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Report

The Cullin Rtt101p Promotes Replication Fork Progression through Damaged DNA and Natural Pause Sites

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Summary

Accurate and complete DNA replication is fundamental to maintain genome integrity. While the mechanisms and underlying machinery required to duplicate bulk genomic DNA are beginning to emerge, little is known about how cells replicate through damaged areas and special chromosomal regions such as telomeres, centromeres, and highly transcribed loci [1]. Here, we have investigated the role of the yeast cullin Rtt101p in this process. We show that *rtt101Δ* cells accumulate spontaneous DNA damage and exhibit a G_2/M delay, even though they are fully proficient to detect and repair chromosome breaks. Viability of *rtt101Δ* mutants depends on Rrm3p, a DNA helicase involved in displacing proteinaceous complexes at programmed pause sites [2]. Moreover, *rtt101Δ* cells show hyperrecombination at forks arrested at replication fork barriers (RFBs) of ribosomal DNA. Finally, *rtt101Δ* mutants are sensitive to fork arrest induced by DNA alkylation, but not by nucleotide depletion. We therefore propose that the cullin Rtt101p promotes fork progression through obstacles such as DNA lesions or tightly bound protein-DNA complexes via a new mechanism involving ubiquitin-conjugation.

Results and Discussion

Cullin-based E3-ligases control many cell-cycle transitions and checkpoint pathways through the regulated ubiquitinylation of specific substrates [3]. The budding yeast genome encodes for three cullin proteins: Cul1p/Cdc53p, which is part of the SCF complex [4], and Cul3p and Rtt101p/Cul8p, whose function is currently unknown. Cells deleted for *RTT101* are viable but display an increased rate of *Ty1* elements transposition and are hypersensitive to genotoxic agents [5–7]. They also show a delay in anaphase progression, which is suppressed by deleting the intra-S-phase checkpoint gene *RAD9* [6]. To further characterize this mitotic delay, we have monitored individual wild-type and *rtt101Δ* cells expressing tubulin-GFP by time-lapse microscopy. This analysis revealed that *rtt101Δ* cells accumulate with a short spindle and the nucleus positioned at the bud neck, and the onset of anaphase is delayed for up to 2 hr (Figures 1A and 1B and Supplemental Movies S1 and S2 available with this article online). In all cases, the subsequent events, including cytokinesis, cell separation, and exit from mitosis, occurred with normal timing.

To determine when in the cell cycle Rtt101p was required, we expressed fully functional HA-Rtt101p in *rtt101Δ* cells from the regulatable *GAL1,10*-promoter (Figure 1C). Cells were grown in galactose and arrested in G_1 with α-factor. The culture was divided and glucose was added to one half to inhibit the expression of HA-Rtt101p. After 3 hr of depletion, HA-Rtt101p was no longer detectable by immunoblotting, indicating that it is a rather unstable protein. The two cultures were released by washing out α-factor, and cell-cycle progression was followed by immunoblotting of marker proteins. Cells lacking Rtt101p accumulated at the metaphase-anaphase transition, as indicated by the delayed degradation of Pds1p-myc and Clb2p. These results suggest that Rtt101p activity is necessary during every S or G_2 phase of the cell cycle and that lack of Rtt101p results in a specific delay prior to the onset of anaphase. Moreover, because no Clb2p or Pds1p-myc accumulated in the G_1-arrested cells after depletion of Rtt101p, we conclude that Rtt101p is not required to maintain APC-Cdh1 activity.

Degradation of Pds1p by the APC is inhibited by either the Mec1p-dependent checkpoint in case of damaged and/or unreplicated DNA or by the Mad2p-dependent mitotic checkpoint in case of spindle defects or unattached chromosomes [8]. To determine whether the mitotic delay of *rtt101Δ* cells was caused by activation of one of these checkpoints, we measured the mitotic index of wild-type, *rtt101Δ*, *rtt101Δ mec1-1*, and *rtt101Δ mad2Δ* cells (Figure 1D). Consistent with the mitotic delay, 23% of *rtt101Δ* cells accumulated with their DNA at the mother/bud neck, compared to 8% in wild-type cells. Importantly, this G_2/M delay was suppressed to wild-type levels by inactivating Mec1p, while deleting *MAD2* had no effect. This suggests that the G_2/M delay results from DNA damage occurring in S phase.

To test this possibility, we scored cells for the formation of Ddc1p-GFP repair foci [9]. We found that 15% of *rtt101Δ* cells formed Ddc1p-GFP foci in unchallenged growth conditions (Figure 1E), which compares well to the 9% observed in the *sic1Δ* mutant, another strain exhibiting spontaneous S phase defects [10]. Rtt101p-deficient cells also showed increased Rad52p-GFP foci (D. Alvaro and R. Rothstein, personal communication), further supporting the view that DNA damage accumulates

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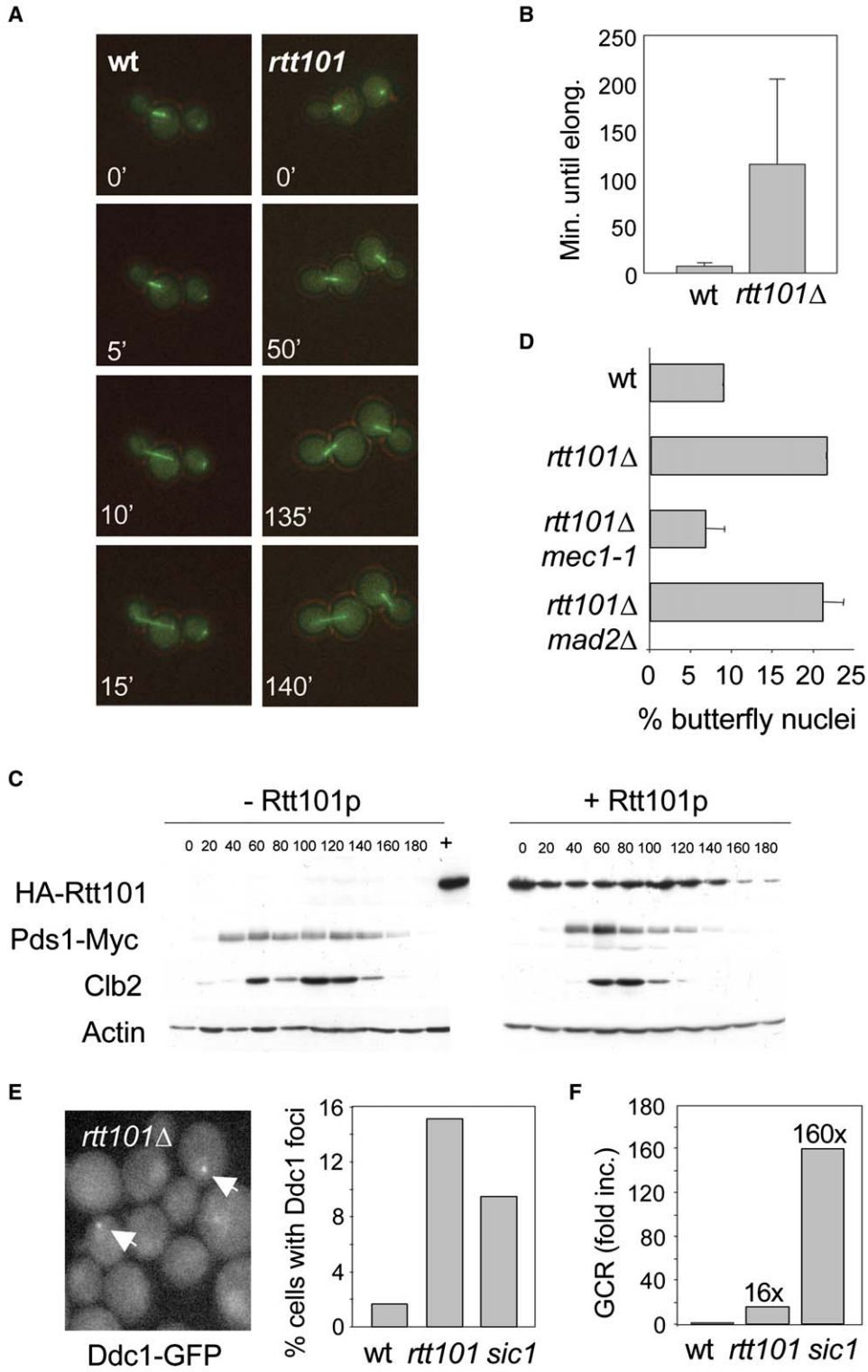

Figure 1. *rtt101⊿* Cells Exhibit a Mec1p-Dependent G2/M Delay

(A) The onset of anaphase was determined by live-cell fluorescence imaging in wild-type (BY4741, left) and *rtt101*Δ (Y01376, right) cells expressing tubulin-GFP (Movies S1 and S2).

(B) The average time between positioning of the preanaphase spindle at the bud neck and onset of spindle elongation was approximately 100 min in *rtt101*Δ cells (n = 5) and 7 min in wild-type cells (n = 4), although the cell-to-cell variation was quite significant.

(C) Y01376 cells harboring a galactose-inducible construct to express HA-Rtt101p were synchronized in G_1 with α factor (time 0) and released into media containing either galactose (+Rtt101p) or glucose (−Rtt101p) to shut off the expression of HA-Rtt101p. Cell-cycle progression was monitored at the times indicated (in minutes) by immunoblotting for Pds1p-myc and Clb2p, while actin served as a loading control.

(D) The accumulation of exponentially growing wild-type (DCY1723), *rtt101*Δ (yTK9), *rtt101*Δ *mec1-1* (yTK21), and *rtt101*Δ *mad2*Δ (yTK11) cells in mitosis was monitored by scoring the number of cells with a single nucleus at the bud-neck visualized by DAPI staining (butterfly nuclei).

spontaneously in these cells. To assess the incidence of these defects on genome integrity, we monitored the rate of spontaneous gross chromosomal rearrangements (GCR) in *rtt101Δ* and *sic1Δ* cells by using a genetic assay developed by the Kolodner lab [11]. We measured a 16-fold increase of the GCR frequency in *rtt101Δ* cells and a 160-fold increase in *sic1Δ* mutants (Figure 1F). This difference is likely to reflect the fact that *rtt101Δ* cells arrest in G_2/M in response to spontaneous DNA damage while *sic1Δ* cells progress through anaphase with partially replicated chromosomes [10].

As shown in Figure 2A, *rtt101Δ* mutants are hypersensitive to the DNA alkylating agent methylmethane sulfonate (MMS) and the topoisomerase I inhibitor camptothecin (CPT), but they are only moderately sensitive to the nucleotide-depleting agent hydroxyurea (HU). MMS and CPT cause various types of lesions that impede fork progression and may lead to the formation of double-strand breaks (DSBs) [12, 13]. In contrast, HU generates DSBs only after extended exposure. Nonhomologous end joining (NHEJ) and homologous recombination (HR) are the two major pathways involved in the repair of DSBs in eukaryotes [14]. To test whether Rtt101p was involved in one of these pathways, we analyzed repair of a site-specific DSB in strains where NHEJ and HR could be assessed individually. As shown in Figure 2B, we expressed the HO endonuclease in *rtt101Δ* cells in which the *HML* and *HMR* sequences were deleted to eliminate the possibility of repair by HR [15]. Interestingly, we found no difference in survival between wild-type and *rtt101Δ* cells, whereas viability of the NHEJ mutant *yku70Δ* was severely compromised. To assess HR efficiency, we used a similar strain, which contained a noncleavable copy of the *MAT* locus (*MAT-inc*) on chromosome V [16]. In HR-competent cells, the cleaved *MAT* locus recombines with *MATinc*, thereby rendering the cells HO resistant. As shown in Figure 2C, *rtt101Δ* cells are fully HR competent and grow like wild-type cells when HO is constitutively induced. In contrast, *rad52Δ* cells were unable to form colonies under the same conditions. Taken together, these results demonstrate that Rtt101p is not required for DSB repair in vivo.

Cells respond to replication stress by repressing late replication origins in a Rad53p-dependent manner [17, 18]. To test whether Rtt101p is involved in the activation of this checkpoint response, cells were released from G_1 in a medium containing either HU or MMS, and the phosphorylation state of Rad53p, indicative of its activation, was monitored by immunoblotting [19]. Cells lacking Rtt101p were able to efficiently signal to Rad53p in response to both HU and MMS (Figure 2D). Importantly, *rtt101Δ* cells were also able to prevent fork collapse and to repress late origins in response to HU, which is not the case for *rad53* mutants (Figure 2E). These data confirm that the intra-S phase checkpoint can be activated and executed independently of Rtt101p and that the genomic instability and sensitivity to genotoxic agents is not due to a failure in checkpoint signaling.

Natural replication pausing sites occur at ~1400 nonnucleosomal proteinaceous areas throughout the genome [2]. The pausing is enhanced in *rrm3Δ* cells, which often leads to unstable replication intermediates and DSBs. It has therefore been proposed that Rrm3p prevents fork collapse by removing proteins at pause sites. As a result, *rrm3Δ* is synthetic lethal with mutations affecting either checkpoint responses, DSB repair, or fork stability [20, 21]. Interestingly, *rrm3Δ* is also synthetic lethal with *rtt101Δ* (Figure S1A; [21]). Since *rtt101Δ* cells are proficient for both checkpoint response and DSB repair, we next tested whether Rtt101p is acting together with Rrm3p to promote fork progression through nonnucleosomal proteinaceous regions of the genome. We monitored fork pausing by 2D gel electrophoresis at two well-characterized programmed pause sites, the centromeric region *CEN4*, and the replication fork barrier (RFB) of the rDNA (Figures 3A and 3B). As described previously [2], we observed an accumulation of arrested and converging forks in *rrm3Δ* cells (Figure 3A). However, we found no significant difference in the amount of pausing between wild-type and *rtt101Δ* cells at either *CEN4* or RFBs. We therefore conclude that the function of Rrm3p at natural pause sites is not shared by Rtt101p.

We next monitored the integrity of forks arrested at natural pause sites in the absence of Rtt101p. The consequences of destabilized forks can be measured at the rDNA locus through the formation of extrachromosomal rDNA circles (ERCs). Interestingly, we found that *rtt101Δ* cells exhibit a 3-fold increase of ERC levels (Figures 3C and 3D). Inactivating the RFB by deleting *FOB1* in the *rtt101Δ* background significantly decreased ERC accumulation [22], even though residual levels were detected, which is reminiscent of *rrm3Δ* mutants [23].

The RecQ-like helicase Sgs1p suppresses toxic recombination intermediates at stalled forks [24] and is required for survival in an *rrm3Δ* background [20]. Interestingly, the synthetic lethality between *sgs1Δ* and *rrm3Δ* can be relieved by further inactivating the HR pathway [20]. To test whether the role of Rtt101p at stalled forks is also to suppress HR, we deleted *RAD51*, *RAD55*, or *RAD52* in *rtt101Δ rrm3Δ* cells. However, this was not sufficient to rescue the lethality *rtt101Δ rrm3Δ* cells (Figure S1B), indicating that hyperrecombination is not responsible for the loss of viability of these cells.

The alkylating agent MMS is known to cause damage specifically in S phase by blocking the progression of replication forks [13]. In wild-type cells, MMS induces a 5- to 10-fold reduction of fork speed in a checkpoint-independent manner [25]. If Rtt101p is indeed required to promote fork progression through replication-impeding obstacles, we predicted that deletion of *RTT101* would exacerbate the effect of MMS and would therefore prevent completion of DNA replication, in a manner comparable to *rrm3Δ rtt101Δ* double mutants. To test this possibility, wild-type and *rtt101Δ* cells were

(E) The accumulation of Ddc1p-GFP foci (arrows) was determined in exponentially growing wild-type (E1528), *rtt101Δ* (yBL251), and *sic1Δ* (E1565) cells, used here as positive control. At least 500 cells were analyzed for each strain, by projecting stacks of 7 z-sections.
(F) The frequency of gross chromosomal arrangements (GCR) was determined in wild-type (E1557), *rtt101Δ* (YBL250), and *sic1Δ* (E1601) cells harboring *CAN1* and *URA3* marker on a nonessential region of the right arm of chromosome V [32]. Survival on selective media is scored when both markers are lost due to chromosomal breakage or rearrangement.

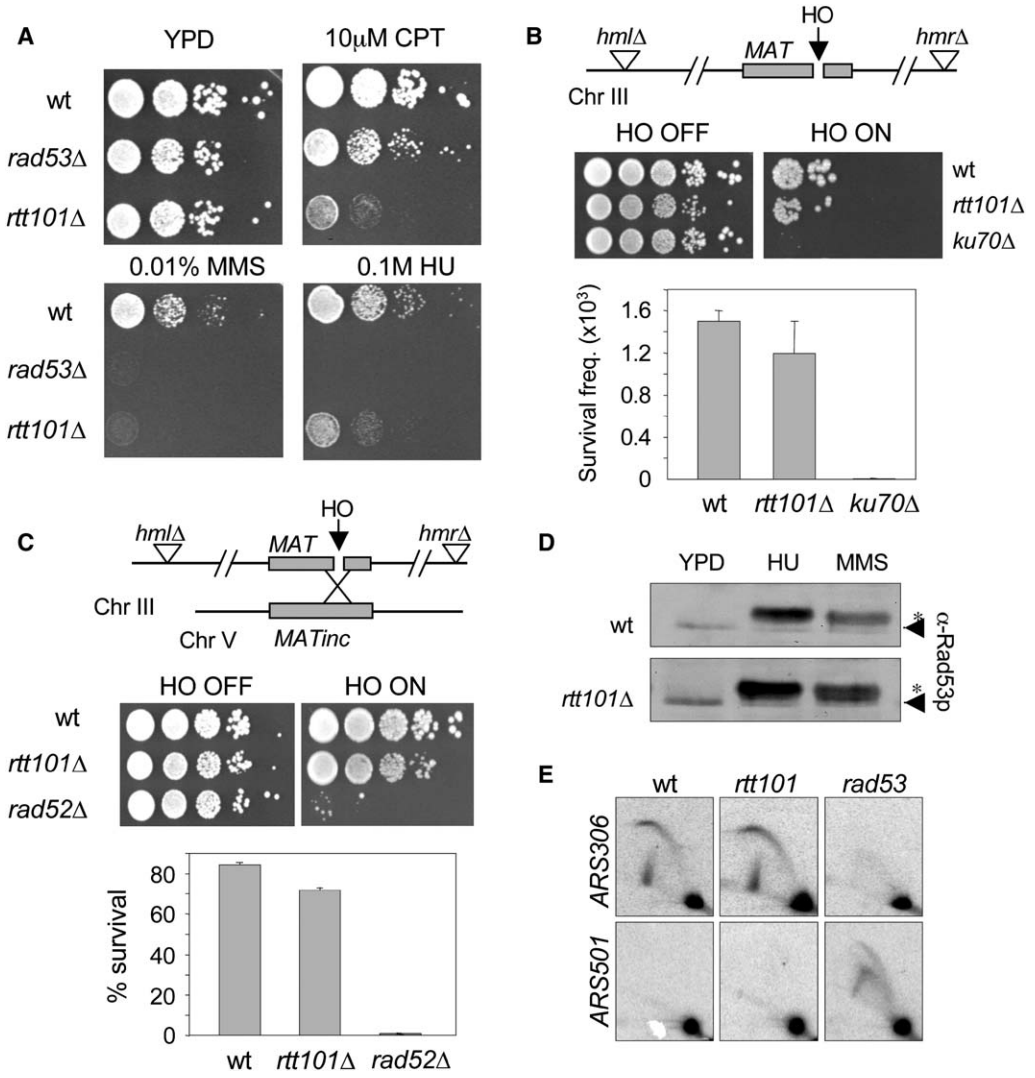

Figure 2. *rtt101⊿* Cells Are Sensitive to DNA Damage but Are Competent to Repair Chromosome Breaks and to Activate the Replication Checkpoint

(A) 10-fold serial dilutions of wild-type and the indicated deletion strains were spotted on YPD plates containing either no drug or the indicated concentrations of the genotoxic agents MMS, CPT, and HU. The plates were photographed after 3 days at 30ºC.

(B) A schematic representation of the strain JKM179 where both *HML and HMR* have been deleted. Wild-type (JKM179), *rtt101⊿* (yBL545), and control *ku70⊿* (JKM181) cells, which all express the HO-endonuclease from the inducible *GAL1,10*-promoter, were plated on media containing galactose (HO ON) or glucose (HO OFF), and the ratio of the number of colonies grown under the two conditions was determined by serial dilution spottings and quantitatively by cell counting.

(C) Schematic representation of the strain GA2321, in which both *HML* and *HMR* have been deleted and a noncleavable copy of the *MAT* locus has been integrated on chromosome V. Wild-type (GA2321), *rtt101⊿* (*MATinc*), and for control *rad52⊿* (GA2368) cells, which all express the HO-endonuclease from the inducible *GAL1,10* promoter, were plated on media containing galactose (HO ON) or glucose (HO OFF), and the ratio of the number of colonies grown under the two conditions was determined.

(D) Wild-type (BY4741) and *rtt101⊿* (Y01376) cells were arrested in G_1 with α-factor and released in rich medium (YPD) or media containing as indicated 0.2 M HU or 0.02% MMS. Protein extracts were prepared after 30 min, and the phosphorylation state of Rad53p was determined by immunoblotting with Rad53p-specific antibodies. Asterisks indicate hyperphosphorylated Rad53p.

(E) 2D gel analysis of the early *ARS306* and the late *ARS501* replication origins in wild-type (BY4741), *rtt101⊿* (Y01376), and *rad53-11* (PP022) cells released into S phase in the presence of 200 mM HU.

released from G_1 in medium containing MMS. After 1 hr, MMS was quenched and cells were released in drug-free medium. Completion of chromosome replication was analyzed by pulse field gel electrophoresis (PFGE) 50, 90, and 120 min after release, as described previously [26]. Whereas wild-type cells were able to complete replication after MMS removal, *rtt101⊿* cells were severely delayed (Figure 3E), consistent with sustained phosphorylation of Rad53p (Figure 3F). This defect is reminiscent of *rtt107*Δ cells, which were previously shown to be defective for recovery from the checkpoint arrest [27, 28]. Strikingly, PFGE analysis of wild-type and *rtt101⊿* cells after block of the replication fork by HU instead of MMS demonstrated that DNA replication resumed with similar kinetics (Figures 3G and 3H). These data are consistent with the fact that *rtt101⊿* cells are

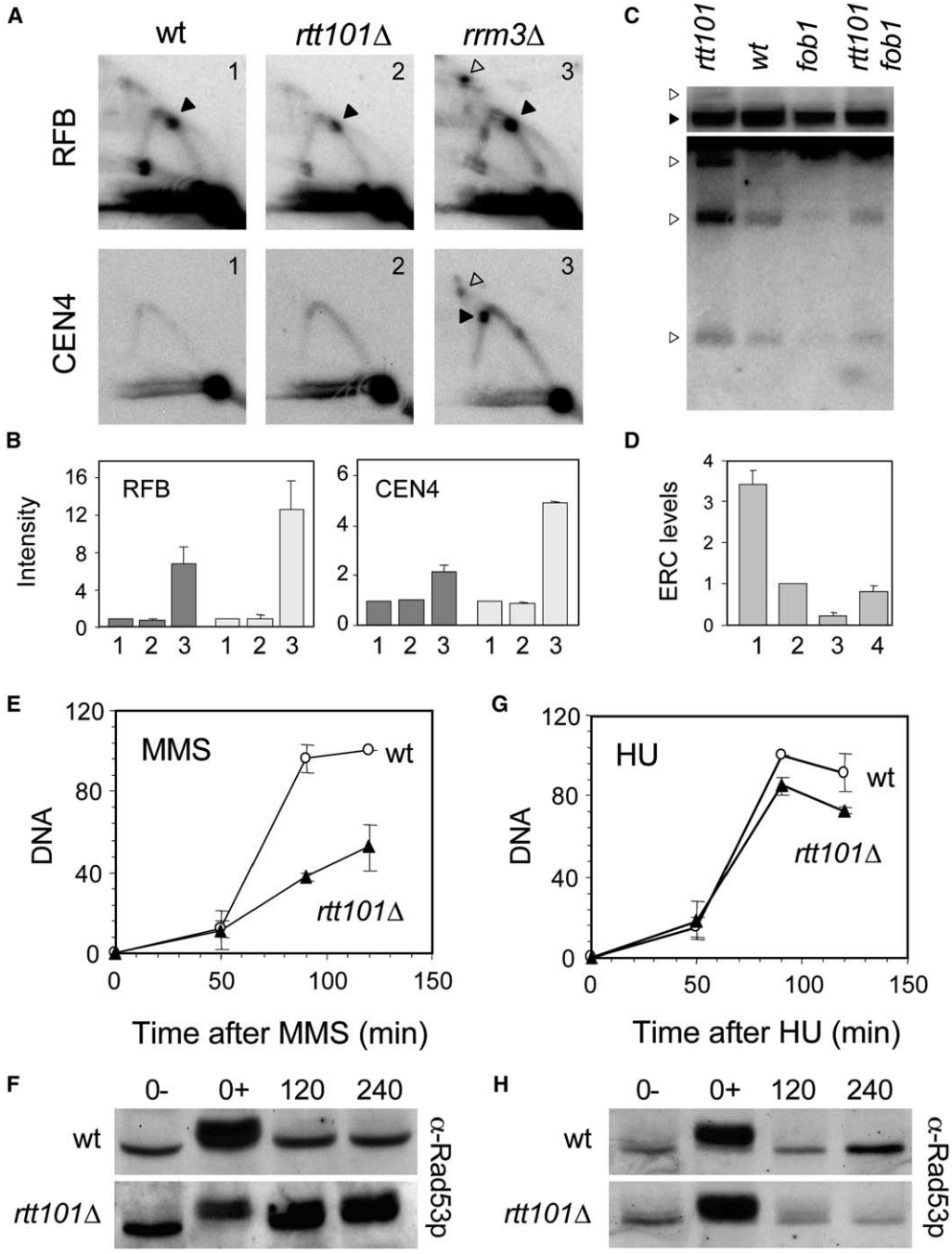

Figure 3. Rtt101p Prevents Fork Collapse at Natural Pause Sites and Promotes Recovery from MMS

(A) Replication fork pausing at *CEN4* and at the rDNA replication fork barrier (*RFB*) was monitored by 2D gel analysis. The DNA of wild-type (BY4741), *rrm3Δ* (Y00994), and *rtt101Δ* (Y01376) cells synchronized in S phase was digested with *Xba*I, separated on 2D gels, and probed with specific probes for *RFB* (top) and *CEN4* (bottom). Extended fork pausing (solid arrowheads) and converging forks (empty arrowheads) were detected in *rrm3Δ* mutants, but not in wild-type or *rtt101Δ* cells.

(B) Relative amount of arrested (dark gray) and converging (light gray) forks at the rDNA *RFB* and *CEN4* in three independent experiments. 1, wild-type; 2, *rtt101Δ*; 3, *rrm3Δ*.

(C) Extrachromosomal rDNA circles (ERCs) in exponentially growing wild-type (BY4741), *rtt101Δ* (Y01376), *fob1Δ* (Y04044), and *rtt101Δ fob1Δ* (yBL415) cells (empty arrowheads) were detected by Southern blot with an rDNA probe. A shorter exposure of the rDNA array (filled arrowhead) is shown.

(D) ERC levels in three independent experiments were normalized to the amount of chromosomal rDNA and expressed relative to the amount of ERCs in wild-type cells. 1, *rtt101Δ*; 2, wild-type; 3, *fob1Δ*; 4, *rtt101Δ fob1Δ*.

(E) Wild-type (BY4741) and *rtt101Δ* (Y01376) cells were arrested in G_1 with α-factor (0^-) and released as indicated for 1 hr (0^+) into medium containing 0.033% MMS. The drug was washed out and completion of DNA replication was monitored by PFGE analysis. The average intensity and standard deviation calculated for five representative chromosomes is shown.

(F) The phosphorylation state of Rad53p was determined by immunoblotting at the indicated time points (in minutes).

(G and H) The experiment described above was repeated with 0.2 M HU instead of MMS.

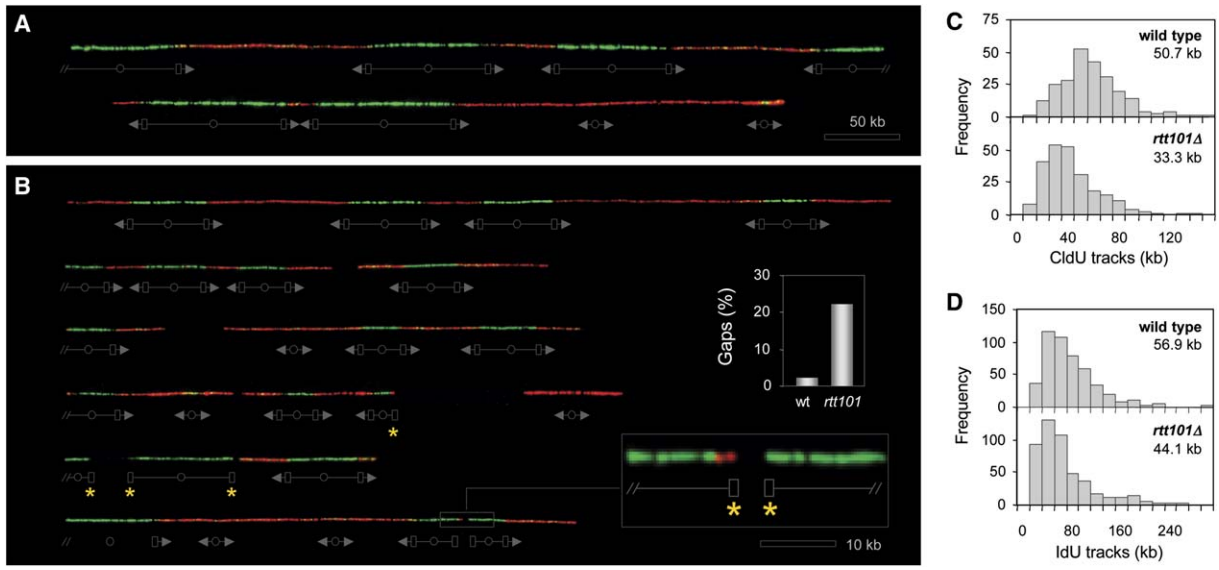

Figure 4. Rtt101p Promotes Fork Progression through Alkylated DNA Templates

(A and B) Wild-type (PP108) and *rtt101Δ* (yBL317) cells were arrested in G_1 with α-factor and released for 60 min into S phase in the presence of 0.033% MMS. Early-firing origins were labeled with CldU (green), and fork recovery after release from MMS was detected after IdU incorporation (red). Chromosomes were stretched by DNA combing and representative DNA fibers are shown. Open boxes indicate the position of individual forks (short IdU track, or no IdU at all). The inset shows a 5× enlargement of a segment of fiber #6. The inset histogram depicts the frequency of fibers greater than 150 kb with unreplicated gaps.

(C and D) Size distribution of CldU and IdU tracks in wild-type and *rtt101Δ* cells. Median values are indicated. The Wilcoxon rank sum test indicates that distributions are different for both CldU and IdU tracks ($p < 10^{-5}$) and the populations are different for all reasonable confidence intervals.

less sensitive to HU than to MMS (Figure 2A) and suggest that fork arrest induced by DNA lesion, but not fork pausing per se, is toxic in the absence of Rtt101p.

To check whether the slow recovery of *rtt101Δ* cells from MMS is due to replication fork defects, we monitored replication of individual chromosomes by DNA combing [29]. To this aim, G_1 cells were released synchronously into MMS-containing medium in the presence of chlorodeoxyuridine (CldU). After 90 min, MMS was washed out and cells were incubated for another 90 min in fresh medium containing iododeoxyuridine (IdU). After DNA combing, specific antibodies were used to discriminate between DNA synthesis occurring in the presence of MMS (CldU, green) or after release (IdU, red; Figures 4A and 4B). As shown in Figure 4C, CldU tracks were 50% shorter in *rtt101Δ* compared to wild-type cells, supporting the view that Rtt101p promotes fork progression through alkylated DNA. After 90 min release from MMS, we observed that more than 90% of the forks resumed DNA replication in the absence of Rtt101p (Figures 4A and 4B). Yet unreplicated gaps were detected in 22% of the fibers in *rtt101Δ* mutants, compared to only 2% in wild-type cells (Figure 4B). These unreplicated gaps after release from MMS could reflect a dependence on late origins, which are repressed in *rtt101Δ* cells due to the persistent activation of Rad53p. However, recent studies indicate that late origins are dispensable for viability [30] and that their regulation in the presence of MMS is not an essential function of the replication checkpoint [25]. It is possible that the repair of alkylated DNA is not rapid enough to avoid further collisions with restarting replication forks during the IdU chase, which may cause premature fork arrest/collapse in *rtt101Δ* cells. However, *rtt101Δ* cells show no defects in DSB repair, and in contrast to repair proteins, Rtt101p is required to prevent hyperrecombination at natural pause sites, such as the rDNA RFB. Rtt101p could act at stalled forks to prevent the uncoupling of the replication machinery from sites of DNA synthesis, as is the case for Mrc1p and Tof1p [31]. However, the fact that this uncoupling is detected only in the presence of HU and does not occur in *mrc1Δ* cells exposed to MMS [26] does not support this view. We therefore favor the hypothesis that Rtt101p may, via an ubiquitination reaction, modify component(s) of the replication machinery to promote fork progression through damaged DNA or natural replication-impeding loci. Preliminary evidence indicates that Rtt101p forms a complex with Mms1p and Mms22p, two proteins involved in the tolerance to MMS-induced DNA damage. Further characterization of this complex and the identification of the critical target(s) of Rtt101p will certainly shed light on this important new mechanism maintaining genome integrity during DNA replication.

Supplemental Data

Supplemental Data include one figure, one table, two movies, and Supplemental Experimental Procedures and can be found with this article online at http://www.current-biology.com/cgi/content/full/16/8/786/DC1/.

Acknowledgments

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