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Exo1 Processes Stalled Replication Forks and Counteracts Fork Reversal in Checkpoint-Defective Cells

Short Article

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Summary

The replication checkpoint coordinates the cell cycle with DNA replication and recombination, preventing genome instability and cancer. The budding yeast Rad53 checkpoint kinase stabilizes stalled forks and replisome-fork complexes, thus preventing the accumulation of ss-DNA regions and reversed forks at collapsed forks. We searched for factors involved in the processing of stalled forks in HU-treated *rad53* cells. Using the neutral-neutral two-dimensional electrophoresis technique (2D gel) and psoralen crosslinking combined with electron microscopy (EM), we found that the Exo1 exonuclease is recruited to stalled forks and, in *rad53* mutants, counteracts reversed fork accumulation by generating ss-DNA intermediates. Hence, Exo1-mediated fork processing resembles the action of *E. coli* RecJ nuclease at damaged forks. Fork stability and replication restart are influenced by both DNA polymerase-fork association and Exo1-mediated processing. We suggest that Exo1 counteracts fork reversal by resecting newly synthesized chains and resolving the sister chromatid junctions that cause regression of collapsed forks.

Introduction

Cells coordinate chromosome replication with cell cycle progression, repair, recombination, and sister chromatid cohesion to prevent genome instability (Bell and Dutta, 2002; Muzi-Falconi et al., 2003; Nasmyth, 2001). A failure in the tuning of these pathways leads to chromosome lesions, mutations, genome rearrangements, and cancer. To avoid such problems, eukaryotic cells have developed the replication checkpoint (Muzi-Falconi et al., 2003) that controls genome integrity by preventing mitosis until replication has been completed (Nyberg et al., 2002; Zhou and Elledge, 2000) and by controlling the stability of stalled forks (Lopes et al., 2001; Sogo et al., 2002; Tercero and Diffley, 2001) and replisome-fork association (Cobb et al., 2003; Lucca et al., 2004). The checkpoint also coordinates replication fork progression with recombination following DNA synthesis blocks or intra-S DNA damage (Foiani et al., 2000; Rhind and Russell, 2000). In yeast, the replication checkpoint is mediated by the Mec1 and Rad53 protein kinases (Zhou and Elledge, 2000). Active Mec1 and Rad53 modify the phosphorylation state of proteins implicated in recombination and replication (Muzi-Falconi et al., 2003). In particular, the DNA polymerase α-primase complex (pol-prim) and the single-strand DNA binding protein RPA (Brush et al., 1996; Pellicioli et al., 1999) are targeted by the checkpoint, probably to stabilize the replisome-fork association when forks stall (Lucca et al., 2004). Checkpoint-defective cells experiencing a hydroxyurea (HU)-induced replication block or intra-S DNA damage exhibit a variety of abnormal events: (i) the firing of dormant and pseudo origins (Santocanale and Diffley, 1998; Shirahige et al., 1998; Sogo et al., 2002) that accelerates the completion of DNA synthesis in the presence of a damaged template (Tercero and Diffley, 2001) (it should be noted however that this phenotype contributes only modestly to cell viability [Tercero et al., 2003]); (ii) the progressive dissociation of DNA polymerases from stalled replication forks that affects replication resumption (Cobb et al., 2003; Lucca et al., 2004); (iii) and the unscheduled formation of abnormal DNA structures at replication forks that gives rise to recombination intermediates and DNA breaks (Cha and Kleckner, 2002; Lopes et al., 2001; Sogo et al., 2002). In particular, checkpoint mutants accumulate single-stranded DNA molecules (gapped and hemireplicated molecules) and four-branched structures (reversed forks) (Sogo et al., 2002). The gapped and hemireplicated molecules result from lagging strand defects and, possibly, from nucleolytic events (Lopes et al., 2001; Sogo et al., 2002). Reversed forks appear to result from the conversion of specialized sister chromatid junctions (SCJs) into four-branched structures at collapsed forks (Lopes et al., 2003). The X-shaped SCJ molecules form during origin firing under physiological conditions and branch migrate chasing replication forks (Lopes et al., 2003). The SCJs resemble hemicatenanes and likely contribute to the establishment of sister chromatid cohesion during S phase and assist sister-chromatid-mediated recombination and replication bypass processes (Lopes et al., 2003). It has been suggested that, in the absence of stable replisome-fork complexes, the SCJs run off at stalled forks, allowing the ends of the daughter strands to pair together, thus giving rise to reversed forks that can be further processed by nucleolytic events (Lopes et al., 2003).

It is still unclear whether the formation of gapped molecules can influence the accumulation of reversed forks or, vice versa, whether reversed fork formation is a prerequisite for the accumulation of single-stranded regions at stalled forks. Further, it is unknown whether the pathological events at stalled forks in checkpoint-

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defective cells occur spontaneously, as a result of replisome-fork collapse or, rather, are mediated by enzymatic activities. It should be noted that the accumulation of gapped molecules and reversed forks, by providing potential substrates for enzymes normally implicated in recombination, could also engage replicating chromosomes in recombination processes. This is the case in *E. coli* where the RuvABC, the RecBC, and the RecQJ recombination pathways have been directly implicated in the processing of reversed forks (Courcelle et al., 2003; McGlynn and Lloyd, 2002; Seigneur et al., 1998). The aberrant transitions occurring at replication forks owing to checkpoint failures may very well account for the genomic rearrangements taking place at fragile sites (Cimprich, 2003) and slow replication zones (Cha and Kleckner, 2002) and, more in general, for the genome instability of cancer cells, the vast majority of which sooner or later accumulate mutations in checkpoint genes (Kolodner et al., 2002; Weinert, 1997). We searched for enzymatic activities implicated in stalled fork processing in *rad53* mutants experiencing replication pausing and found that the Exo1 exonuclease is recruited to stalled replication forks and, in *rad53* cells, promotes the formation of single-stranded DNA intermediates, thus counteracting fork reversal.

Exo1 is a DNA repair nuclease of the Rad2 gene family, originally identified as 5′-3′ exonuclease physically interacting with the mismatch repair (MMR) protein Msh2 (Dzantiev et al., 2004; Fiorentini et al., 1997; Tishkoff et al., 1997). Exo1 has also been implicated in mitotic and meiotic recombination (Fiorentini et al., 1997), double-strand break (DSB) processing (Lewis et al., 2002), Okazaki fragment processing (Tishkoff et al., 1997), and telomere processing, at least in certain genetic backgrounds (Jia et al., 2004; Maringele and Lydall, 2002).

Results and Discussion

Replication forks can be visualized by the 2D gel method (Friedman and Brewer, 1995) that has been also used to study abnormal transitions occurring at stalled forks in *rad53* mutants (Lopes et al., 2001). Wild-type (wt) cells released from G1 in the presence of HU accumulate (i) bubble structures, which result from bidirectional origin firing; (ii) large Y molecules, which arise from asymmetric progression of replication forks; and (iii) X-shaped sister chromatid junctions, which may represent hemicatenanes (Figure 1A) (Lopes et al., 2003). Replication intermediates peak at 60 min and then drop off as a result of fork and X spike movement. Conversely, in HU-treated *rad53-K227A (rad53)* cells bubbles, large Ys and X molecules degenerate into small Ys, possibly as a result of nucleolytic processing, and into a cone signal, likely representing reversed forks (Figure 1A) (Lopes et al., 2001). These abnormalities are thought to arise from the run off of the X-shaped junctions at collapsed forks (Lopes et al., 2003). Accordingly, with time, the defined X structures in *rad53* cells are converted into X molecules with a smaller mass migrating more diffusely within the cone signal, as measured by the gradual reduction of the X spike/cone ratio (Figures 1A and 1B).

By 2D gel we screened for mutations in repair and/or recombination genes able to alter the distribution of abnormal replication structures in a *rad53* background (data not shown) and found the gene encoding the Exo1 exonuclease (Fiorentini et al., 1997; Tishkoff et al., 1997). In *rad53exo1*Δ mutants, bubbles, large Ys and X molecules accumulate at 60 min and remain stable and constant throughout the treatment (Figure 1B). Since the X-shaped intermediates give rise to the cone signal in *rad53* mutants, we measured the X spike-cone ratio in both *rad53* and *rad53exo1* cells to evaluate the progressive degeneration of SCJs into reversed forks. As shown in Figure 1B, while in *rad53* mutants, the spike-cone ratio progressively diminishes, in *rad53exo1* cells, it remains constant. We compared fork and X spike movement in *rad53exo1*Δ and *rad53* cells. In both strains, forks invade the restriction fragment positioned 2.65 kb to the left of *ARS305* but fail to proceed further (Figure 1C, data not shown). However, differently from *rad53* cells, in *rad53exo1*Δ mutants, a defined X spike is visualized on the fragment proximal to *ARS305* (Figure 1C). Conversely, in HU-treated wt cells, forks can achieve residual DNA synthesis for at least 17 kb (Lopes et al., 2001) (data not shown). We failed to detect any difference in the level of replication intermediates and fork movement between HU-treated wt and *exo1*Δ cells (Figure 1A, data not shown). We conclude that the stability of the X-shaped junctions and fork processing in *rad53* mutants is influenced by Exo1. Since Exo1 interacts physically with Msh2 and genetically with Rad27 and Mre11 (Dzantiev et al., 2004; Fiorentini et al., 1997; Lewis et al., 2002; Tishkoff et al., 1997), we tested whether ablation of any of these genes would affect the 2D gel profile of HU-treated *rad53* cells. We failed to detect any difference in 2D gel profiles between *rad53msh2*Δ, *rad53rad27*Δ, *rad53mre11*Δ, and *rad53* mutants cells (data not shown). We therefore conclude that Exo1 specifically affects the stability of replication forks in *rad53* mutant cells.

A logical expectation from the previous result is that Exo1 physically interacts with stalled forks, at least in *rad53* cells. We addressed this issue by chromatin-IP (ChIP). ChIP analysis was carried out on the chromosomal region containing *ARS305* and on three other regions positioned 9 kb to the left and 8 and 17 kb to the right, respectively, of *ARS305* (Figure 1D) (Kamimura et al., 2001). We found that, at early time points, Exo1 specifically associates with the *ARS305* fragment both in HU-treated wt and *rad53* cells. At later times in wt, but not *rad53* cells, the *ARS305* adjacent fragments can be also amplified, perhaps due to residual fork movement. Hence, Exo1 associates with stalled forks both in wt and *rad53* cells; this observation may suggest that Exo1 directly interacts with the replisome (at least in wt cells), perhaps to mediate specialized replication and or replication-coupled repair steps (Dzantiev et al., 2004; Surtees and Alani, 2004). The persistence of Exo1 at the *ARS305* region in *rad53* cells also suggests that the rate of Exo1-mediated nucleolytic processing is very slow. The inability of *rad53* cells to restart fork progression following HU removal (Lopes et al., 2001) is not rescued by *EXO1* ablation, while *exo1*Δ mutants behave like wt cells during HU recovery (data not shown). We therefore tested whether in *rad53exo1* mutants stalled forks were still deprived of DNA polymerase α. We analyzed by ChIP the stability of DNA polymerase α-fork association in wt, *rad53*, and *rad53exo1*Δ cells. In wt cells, DNA

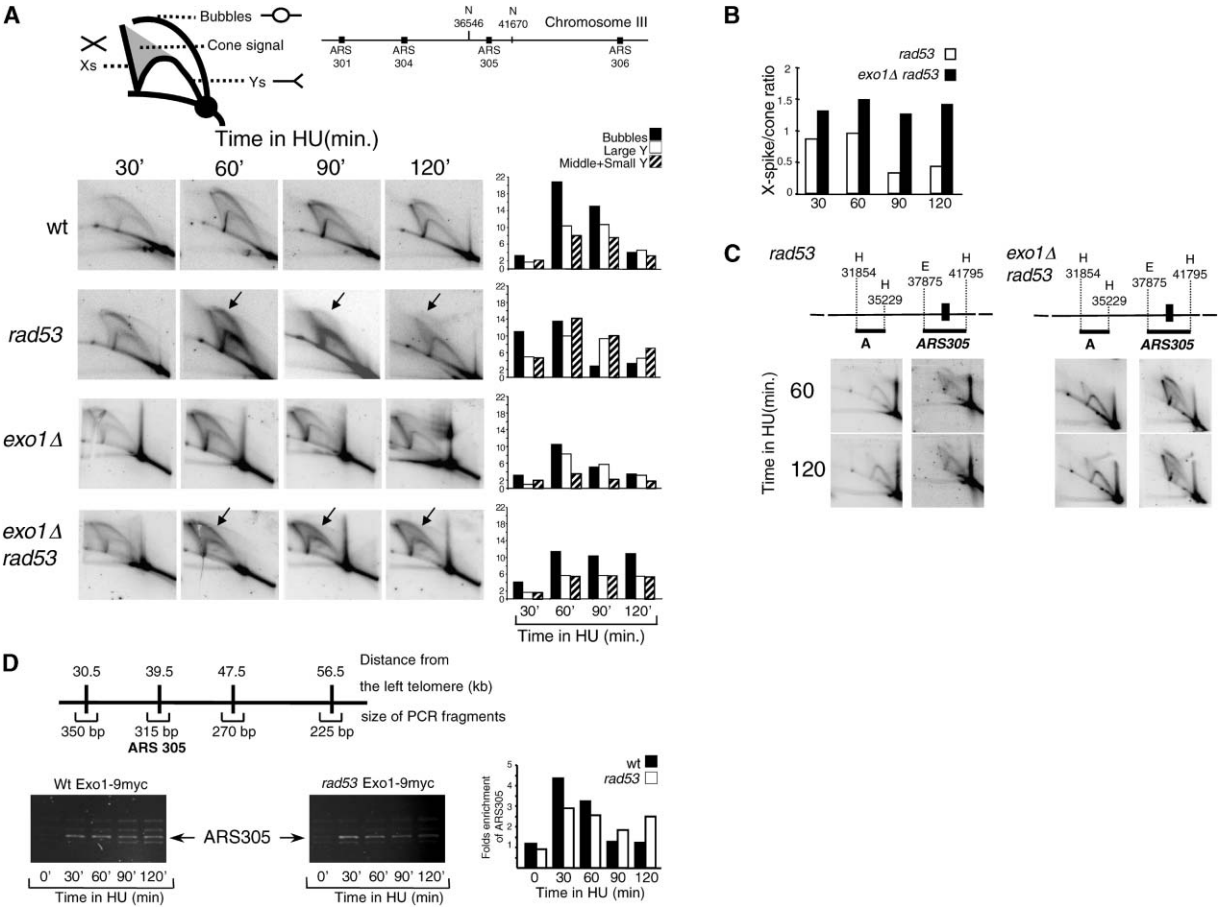


Figure 1. Exo1 Is Recruited to Stalled Forks and Processes Them in HU-Treated *rad53* Mutants

(A) Neutral/neutral 2D gels at *ARS305* origin in wild-type (W303-1A), *rad53* (CY2034), *exo1*Δ (CY5145), and *rad53exo1*Δ (CY5469) cells. Cells were grown in YPD medium, presynchronized by α factor (αF) treatment, and released from the G1 block in fresh medium containing 0.2 M HU. DNA was prepared from cells collected at the indicated times; 10 μg of total DNA were digested with NcoI, electrophoresed, and transferred to nylon membranes probed with a ^{32}P- labeled BamH1-NcoI 3.0 kb fragment, spanning the *ARS305* origin. Relative quantifications are also presented. A schematic representation of the replication intermediates discussed in the text is presented.

(B) Analysis of the X spike/cone signal ratio relative to the experiment described in (A) has been carried out by measuring the relative intensity of the X spike and the cone area as previously described (Lopes et al., 2001, 2003).

(C) Analysis of replication intermediates in the regions adjacent to *ARS305* origin in CY2034 and CY5469. The same DNA preparation analysed in (A) was used to monitor replication intermediates in the regions flanking *ARS305* following digestion with EcoRV-HindIII and hybridization with the relative probes.

(D) Exo1 is recruited at stalled forks. Samples from cells grown as in (A) were taken at the indicated time points and processed by ChIP. Quantifications were carried out using the NIH Image 1.62 software. Schematic representation of Chromosome III showing the PCR fragments used for ChIP analysis is also presented.

polymerase α associates with the *ARS305* restriction fragment at 40 min and remains associated throughout the treatment (Lucca et al., 2004) (Figure 2). Conversely, in *rad53* and *rad53exo1*Δ cells, the level of DNA polymerase α association with the *ARS305* fragment is comparable and reduced relative to wt cells (Figure 2). Hence, *EXO1* deletion in *rad53* cells is unable to rescue the inability to maintain DNA polymerase α stably associated with stalled forks, thus explaining the inability of *rad53exo1*Δ cells to resume replication following HU removal. We conclude that the replication fork restart defect in checkpoint mutants likely depends on the loose association between DNA polymerases and stalled forks, while the instability of the replication forks is influenced by the Exo1-mediated processing of replication intermediates. This is in accordance with the finding that in *rad53exo1*Δ mutants the entire population of replication intermediates remains stable and constant throughout the treatment but is unable to move further following HU removal.

We then analyzed the replication intermediates using psoralen crosslinking combined with EM (Sogo et al., 2002) in wt, *rad53*, *exo1*Δ, and *rad53exo1*Δ strains released from G1 in the presence of HU. In wt and *exo1*Δ cells, more than 96% of the intermediates are represented by normal bubbles containing short regions of ssDNA at the forks (Sogo et al., 2002) (data not shown). Conversely, in *rad53* cells, most of the intermediates are represented by hemireplicated structures and gapped molecules (Sogo et al., 2002) (Figures 3B–3D). Further, 9.2% of the forks are reversed (Figures 3D and 3F). *rad53exo1*Δ cells, compared to *rad53* mutants, exhibit

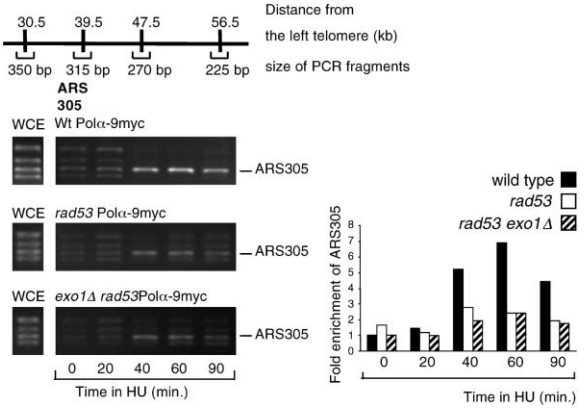
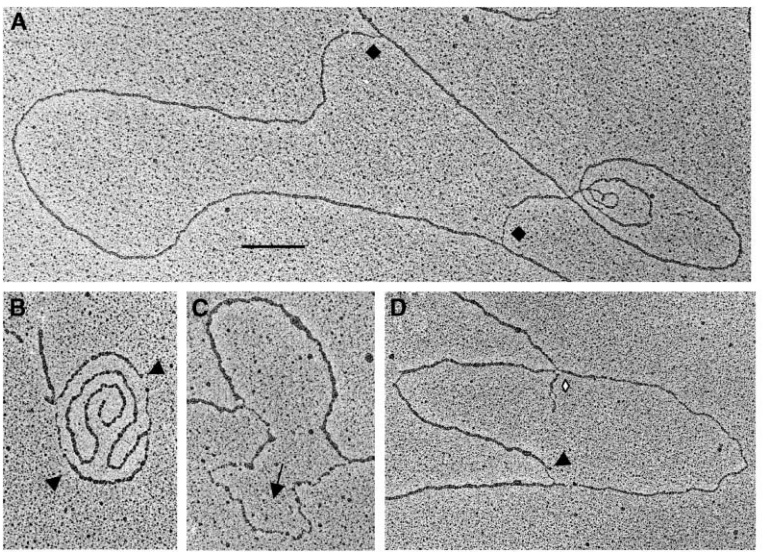


Figure 2. *rad53exo1*Δ Cells Exhibit a Loose DNA Polymerase α Fork Association

ChIP analysis of DNA polymerase α was carried out in wild-type, *rad53*, and *rad53exo1*Δ cells grown in YP 2% raffinose medium, presynchronized by α-factor (αF) treatment, and released into fresh medium containing 0.2 M HU. Quantifications are also presented.

a lower amount of gapped molecules (29.6% versus 44%; $p < 0.01$ [contingency $\chi^2 = 7.7$]) and, concomitantly, a higher level of normal bubbles (35.5% versus 19%; $p < 0.01$ [contingency $\chi^2 = 11.9$]) (Figure 3E) and reversed forks (30.2% versus 9.2%; $p < 0.01$ [contingency $\chi^2 = 24.8$]) (Figure 3F). Hence, both the 2D gels and the EM analysis support the conclusion that in checkpoint-deficient mutants Exo1 mediates the nucleolytic processing of stalled forks, probably by resecting newly synthesized strands deprived of DNA polymerases, thus leading to the formation of structures that, on 2D gels, migrate with a lower mass (small Ys) and by EM are visualized as gapped molecules. The EM data also indicate that Exo1 counteracts the accumulation of reversed forks. We note that reversed fork resection would cause accumulation of gapped molecules and single-stranded regressed arms (Figure 4A) (Sogo et al., 2002). Similarly, gapped molecules might result from resection of newly synthesized strands at stalled forks (Figure 4A). The simplest model to explain our data is that Exo1-mediated resection of newly synthesized filaments resolves the sister chromatid junctions, thus generating gapped intermediates (Figure 4A). Since the run-off of the sister chromatid junctions at stalled forks mediates the formation of reversed forks, Exo1 could prevent reversed fork formation by limiting the amount of these joint molecules. Hence, *rad53exo1*Δ mutants, compared to *rad53* cells, would concomitantly accumulate X molecules and reversed forks and exhibit a reduced level of gapped intermediates (Figure 4A). According to this hypothesis, the X spike/cone ratio remains constant in *rad53exo1*Δ cells. We cannot exclude the possibility that Exo1 is at the same time implicated in the resection of reversed forks (Figure 4A) as the regressed arm of the reversed fork mimics a double-stranded end that represents a substrate of Exo1 (Fiorentini et al., 1997). In any case, the model we propose implicates that reversed forks and a fraction of gapped molecules do not arise independently from each other but, rather, represent two steps of the same processing pathway mediated by the Exo1 nuclease. We note that, so far, we have been un-



E

	Normal molecules	Gapped molecules	Hemi-replicated molecules
rad53 HU 2h (184)	19%	44%	37%
*exo1*Δ *rad53* HU 2h (195)	35.5%	29.6%	34.9%

F

	Reversed forks
rad53 HU 2h (184)	9.2%
*exo1*Δ *rad53* HU 2h (195)	30.2%

Figure 3. Analysis of Replication Intermediates in *rad53* and *rad53exo1*Δ Mutants

Replication intermediates were isolated from in vivo psoralen crosslinked chromatin prepared from wild-type, *rad53*, and *rad53exo1*Δ cells released from a G1 block into YPD medium containing 0.2 M HU. Electron micrographs of normal replicating bubbles from wt cells (A), gapped molecules from *rad53* cells (B), hemireplicated bubbles from *rad53* cells (C), and gapped and reversed forks from *rad53* cells (D). The percentages of the different replication intermediates in *rad53* and *rad53exo1*Δ strains are shown in (E) and (F). The same set of molecules (in brackets) was analyzed to measure the percentages of normal, gapped, and hemireplicated forks (E) or reversed forks (F). Diamonds represent short gaps (ssDNA). Arrowheads represent the transition from double-stranded DNA to single-stranded DNA, arrows indicate the single-stranded arms of hemireplicate bubbles, and the white rhombus indicates the regressed arm of the reversed fork. The black bar in (A) represents 0.5 kb.

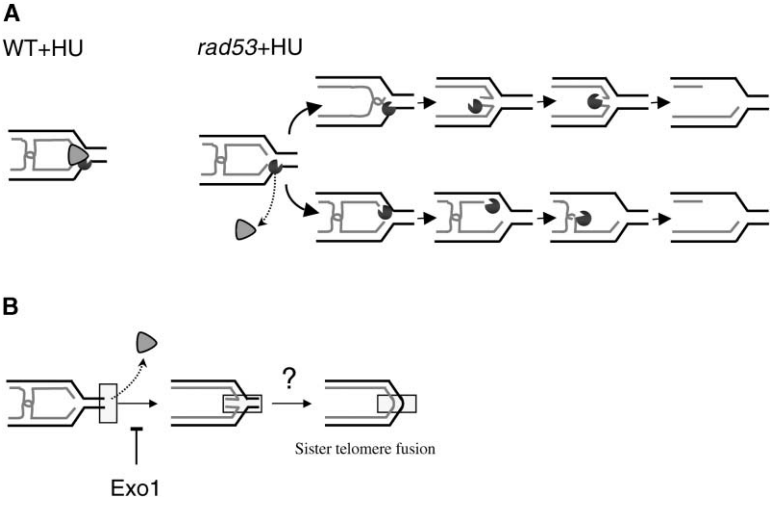


Figure 4. A Model for Exo1-Mediated Processing of Stalled Forks

(A) HU-treated wild-type cells accumulate a stable replisome and Exo1 exonuclease at stalled forks. The replisome is indicated by the grey triangle and Exo1 by the dark semicircular shape. The presence of the replisome and the functional checkpoint prevent Exo1 from resecting newly synthesized chains, at least at canonical stalled forks. In HU-treated *rad53* cells, the replisome dissociates from stalled forks and Exo1 resects newly synthesized strands and, possibly, reversed forks. Resection of newly synthesized chains resolves the structure of the sister chromatid junctions (resembling hemicatenanes) that promote fork reversal at collapsed forks (for further details, see Lopes et al. 2003).

(B) Replication forks collapse when reaching a double-strand break on the template or a telomere (both represented by the grey rectangle); the sister chromatid junctions run off, engaging the ends of the newly synthesized strands into pairing and generating reversed forks. Reversed fork formation at a broken chromosome (or at a telomere) could then allow the formation of sister chromatid fusions. Exo1, by resecting newly synthesized chains, counteracts fork reversal and sister chromatid fusions at forks collapsing at DSBs or telomeres.

able to visualize by EM intermediates with single-strand junctions on newly synthesized strands. Since these structures are particularly labile (Lopes et al., 2003), we cannot exclude a specific loss of such intermediates during the enrichment procedure, possibly due to the formation of nicks (that would cause catenate resolution) and/or reduced affinity for BND cellulose (Benard et al., 2001; Linskens and Huberman, 1988; Lucchini and Sogo, 1994).

*rad53exo1*Δ mutants still exhibit a significant fraction of gapped and hemireplicated molecules. Since gapped and hemireplicated structures likely result from two superimposed defects, a lagging strand synthesis defect, and unscheduled nucleolytic events (Lopes et al., 2001; Sogo et al., 2002), likely *EXO1* deletion rescues the nucleolytic processing but not the lagging strand defect of *rad53* cells. This is in accordance with the finding that *rad53exo1*Δ cells fail to restore a stable DNA polymerase α fork association. Alternatively, fork resection in *rad53exo1*Δ cells might be mediated by other unknown exonucleases. We note, however, that neither Mre11 nor Rad2 nucleases affect the 2D gel profile of HU-treated wt and *rad53* cells (data not shown). We failed to detect accumulation of reversed forks in HU-treated *exo1*Δ mutants. This is in accordance with previous findings indicating that fork reversal, if occurring, represents a rare event in wt cells or, rather, a pathological situation (Lopes et al., 2001, 2003; Sogo et al., 2002).

The role of Exo1 in counteracting fork reversal and in generating single-stranded intermediates at stalled forks presents an intriguing similarity with the role proposed for the RecJ nuclease in *E. coli* cells experiencing DNA damage induced replication blocks (Courcelle et al., 2003). However, it is unlikely that the Exo1-mediated resection of reversed forks plays any physiological role in promoting fork restart as there is no evidence that fork reversal indeed occurs in wt and *exo1*, at least at canonical replication forks. Moreover, it should be noted that replication resumption at collapsed forks may not be crucial in eukaryotes, as the presence of multiple origins would allow a fork from an adjacent origin to converge on a damaged fork to complete replication (McGlynn and Lloyd, 2002).

A key issue is why Exo1 associates with forks even in wt cells. The most likely possibility is that, in the presence of a stable replisome-fork association, Exo1 mediates certain steps of lagging strand synthesis or mismatch repair (Surtees and Alani, 2004). This is in accordance with the findings that *EXO1* deletion causes synthetic growth defects with mutations in the *RAD27* (Tishkoff et al., 1997) or in the *PRI1* (data not shown) genes implicated in lagging strand synthesis, and with the observation that Exo1 and PCNA functionally and physically interact (Dzantiev et al., 2004). Another possibility is that Exo1 prevents abnormal transitions, specifically at those forks collapsing at double-stranded breaks (DSBs) or at telomeres where replisomes dissociate, thus allowing the sister chromatid junctions to promote fork reversal at newly synthesized ends and, possibly, sister chromatid fusions (Figure 4B). Given that in wt cells grown under physiological conditions DSBs are very rare (Fabre et al., 2002) and telomere ends are efficiently capped, it could be difficult to visualize these transitions. However, this might be a relevant problem in certain genetic backgrounds and, particularly, in checkpoint mutants. Interestingly, in *S. pombe* cells lacking a functional checkpoint, a fraction of sister telomeres fuse together (Naito et al., 1998), and it would be relevant to address whether this process is influenced by Exo1. In *cdc13* mutants that leave telomeres unprotected and are thought to be defective in DNA polymerase α loading at telomeres (Qi and Zakian, 2000) (thus mimicking the situation at stalled forks in *rad53* mutants), Exo1 has been implicated in the processing of telomeres (Maringele and Lydall, 2002). Further, it has recently been suggested that Exo1 is targeted by the Mec1-Rad9-Rad53 checkpoint pathway at telomeres (Jia et al., 2004).

In *rad53* cells, Exo1 activity is expected to affect the coordination between replication and recombination and, in general, genome stability by preventing the accumulation of reversed forks and therefore by counter-

acting the unscheduled recruitment of HJ-resolution activities at the forks (McGlynn and Lloyd, 2002). On the other hand, Exo1 activity, by contributing to the formation of single-stranded DNA at stalled forks, could generate a variety of recombination substrates, particularly when forks collapse at double-strand breaks or at chromosome ends. Since the *rad53* cellular context has an interesting analogy with cancer cells that are often characterized by checkpoint defects and abnormal recombination events, we suspect that the mammalian Exo1 could influence the molecular events leading to genome instability, particularly in those cells that have lost a functional checkpoint.

Altogether, our findings have contributed to unmask a role for the Exo1 nuclease in the processing of replication forks in the absence of a functional checkpoint response.

Experimental Procedures

Yeast Strains

The yeast strains used in this study are isogenic to W303-1A (wt) (Thomas and Rothstein, 1989), CY2034 (*rad53-K227A*), CY5145 (*exo1*Δ), CY5469 (*rad53-K227A exo*1Δ), CY5699 (*EXO1-9MYC*), CY5700 (*rad53-K227A EXO1-9MYC*), CY3211 (*POL1-9MYC*), CY5242 (*rad53-K227A POL1-9MYC*), and CY5746 (*rad53-K227A exo1*Δ *POL1-9MYC*).

Protein Extraction, Western Blotting, and Cell Cycle Analysis

The procedures for protein extraction, Western blotting, and FACS analysis were already described (Pellicioli et al., 1999). Anti-Rad53 antibodies were kindly provided by J. Diffley.

2D Gel, EM, and ChIP Analysis

DNA extraction with the CTAB method and neutral-neutral two-dimensional gel electrophoresis were performed as described (Lopes et al., 2003). Replication intermediates were quantified as described (Lopes et al., 2001). ChIP and EM analysis were performed as described (Lucca et al., 2004; Sogo et al., 2002).

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