Tel1/ATM prevents degradation of replication forks that reverse after topoisomerase poisoning

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Abstract

In both yeast and mammals, the topoisomerase poison camptothecin (CPT) induces fork reversal, which has been proposed to stabilize replication forks, thus providing time for the repair of CPT-induced lesions and supporting replication restart. We show that Tel1, the *Saccharomyces cerevisiae* orthologue of human ATM kinase, stabilizes CPT-induced reversed forks by counteracting their nucleolytic degradation by the MRX complex. Tel1-lacking cells are hypersensitive to CPT specifically and show less reversed forks in the presence of CPT. The lack of Mre11 nuclease activity restores wild-type levels of reversed forks in CPT-treated *tel1Δ* cells without affecting fork reversal in wild-type cells. Moreover, Mrc1 inactivation prevents fork reversal in wild-type, *tel1Δ*, and *mre11* nuclease-deficient cells and relieves the hypersensitivity of *tel1Δ* cells to CPT. Altogether, our data indicate that Tel1 counteracts Mre11 nucleolytic activity at replication forks that undergo Mrc1-mediated reversal in the presence of CPT.

Keywords camptothecin; fork reversal; Mrc1; MRX; Tel1

Subject Categories DNA Replication, Repair & Recombination

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Introduction

The *Saccharomyces cerevisiae* Tel1 (ATM in humans) and Mec1 (ATR in humans) kinases act as master regulators of the DNA damage response, which is activated by DNA lesions or replication stress and preserves genome stability by promoting DNA repair and regulating cell cycle progression [1,2]. Tel1/ATM is activated by DNA double-strand breaks (DSBs), where it is recruited through the interaction with the MRX (Mre11-Rad50-Xrs2)/MRN (MRE11-RAD50-NBS1) complex. By contrast, Mec1/ATR primarily recognizes single-stranded DNA (ssDNA) regions coated by replication protein A (RPA). Activated Tel1/ATM and Mec1/ATR trigger a checkpoint response that temporarily arrests cell cycle progression

through phosphorylation of the effector kinases Rad53 (CHK2 in humans) and Chk1, whose activation also requires the adaptors Rad9/53BP1 and Mrc1/Claspin [1,2].

Tel1/ATM also participates in DSB repair. DSBs can be repaired by either non-homologous end joining (NHEJ) or homologous recombination (HR), with HR being triggered by nucleolytic degradation of the 5' DSB ends in a two-step process called resection. MRX/MRN and Sae2/CtIP bind to DSB ends and initiate resection, thus generating a substrate for the extensive processing carried on by the nucleases Exo1 and Dna2 [3]. Tel1 assists MRX in initial resection [4] and promotes MRX association at DSBs, which is needed for keeping DNA ends in close proximity to favor repair [5].

Despite the multiple functions of Tel1/ATM in the stress response, Tel1-deficient cells do not show obvious hypersensitivities to several genotoxic agents. However, *tel1Δ* cells are hypersensitive to camptothecin (CPT) [5,6], which inhibits the type I topoisomerase Top1 and whose derivatives are currently used in cancer therapy [7]. Type I topoisomerases relax DNA supercoiling by nicking the DNA, enabling broken strand rotation and re-ligating the break. CPT binds to the Top1-DNA intermediate and delays the re-ligation reaction, thus blocking Top1 on DNA and creating a physical barrier to replication fork progression [7].

Recent work in both yeast and mammals has refined the classical model of CPT cytotoxicity, showing that Top1 poisoning prevents the release of torsional stress [8] and induces replication fork slowing down and reversal [9]. Reversed forks are four-way junctions generated by both the annealing of the two newly synthesized strands and the re-annealing of the parental strands [10,11]. Although reversed forks have long been considered pathological structures generated in checkpoint-defective mutants [12], they recently have been observed also in checkpoint-proficient CPT-treated yeast cells [9], as well as in human cells exposed to different replication stress [13]. These findings led to the idea that fork reversal actively pauses replication forks in a stable conformation, thus providing time for lesion removal and replication restart [10]. Remarkably, reversed forks were identified as entry points for nucleases [14–18], which allow extensive degradation of chromosomal DNA in the absence of human FANCD2, BRCA1, and BRCA2 proteins

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[19,20] and contribute to the chemosensitivity of BRCA-defective tumors [21]. However, description of the factors that mediate formation and stability of reversed forks is only at the beginning, and mechanistic insights into these transactions at replication forks are of great importance to improve cancer treatments.

By investigating the molecular basis of the hypersensitivity to CPT of *tel1* mutants, here we show that Tel1/ATM protects reversed replication forks from nucleolytic degradation by Mre11. Furthermore, inactivation of the replisome component Mrc1 prevents fork reversal in wild-type, *tel1Δ* and *mre11* nuclease-deficient cells, and relieves CPT hypersensitivity of *tel1Δ* cells, indicating that fork reversal triggered by Mrc1 is detrimental in the absence of Tel1.

Results

Tel1 specifically supports resistance to camptothecin

We further extended the previous observation that *tel1Δ* cells are hypersensitive to CPT [5,6] by showing that Tel1 kinase activity is specifically required for CPT resistance. In fact, either the lack of Tel1 or the expression of a *tel1-kd* allele, which causes G2611D, D2612A, N2616K and D2631E amino acid substitutions that abolish Tel1 kinase activity *in vitro* [22], decreased cell viability in the presence of CPT, but not in the presence of the alkylating agent methyl methanesulfonate (MMS), the DNA replication inhibitor hydroxyurea (HU) or the radiomimetic drug phleomycin (Fig 1A and B). When we carried out Western blot analyses of protein extracts from untreated or CPT-treated cells expressing a fully functional Top1-HA-tagged protein, we detected similar amount of Top1 in both wild-type and *tel1Δ* cells (Fig 1C). Furthermore, *TOP1* deletion completely relieved CPT hypersensitivity of *tel1Δ* cells (Fig 1D), indicating that Top1 poisoning is responsible for the hypersensitivity to CPT of *tel1* mutant cells.

Tel1 is important to maintain the length of telomeres, the nucleoprotein structures at the ends of eukaryotic chromosomes [1]. Telomere shortening in *tel1Δ* cells does not account for the CPT hypersensitivity of these cells. In fact, ectopic expression of a Cdc13-Est1 fusion, which re-elongates telomeres in *tel1Δ* cells [23], did not restore CPT resistance in the same cells (Fig EV1).

Tel1 is recruited to DSBs by the MRX complex [24], which acts together with Tel1 in promoting DSB resection, with MRX playing the major role [4]. Consistent with a more severe repair defect observed upon MRX than Tel1 inactivation [4,5], cells lacking the Mre11 subunit of MRX were more sensitive to CPT than *tel1Δ* cells (Fig 1E and F). Furthermore, *TEL1* deletion did not increase the CPT hypersensitivity of *mre11Δ* cells (Fig 1E and F), strongly suggesting that Tel1 and MRX act in the same pathway also to support CPT resistance.

The collision between replication forks and Top1-DNA intermediates has been proposed to generate DSBs [7], which require resection to be repaired by HR [3]. Thus, Tel1 might promote CPT resistance by initiating resection of CPT-induced DSBs. However, a resection defect is unlikely to be the cause of the CPT hypersensitivity of *tel1Δ* cells. In fact, overexpression of the exonuclease *EXO1*, which restores DSB resection in *tel1Δ* cells [4] and partially suppresses both the resection defect and the CPT hypersensitivity of

mre11Δ and *sae2Δ* cells (Fig 2A) [25,26], did not restore CPT resistance in *tel1Δ* cells (Fig 2A).

As *tel1Δ* cells are hypersensitive to CPT, but not to other genotoxic treatments that cause DSBs (Fig 1A), Tel1 might prevent conversion of Top1-DNA intermediates into DSBs. These intermediates can be removed by redundant pathways (Tdp1, Rad1/Rad10, Slx1/Slx4, Mus81/Mms4, and Rad27), whose action creates single-strand breaks and/or single-strand gaps that are mainly repaired by recombination [7]. The lack of Tel1 increased the hypersensitivity to CPT of *slx4Δ* (Fig 2B), *rad27Δ* (Fig 2C), *mms4Δ* (Fig 2D), and *rad52Δ* cells (Fig 2E), indicating that Tel1 supports cell viability in CPT by acting in a pathway that is different from those involving the above genes.

The lack of either Tdp1 or Rad1, which caused very mild sensitivity only to high CPT doses, did not increase the hypersensitivity to CPT of *tel1Δ* cells (Fig 2F). As both *tel1Δ* (Fig 2F) and *rad1Δ tdp1Δ* cells (Fig 2F) [27–29] were more sensitive to CPT than either *rad1Δ* or *tdp1Δ* single mutant cells, Tel1 might control both the Tdp1- and the Rad1-dependent pathways, which are known to induce the removal of Top1-DNA intermediates in a redundant manner [27,28,30]. However, *tel1Δ* cells were less sensitive to CPT than *rad1Δ tdp1Δ* double mutant cells (Fig 2F), whose hypersensitivity was not further increased by the lack of Tel1 (Fig 2F and G). These genetic interactions suggest that Tel1 does not simply promote the action of Tdp1 and Rad1/Rad10. Rather, Top1 removal by these two pathways appears important to allow Tel1 action at Top1-induced DNA damage.

Tel1 counteracts the activation of a Mec1-dependent checkpoint in CPT

Tel1 might support cell survival to CPT treatment by activating a checkpoint response. Alternatively, Tel1 may counteract the accumulation of CPT-induced damage through an unknown pathway, and its absence could cause prolonged checkpoint activation due to persistence of CPT-induced checkpoint signals. Thus, we analyzed checkpoint activation in wild-type, *tel1Δ*, and *tel1-kd* cells arrested in G1 and released either in the presence or in the absence of CPT. Consistent with the finding that CPT delays nuclear division without affecting bulk DNA replication [31], CPT-treated and CPT-untreated wild-type cells completed DNA replication with similar kinetics (Fig 3A), whereas nuclear division in CPT-treated wild-type cells took place 30 min later than that in untreated cells (Fig 3B). Conversely, CPT-treated *tel1Δ* and *tel1-kd* cells arrested with 2C DNA content (Fig 3A) and undivided nuclei (Fig 3B). This cell cycle arrest correlated with hyperphosphorylation of the checkpoint kinase Rad53, which accumulated as slowly-migrating phosphorylated forms in CPT-treated *tel1Δ* and *tel1-kd* cells, while only a mild reduction in Rad53 electrophoretic mobility can be detected in wild-type cells (Fig 3C). Furthermore, Rad53 phosphorylation persisted longer in *tel1Δ* cells than in wild-type after a transient CPT treatment (Fig 3D).

Rad53 phosphorylation in CPT-treated *tel1Δ* cells depends on the checkpoint kinase Mec1. In fact, it was abolished in both CPT-treated *mec1Δ* and *tel1Δ mec1Δ* cells (Fig 3E), which were kept viable by the lack of the ribonucleotide reductase inhibitor Sml1 [32]. On the contrary, treatment with the microtubule-depolymerizing drug nocodazole did not reduce Rad53 hyperphosphorylation in CPT-treated

Figure 1. The lack of Tel1 causes a specific hypersensitivity to CPT.

- A** Exponentially growing cell cultures were serially diluted (1:10) before being spotted out onto YEPD plates with or without methyl methanesulfonate (MMS), hydroxyurea (HU), phleomycin (Phleo), or camptothecin (CPT) at the indicated concentrations.
- B** Appropriate dilutions of cell cultures were distributed on YEPD plates with or without different concentrations of CPT. Plates were incubated 3 days at 25°C to determine the colony-forming units. Plotted values are the mean values with error bars denoting SD ($n = 3$).
- C** Wild-type and *tel1Δ* strains expressing a fully functional Top1-HA-tagged protein were arrested in G1 with α -factor (α f) and released into YEPD supplemented with CPT (50 μM). Western blot with anti-HA antibodies (top) and Coomassie staining (bottom) of protein extracts prepared at the indicated time points.
- D, E** Exponentially growing cell cultures were serially diluted (1:10) before being spotted out onto YEPD plates with or without CPT.
- F** Colony-forming unit assay as in (B).

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tel1Δ cells (Fig 3F). This result indicates that the signals activating Mec1 in these cells are not generated by the attempt to separate sister chromatids with trapped Top1.

Altogether, these findings indicate that Tel1 is not required to activate the checkpoint upon CPT treatment. Rather, a Mec1-dependent checkpoint is hyperactivated in CPT-treated *tel1* cells, suggesting that CPT-induced lesions persist longer in *tel1* mutants than in wild-type cells. As ssDNA is proposed to be the signal for Mec1 activation [33], Tel1 might counteract the accumulation of ssDNA after Top1 inhibition.

The lack of Mrc1 suppresses the hypersensitivity to CPT of *tel1Δ* cells

Mec1 activates both the DNA replication checkpoint and the DNA damage checkpoint, which require the mediators Mrc1 and Rad9, respectively [2]. Activation of the CPT-induced checkpoint in the

presence of Tel1 is almost completely Rad9-dependent. In fact, wild-type and *mrc1Δ* cells showed similar CPT-induced Rad53 phosphorylation, which was instead almost abolished in *rad9Δ* cells (Fig 4A and B). Conversely, both Rad9 and Mrc1 appear to participate in checkpoint activation in the absence of Tel1, as the lack of either Mrc1 or Rad9 reduced Rad53 phosphorylation (Fig 4A and B) and allowed nuclear division (Fig 4C) in CPT-treated *tel1Δ* cells, with *RAD9* deletion showing the strongest effect.

The lack of Rad9, which strongly reduced checkpoint activation in CPT-treated *tel1Δ* cells (Fig 4A–C), slightly increased the CPT hypersensitivity of the same cells (Fig 4D), suggesting that a Rad9-dependent DNA damage checkpoint supports cell survival both in the presence and in the absence of Tel1 by delaying progression through mitosis. Strikingly, *tel1Δ mrc1Δ* double mutant cells were less sensitive to CPT than *tel1Δ* cells and lost viability only at high CPT doses, similar to *mrc1Δ* cells (Fig 4D and E), indicating that *MRC1* deletion is epistatic to *TEL1* deletion for CPT sensitivity.

Figure 2. The lack of Tel1 increases the hypersensitivity to CPT of repair mutants.

A Exponentially growing cell cultures were serially diluted (1:10) before being spotted out onto YEPA plates with or without CPT at the indicated concentrations.
B–E Colony-forming units on YEPA plates with or without different concentrations of CPT. Plates were incubated 3 days at 25°C. Plotted values are the mean values with error bars denoting SD ($n = 3$).
F Exponentially growing cell cultures were serially diluted (1:10) before being spotted out onto YEPA plates with or without CPT.
G Colony-forming unit assay as in (B–E).

The lack of Tof1 or Csm3, both forming a complex with Mrc1 [34], exacerbated the CPT sensitivity of *tel1* Δ cells (Fig EV2A). Therefore, the absence of Mrc1, but not of Csm3 or Tof1, relieves the CPT hypersensitivity caused by the lack of Tel1. Furthermore, suppression of the CPT hypersensitivity by the lack of Mrc1 is specific for *tel1* Δ cells, as *MRC1* deletion did not suppress, but rather increased, the CPT hypersensitivity of *rad27* Δ , *sae2* Δ , and *tdp1* Δ *rad1* Δ cells (Fig EV2B–D). Altogether, these results indicate that Mrc1 action is detrimental when Top1 is inhibited in the absence of Tel1.

The lack of Mrc1 DNA replication function relieves hypersensitivity and checkpoint hyperactivation of CPT-treated *tel1* Δ cells

Besides promoting checkpoint activation, Mrc1 supports unperturbed DNA replication [35,36]. These two Mrc1 functions are genetically separable. In fact, the *mrc1*-AQ allele, which abolishes the Mec1-dependent phosphorylation of Mrc1, causes checkpoint defects without affecting unperturbed replication. Conversely, cells expressing either the *mrc1*-C15 or the *mrc1*-C14 allele, which both cause

Figure 3. CPT triggers the hyperactivation of a Mec1-dependent checkpoint in the absence of Tel1.

A–C Exponentially growing cell cultures (exp) were arrested in G1 with α -factor (α) and released into YEPD with or without CPT (50 μ M). Cell samples were harvested at the indicated time points to evaluate DNA contents by flow cytometry (A), nuclear division by fluorescence microscopy (B), and Rad53 phosphorylation by Western blot with anti-Rad53 antibodies (C).
 D Exponentially growing cell cultures (exp) were treated with CPT (50 μ M) for 1 h, before being released in fresh medium without CPT. Western blot analysis with anti-Rad53 antibodies of protein extracts prepared at the indicated time points.
 E, F G1-arrested cell cultures (α) were released in YEPD supplemented with CPT (50 μ M) (E) or in YEPD supplemented with CPT (10 μ M) with (+noc) or without (–noc) nocodazole (F). Western blot analysis with anti-Rad53 antibodies.

Source data are available online for this figure.

C-terminal truncations of 193 and 253 amino acids, respectively, are checkpoint proficient but replicate their DNA as slowly as *mrc1Δ* cells [35–37]. *tel1Δ mrc1-AQ* cells were as sensitive as *tel1Δ* cells to CPT (Fig 5A), and both these strains hyperactivated the CPT-induced checkpoint compared to wild-type and *mrc1-AQ* cells (Fig 5B and C). By contrast, both *mrc1-C15* (Fig 5A) and *mrc1-C14* (Fig EV3) alleles completely relieved the sensitivity to CPT of *tel1Δ* cells, with *mrc1-C15* also reducing Rad53 hyperphosphorylation (Fig 5D) and allowing nuclear division in CPT-treated *tel1Δ* cells (Fig 5E). Thus, Mrc1 replication function, but not its checkpoint function, is responsible for the hypersensitivity of *tel1Δ* cells to Top1 inhibition.

MRC1 deletion was proposed to suppress the hypersensitivity to MMS and CPT of cells lacking the ubiquitin ligase component Rtt107

by increasing origin firing [38]. However, increasing origin firing is likely not sufficient to alleviate CPT hypersensitivity of *tel1Δ* cells, as inactivation of the histone deacetylase Rpd3, which causes precocious late-origin firing [39], exacerbated the CPT sensitivity of *tel1Δ* cells (Fig 5F). According to these findings, the lack of Tel1 increased CPT hypersensitivity of *rtt107Δ* cells (Fig 5G), indicating that Tel1 and Rtt107 play different functions in CPT resistance.

Tel1 counteracts Mre11-mediated degradation of CPT-induced reversed forks

Top1 inhibition by CPT slows down replication fork progression and causes reversal of a significant percentage of replication forks in

Figure 4. Mrc1 inactivation restores CPT resistance in the absence of Tel1.

A–C Exponentially growing cell cultures were arrested in G1 with α -factor (α f) and released in YEPD supplemented with CPT (50 μ M). (A) Western blot analysis with anti-Rad53 antibodies. (B) Quantitative analysis of Rad53 phosphorylation was performed by calculating the ratio of band intensities for slowly-migrating bands to the total amount of protein. Plotted values are the mean values with error bars denoting SD ($n = 3$). Statistical analysis: Student's t -test; ns, not significant; ** $P < 0.01$; *** $P < 0.001$; **** $P < 0.0001$. (C) Nuclear division determined by fluorescence microscopy.

D Exponentially growing cell cultures were serially diluted (1:10) before being spotted out onto YEPD plates with or without CPT at the indicated concentrations.

E Cells were distributed on YEPD plates with or without CPT at different concentrations to determine colony-forming units after 3 days at 25°C. Plotted values are the mean values with error bars denoting SD ($n = 3$).

Source data are available online for this figure.

both yeast and mammals [9]. Importantly, Top1 inhibition is the only genotoxic treatment, among those tested (HU, UV, MMS), to be reported to induce frequent fork reversal in checkpoint-proficient *S. cerevisiae* cells [9,12,40], providing a hint toward the specific CPT sensitivity observed in *tel1Δ* cells. We directly visualized fork architecture by *in vivo* psoralen cross-linking and electron microscopy (EM) in wild-type and *tel1Δ* cells synchronously released from an α -factor arrest into S phase in the presence of CPT (Fig EV4A and B). Reversed forks appeared as replication forks with a regressed arm forming a four-way junction (Fig 6A). As expected [9], about 20% of replication forks reversed in CPT-treated wild-type

cells (Fig 6B and C). Their frequency was twofold decreased in the absence of Tel1 (Figs 6B and C, and EV4C), suggesting that Tel1 promotes formation or stability of CPT-induced reversed forks.

Nucleolytic degradation of reversed forks was observed in both yeast and mammals [14–19,41]. Thus, Tel1 might counteract nucleolytic action at reversed forks, thereby limiting Mec1 activation upon CPT treatment. We then directly visualized fork reversal by EM in CPT-treated (Fig EV4A and B) wild-type and *tel1Δ* cells optionally carrying the *EXO1* deletion or the *mre11-H125N* allele, which abolishes Mre11 nuclease activity without affecting checkpoint activation and MRX recruitment to DNA [42,43]. Both Exo1 and MRX

Figure 5. The lack of Mrc1 function in DNA replication restores CPT resistance in the absence of Tel1.

A Exponentially growing cell cultures were serially diluted (1:10) before being spotted out onto YEPA plates with or without CPT at the indicated concentrations.
B–E G1-arrested cell cultures (α f) were released in YEPA supplemented with CPT (50 μ M). Samples collected at the indicated time points were subjected to Western blot analysis with anti-Rad53 antibodies (B, D) or stained with propidium iodide to analyze the kinetics of nuclear division (C, E).
F Exponentially growing cell cultures were serially diluted (1:10) before being spotted out onto YEPA plates with or without CPT.
G Colony-forming units on YEPA plates with or without CPT at different concentrations. Plates were incubated 3 days at 25°C. Plotted values are the mean values with error bars denoting SD ($n = 3$).

Source data are available online for this figure.

Figure 6. The lack of Tel1 allows Mre11-dependent degradation of reversed forks in CPT-treated cells.

A–E G1-arrested cell cultures were released in YEPD supplemented with CPT (50 μ M). (A, D) Electron micrographs of a representative reversed fork with the four-way junction magnified in the inset (A) and a three-way replication fork exposing ssDNA at the junction (D). In (D) the black arrow indicates the ssDNA region, which is magnified in the inset. Scale bars: 200 nm, 50 nm in the insets. (B, C) Frequency of reversed replication forks in CPT (40 min, 50 μ M CPT). Two independent experiments were performed with very similar results. The results of the individual biological replicates and the number of analyzed molecules are shown in Fig EV4C. (E) Graphical distribution of ssDNA length at the junction (black arrow in (D)) in forks isolated after CPT treatment (40 min, 50 μ M CPT). Only molecules with detectable ssDNA stretches are included in the analysis. The lines show the median length of ssDNA regions at the fork in the specific set of analyzed molecules. Statistical analysis: Mann–Whitney test; ns, not significant; * P < 0.05; ** P < 0.01; **** P < 0.0001. The number of analyzed molecules is in brackets. F, G G1-arrested cell cultures (α) were released into YEPD supplemented with CPT (50 μ M). Western blot analysis with anti-Rad53 antibodies.

Source data are available online for this figure.

nuclease activity turned out to be dispensable for fork reversal. In fact, both CPT-treated *exo1Δ* and *mre11-H125N* cells showed wild-type levels of reversed forks (Fig 6B and C). Remarkably, the *mre11-H125N* mutation restored wild-type amount of fork reversal even in *tel1Δ* cells (Fig 6B), while we observed very similar frequencies of reversed forks in *exo1Δ* and *exo1Δ tel1Δ* cells (Fig 6C). These results strongly suggest that decreased reversed fork levels in the absence of Tel1 are due to unscheduled nucleolytic processing that depends mainly on Mre11.

In line with unscheduled Mre11-dependent fork processing, *tel1Δ* cells showed extended ssDNA stretches at replication forks (Fig 6D and E) compared to wild-type cells. This phenotype was fully suppressed by the lack of Mre11 nuclease activity (*mre11-H125N*; Fig 6E). As ssDNA was proposed to trigger Mec1 activation [33], this unscheduled nucleolytic processing could also account for checkpoint hyperactivation in CPT-treated *tel1Δ* cells. Indeed, *TEL1* deletion in *mre11-H125N* cells did not lead to the same extent of CPT-induced Rad53 hyperphosphorylation observed when Mre11 is functional (Fig 6F). A similar decrease of Rad53 phosphorylation was observed upon inactivation of Sae2 (Fig 6F), which stimulates Mre11 nuclease activity but not its recruitment to DNA ends [43–45]. Conversely, the lack of Exo1, which did not restore fork reversal in the absence of Tel1 (Fig 6C), did not affect Rad53 phosphorylation in CPT-treated wild-type or *tel1Δ* cells (Fig 6G). Thus, MRX likely generates long ssDNA stretches at replication intermediates in the absence of Tel1. This fork degradation seems to primarily target reversed replication forks and to be responsible for checkpoint hyperactivation in CPT-treated *tel1Δ* cells.

Mrc1-dependent fork reversal triggers fork degradation in CPT-treated *tel1Δ* cells

Recent reports in metazoan show that preventing fork reversal by inactivating fork remodeling enzymes suppresses unscheduled MRE11-dependent fork degradation in BRCA1- or BRCA2-defective cells [15–18]. We asked whether a similar mechanism could underlie the suppression of CPT hypersensitivity by Mrc1 inactivation in *tel1Δ* cells (Fig 4). EM analysis upon CPT treatment revealed that reversed fork levels were markedly reduced by *MRC1* deletion, both in the presence and in the absence of Tel1, and regardless of Mre11 nuclease activity (Fig 6B). Thus, Mrc1 likely works upstream of Tel1 and Mre11, promoting CPT-induced fork reversal and thereby generating the structures that are then targeted by Tel1 and/or Mre11, in agreement with the epistatic relationship observed for CPT sensitivity (Fig 4D and E).

As for other genetic perturbations that impair fork reversal [13], in *mrc1Δ* cells we observed long replication fork-associated ssDNA stretches (Fig 6E), which were not reduced by the lack of Mre11 nuclease activity (Fig 6E). Therefore, this ssDNA likely foregoes Mrc1-mediated fork reversal and could be generated by uncoupled activities at the replication fork in the absence of Mrc1. Whether the lack of Mrc1 causes uncoupling between helicase and polymerase activity is still unclear [34,46]. However, recent work showed that the checkpoint kinase Rad53, which requires Mrc1 to be fully activated upon replication stress [2], couples leading- and lagging-strand synthesis in these conditions [47]. This finding, together with the observation that Mrc1 directly increases the rate

of leading-strand synthesis [48], suggests that *mrc1Δ* cells accumulate ssDNA on the under-replicated leading-strand template.

While Mrc1 inactivation relieved the hypersensitivity to CPT of *tel1Δ* cells (Fig 4D and E), it was unable to suppress CPT sensitivity of *mre11-H125N* cells (Fig EV4D). Furthermore, both *tel1Δ mre11-H125N* and *tel1Δ sae2Δ* double mutants were more sensitive to CPT than *tel1Δ* cells (Fig EV4E and F). This is expected, as Mre11 cleaves DNA ends that are covalently linked by Top1 [30,49], and Sae2 participates in DSB processing and repair together with MRX [3]. Thus, the CPT hypersensitivity caused by either the *mre11-H125N* or *sae2Δ* alleles is likely due to repair defects rather than to abnormal fork metabolism. Our results indicate that, besides being involved in later steps of CPT-induced damage repair, the MRX nuclease activity processes reversed forks and generates ssDNA in CPT-treated *tel1Δ* cells.

Discussion

Here, we describe a novel function of Tel1/ATM in stabilizing CPT-induced reversed forks by counteracting the nucleolytic activity of MRX. This Tel1 function becomes dispensable when fork reversal is prevented by the lack of Mrc1, suggesting that Tel1 and MRX target replication forks after their reversal (Fig 7). Alternatively, Tel1 and Mre11 might function before fork reversal, with Tel1 promoting this transition at stalled forks and MRX triggering fork degradation that prevents fork reversal in the absence of Tel1. We favor the first hypothesis because purified MRE11-RAD50 complex was found to degrade reversed forks *in vitro* [18]. Furthermore, four independent groups recently demonstrated that in metazoan, fork reversal is required for MRE11-dependent degradation of stalled forks in the absence of the protective function exerted by BRCA1 or BRCA2 [15–18]. Tel1 may counteract MRX activity either directly or through a still unknown factor that prevents MRX action at reversed forks (Fig 7).

Reversed forks stabilization by Tel1 can support the completion of DNA replication after the repair of CPT-induced damage by either reversed fork reactivation or fusion with an incoming fork (Fig 7). Consistently, we found that Tel1 inactivation causes a Mre11-dependent increase in the length of ssDNA gaps at three-way fork junctions. A similar phenotype was observed in BRCA-depleted cells, which also displayed MRE11-dependent extensive degradation of nascent DNA that proceeds behind the stalled forks and can be suppressed by preventing fork reversal [17,18]. Similarly, extended ssDNA at three-way fork junctions in *tel1Δ* cells might result from nucleolytic degradation starting from the regressed arm of the reversed forks, and proceeding behind the fork.

Mrc1 inactivation prevents fork reversal and relieves the hypersensitivity to CPT of *tel1Δ* cells, suggesting that unscheduled MRX-dependent processing of reversed forks in the absence of Tel1 may account for the CPT hypersensitivity of these cells. Interestingly, the lack of Tel1 does not increase the CPT hypersensitivity of *rad1Δ tdp1Δ* double mutant cells, indicating that Tel1 somehow participates in the CPT response together with Tdp1 and Rad1. As a strong retention of Top1 on DNA was observed in CPT-treated *rad1Δ tdp1Δ* cells compared to wild-type [30], our results suggest that Top1 removal is required to repair the CPT-induced damage even when the integrity of stalled forks is preserved by Tel1.

Figure 7. Working model for Tel1 function at replication forks in CPT.

CPT-induced Top1 trapping on DNA results in the accumulation of DNA supercoiling ahead of an incoming replication fork. In the presence of this torsional stress Mrc1 promotes fork reversal. Reversed forks can be targeted by nucleases, mainly MRX-Sae2, which nucleolytically process the regressed arm. Tel1 counteracts the action of MRX-Sae2 either directly or through a still unknown factor (X), thus preventing unscheduled fork degradation. Reversed forks stabilization by Tel1 can support the completion of DNA replication after the repair of CPT-induced damage by either reversed fork reactivation or fusion with an incoming fork.

We show that Mrc1 plays an unexpected function in promoting fork reversal in CPT, which is the only condition that was found to trigger fork reversal in checkpoint-proficient yeast cells [9,12,40]. As CPT prevents the release of DNA supercoiling by Top1 [8], torsional stress is expected to participate in CPT-induced fork reversal [9]. Therefore, Mrc1 may indirectly promote fork reversal in CPT by supporting fork progression despite the accumulation of torsional stress and/or by avoiding the redistribution of supercoiling behind the moving fork. Alternatively, Mrc1 may directly promote fork reversal by assisting specialized enzymes in fork remodeling. Interestingly, although several fork remodeling activities were identified in both yeast and mammals [10,11], a potential role in fork reversal for the Mrc1 human orthologue Claspin has not been assessed to date.

Our finding that MRX degrades reversed forks in CPT-treated *tel1Δ* cells, while Exo1 is dispensable, is somehow surprising. In fact, Exo1 was found to nucleolytically process reversed forks generated in *mec1* and *rad53* checkpoint mutants treated with HU [14,41]. Furthermore, human EXO1 contributes to MRE11-dependent degradation of stalled forks in BRCA-defective cells [16]. As a Rad53-dependent phosphorylation was proposed to inhibit Exo1 activity [50] and a Mec1-dependent checkpoint is activated in CPT-treated *tel1Δ* cells, checkpoint activation may limit Exo1 action in these cells. Alternatively, different structures generated at replication forks that stall because of nucleotide depletion or of torsional constraints may be targeted by different nucleases. Indeed, nucleotide depletion in checkpoint mutants causes both uncoupling of leading- and lagging-strand synthesis and re-annealing of the daughter strands in a four-way junction with extensive fork-associated ssDNA regions, which can be the entry point for Exo1 [12,14]. By contrast, torsional stress ahead of a replication fork was proposed to trigger parental strand re-winding and extrusion of the newly synthesized filaments [9,51]. MRX and Tel1 participate in the metabolism of replication forks arrested by a DSB or by transcription [52,53], or involved in the replication of telomeric regions [54,55]. The same factors can also recognize abnormal forks generated in CPT. One possibility is that the regressed arms of CPT-induced reversed forks terminate with blunt or minimally processed DNA

ends, which are known to recruit MRX and Tel1 [24]. However, as Mre11 triggers Top1 release from DNA [30,49], we cannot exclude the possibility that MRX is recruited to the Top1-DNA intermediates and might be able to attack replication forks that reach the Top1-binding sites and reverse in close proximity to these intermediates.

In mammals, reversed forks accumulate in response to different stress [13] and are protected against degradation by mechanisms that involve tumor suppressors and HR proteins [15,19,20]. Unscheduled degradation of reversed forks in the absence of these protective mechanisms is mainly MRN-dependent and causes genomic instability and cell death [16–19,21]. It will be interesting to test whether ATM also contributes to reversed fork protection by preventing MRN action. A detailed understanding of ATM functions in CPT is important to develop novel therapies based on Top1 poisoning against cancers with defective ATM function.

Materials and Methods

Yeast strains and media

All the strains used in this work are listed in Table EV1 and are isogenic to W303 (*MATa/α ade2-1 can1-100 his3-11,15 leu2-3,112 trp1-1 ura3-1 rad5-535*). All genetic manipulations were verified by polymerase chain reaction (PCR) and/or Southern blot analyses. Gene deletions were carried out by one-step PCR methods. A PCR one-step tagging method was used to obtain strains carrying the *TOP1-3HA* allele, which express a fully functional HA-tagged Top1 variant. The centromeric pVL1091 plasmid, carrying a *CDC13-EST1* gene fusion [56], was kindly provided by V. Lundblad (Salk Institute for Biological Sciences, La Jolla, CA, USA). A 2μ *EXO1* plasmid [57] was kindly provided by E. Alani (Cornell University, New York, NY, USA). Centromeric plasmids carrying either the wild-type *MRC1* allele (pMRC1) or the *mrc1-AQ* mutant allele (pAO138), as well as yeast strains carrying *mrc1-C14* and *mrc1-C15* alleles at the endogenous chromosomal locus, were kindly provided by S. Elledge (Harvard Medical School, Boston, MA, USA). Strains carrying either the *tel1-kd*

[22] or the *mre11-H125N* [42] alleles were kindly provided by T.D. Petes (Duke University School of Medicine, Durham, NC, USA) and L. Symington (Columbia University, New York, NY, USA), respectively. Cells were grown in YEP medium (1% yeast extract, 2% peptone) supplemented with 2% glucose (YEPD). (S)-(+)-Camptothecin was dissolved in 1.2% DMSO before addition to the medium.

Analysis of cell cycle progression and Western blotting

Exponentially growing cells were synchronized in the G1 phase of the cell cycle with 3 $\mu\text{g}/\text{ml}$ α -factor. G1-arrested cells were then released into fresh YEPD medium with or without CPT. DNA content was evaluated by fluorescence-activated cell sorting (FACS) analysis of cells stained with propidium iodide. The same samples were analyzed by fluorescence microscopy to evaluate nuclear division. Protein extracts for Western blotting were prepared following cell fixation with trichloroacetic acid (TCA) and were separated in 10% polyacrylamide gels, and then transferred to a nitrocellulose membrane. Top1-HA was detected with 12CA5 anti-HA antibodies (GE Healthcare). Rad53 was detected with anti-Rad53 antibodies (AB104232, Abcam). Quantitative analysis of phosphorylation was performed by calculating the ratio of band intensities for slowly-migrating bands to the total amount of protein.

EM analysis of reversed forks

This procedure was performed as recently described [58], with the modifications below. Cell cultures were arrested in G1 phase with α -factor and then released into fresh medium supplemented with 50 μM CPT (CPT was dissolved in 2% DMSO). After 40 min of CPT treatment, 5×10^9 cells were harvested, rinsed with ice-cold water, and suspended in 20 ml of ice-cold water. Each sample was transferred into two petri dishes (diameter 4.5 cm) and cross-linked with three cycles of treatment with 3,5,8-trimethylpsoralen (TMP, dissolved in 100% EtOH, 10 $\mu\text{g}/\text{ml}$ final concentration). After TMP addition, samples were incubated for 5 min at 4°C in a dark room and then irradiated with 365 nm UV monochromatic light (UV Lamps CAMAG 2 $\times$ 366 8W) in a customized chamber for 3 min. Cells suspension was then transferred into a 50-ml tube and rinsed with ice-cold water to remove ethanol. DNA extraction was carried out with Qiagen Genomic-tip 100/G. Briefly, cells were lysed with 3.12 mg/ml zymolyase 20T in Y1 Qiagen Buffer with β -mercaptoethanol at 30°C for 40 min and then incubated in G2 Qiagen Buffer with 0.2 mg/ml RNaseA and 0.4 mg/ml proteinaseK at 37°C for 2 h. After column purification with Qiagen Genomic-tip 100/G, DNA was eluted with QF Qiagen Buffer and precipitated with 0.7 volumes of isopropanol. Finally, DNA was dissolved in 200 μl TE 1 $\times$ (10 mM Tris-HCl, 1 mM EDTA, pH 8). 100 U of PvuII restriction enzyme (New England Biolabs) was used to digest 12 μg of yeast genomic DNA for 4–5 h. Replication intermediates enrichment was performed by Qiagen Plasmid Mini Kit columns. The Qiagen-tip 20 surface tension was reduced by applying 1 ml QBT buffer. The columns were rinsed with 10 mM Tris-HCl (pH 8.0), 1 M NaCl, followed by equilibration with 10 mM Tris-HCl (pH 8.0), 300 mM NaCl. DNA was then loaded onto the columns. The columns were rinsed with high NaCl solution (10 mM Tris-HCl pH 8.0 and 900 mM NaCl) and eluted with caffeine solution (10 mM Tris-HCl pH 8.0, 1 M NaCl, and 1.8% w/v caffeine). To purify and

concentrate the DNA, an Amicon size-exclusion column was used. DNA was then resuspended in TE 1 $\times$ buffer. The benzyldimethylalkylammonium chloride (BAC) method was used to spread the DNA on the water surface and then load it on carbon-coated 400-mesh copper grids. Subsequently, DNA was coated with platinum using a High Vacuum Evaporator MED 020 (BalTec). Microscopy analysis was performed with a transmission electron microscope (Tecnai G2 Spirit; FEI; LaB6 filament; high tension ≤ 120 kV) and picture acquisition with a side mount charge-coupled device camera ($2,600 \times 4,000$ pixels; Orius 1000; Gatan, Inc.). For each experimental condition, at least 70 replication fork molecules were analyzed. DigitalMicrograph version 1.83.842 (Gatan, Inc.) and ImageJ (National Institutes of Health) were used to process and analyze the images. Every EM experiment was repeated twice, and at least 70 molecules per sample were analyzed (see the table in Fig EV4C). The results were analyzed using GraphPad Prism 6 for Mac OS X, using Mann–Whitney test. Whiskers: 10–90th percentile (**** $P < 0.0001$; *** $P < 0.001$; ** $P < 0.01$; * $P < 0.05$; ns, not significant).

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Author contributions

MC conceived the project; LM, ML, MPL, and MC designed the experiments and analyzed data; LM and CT conducted the experiments; SU and RZ performed the EM analyses; ML, MPL, and MC wrote the paper.

Conflict of interest

The authors declare that they have no conflict of interest.

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