

Temporal separation of replication and recombination requires the intra-S checkpoint

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In response to DNA damage and replication pausing, eukaryotes activate checkpoint pathways that prevent genomic instability by coordinating cell cycle progression with DNA repair. The intra-S-phase checkpoint has been proposed to protect stalled replication forks from pathological rearrangements that could result from unscheduled recombination. On the other hand, recombination may be needed to cope with either stalled forks or double-strand breaks resulting from hydroxyurea treatment. We have exploited fission yeast to elucidate

the relationship between replication fork stalling, loading of replication and recombination proteins onto DNA, and the intra-S checkpoint. Here, we show that a functional recombination machinery is not essential for recovery from replication fork arrest and instead can lead to non-functional fork structures. We find that Rad22-containing foci are rare in S-phase cells, but peak in G2 phase cells after a perturbed S phase. Importantly, we find that the intra-S checkpoint is necessary to avoid aberrant strand-exchange events during a hydroxyurea block.

Introduction

When replication pauses, the stability of stalled replication forks is thought to be maintained by the intra-S-phase checkpoint (Lopes et al., 2001; Tercero and Diffley, 2001; Noguchi et al., 2003). Indeed, aberrant fork structures accumulate in checkpoint-deficient strains after replication block by nucleotide depletion. This observation led to the speculation that unscheduled recombination pathways might process abnormal replication intermediates in these mutants (Sogo et al., 2002). Genetic data in budding yeast suggest that when replication forks are stalled, helicases Sgs1 and Srs2 act to hinder recombinogenic repair pathways at these forks (Fabre et al., 2002). Using purified Srs2, it was shown that Srs2 is able to disassemble a Rad51 nucleofilament *in vitro* and avoid the formation of joint molecules, one of the first steps of recombination (Krejci et al., 2003; Veaute et al., 2003). Indeed, if cells lack the Rrm3 helicase, which helps promote fork movement through protein-induced barriers, either Sgs1 or Srs2 becomes essential unless recombination is suppressed by the deletion of *RAD51* (Schmidt and Kolodner, 2004; Torres et al., 2004). The complex regulation of anti-recombinogenic helicases and the intra-S checkpoint is underscored by the fact that Sgs1 contributes to

the S-phase activation of Rad53 in response to fork stalling on hydroxyurea (HU; Frei and Gasser, 2000), as does Srs2 in response to strand breaks (Liberi et al., 2000).

On the other hand, several recombination-deficient strains have been reported to be sensitive to HU, which induces replication fork stalling by limiting dNTP pools, or to MMS, which induces fork-associated damage (Bjergbaek et al., 2005). This sensitivity has been interpreted as a need for recombination to cope either with stalled replication forks or with double-strand breaks created by drug treatment (Chang et al., 2002).

Using fission yeast, we have explored the relationship of recombination process to stalled fork collapse by monitoring recombination foci formation under conditions that do or do not allow S-phase checkpoint activation. We demonstrate a temporal separation of recombination and replication, which appears compromised in *cds1* (CHK2)-deficient yeast strains.

Results and discussion

To clarify the relationship between recombination and intra-S checkpoint pathways we have used fission yeast, which has two genetically distinct checkpoint-signaling pathways that respond to DNA damage (Fig. 1 A). The CHK2 kinase homologue Cds1 mediates the intra-S checkpoint in response to stalled replication forks and DNA damage during S phase, whereas the G2/M checkpoint is mediated by Chk1 and responds to strand breaks and other damage during G2 phase (Carr,

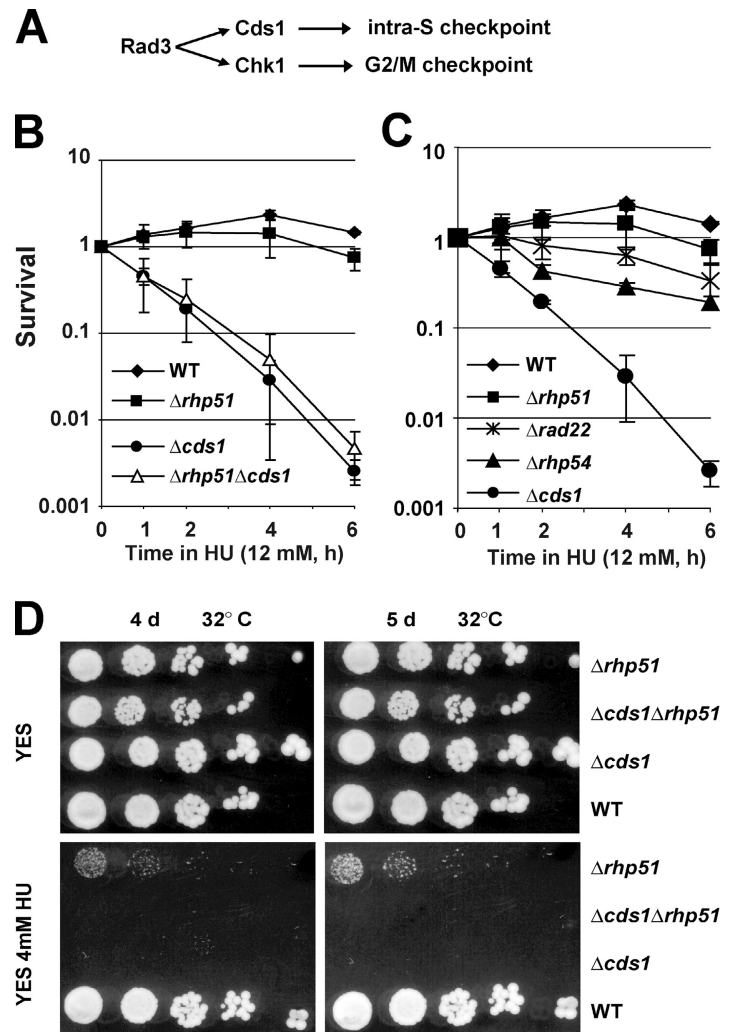
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Abbreviation used in this paper: HU, hydroxyurea.

Figure 1. **Functional recombination machinery is not essential for recovery from stalled replication forks.** (A) Outline of checkpoint pathways in *S. pombe*. (B) Survival of recombination and S-phase checkpoint mutants after acute HU treatment. Isogenic cells of the confirmed genotypes were treated with 12 mM HU during the indicated times before plating in triplicate on YES medium. (C) Sensitivity of the indicated mutants in recombination pathways are compared with that of wild-type and *cds1* cells after exposure to 12 mM HU for the indicated time and plating on solid YES medium for outgrowth. Experiments were performed at least twice and error bars are shown. (D) Sensitivity of the $\Delta rhp51$ cells to chronic HU exposure (4 mM).



2002). This separation of function allows us to examine the outcome of suppressing checkpoint activation in S phase without compromising G2 checkpoint function. This is unlike the situation in budding yeast, in which both the intra-S and the G2/M checkpoints depend on the CHK2 homologue, Rad53^{Sc}. The ATR kinase homologue, called Rad3 in fission yeast, acts upstream of both pathways throughout the cell cycle, activating downstream kinases in response to fork stalling or DNA lesions.

First, we investigated the relationship between the S-phase checkpoint and recombination pathways genetically. Cell survival was monitored after acute HU treatments in a wild-type background or isogenic strains defective for the intra-S-phase checkpoint ($\Delta cds1$), recombination ($\Delta rhp51$), or both ($\Delta cds1\Delta rhp51$; Fig. 1 B). As expected, both the $\Delta cds1$ and $\Delta cds1\Delta rhp51$ strains are highly sensitive to the HU-induced replication block, whereas the $\Delta rhp51$ and $\Delta rad22$ mutants show little if any sensitivity to acute HU treatment (Fig. 1, B and C). Among other known recombination-deficient mutants, $\Delta rhp54$ is the most sensitive with 18% survival after 6 h exposure to HU, whereas <0.5% of the $\Delta cds1$ cells survive this treatment (Fig. 1, B and C). This suggests that functional recombination machinery is not essential for recovery from a stalled replication fork in fission yeast.

In contrast to the healthy recovery from fork arrest detected for the $\Delta rhp51$ mutant, others have reported a pronounced hypersensitivity to chronic HU treatment (Zolezzi et al., 2002). Indeed, when $\Delta rhp51$ cells are plated on 4 mM HU, the mutant is extremely slow growing (Fig. 1 D). Nonetheless, ~50% of the $\Delta rhp51$ -deficient cells give rise to small colonies visible after 5 d, while no *cds1*-deficient cells survive. Moreover, $\Delta rhp51$ cells appear elongated during exposure to HU, indicating a prolonged G2 checkpoint arrest. Together, these results suggest that the recombination machinery is not essential for recovery from stalled replication forks, although recombination may well facilitate repair of the strand breaks generated during replication on low concentrations of HU. Fundamental differences in the cellular response to low levels of HU and high, fork-arresting concentrations have also been characterized in *Saccharomyces cerevisiae*, where chronic HU treatment induces Chk1 and acute levels of HU do not (Schollaert et al., 2004).

Independent visualization of replication and recombination foci

To further analyze the relationship between replication and recombination pathways during HU arrest/release, we visualized

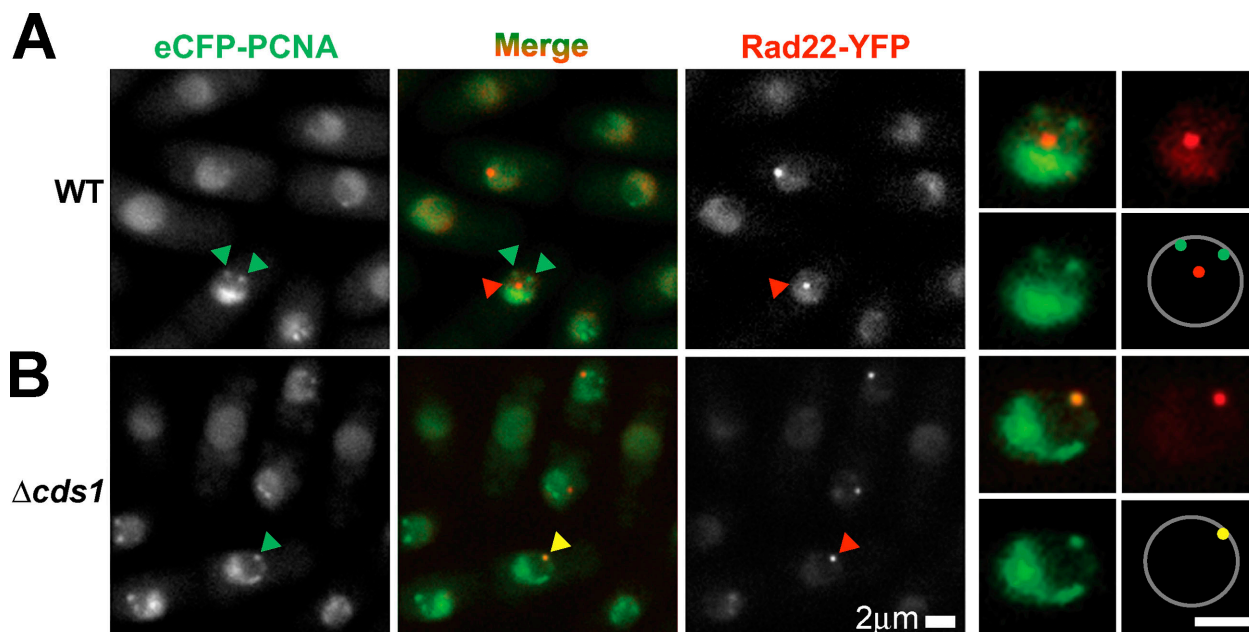


Figure 2. **Spatial separation of replication and recombination factories is affected by loss of the S-phase checkpoint.** Low-level diffuse PCNA signals are typical of non-S-phase cells, whereas S-phase cells have a bright PCNA pattern. Although Rad22 foci are rare in wild-type cells, we show an example to illustrate the absence of colocalization with PCNA (enlarged to right). An example of colocalization is shown for *cds1*-deficient cells (enlarged in the right-most panel).

both recombination and replication foci in HU-treated cells. YFP fusions to Rad22, the Rad52^{Sc} homologue in *Schizosaccharomyces pombe*, allow us to identify subnuclear sites of loading of recombination proteins onto DNA, and a CFP-tagged version of PCNA was used to reveal replication foci. As in budding yeast, induced recombination leads to a concentration of the normally diffuse Rad22 fluorescence into a few bright nuclear spots (Lisby et al., 2001; Du et al., 2003; Meister et al., 2003). In the absence of HU, 14% of a nonsynchronized wild-type population harbour a single Rad22-YFP nuclear spot, which most likely corresponds to sites of post-

replicative DNA repair (Meister et al., 2003; Noguchi et al., 2003). Only 1% of wild-type cells harbour more than one spot. These Rad22 foci appear in G2 or very late S phase. This observation parallels a report from Lisby et al. (2001), who showed in *S. cerevisiae* that 22% of the large-budded yeast cells harbour Rad52^{Sc} foci. However, in budding yeast the distinction between S and G2 phases of the cell cycle could not be made, nor was the relationship of these foci to replication foci determined. Importantly, we show here that Rad22 foci rarely coincide with bright foci of PCNA in wild-type fission yeast cells (<4%; Fig. 2 A).

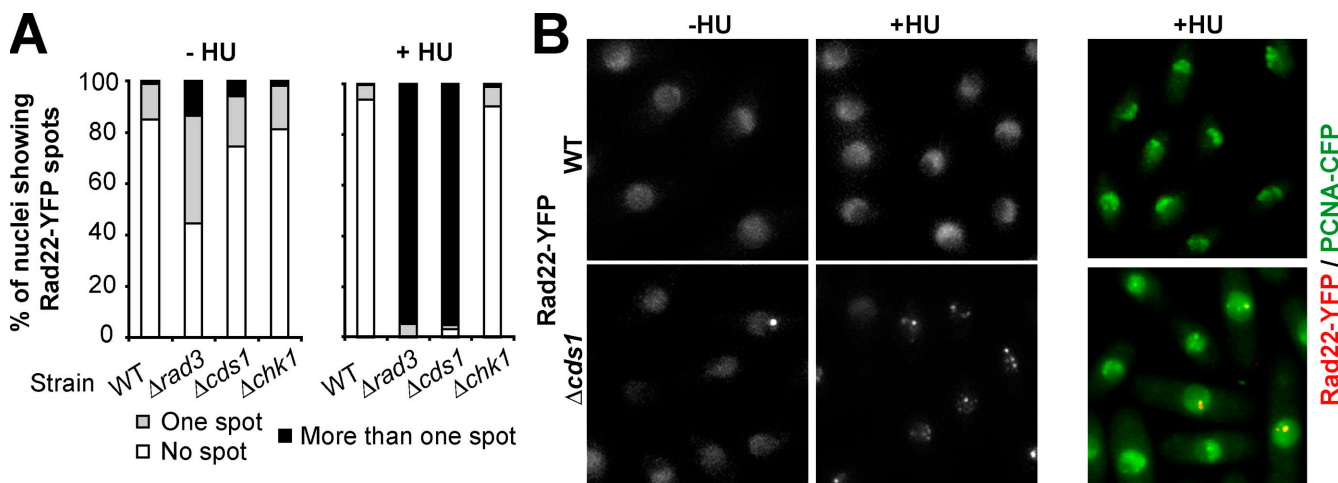


Figure 3. **Hydroxyurea induces recombination foci in S-phase checkpoint mutants, not in wild-type or G2/M mutant cells.** (A) Quantitation of the fraction of nuclei containing Rad22-YFP foci in asynchronously growing cells (-HU) or in cells treated with 12 mM HU for 2 h (+HU) in wild-type and checkpoint mutants. (B) Wild-type and $\Delta cds1$ strains were imaged either in the absence of HU or after a 2-h exposure to 12 mM HU. Left panels show Rad22-YFP foci are suppressed in cells with an intact S-phase checkpoint response during HU replication arrest, whereas the color image confirms that both cells have bright PCNA-CFP signals typical of S-phase cells.

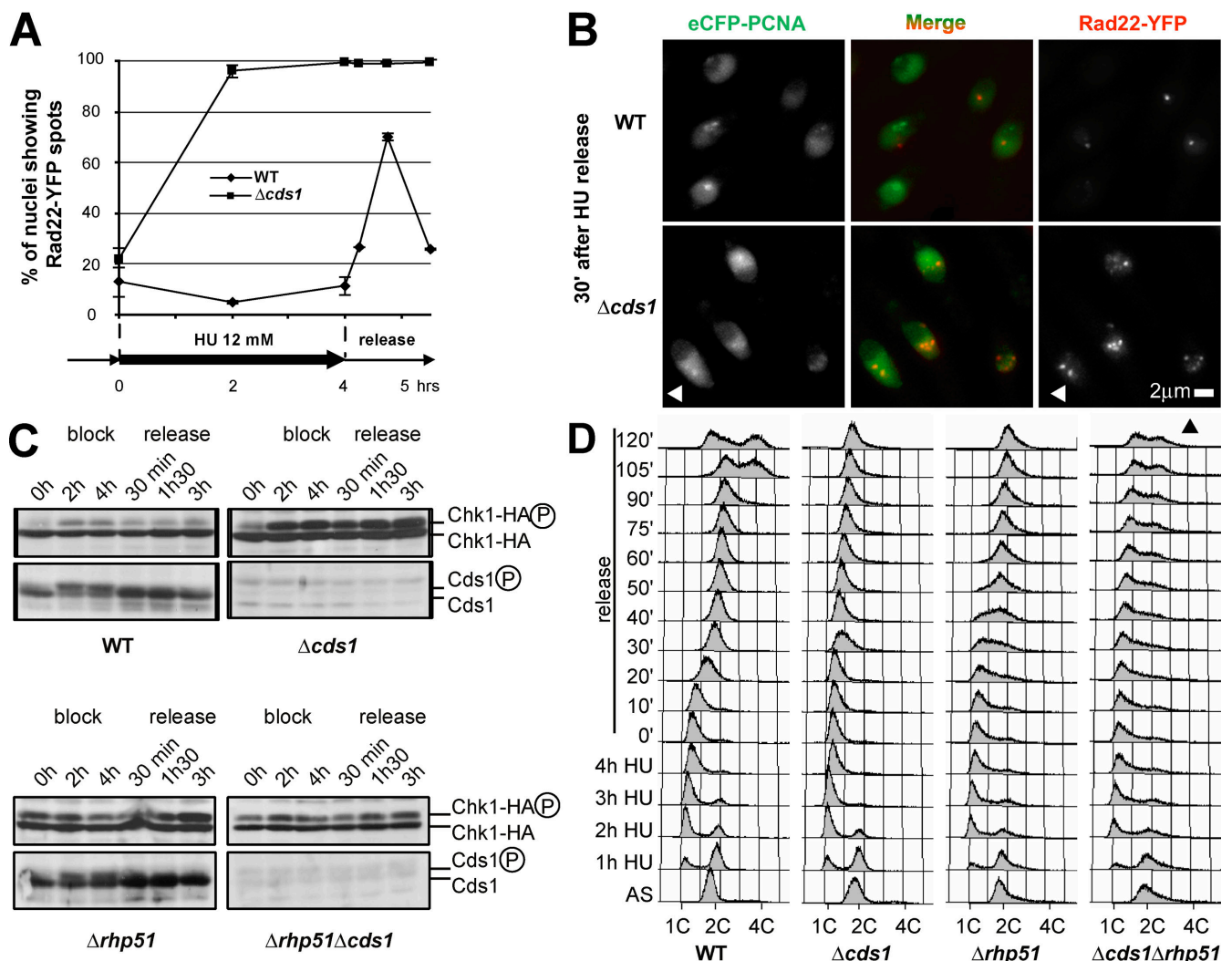


Figure 4. Temporal separation of replication and recombination is affected by loss of the S-phase checkpoint. (A) The appearance of Rad22-YFP spots was monitored during an HU block and after release into fresh medium in wild-type and $\Delta cds1$ cells. Loss of the Cds1-mediated checkpoint leads to the accumulation of persistent Rad22 foci in S phase. In wild-type cells, Rad22 foci do not accumulate until release from HU arrest, and then rapidly disappear. This suggests a temporal separation of replication and recombination ensured by the S-phase checkpoint. Experiments were performed twice and error bars are shown. (B) Live imaging of ECFP-PCNA (green) and Rad22-YFP (red) in cells 30 min after release from HU arrest. Note that wild-type cells have few replication (PCNA) factories remaining when Rad22 foci appear, whereas the S-phase pattern persists in checkpoint-deficient cells, where PCNA and Rad22 foci often colocalize (white arrowheads). (C) Cds1 and Chk1 activation upon HU treatment and release in S-phase checkpoint or/and recombination mutants is monitored by Western blots. In each blot the top band represents the modified (activated) form of the kinase. (D) S-phase progression after release from HU treatment is restored by *rhp51* deletion in checkpoint mutants. Cells deficient for S-phase checkpoint ($\Delta cds1$) are unable to resume replication after release from HU treatment, whereas strains deficient for both checkpoint and recombination can ($\Delta cds1 \Delta rhp51$, black arrowhead).

In asynchronously growing $\Delta cds1$ cells, we observe a slight increase in the frequency of cells showing one Rad22 spot (20%, Fig. 2 B; quantified in Fig. 3 A) and a fivefold increase of cells with several spots (6%, Fig. 3 B), even in the absence of HU. This is consistent with previous reports (Meister et al., 2003; Noguchi et al., 2003). Moreover, a larger number of these Rad22 spots coincide with foci of PCNA (13%, $n = 102$ vs. <4%, $n = 82$, in wild-type cells).

When cells are exposed to HU for 2 h, the proportion of $\Delta cds1$ or $\Delta rad3$ cells harbouring multiple Rad22-YFP foci increases to 95%, whereas in wild-type and $\Delta chk1$ cells this value remains at 0% and 1.2%, respectively (Fig. 3). Importantly, in S-phase checkpoint-deficient strains, we observe Rad22 foci in cells that harbour a bright, granular PCNA distri-

bution, indicating that these cells are still in S phase (Fig. 2). Among the brightest foci a significant fraction colocalizes with PCNA foci (34%, $n = 115$).

After HU release, the Rad22 foci persist for at least 2 h in almost all of the $\Delta cds1$ cells, whereas in wild-type strains Rad22 foci accumulate and peak at 30 min after release, and then rapidly disappear (Fig. 4 A). This peak of Rad22 foci in wild-type cells correlates with the dephosphorylation of Cds1 and near completion of DNA replication which, as judged by FACS profile analysis and PCNA foci disappearance, is completed between 30 and 40 min (Fig. 4, C and D). Moreover, 60% of the Rad22 foci-containing cells lack PCNA foci, indicating that they have moved into G2 phase, whereas 40% of these cells have a few bright perinucleolar PCNA foci, which

we show elsewhere to be representative of late S phase (unpublished data). Thus, Rad22 focus formation can occur during late S phase, but seems to be delayed as long as the intra-S checkpoint is activated. Consistently, we see little colocalization of Rad22 with the residual PCNA foci in a wild-type strain (<12%, Fig. 4 B). We conclude that there is a temporal and a spatial separation of recombination and replication events in cells that have an intact intra-S checkpoint.

Recombination leads to nonfunctional replication forks in checkpoint-deficient cells

Whereas others interpreted similar observations in *S. cerevisiae* as an attempt made by checkpoint-deficient strain to restart collapsed replication forks through recombination (Lisby et al., 2004), the results are also consistent with other interpretations. On one hand, unprotected, stalled replication forks may expose single-stranded DNA that is recognized and inappropriately used by the recombination machinery. Alternatively, unprotected replication forks may “collapse” in the absence of intra-S checkpoint, creating structures or breaks that in turn recruit the recombination machinery. In other words, Rad22 foci formation during S phase could be either a cause or a consequence of replication fork collapse.

To discriminate between these two scenarios we monitored cell cycle progression and checkpoint activation after HU release in the $\Delta cds1$ strain, comparing it with $\Delta rhp51$ and the double mutant (Fig. 4, C and D). As previously described, both wild-type and $\Delta rhp51$ cells activate Cds1 kinase and accumulate with a 1C DNA content in the presence of HU. After release, Cds1 is rapidly dephosphorylated, and cells complete S phase and enter the following cell cycle. The $\Delta cds1$ mutant, on the other hand, activates Chk1 in response to HU, fails to complete S phase after release, and dies with unreplicated DNA (Lindsay et al., 1998; Brondello et al., 1999; Fig. 4, C and D). Intriguingly, a significant fraction of the $\Delta cds1\Delta rhp51$ cells progress through S phase from 1C to 2C DNA content only slightly slower than $\Delta rhp51$ -deficient cells (Fig. 4 D), suggesting that at least some replication forks are still functional after HU release in the absence of both Cds1 and Rhp51, but not in the absence of Cds1 alone. Therefore, aberrant Rhp51-dependent recombination may actually create nonfunctional structures at replication forks in the $\Delta cds1$ mutant. This, together with the low sensitivity to HU arrest monitored in recombination-deficient mutants, further stresses that the recombination machinery is not essential for recovery, but may instead lead to aberrant nonfunctional structures at stalled forks.

To further confirm the effect of recombination on stalled replication forks in the absence of checkpoint, 2D gel analysis was performed to detect replication intermediates at the early firing origin *ars2.1* (Kim and Huberman, 2001) in wild-type, $\Delta cds1$, $\Delta rhp51$, and $\Delta cds1\Delta rhp51$ mutants upon HU block and release (Fig. 5). In wild-type cells arrested in HU, both replication bubbles (Fig. 5, black arrowhead) and Y-forks (Fig. 5, white arrowhead) are stabilized and can be observed at 2 h and 4 h after HU addition. 1 h after release from HU, replication intermediates are nearly absent, indicating that cells have entered

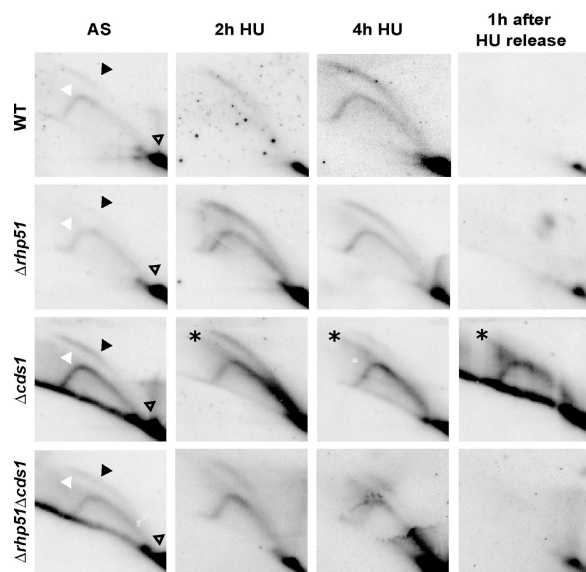


Figure 5. *rhp51* deletion partially rescues replication forks in intra-S checkpoint-deficient strain upon HU treatment. 2D gels analysis of replication intermediates at *ars2.1* upon HU block and release in wild-type, $\Delta cds1$, $\Delta rhp51$, and $\Delta cds1\Delta rhp51$ cells are shown. Note that DNA replication intermediates were enriched by BND-cellulose chromatography, which is responsible for the low signal obtained in G2 cells (30 min after HU release) and the variable levels of 1n DNA (open arrowhead). In wild-type, $\Delta rhp51$, and $\Delta cds1\Delta rhp51$ cells replication intermediates (replication bubbles, black arrowhead; Y-forks, white arrowhead) persist upon HU treatment and disappear after release. In $\Delta cds1$ cells the bubble arc progressively disappears, and is replaced by double-Ys arcs and X-shaped forms (asterisk) that persist after HU release.

G2 by this time, consistent with the FACS profile. In $\Delta rhp51$ cells, both bubble and Y arcs are clearly visible during an HU arrest. After HU release, replication intermediates disappear and cells enter G2 phase (see FACS analysis), demonstrating that restart from stalled replication fork does not require Rhp51-dependent recombination events. This is consistent with the weak sensitivity to acute HU treatment shown by $\Delta rhp51$ cells and the FACS profiles detected after HU release. In intra-S-phase checkpoint mutant $\Delta cds1$, both bubble and Y arcs are present in asynchronously growing cells. When cells are blocked in HU, the bubble arc progressively disappears, and is replaced by double-Ys arcs and X-shaped intermediates forming a conical signal (Fig. 5, asterisk). After HU removal, replication intermediates and the conical signal do not disappear, suggesting that these aberrant fork structures are unable to complete replication, failing to restart stalled forks or initiating new forks from unfired replication origins (Fig. 5; see also Fig. 4 D, FACS). These observations are consistent with previous findings in both budding and fission yeast (Lopes et al., 2001; Tercero and Diffley, 2001; Noguchi et al., 2003), and have been interpreted as replication fork collapse. Strikingly, in the $\Delta cds1\Delta rhp51$ double mutant, replication intermediates disappear by 1 h after HU release. This disappearance together with the increase in DNA content observed by FACS suggests that DNA replication restarts after release from HU in this strain. This replication can be performed in two ways: either by restarting stalled forks or by firing neighboring origins that did

not fire during the HU block. We favor the former hypothesis because the delay of late origin firing depends on the intra-S checkpoint (Kim and Huberman, 2001), and therefore late origins should have fired before arrest in this mutant. In summary, we propose a model in which the deletion of *rhp51* actually rescues replication fork collapse of the intra-S checkpoint-deficient strain by preventing inappropriate strand pairing. This strongly suggests that aberrant loading of recombination proteins onto DNA can be toxic during a perturbed S phase in the absence of the intra-S checkpoint.

Putative regulation mechanism of recombination during DNA replication

Although some of the $\Delta cds1\Delta rhp51$ cells seem to progress through S phase after HU release, no cell survives this treatment. This is similar to the $\Delta cds1$ single mutant. However, whereas $\Delta cds1$ cells die with nonfunctional replication intermediates, at least a fraction of the $\Delta cds1\Delta rhp51$ cells die with a 2C DNA content, indicating that they have been able to resume DNA replication. We propose that the lethality of the double mutant upon HU treatment is due to DNA damage incurred at “unprotected” replication forks, as if, due to the absence of Cds1, stalled forks are exposed to different kinds of insults. One of them, as we show here, appears to lead to an aberrant Rhp51-dependent strand exchange that hinders further replication. However, several other lethal reactions could happen at those forks, and some may actually require recombination to be repaired in G2. Thus, although recombination is not required to recover from stalled replication forks as long as the intra-S checkpoint is functional (Fig. 1), it could nonetheless contribute to a G2 phase recovery from insults arising from an HU-induced arrest in $\Delta cds1$ strains. Because the presence of Rhp51 during S phase leads to nonfunctional structures in the *cds1* mutant (see 2D gels in Fig. 5) and yet may be needed for G2 phase repair events, the recombination apparatus must be tightly regulated during DNA replication. We argue that during DNA replication this is achieved through Cds1 and the intra-S checkpoint.

We envision two nonexclusive means through which the intra-S checkpoint can protect replication forks from recombination. First, the checkpoint may stabilize the replication machinery at the stalled fork. In budding yeast the ATR homologue Mec1^{Sc} and a fork-associated mediator of checkpoint activation Mrc1^{Sc} and Tof1^{Sc}, but not Rad53^{Sc}, are involved in DNA pol ϵ stabilization upon exposure to HU (Aparicio et al., 1999; Cobb et al., 2003; Katou et al., 2003; Bjergbaek et al., 2005). Equivalent experiments have not been performed in fission yeast to date, although the Tof1^{Sc} homologue, Swi1, and an interacting protein, Swi3, are both required for fork stabilization after replication stalling (Noguchi et al., 2004). A second mechanism involves the direct regulation of recombination proteins by the intra-S checkpoint. In support of this, Cds1 activation was shown to trigger the phosphorylation and nuclear delocalization of the recombination protein Rad60 (Boddy et al., 2003), providing the means to regulate the recombination apparatus. A further target of Cds1 is Mus81, a subunit of a heterodimeric Holliday-junction structure-specific endonuclease complex (Boddy et al., 2000). Cds1-mediated phosphor-

ylation of Mus81 in response to HU reduces its chromatin-binding activity. This may in turn reduce the frequency of Mus81/Erme1-dependent deletions in replication-stressed cells (Kai and Wang, 2003).

In other experimental systems (mammalian cells, *Xenopus* egg extracts) two Rad51-interacting proteins, BRCA2^{Hs} and XBlm, help HU-arrested cells avoid pathological rearrangements and double-strand breaks at stalled replication forks, potentially by regulating recombination (Lomonosov et al., 2003; Li et al., 2004; Shivji and Venkitaraman, 2004). Moreover, overexpressed Rad51 leads to apoptosis in *Drosophila* (Yoo and McKee, 2004). Finally, in *S. cerevisiae* the anti-recombinase helicase Srs2 is phosphorylated in a checkpoint-dependent manner, and may contribute to a reduction of fork-associated recombination (Liberi et al., 2000; Veaute et al., 2003). Both Srs2 and Sgs1 helicase mutations have Rad51-reversible synthetic defects when combined with the loss of Rrm3, a DNA helicase that promotes replication fork progression through ribosomal DNA repeats and telomeric DNA (Ooi et al., 2003; Schmidt and Kolodner, 2004; Torres et al., 2004). All these observations indicate the detrimental impact that Rad51-mediated recombination can have on stalled forks, a fact we have demonstrated here genetically.

Our results indicate a spatio-temporal separation of replication and recombination events that specifically requires the intra-S checkpoint. We show that the recombination machinery is not required to restart stalled forks, and can even be toxic when there is a massive stalling of unprotected replication forks. However, recombination proteins are loaded onto DNA after a perturbed S phase probably to deal efficiently with structures that arise from replication fork stalling. Because the $\Delta cds1$ mutant accumulates more Rad22 foci in S phase than wild-type cells do even in the absence of HU, the intra-S checkpoint may prevent DNA breakage and/or delay recombination activation until G2 phase during normal cell cycle progression.

Materials and methods

S. pombe strains and culture procedures

All constructs used in this study are derived from previously described strains (Meister et al., 2003; Smeets et al., 2003). Classical genetic procedures were used to generate double-tagged strains expressing both ECFP-PCNA and Rad22-YFP for imaging, or Chk1-HA for Western blotting. Cells were cultured in YES (supplemented rich medium, yeast extract, 5 g/l; Difco). Exponentially growing cultures were treated with hydroxyurea (12 mM final concentration; US Biologicals) during the indicated time at 30°C. For release experiments, cells were recovered by centrifugation and washed once in water before dilution in fresh YES. For drop tests, cells were grown to log phase in rich medium at 30°C and then resuspended in YES at a density of 4×10^5 cells/ml. 4- μ l aliquots of 1:5 serial dilutions were spotted on solid YES medium containing or not HU as indicated and allowed to grow at 32°C for either 4 or 5 d in the dark.

Western blot

Whole-cell extracts were performed as described previously (Smeets et al., 2003). Chk1-HA was revealed using monoclonal anti-HA antibody (Roche), whereas Cds1 was detected with a polyclonal anti-Cds1 antibody (a gift of A. Carr, University of Sussex, Brighton, UK) using chemiluminescence (Lightning Plus; PE Corp.).

Survival curve

For survival curves, cells deleted for the indicated genes were adjusted to a final concentration of 2×10^6 cells/ml before HU addition. After dilu-

tion, cells were plated on YES and incubated at 30°C. Survival was estimated as the ratio of treated versus untreated cells.

FACS staining

Ethanol-fixed cells were treated as described previously (Sazer and Sherwood, 1990). DNA was stained with Sytox green (Molecular Probes, Inc.) at a final concentration of 1 μ M. Acquisition was performed on a BD FACS Calibur.

Imaging of cells

Cells were imaged on an 1.4% agarose pad in YE medium at 30°C using a microscope (model IX70; Olympus) equipped with a 100 \times objective (NA 1.4; Carl Zeiss MicroImaging, Inc.), a CoolSnapHQ cooled CCD camera and a TillVision polychrome II monochromator as light source (two sequential wavelength 430/515 nm, Chromas filter cube CFP/YFP). Three to eight 12-plane stacks were acquired using Metamorph (Universal Imaging Corp.), with a step size of 0.2 μ m. Stacks were Z-projected using Metamorph and nuclei types were counted on projections. An average of 400 nuclei from two separate experiments was examined for each time point.

2D-gel electrophoresis

DNA extraction was performed as described previously (Segurado et al., 2002), except that DNA replication intermediates were enriched by BND-cellulose chromatography. 2D-gels were realized as described in Brewer and Fangman (1987), modified after Hyrien and Mechali (1992). First dimension was performed at RT during 68 h at 0.65 V/cm on a 0.4% agarose gel, whereas second dimension was performed at 4°C and 0.95 V/cm for 17h30. Probe engulfing *ars2.1* (Kim and Huberman, 2001) was obtained by PCR using oligos 5'-AAGCTTTAGCTAAGGTTCCGGT-TGTCATTGGATGATACCC-3' and 5'-AAGCTTCACTCTGTGATAAATTC-ATGAAAAGAAAACATGA-3'.

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