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,
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, Rad53Sc.

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 (Δcds1), recombination (Δrhp51), or both (Δcds1Δrhp51; Fig. 1 B). As expected, both the Δcds1 and Δcds1Δrhp51 strains are highly sensitive to the HU-induced replication block, whereas the Δrhp51 and Δrad22 mutants show little if any sensitivity to acute HU treatment (Fig. 1, B and C). Among other known recombination-deficient mutants, Δrhp54 is the most sensitive with 18% survival after 6 h exposure to HU, whereas <0.5% of the Δcds1 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 Δrhp51 mutant, others have reported a pronounced hypersensitivity to chronic HU treatment (Zolezzi et al., 2002). Indeed, when Δrhp51 cells are plated on 4 mM HU, the mutant is extremely slow growing (Fig. 1 D). Nonetheless, ~50% of the Δrhp51-deficient cells give rise to small colonies visible after 5 d, while no cds1-deficient cells survive. Moreover, Δrhp51 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 (Schol-laert 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
both recombination and replication foci in HU-treated cells. YFP fusions to Rad22, the Rad52 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 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 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).

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 Δcds1 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.
In asynchronously growing Δcds1 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 = 115).

After HU release, the Rad22 foci persist for at least 2 h in almost all of the Δcds1 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 Δcds1 strain, comparing it with Δrhp51 and the double mutant (Fig. 4, C and D). As previously described, both wild-type and Δrhp51 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 Δcds1 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 Δcds1Δrhp51 cells progress through S phase from 1C to 2C DNA content only slightly slower than Δrhp51-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 Δcds1 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, Δcds1, Δrhp51, and Δcds1Δrhp51 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 G2 by this time, consistent with the FACS profile. In Δrhp51 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 Δrhp51 cells and the FACS profiles detected after HU release. In intra-S-phase checkpoint mutant Δcds1, 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 forms (asterisk) that persist after HU release.

![Figure 5](image-url)
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 Δcds1Δrhp51 cells seem to progress through S phase after HU release, no cell survives this treatment. This is similar to the Δcds1 single mutant. However, whereas Δcds1 cells die with nonfunctional replication intermediates, at least a fraction of the Δcds1Δrhp51 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 Δcds1 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 Mc1Sc and a fork-associated mediator of checkpoint activation Mc1Sc and Tof1Sc, but not Rad53Sc, 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 Tof1Sc homologue, Sw1, 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 phosphorylation of Mus81 in response to HU reduces its chromatin-binding activity. This may in turn reduce the frequency of Mus81/Eme1-dependent deletions in replication-stressed cells (Kai and Wang, 2003).

In other experimental systems (mammalian cells, X. laevis egg extracts) two Rad51-interacting proteins, BRCA2A2 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; Veante 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 Δcds1 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, supplemented with 2% glucose). 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 × 10⁶ cells/ml. 4-μl 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 × 10⁶ cells/ml before HU addition. After dilu-
tion, and NCCR program “Frontiers in Genetics”.

P. Meister received a scholarship from the French MRT and A. Baldacci “Épigénétiques dans la réponse aux agents génotoxiques et le contrôle du cycle cellulaire”. P. Meister to the Gasser lab, and by the Institut Curie PIC “Paramètres et conditionnement des cellules HU11032...”

Critical readings of the manuscript.

We thank the Baldacci and Gasser laboratories for helpful discussions and critical reading of the manuscript.

This work was supported in part by an EMBO Shortterm Fellowship to P. Meister to the Gasser lab, and by the Institut Curie PIC “Paramètres épigénétiques dans la réponse aux agents génotoxiques et le contrôle du cycle cellulaire”. P. Meister received a scholarship from the French MRT and A. Taddei is an EMBO longterm fellow. The Gasser laboratory is supported by the WW TMR Tumors and Cancer, the Swiss National Science Foundation, and NCCR program “Frontiers in Genetics”.

Submitted: 18 October 2004
Accepted: 5 January 2005

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