **b**. **d**, Mrc1 is modified by phosphorylation. Wild-type cells containing Mrc1–13myc (Y1134) were incubated with 200 mM HU for 1.5 h. Protein extracts were immunoprecipitated with anti-Myc antibodies and incubated with or without calf intestinal phosphatase (CIP) or lambda phosphatase (λ) for 30 min. Western blots were performed using anti-Myc antibody. **e**, Log-phase (filled symbols) and nocodazole-arrested (open symbols) cultures of wild type (Y300; black squares), *rad53-21* (Y301; red circles) and Δ*mrc1-3* (Y1123; blue triangles) were plated on YPD and irradiated with 20 J m^{-2} or 60 J m^{-2} of ultraviolet light. Surviving colonies were counted and are shown as a percentage of untreated culture. **f**, G1-arrested cultures of wild type (Y300; black squares), Δ*mrc1* (Y1123; blue triangles) and *rad53-21* (Y301; filled red circles) cells were G1-arrested with α-factor and released into media containing 0.033% MMS. Samples were taken at 1-h intervals and plated onto YPD to assess viability.

this subpopulation artificially resistant to the effects of HU. When these differences in cell-cycle distribution are taken into account, *mrc1-1 rad53-21* mutants displayed a similar lethality rate in HU as *rad53-21* single mutants (Fig. 2e, viability). Furthermore, if one takes into account the slower kinetics of bud emergence, the *mrc1-1 rad53-21* mutants had a similar extent and timing of spindle elongation in HU as *rad53-21* and *mrc1-1* single mutants (Fig. 2e, elongated spindles, *mrc1 rad53* normalized). The lack of an enhanced phenotype, with respect to either the rate of appearance or the total number of elongated spindles, in *mrc1 rad53* cells suggests that Mrc1 and Rad53 function in the same pathway. These experiments were performed at 24 °C because of the temperature-sensitive phenotype of the *mrc1-1 rad53-21* double mutant at 37 °C.

Mrc1 is required for full activation of Rad53 in response to HU. Rad53 is hyperphosphorylated in the presence of HU in a Mec1-dependent manner[6,7], and this phosphorylation correlates with an increase in Rad53 kinase activity[10]. To determine whether Mrc1 regulates Rad53 phosphorylation, G1-arrested cultures of wild-type and Δ*mrc1-2* mutants were released into media containing HU, and samples were taken every 30 min. Rad53 hyperphosphorylation can be observed in wild-type cells as early as 30 min, and full activation occurs at 60 min. The *mrc1* mutant showed a delay in phosphorylation of Rad53 (Fig. 3a, + HU lanes). In contrast, no delay in Rad53 phosphorylation was detected in *cdc13-1*

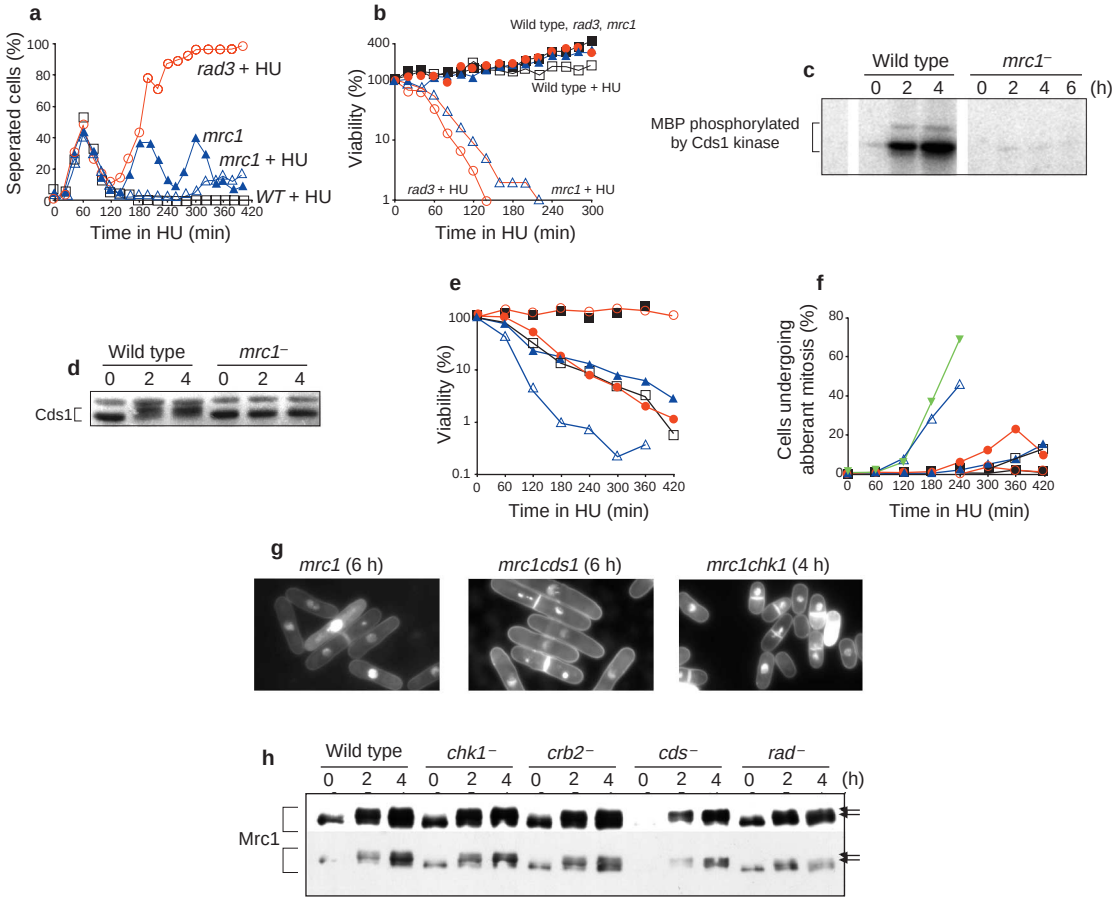

Figure 5. ***S. pombe*** **Mrc1 functions in the replication checkpoint.** **a**, G2 cells of wild-type (501; black squares), *mrc1*⁻ (1969; blue triangles) and *rad3*⁻ (1378; red circles) *S. pombe* strains prepared by lactose gradient centrifugation were incubated in the presence (open symbols) or absence (closed symbols) of 20 mM HU. Samples were extracted every 20 min, fixed in methanol and stained with DAPI and Calcofluor to determine the percentage of septated cells. In the absence of HU, wild-type and *rad3*⁻ cells behaved identically to *mrc1*⁻ cells and are not shown. **b**, Cells from **a** were removed, diluted and plated to assay the number of viable cells. Viability at each time point was normalized to 100% at time 0. **c**, We assessed the activity of the Cds1 kinase towards the myelin basic protein (MBP) from extracts from wild-type (501) and *mrc1*⁻ (1969) cells that had been treated with 20 mM HU for the indicated times. **d**, Extracts from cells used in **c** were prepared for SDS–PAGE and immunoblotted with anti-Cds1 antibodies. **e**, Asynchronous log-phase cultures of wild type (501, black filled squares), *chk1*⁻ (1098, red open circles), *mrc1*⁻ (1969, blue filled triangles), *cds1*⁻ (1561, red filled circles), *mrc1*⁻ *cds1*⁻ (1972, black open squares) and *mrc1*⁻ *chk1*⁻ (blue open triangles) were treated with 20 mM HU and cells extracted hourly to assess the percentage of viable cells remaining (normalized to 100% at time 0). **f**, In a parallel experiment the percentage of aberrant mitoses in the culture was scored microscopically by fixing cells at the indicated time points after staining with DAPI and Calcafluor. Strains and symbols used are the same as in **e**, with the addition of *cds1*⁻ *chk1*⁻ (green filled inverted triangles). **g**, Samples of *mrc1*⁻ (1969), *mrc1*⁻ *cds1*⁻ (1972), *mrc1*⁻ *chk1*⁻ (1971) cells from the experiment shown in **f**. These data show typical example of cells at 6, 6 and 4 h respectively after HU addition. **h**, An exponential-phase culture of wild-type cells containing the *mrc1*⁺–TAP construct (the wild-type *mrc1* gene linked to the tandem affinity purification epitope; 1970) was incubated in YE medium containing 20 mM HU at 30 °C. Cell extracts were prepared for SDS–PAGE and immunoblotted with rabbit anti-mouse IgG. Dark (top) and light exposures (bottom) of the same immunoblot are shown. Arrows at the right indicate modified Mrc1 protein.

mrc1 mutants released from a G1 block into the nonpermissive temperature (data not shown), indicating that in the *mrc1* background, signalling through Rad53 in response to DNA damage is intact.

Rad53 activation in *mrc1* mutants requires Rad9. Rad53 activation does not occur in wild-type cells during a normal cell cycle but was observed in late S phase in untreated Δ*mrc1-2* cells (Fig. 3a, no HU lanes). This transient activation of Rad53 may indicate the presence of a DNA replication problem that leads to DNA damage, which is consistent with the slower rate of S-phase progression observed in this mutant (Fig. 2b).

Because Rad53 activation in response to DNA damage is dependent on Rad9, we examined Rad9 modification in synchronized cultures of *mrc1* mutants. Rad9 undergoes Mec1-dependent hyperphosphorylation in the presence of DNA damage, significantly reducing Rad9 mobility in SDS polyacrylamide gel electrophoresis (SDS–PAGE)[21,22]. We found that Rad9 is hyperphosphorylated in *mrc1* cells at 60 min (Fig. 3b), coincident with Rad53 activation. This result supports the idea that DNA damage is present in Δ*mrc1* mutants late in S phase, leading to Rad9-dependent activation of Rad53.

To test whether the partial activation of Rad53 observed in HU-treated *mrc1* is due to Rad9 activation, Rad9 phosphorylation was examined in log-phase wild-type and Δ*mrc1* cultures treated with 200 mM HU (Fig. 3c). HU does not cause Rad9 modification in wild-type cells. However, in Δ*mrc1* mutants, a shift in the Rad9 protein band is observed at 60 and 120 min. Activation of Rad9 is thought to lead to hyperphosphorylation of the Chk1 protein[13]. Consistent with this idea, we observed that Δ*mrc1*, but not wild

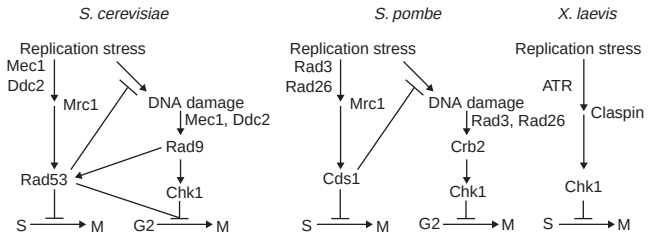

Figure 6. **Comparison of the replication checkpoint pathways in different organisms.** ScMrc1, SpMrc1 and XClaspin relay replication stress signals from ATR kinase homologues to checkpoint kinases ScRad53, SpCds1 and XChk1, respectively.

type, cells phosphorylate Chk1 in the presence of HU (Fig. 3d). These data strengthen the hypothesis that the absence of Mrc1 leads to DNA damage, which activates the Rad9 pathway, leading to phosphorylation of both Rad53 and Chk1.

Consistent with these observations, *mrc1* mutants require Rad9 for survival (data not shown). We were able to suppress the synthetic lethality of the Δ*mrc1* Δ*rad9* double mutant by overexpressing *RNR1,* which also rescues Δ*rad53* lethality[12]. The HU sensitivity of the double mutant is much greater than that of *mrc1* and is comparable to that of a *rad53* null mutant (data not shown). We examined HU-induced Rad53 phosphorylation treatment in synchronous cultures of wild type, Δ*mrc1* Δ*rad9* and Δ*mrc1* Δ*rad9* double mutants carrying *RAD9* or *MRC1* on a plasmid (Fig. 3e). Phosphorylated forms of Rad53 are present in wild-type cells at 60 and 90 min but are completely absent in the Δ*mrc1* Δ*rad9* double mutant. As expected, adding *MRC1* on a plasmid restores Rad53 activation in this mutant. Δ*mrc1* Δ*rad9* + *RAD9* cells behave like the Δ*mrc1* single mutant (Fig. 3a, Δ*mrc1* + HU lanes), Rad53 modification is present but is delayed and at reduced levels. It should be noted that although overexpression of *RNR1* suppresses the lethality of *mrc1rad9* mutants, this overexpression has no effect on the rate of DNA synthesis in *mrc1* and *mrc1rad9* mutants as these strains still exhibit slower kinetics of progression through S phase (data not shown).

Mrc1 is phosphorylated in a Mec1- and Rad53-dependent manner. Many key signal-transducing proteins in the DNA replication checkpoint are phosphorylated in response to replication stress. To test whether the Mrc1 protein is modified in response to DNA damage or HU treatment, we performed western analyses using antibodies to Mrc1. In asynchronous wild-type cells, treatment with 200 mM HU or 0.1% MMS results in a modified Mrc1 protein with reduced electrophoretic mobility (Fig. 4a). This modification is not observed in an unperturbed cell cycle (Fig. 4b,c), suggesting that the Mrc1 shift is not a result of partial synchronization of cells in S phase caused by HU and MMS treatment. Additionally, this analysis reveals that the Mrc1 protein is present throughout the cell cycle, with a modest increase in levels during S phase.

After protein extracts from HU-treated wild-type cells were incubated with phosphatase, Mrc1 reverted back to the faster-migrating form, indicating that the observed Mrc1 shift is due to phosphorylation (Fig. 4d). Mrc1 phosphorylation is greatly reduced in both HU-treated Δ*mec1* and Δ*rad53* strains, demonstrating that this modification is largely dependent on the Mec1 and Rad53 kinases (Fig. 4a). We also examined Mrc1 phosphorylation in Δ*rad9,* Δ*ddc1* and Δ*chk1* mutants (Fig. 4a). These mutants are defective in the DNA damage response but do not affect the DNA replication checkpoint pathway[23]. As expected, they showed wild-type levels of Mrc1 phosphorylation in the presence of HU. In response to MMS, *rad9* and *ddc1* mutants showed reduced Mrc1 phosphorylation, consistent with their impairment in transducing DNA damage signals. Mrc1 therefore appears to be an effector of the DNA damage signal transduction pathway, but is not required for the cell-cycle-arrest branch of the pathway. Consistent with a role in responding to DNA damage, the Δ*mrc1* mutant is moderately sensitive to ultraviolet light (Fig. 4e) and MMS (Fig. 4f). Both asynchronous and G2-arrested Δ*mrc1* cells were slightly sensitive to ultraviolet light relative to the wild type, revealing a minor role for Mrc1 in G2 DNA damage response. Furthermore, the Chk1 kinase, which is required for the cell-cycle-arrest response to DNA damage, seems to be properly regulated in MMS-treated *mrc1* mutants (Fig. 3d).

***S. pombe* Mrc1 is required for Cds1 activation and the replication checkpoint.** If Mrc1 is conserved functionally, its *S. pombe* homologue should regulate Cds1. To test this idea, we replaced *mrc1*$^+$ in *S. pombe* with *ura4* (in *S. pombe mrc1*$^+$ designates the wild-type gene and *mrc1*$^-$ designates a mutant gene). These *mrc1*$^-$ mutants are sensitive to HU and were examined for defects in the S-phase checkpoint. Cells were synchronized in G2 using lactose gradient centrifugation[24] and incubated with 20 mM HU. Whereas wild-type cells arrested before the second synchronous mitosis in HU, 20% of *mrc1*$^-$ cells entered mitosis after 320 min (Fig. 5a). This defect is not as severe as that observed in *rad3*$^-$ mutants, but is similar to the defect seen in *cds1*$^-$ cells. *mrc1*$^-$ mutants also rapidly lost viability as cells entered the first S phase, many hours before the onset of the aberrant mitosis, as has been previously reported for checkpoint *rad* mutants[25] (Fig. 5b).

We next tested whether the Cds1 kinase can be activated in *S. pombe mrc1*$^-$ cells by HU. In this background, no increase in Cds1 kinase activity is observed even after 6 h of HU treatment, whereas wild-type cells activate Cds1 by >15-fold within 2–4 h (Fig. 5c)[8]. This activation coincides with the Cds1 hyperphosphorylation[8] that is observed in the wild type but not *mrc1*$^-$ mutants (Fig. 5d). In support of our interpretation that Mrc1 is required to activate Cds1 in HU-treated cells, we find that *mrc1*$^-$ *chk1*$^-$ cells are hypersensitive to HU (Fig. 5e), similar to *cds1*$^-$ *chk1*$^-$ double mutants. Furthermore, *mrc1*$^-$ *chk1*$^-$ double mutants exhibit a rapid accumulation of cells entering aberrant mitosis, again reminiscent of *cds1*$^-$ *chk1*$^-$ double mutants (Fig. 5f and g). These data indicate that, in the absence of Mrc1, Cds1 is not activated, causing DNA replication errors in response to HU. This leads to the activation of the Chk1- and Crb2-dependent DNA damage checkpoint as previously reported for Cds1 null mutants[8].

Similar to *S. cerevisiae* Mrc1, *S. pombe* Mrc1 is also modified by HU treatment (Fig. 5h). This is partially dependent on the presence of Rad3, the *S. pombe* homologue of Mec1. In contrast to ScMrc1, SpMrc1's alteration in electrophoretic mobility is independent of Cds1, the *S. pombe* counterpart of Rad53. Chk1 and Crb2, which are specific for DNA damage response, are not required for Mrc1 modification.

Discussion

Mrc1 is a phylogenetically conserved protein that functions to transduce DNA replication stress signals to Chk2 kinase homologues in *S. cerevisiae* and *S. pombe* (these pathways are depicted in Figure 6). With respect to DNA replication stress, Mrc1 functions in a manner analogous to the role of ScRad9 and SpCrb2 in responding to DNA damage. This idea is further supported by Mrc1's sequence similarity to Crb2. It is not clear whether Mrc1 uses the same mechanism as Rad9 to activate Rad53 because, unlike Rad9, which binds Rad53 in response to damage, we have been unable to co-immunoprecipitate Rad53 with Mrc1 (data not shown). This may be a result of technical difficulties (Mrc1 is largely insoluble), or could reflect a different mechanism for Mrc1 activation of Rad53.

Mrc1 and Mec1 function in the same pathway upstream of Rad53. Similar to Rad9, Mrc1 may be phosphorylated by Mec1 to activate Rad53. Alternatively, Mrc1 might activate Mec1 towards

Rad53. That the phosphorylation of SpMrc1 in response to HU treatment is Rad3 dependent but Cds1 independent suggests that Mrc1 may be a target of the Mec1/Rad3 kinases. It remains to be seen whether phosphorylation of Mrc1, like that of Rad9, is required for Rad53 or Cds1 activation.

mrc1 mutants have a slower S phase, which implies that Mrc1 is involved in DNA replication. This idea (of a second function separate from its checkpoint role) is supported by synthetic phenotypes observed in the *rad53 mrc1* double mutants. Mrc1 is unique in the sense that it is both a regulator of Rad53 and a putative substrate. As an effector, hyperphosphorylation by Rad53 could provide a means for the checkpoint to regulate Mrc1's replication function. Alternatively, hyperphosphorylating Mrc1 could mediate a feedback signal from Rad53 that eventually leads to the kinase's inactivation.

Although quite similar, *mrc1* mutants in different species behave slightly differently. For example, *S. cerevisiae mrc1* mutants show partial activation of Rad53, in contrast to *S. pombe mrc1*$^-$ mutants in which activation of Cds1 is not observed. This is probably related to the fact that Rad53 is involved in both the DNA damage and replication checkpoint pathways, unlike *S. pombe* Cds1, which functions only in the replication checkpoint. Relative to Cds1, Rad53 has an additional forkhead homology-associated (FHA) domain on its C terminus that is required for activation by Rad9 in response to DNA damage[9]. Therefore, because *mrc1* mutants generate DNA damage when treated with HU, Rad9 can activate Rad53 whereas Crb2 cannot activate Cds1. However, activation of Crb2 does result in activation of Chk1, which is supported by the enhanced checkpoint defect observed in the *mrc1chk1* double mutant. In addition, the hyperphosphorylation of Rad9 in HU-treated *mrc1* mutants results in delayed activation of Rad53. These kinetics help to explain why *mrc1* mutants are unable to prevent spindle elongation and late origin firing, which occur in a precipitous fashion, but show an intermediate response to transcriptional induction.

It is likely that Mrc1 is an analogue of *Xenopus* Claspin for both functional and sequence conservation reasons. If so, it reveals an interesting evolutionary change in higher eukaryotes. Claspin, like Mrc1, is required for the DNA replication checkpoint in *Xenopus*[15] (depicted in Fig. 6). In contrast to yeast, however, Claspin regulates Chk1. It might be that vertebrates primarily rely on the Chk1 pathway for the DNA replication checkpoint whereas yeast primarily rely on Chk2 homologues. If so, the activation mechanism of Claspin and Mrc1 may be shared but their downstream functions seem to have diverged. It is clear that the Mrc1 proteins, which mediate the replication checkpoint, are a key missing link in the pathway controlling activation of the checkpoint kinases and provide an important connection between DNA replication and cell-cycle control. Because DNA replicational stress probably makes a significant contribution to genomic instability, mutations in this pathway in humans are likely to contribute to tumorigenesis. □

Methods

S. cerevisiae strains, growth conditions.

S. cerevisiae strains used are isogenic with the W303-derived Y300 strain (*MATa trp1-1 ura3-1 his3-11,15, leu2-3,112 ade2-1 can1-100*)[20]. To construct Δ*mrc1-2* (Y1122), a 2562 base-pair (bp) *Spe*I–*Spe*I fragment within *MRC1* was replaced with a *KANHIS3* cassette from pJA50[26]. Δ*mrc1-3* (Y1123) and a strain expressing a Mrc1–13myc construct (in which 13 tandem copies of the myc epitope are attached to the carboxyl terminus of Mrc1) were constructed using polymerase chain reaction (PCR)-based methods[27].

Experiments were carried out in rich media (YPD) at 30 °C unless otherwise indicated. For G1 synchrony experiments, we arrested cells in YPD pH 3.9 media containing α-factor for 2 or 3 h. We added 5 μg ml^{-1} α-factor at 0 and 1.5 h. All experiments were repeated once or twice.

Viability and checkpoint assays.

HU and ultraviolet killing assays were performed as described previously[12]. To score anaphase entry, fluorescence microscopy was used after staining fixed cells with DAPI (4,6-diamidino-2-phenylindole) and anti-tubulin[20]. Cell preparation for Southern analysis of replication origins[19]and northern blot analysis of *RNR3* (ref. 20) have been described previously. For fluorescence-activated cell sorting (FACS) analysis, we followed a protocol published previously[12], except that 300 μl of cells were fixed by adding them directly to 700 μl ethanol.

Protein methods.

Protein extracts for western blots were prepared by the trichloroacetic acid (TCA) method[28]. Mrc1 was visualized after running protein extracts in 8% SDS–PAGE and immunoblotting with polyclonal anti-Mrc1 antibodies. For the phosphatase experiment, we followed a previously published method[6], but used anti-Myc antibodies to detect Mrc1–13myc.

S. pombe strains, cytology and protein methods.

All *S. pombe* strains used are derivatives of 501 (*leu1-32 ade6-704 ura4-D18 h-*). *mrc1*$^+$ was deleted as described previously[29] and verified using PCR.A C-terminal TAP tag[32] was added to Mrc1 by using methods described previously[29]. To check the sensitivity and mitotic delay defect in HU, cells were synchronized in G2 by lactose gradient centrifugation[24] and incubated in the presence of 20 mM HU. Post-mitotic and septated cells were scored microscopically after DAPI and Calcofluor staining[30].

Protein extracts for western analysis were prepared using the trichloroacetic acid (TCA) method[31]. Cds1 was visualized using anti-Cds1 serum. Rabbit anti-mouse IgG was used to visualize Mrc1–TAP protein. For Cds1 kinase activity, extracts were prepared and assayed as described previously[8].

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1. Zhou, B. B. & Elledge, S. J. The DNA damage response: putting checkpoints in perspective. *Nature* **408,** 433–439 (2000).
2. Hirao, A. *et al.* DNA damage-induced activation of p53 by the checkpoint kinase Chk2. *Science* **287,** 1824–1827 (2000).
3. Shieh, S. Y., Ahn, J., Tamai, K., Taya, Y. & Prives, C. The human homologs of checkpoint kinases Chk1 and Cds1 (Chk2) phosphorylate p53 at multiple DNA damage-inducible sites. *Genes Dev.* **14,** 289–300 (2000).
4. Chehab, N. H., Malikzay, A., Appel, M. & Halazonetis, T. D. Chk2/hCds1 functions as a DNA damage checkpoint in G(1) by stabilizing p53. *Genes Dev.* **14,** 278–288 (2000).
5. Bell, D. W. *et al.* Heterozygous germ line hCHK2 mutations in Li-Fraumeni syndrome. *Science* **286,** 2528–2531 (1999).
6. Sanchez, Y. *et al.* Regulation of RAD53 by the ATM-like kinases MEC1 and TEL1 in yeast cell cycle checkpoint pathways. *Science* **271,** 357–360 (1996).
7. Sun, Z., Fay, D. S., Marini, F., Foiani, M. & Stern, D. F. Spk1/Rad53 is regulated by Mec1-dependent protein phosphorylation in DNA replication and damage checkpoint pathways. *Genes Dev.* **10,** 395–406 (1996).
8. Lindsay, H. D. *et al.* S-phase-specific activation of Cds1 kinase defines a subpathway of the checkpoint response in *Schizosaccharomyces pombe. Genes Dev.* **12,** 382–395 (1998).
9. Sun, Z., Hsiao, J., Fay, D. S. & Stern, D. F. Rad53 FHA domain associated with phosphorylated Rad9 in the DNA damage checkpoint. *Science* **281,** 272–274 (1998).
10. Pellicioli, A. *et al.* Activation of Rad53 kinase in response to DNA damage and its effect in modulating phosphorylation of the lagging strand DNA polymerase. *EMBO J.* **18,** 6561–6572 (1999).
11. Saka, Y., Esashi, F., Matsusaka, T., Mochida, S. & Yanagida, M. Damage and replication checkpoint control in fission yeast is ensured by interactions of Crb2, a protein with BRCT motif, with Cut5 and Chk1. *Genes Dev.* **11,** 3387–3400 (1997).
12. Desany, B. A., Alcasabas, A. A., Bachant, J. B. & Elledge, S. J. Recovery from DNA replicational stress is the essential function of the S-phase checkpoint pathway. *Genes Dev.* **12,** 2956–2970 (1998).
13. Sanchez, Y. *et al.* Control of the DNA damage checkpoint by chk1 and rad53 protein kinases through distinct mechanisms. *Science* **286,** 1166–1171 (1999).
14. Willson, J., Wilson, S., Warr, N. & Watts, F. Z. Isolation and characterization of the *Schizosaccharomyces pombe* rhp9 gene: a gene required for the DNA damage checkpoint but not the replication checkpoint. *Nucleic Acids Res.* **25,** 2138–2146 (1997).
15. Kumagai, A. & Dunphy, G. W. Claspin, a novel protein required for the activation of Chk1 during a DNA replication checkpoint response in *Xenopus* egg extracts. *Mol. Cell* **6,** 839–849 (2000).
16. Weinert, T. A. & Hartwell, L. H. The RAD9 gene controls the cell cycle response to DNA damage in *Saccharomyces cerevisiae. Science* **241,** 317–322 (1988).
17. Paulovich, A. G. & Hartwell, L. H.. A checkpoint regulates the rate of progression through S phase in *S. cerevisiae* in response to DNA damage. *Cell* **82,** 841–847 (1995).
18. Spellman, P. T. *et al.* Comprehensive identification of cell cycle-regulated genes of the yeast *Saccharomyces cerevisiae* by microarray hybridization. *Mol. Biol. Cell* **9,** 3273–3297 (1998).
19. Santocanale, C. & Diffley, J. F. A Mec1- and Rad53-dependent checkpoint controls late-firing origins of DNA replication. *Nature* **395,** 615–618 (1998).
20. Allen, J. B., Zhou, Z., Siede, W., Friedberg, E. C. & Elledge, S. J. The SAD1/RAD53 protein kinase controls multiple checkpoints and DNA damage-induced transcription in yeast. *Genes Dev.* **8,** 2401–2415 (1994).
21. Vialard, J. E., Gilbert, C. S., Green, C. M. & Lowndes, N. F. The budding yeast Rad9 checkpoint protein is subjected to Mec1/Tel1-dependent hyperphosphorylation and interacts with Rad53 after DNA damage. *EMBO J.* **17,** 5679–5688 (1998).
22. Emili, A. MEC1-dependent phosphorylation of Rad9p in response to DNA damage. *Mol. Cell* **2,** 183–189 (1998).
23. Foiani, M. *et al.* DNA damage checkpoints and DNA replication controls in *Saccharomyces cerevisiae. Mutat. Res.* **451,** 187–196 (2000).
24. Barbet, N. C. & Carr, A. M. Fission yeast wee1 protein kinase is not required for DNA damage-dependent mitotic arrest. *Nature* **364,** 824–827 (1993).
25. Enoch, T., Carr, A. M. & Nurse, P. Fission yeast genes involved in coupling mitosis to completion of DNA replication. *Genes Dev.* **6,** 2035–2046 (1992).
26. Allen, J. B. & Elledge, S. J. A family of vectors that facilitate transposon and insertional mutagenesis of cloned genes in yeast. *Yeast* **10,** 1267–1272 (1994).
27. Longtine, M. S. *et al.* Additional modules for versatile and economical PCR-based gene deletion and modification in *Saccharomyces cerevisiae. Yeast* **14,** 953–961 (1998).
28. Paciotti, V., Lucchini, G., Plevani, P., and Longhese, M. P. Mec1p is essential for phosphorylation of the yeast DNA damage checkpoint protein Ddc1p, which physically interacts with Mec3p.

EMBO J. **17,** 4199–4209 (1998).
29. Bahler, J. *et al.* Heterologous modules for efficient and versatile PCR-based gene targeting in *Schizosaccharomyces pombe*. *Yeast* **14,** 943–951 (1998).
30. Russell, P. & Nurse P. cdc25+ functions as an inducer in the mitotic control of fission yeast. *Cell* **45,** 145–153 (1986).
31. Caspari, T. *et al.* Characterization of *Schizosaccharomyces pombe* Hus1: a PCNA-related protein that associates with Rad1 and Rad9. *Mol. Cell Biol.* **20,** 1254–1262 (2000).
32. Tasto, J. J., Carnahan, R. H., McDonald, W. H. & Gould, K. L. Vectors and gene targeting modules for tandem affinity purification in *Schizosaccharomyces pombe*. *Yeast* **18,** 657–662 (2001).

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