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Ctp1-dependent clipping and resection of DNA double-strand breaks by Mre11 endonuclease complex are not genetically separable

Kristi L. Jensen and Paul Russell*

Department of Cell and Molecular Biology, The Scripps Research Institute, La Jolla, CA 92037, USA

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ABSTRACT

Homologous recombination (HR) repair of programmed meiotic double-strand breaks (DSBs) requires endonucleolytic clipping of Rec12^{Spo11}-oligonucleotides from 5′ DNA ends followed by resection to generate invasive 3′ single-stranded DNA tails. The Mre11-Rad50-Nbs1 (MRN) endonuclease and Ctp1 (CtIP and Sae2 ortholog) are required for both activities in fission yeast but whether they are genetically separable is controversial. Here, we investigate the mitotic DSB repair properties of Ctp1 C-terminal domain (*ctp1-CD*) mutants that were reported to be specifically clipping deficient. These mutants are sensitive to many clastogens, including those that create DSBs devoid of covalently bound proteins. These sensitivities are suppressed by genetically eliminating Ku nonhomologous end-joining (NHEJ) protein, indicating that Ctp1-dependent clipping by MRN is required for Ku removal from DNA ends. However, this rescue requires Exo1 resection activity, implying that Ctp1-dependent resection by MRN is defective in *ctp1-CD* mutants. The *ctp1-CD* mutants tolerate one but not multiple broken replication forks, and they are highly reliant on the Chk1-mediated cell cycle checkpoint arrest, indicating that HR repair is inefficient. We conclude that the C-terminal domain of Ctp1 is required for both efficient clipping and resection of DSBs by MRN and these activities are mechanistically similar.

INTRODUCTION

Meiotic recombination relies on the formation of programmed DNA double-strand breaks (DSBs) for faithful segregation of homologous chromosomes during the first meiotic division (1). Spo11 (called Rec12 in *Schizosaccharomyces pombe*) acts in a topoisomerase II-like mechanism to create DSBs, which are subsequently repaired via homologous recombination (HR) to generate DNA crossovers resulting in reciprocal recombination. Spo11 dimers become covalently bound to the 5′ DNA terminus of a DSB. Endonucleolytic cleavage ('clipping') liberates Spo11-bound oligonucleotides from the DSB, which is followed by 5′ to 3′ resection to generate a 3′ single-stranded DNA (ssDNA) overhang required for HR. The Mre11-Rad50-Nbs1 (MRN) endonuclease complex interacts with CtIP/Sae2 (*S. pombe* Ctp1) to catalyze these DNA end-processing events.

In mitotically dividing cells, spontaneous DSBs arise from environmental DNA damaging agents such as gamma radiation or chemical clastogens, or via intrinsic causes such as replication fork collapse. Repair of a DSB during the mitotic cell cycle occurs through ligation of DNA ends by the nonhomologous end-joining (NHEJ) or HR pathways (2–5). Repair pathway choice involves cell cycle regulation of Ctp1/Sae2/CtIP activity through periodic gene expression or CDK-mediated phosphorylation. Efficient HR repair in mitotic fission yeast cells requires the MRN protein complex and Ctp1, which endonucleolytically remove non-covalently bound NHEJ-promoting Ku complex from DNA ends (6–8). Deletion of the Ku complex in MRN or Ctp1-deficient backgrounds rescues the repair defect by allowing Exo1, which is a 5′ to 3′ exonuclease, to access and resect DSBs (6,8,9). Similar genetic and biochemical relationships have been experimentally established for orthologous proteins in *Saccharomyces cerevisiae* and mammals (2,3,5), consistent with the paramount requirement for HR to maintain genome stability, prevent disease and undergo sexual reproduction (1,10).

The first steps of HR repair catalyzed by MRN and CtIP/Sae2/Ctp1 are now well understood in broad outline, but there remains substantial uncertainty and controversy about critical parts of the mechanism. Notably, some studies indicate that CtIP and Sae2 have an intrinsic endonuclease activity that may be important for HR (11–13), whereas others report that Sae2 and Ctp1 lack nuclease activities but provide architectural and DNA binding functions that are critical for activating or coordinating Mre11 endonuclease activity at DSBs (14,15). CtIP and its orthologs might contribute distinct activities required for dif-

*To whom correspondence should be addressed. Tel: +1 858 784 8273; Fax: +1 858 784 2265; Email: prussell@scripps.edu

ferent steps of DNA end processing, such as clipping and resection, which theoretically should be genetically separable. With this idea in mind, Ma *et al*. performed a genetic screen to identify *ctp1* mutants that are defective for clipping but not resection (16). The screen was designed to isolate mutants that were sensitive to camptothecin (CPT), which is a topoisomerase I (Top1) poison, yet comparatively less sensitive to the DNA alkylating agent methyl methanesulfonate (MMS). Endonucleolytic processing of alkylated DNA creates 'clean' DSBs that are devoid of covalently bound proteins, whereas CPT inhibits Top1 by blocking the rejoining step of the cleavage/religation reaction, resulting in stabilization of a covalent reaction intermediate known as the Top1 cleavable complex (Top1cc). The 11 unique *ctp1* alleles isolated by Ma *et al*. all contained mutations that truncate or introduce amino acid coding changes into the C-terminal domain of the protein. Similar to the *ctp1Δ* allele, these *ctp1* C-terminal domain (*ctp1-CD*) alleles greatly decrease spore viability and release of Rec12-bound oligonucleotides, indicating a clipping deficiency, although meiotic recombination rates were only modestly reduced. Resection and recombination were proficient at a meganuclease-induced DSB in a *ctp1-CD rec12Δ* meiosis, indicating that *ctp1-CD* alleles impair clipping but not resection (16).

We undertook the present study to investigate the importance of MRN-Ctp1-mediated clipping to remove Ku from DNA ends in mitotic cells. Key findings to emerge from our studies are that *ctp1-CD* mutants are sensitive to a wide range of genotoxins and these sensitivities are suppressed by genetically eliminating Ku, indicating that Ctp1-dependent clipping by MRN is required to remove Ku from DSBs. However, genetic suppression of *ctp1-CD* by eliminating Ku strictly depends on the resection activity provided by Exo1, indicating that resection is critically impaired in these mutants. From these and other results we conclude that clipping and resection by MRN-Ctp1 are mechanistically similar.

MATERIALS AND METHODS

General *S. pombe* methods

S. pombe strains were generated using previously described methods (17). DNA damage sensitivity was assayed using 5-fold serial dilutions of exponentially growing cells. Dilutions were spotted onto YES (yeast extract, glucose and supplements) agar containing the indicated concentration of DNA damaging agent, and to YES agar followed by exposure to a UV source or a ^{137}Cs source of ionizing radiation (IR). Plates were incubated at 32˚C for 3 days before imaging. Strains used were: PR109 *h⁻ leu1-32 ura4-D18*, OL4121 *h⁻ leu1-32 ura4-D18 ctp1::kanMX6*, SC4083 *h⁻ leu1-32 ura4-D18 pku80::hphMX6*, OL5186 *h⁻ leu1-32 ura4-D18 exo1::natMX6*, KJ5378 *h⁺ leu1-32 ura4-D18 chk1::natMX6*, KJ5379 *h⁺ leu1-32 ura4-D18 pku80::hphMX6 exo1::natMX6*, KJ5380 *h⁻ leu1-32 ura4-D18 ctp1::kanMX6 pku80::hphMX6*, KJ5381 *h⁺ leu1-32 ura4-D18 ctp1::kanMX6 pku80::hphMX6 exo1::natMX6*, KJ5382 *h⁻ leu1-32 ura4-D18 ctp1::kanMX6 exo1::natMX6*, OL5359 *h⁻ ade6-M26 ura4-D18 arg1-14*, OL5360 *h⁻ ade6-M26 ura4-D18 arg1-14 ctp1::kanMX6*, OL5362 *h⁻ ade6-M26 ura4-D18 arg1-14 ctp1-6* [identical to GP7856 reported by Ma *et al*. (16)], OL5365 *h⁻ ade6-M26 ura4-D18 arg1-14 ctp1-25* [identical to GP7862 reported by Ma *et al*. (16)], KJ5383 *h⁻ ade6-M26 ura4-D18 ctp1-6 pku80::hphMX6*, KJ5384 *h⁻ ade6-M26 ura4-D18 arg1-14 ctp1-6 pku80::hphMX6 exo1::natMX6*, KJ5385 *h⁻ ade6-M26 ura4-D18 ctp1-6 exo1::natMX6*, KJ5386 *h⁻ ade6-M26 ura4-D18 arg1-14 ctp1-25 pku80::hphMX6*, KJ5387 *h⁻ ade6-M26 ura4-D18 ctp1-25 pku80::hphMX6 exo1::natMX6*, KJ5388 *h⁻ ade6-M26 ura4-D18 arg1-14 ctp1-25 exo1::natMX6*, KJ5389 *h⁺ leu1-32 ura4-D18 ctp1::kanMX6 chk1::natMX6*, KJ5390 *h⁻ leu1-32 ura4-D18 arg1-14 ctp1-6 chk1::natMX6*, KJ5391 *h⁺ ade6-M26 ura4-D18 arg1-14 ctp1-25 chk1::nat*, YYY4403 *h⁺ leu1-32 ura4-D18 mat2,3::leu2⁺ mat1-P*, YYY4270 *h⁺ leu1-32 ura4-D18 rad2::ura4⁺*, BM3151 *h⁺ leu1-32 ura4-D18 ade6-M216? can1-1? cdc17-K42*, KJ5392 *h⁺ leu1-32 ura4-D18 ctp1-TAP-kanMX6 mat2,3Δ::leu1⁺*, KJ5393 *h⁺ leu1-32 ura4-D18 ctp1-TAP-kanMX6 rad2::ura4⁺* and KJ5394 *h⁺ leu1-32 ura4-D18 ade6-M216? can1-1? ctp1-TAP-kanMX6 cdc17-K42*.

RESULTS

Ctp1-CD strains have an intermediate level of sensitivity to clastogens

We set out to characterize the effects of *ctp1-CD* mutations in mitotic cells and specifically to test whether their clipping deficiency impaired removal of Ku from DNA ends. We chose to focus on two of the *ctp1-CD* alleles that were most extensively characterized in meiotic assays, *ctp1-6* and *ctp1-25*, which are nonsense mutation alleles that truncate the C-terminal domain of *ctp1⁺*, resulting in loss of the conserved RHR or RHR and CxxC motifs, respectively (Figure 1A) (16). Clastogens tested included CPT and MMS, both used at concentrations at which the *ctp1-CD* strains were isolated; hydroxyurea (HU), which arrests replication forks by depletion of deoxyribonucleotides due to inactivation of ribonucleotide reductase; DNA photoproduct formation by ultraviolet light (UV); and ionizing radiation (IR), of which toxicity is mostly caused by direct generation of DSBs. Repair of damage caused by these agents requires functional HR machinery, including the MRN-Ctp1 complex (7,18). Note that we did not test poisons that trap topoisomerase 2 (Top2) on DNA 5′ ends as we assumed that the inability of *ctp1-CD* cells to clip Rec12 from DNA 5′ ends in meiotic cells as reported by Ma *et al*. (16) would also apply to the covalently bound Top2 in mitotic cells, which would interfere with assessment of whether these mutants were defective at removing non-covalently bound Ku from DNA ends.

Exposure to these DNA damaging situations shows that *ctp1-CD* strains are more sensitive to DNA damage than wild type but less sensitive than *ctp1Δ* strains (Figure 1B and C). Thus, *ctp1-CD* alleles cause intermediate sensitivity to the entire set of DNA damaging agents. There was no obvious preferential sensitivity to CPT or MMS, although chronic treatment with these agents appeared to be more toxic than acute exposure to UV or IR. We note that when tested at the higher tested concentrations of CPT and MMS, which can be expected to cause multiple DSBs each cell cycle, there were no differences between the *ctp1-CD* and *ctp1Δ* strains, yet *ctp1⁺* cells maintained high via-

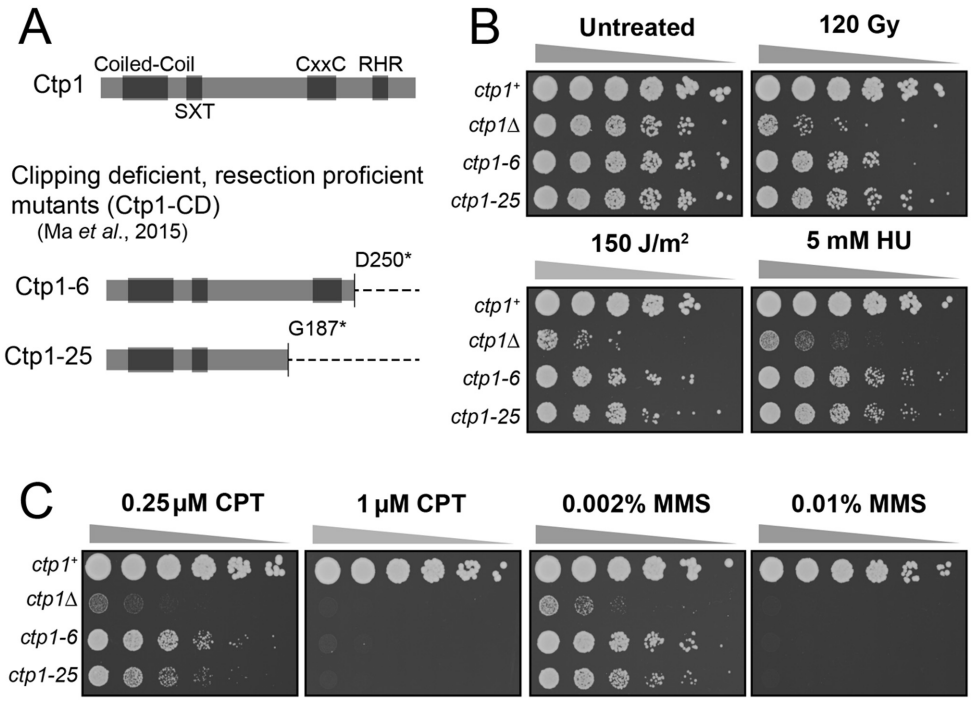

Figure 1. The *ctp1-CD* alleles cause an intermediate level of sensitivity to clastogens. (**A**) The *ctp1-CD* alleles *ctp1-6* and *ctp1-25* are C-terminal truncation mutations that result in loss of regions including the RHR domain or the RHR and CxxC domains, respectively. (**B**) The *ctp1-CD* strains are moderately sensitive to IR, UV and HU. (**C**) C-terminal truncation of $ctp1^+$ results in sensitivity to CPT and MMS, with higher genotoxin concentrations causing lethality comparable to *ctp1Δ*.

bility (Figure 1C). The *ctp1-6* and *ctp1-25* strains used for these studies were obtained directly from Ma *et al.* (16) and genetic crosses confirmed there were no suppressor mutations in these isolates. We further confirmed these findings with endogenous genetic mutations that re-engineered the *ctp1-6* and *ctp1-25* truncations to eliminate the potential readthrough of the nonsense mutations.

The Ku complex blocks HR repair in *ctp1-CD* mutants

The NHEJ promoting Ku complex (composed of Pku70 and Pku80 subunits) and the MRN complex are both able to occupy DNA ends at a DSB regardless of cell cycle stage (6). Removal of MRN and the Ku complex from the ends of a DSB and subsequent repair via HR requires Ctp1 and the endonucleolytic activity of Mre11 (6–9). In the absence of MRN-Ctp1 nuclease activity the Ku complex binds and remains associated with DNA ends. However, NHEJ is an ineffective mechanism of repairing DSBs in actively growing fission yeast cells, and to make matters worse Ku blocks Exo1-mediated resection that can promote HR in the absence of MRN or Ctp1. In the *ctp1-CD* backgrounds, deletion of the Ku complex subunit $pku80^+$ rescues sensitivity to all tested DNA damaging agents (Figure 2). This rescue is especially apparent in cells exposed to CPT and MMS. These data indicate that *ctp1-CD* mutants are unable to efficiently remove non-covalently bound Ku from DNA ends in mitotic cells, just as they are defective at removing covalently bound Rec12 from DNA ends in meiotic cells (16). The fact that eliminating Ku substantially rescues the CPT sensitivity of *ctp1-CD* mutants indicates that these mutants have no defect in removing Top1cc from DNA.

Resection activity provided by Exo1 is required for efficient DSB repair in *ctp1-CD* strains

Exo1 is a 5′ to 3′ exonuclease that is required for the long-range resection of DSBs (6,19–21). Exo1 is not typically required for DSB repair in mitotic cells but it becomes essential for repair when defects in MRN-Ctp1 activity are suppressed by eliminating Ku (7,9). If *ctp1-CD* strains were defective for clipping but not resection we would not expect elimination of Exo1 to enhance *ctp1-CD* genotoxin sensitivity, nor would we expect suppression of *ctp1-CD* genotoxin sensitivity by eliminating Ku to depend on Exo1. However, we observed these effects – the *exo1Δ* mutation enhanced DNA damage sensitivity in *ctp1-CD* backgrounds and ablated rescue of *ctp1-CD* by *pku80Δ* (Figure 3). These effects were detected with all DNA damaging agents but were most obvious in cells chronically treated with MMS or CPT (Figure 3). Ctp1-CD mutants being substantially defective in resection and therefore reliant on Exo1 to complete DSB repair most straightforwardly explain these genetic interactions.

Ctp1-CD strains efficiently repair a single DSB but cannot cope with multiple DSBs

Our genetic investigations indicated that *ctp1-CD* mutations are generally hypomorphic, being partially deficient for both clipping and resection. In such circumstances one

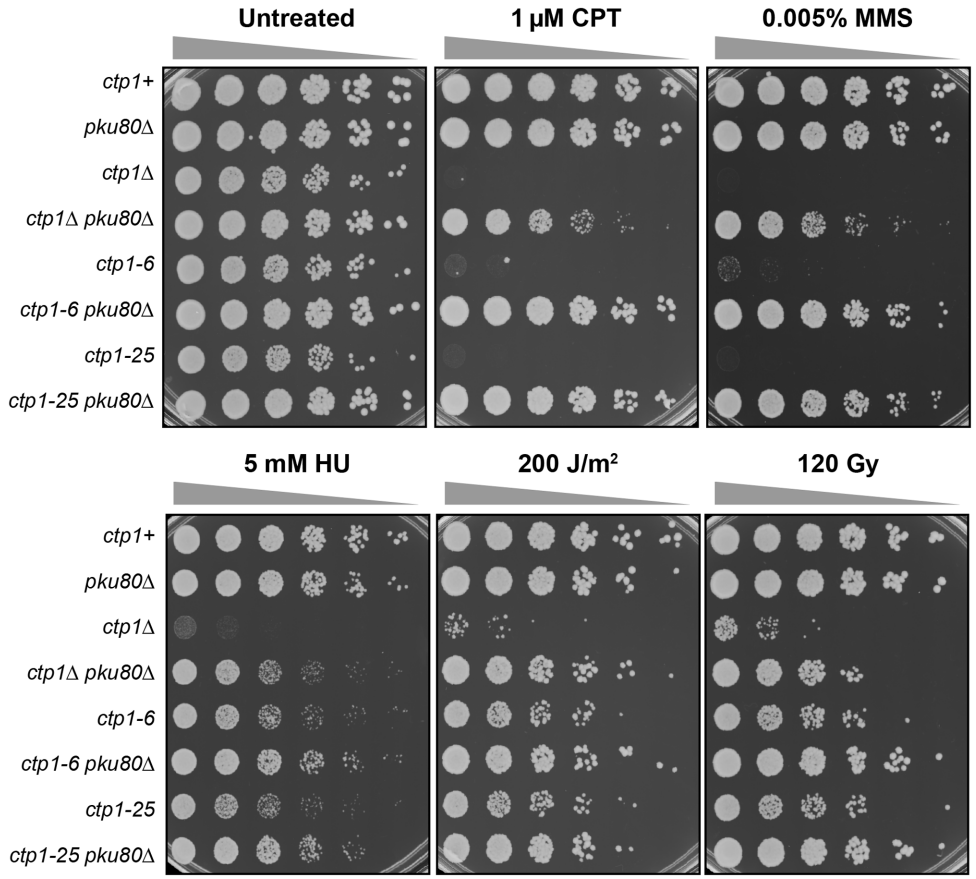

Figure 2. Genetic ablation of the Ku complex suppresses clastogen sensitivity in *ctp1-CD* strains. Strong DNA damage rescue of *ctp1Δ* and *ctp1-CD* strains is achieved by deletion of *pku80*$^{+}$ in the presence of CPT and MMS. In the presence of HU, or following exposure to UV or IR, a mild rescue is achieved.

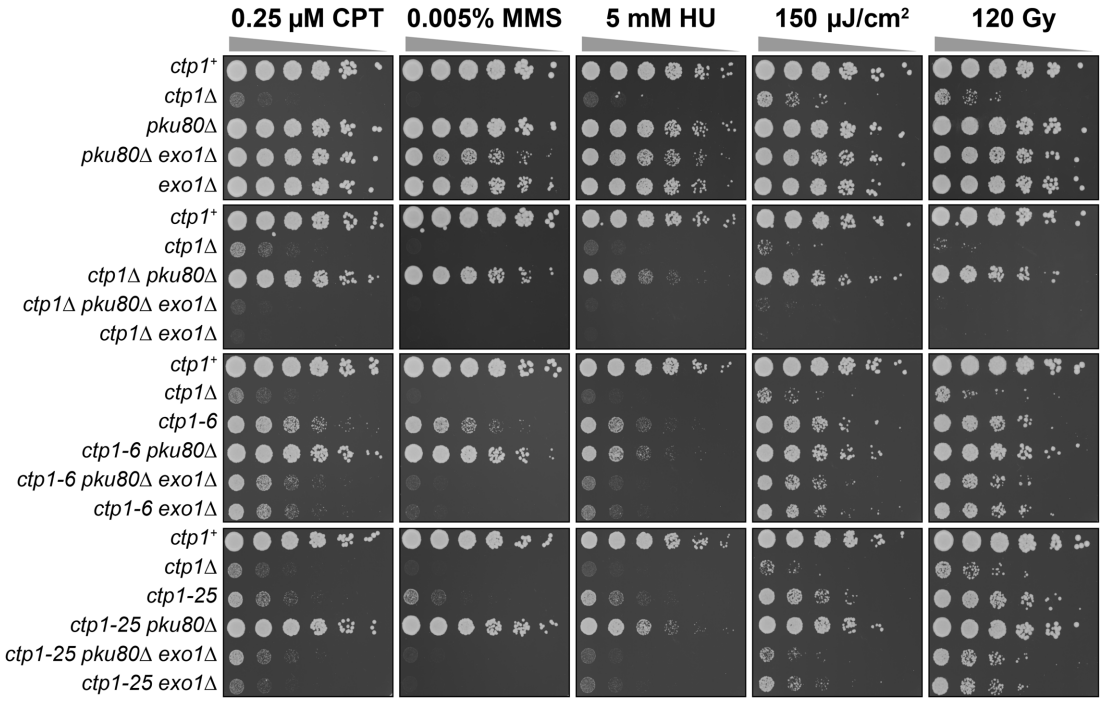

Figure 3. Exo1 is required for rescue of *ctp1-CD* by *pku80Δ*. In the absence of *exo1*$^{+}$, *ctp1-CD* strains are more sensitive to CPT, MMS, HU, UV and IR than in the presence of *exo1*$^{+}$. This indicates that unlike the *ctp1*$^{+}$ background, function of Exo1 is required for DNA repair in the *ctp1-CD* background. This is true regardless of Ku binding to DSBs. These data indicate that *ctp1-CD* mutants have a resection defect.

might expect Ctp1 activity in *ctp1-CD* cells to be sufficient to repair a single DSB but insufficient for multiple breaks. We decided to test this prediction.

The *mat2,3Δ* ('donorless') system takes advantage of the biology of mating-type switching in fission yeast to generate a site-specific broken replication fork that is repaired by HR between sister chromatids (22). Mating type switching is achieved by an imprinting mechanism that creates a programmed single-strand break on the lagging strand during S phase (23). This break is protected from ligation through the entire cell cycle and is converted to a DSB when the replication machinery encounters the break in the subsequent S phase. In this paradigm, of the four cells produced from two cell divisions, one will be like the progenitor cell without an imprint site, one will have repaired a DSB and switched mating types, and two will have an imprint that will be converted to a DSB and repaired during the next cell cycle. This pattern is known as the one-in-four rule. In wild type (h^{90} mating-type) cells, the DSB is repaired using one of two DNA cassettes called *mat2P* and *mat3M* located within the mating type locus. In the *mat2,3Δ* strain, the break is repaired efficiently from the sister chromatid. Key HR repair enzymes, including the MRN-Ctp1 complex, are required for repair of the break with or without the donor cassettes (24). It has previously been shown that *ctp1Δ mat2,3Δ* spores produce microcolonies as predicted if the programmed DSBs at the mating type locus cannot be repaired leading to cell death. This growth defect is largely alleviated in *ctp1Δ mat2,3Δ pku80Δ* strains (6). As expected, of ten tetrads from *ctp1Δ* crossed to *mat2,3Δ*, the *ctp1Δ mat2,3Δ* spores produced only microcolonies that were invisible to the naked eye (Figure 4A). In contrast, *ctp1-6 mat2,3Δ* and *ctp1-25 mat2,3Δ* spores produced visible colonies that in most instances were only modestly smaller than their *ctp1*$^+$ *mat2,3Δ* counterparts. These observations indicate that *ctp1-CD* mutants have a relatively high probability of repairing a single broken fork whereas repair in *ctp1Δ* cells is negligible.

Next, to test the ability of *ctp1-CD* strains to repair multiple broken replication forks, genetic crosses were performed with *cdc17-K42* and *rad2Δ*. The *cdc17*$^+$ gene encodes the essential DNA ligase required for ligation of DNA fragments created during DNA replication (25). Partial defects in Cdc17 activity are expected to leave single-strand DNA nicks that will subsequently lead to replication fork breakage. Although we observed that *cdc17-K42* temperature sensitive mutants grew well at the permissive temperature of 25°C, we found that under the same conditions *cdc17-K42* caused acute lethality in *ctp1Δ* and *ctp1-CD* backgrounds (Figure 4B). This result was corroborated by our analyses of genetic interactions with *rad2Δ*. Rad2 is the FEN-1 flap endonuclease ortholog required for removal of the RNA primer during DNA replication (26). Deletion of *rad2*$^+$ results in persistence of Okazaki DNA fragments that trigger replication fork collapse in the following S-phase (27). We observed that *rad2Δ* has synthetic lethal interactions with *ctp1Δ* and *ctp1-CD* alleles (Figure 4C). Taken together, these data indicate that *ctp1-CD* alleles lower the threshold of lethal DNA damage without making cells acutely sensitive to a single DSB.

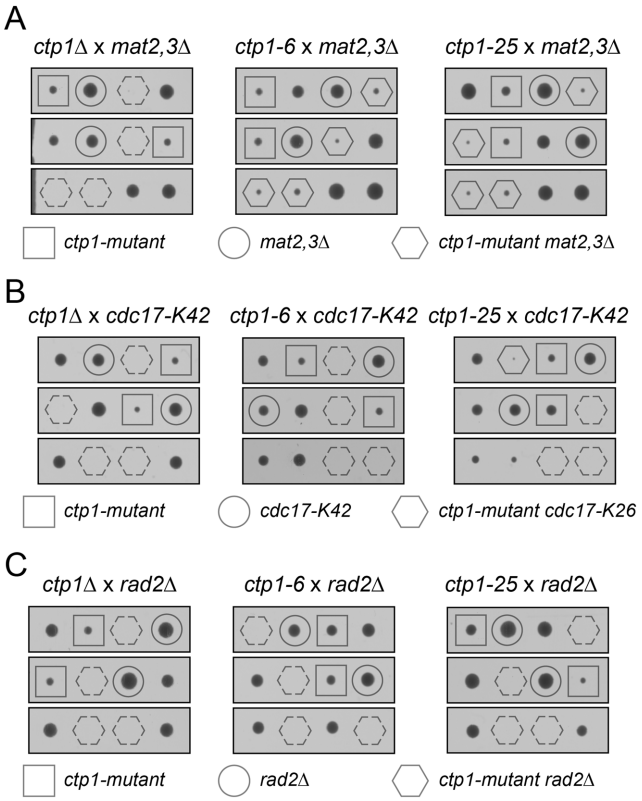

Figure 4. The *ctp1-CD* strains are able to repair a single broken fork but fail to complete repair of more abundant breaks caused by deletion of *rad2*$^+$or a *cdc17-K42* allele. (**A**) Unlike *ctp1Δ mat2,3Δ* (0/7 known double mutant spores formed colonies), the *ctp1-6 mat2,3Δ* and *ctp1-25 mat2,3Δ* spores were able to form colonies (16/17 and 13/16, respectively). (**B**) In contrast, , *ctp1-6 rad2Δ and ctp1-25 rad2Δ spores failed to form colonies (0/25, 0/19 and 0/8 spores formed colonies respectively). (**C**) Similarly, ctp1Δctp1 cdc17-K42*, *ctp1-6 cdc17-K42,* and *ctp1-25 cdc17-K42* spores failed to form colonies or formed microcolonies (0/17, 0/10, and 1/14 respectively) at 25°C. (**C**) Similarly, *ctp1 rad2, ctp1-6 rad2,* and *ctp1-25 rad2* spores failed to form colonies (0/25, 0/19, and 0/8 respectively). Together, these data indicate that *ctp1-CD* mutants are usually able to repair a single broken fork in S-phase, but cannot successfully repair multiple DSBs.

An intact checkpoint is required for repair of DNA damage in *ctp1-CD* strains

In survival assays, it appeared that *ctp1-CD* mutants were less sensitive to IR or UV in comparison to CPT or MMS (Figure 1B and C). This difference might reflect the acute application of DNA damage during IR or UV treatment versus chronic treatment with the other genotoxins. We decided to investigate the role of the DNA damage checkpoint in these situations, as a checkpoint delay of cell cycle progression might be especially important if HR repair is slowed but not ablated in *ctp1-CD* cells. Indeed, we found that elimination of the checkpoint kinase Chk1 dramatically increased DNA damage sensitivity of *ctp1-CD* mutants (Figure 5). The effects were noticeable for all genotoxins although the synergistic effects of combining *chk1Δ* with *ctp1-CD* alleles were especially apparent with IR and UV. Genetic interactions between *ctp1Δ* and *chk1Δ* were

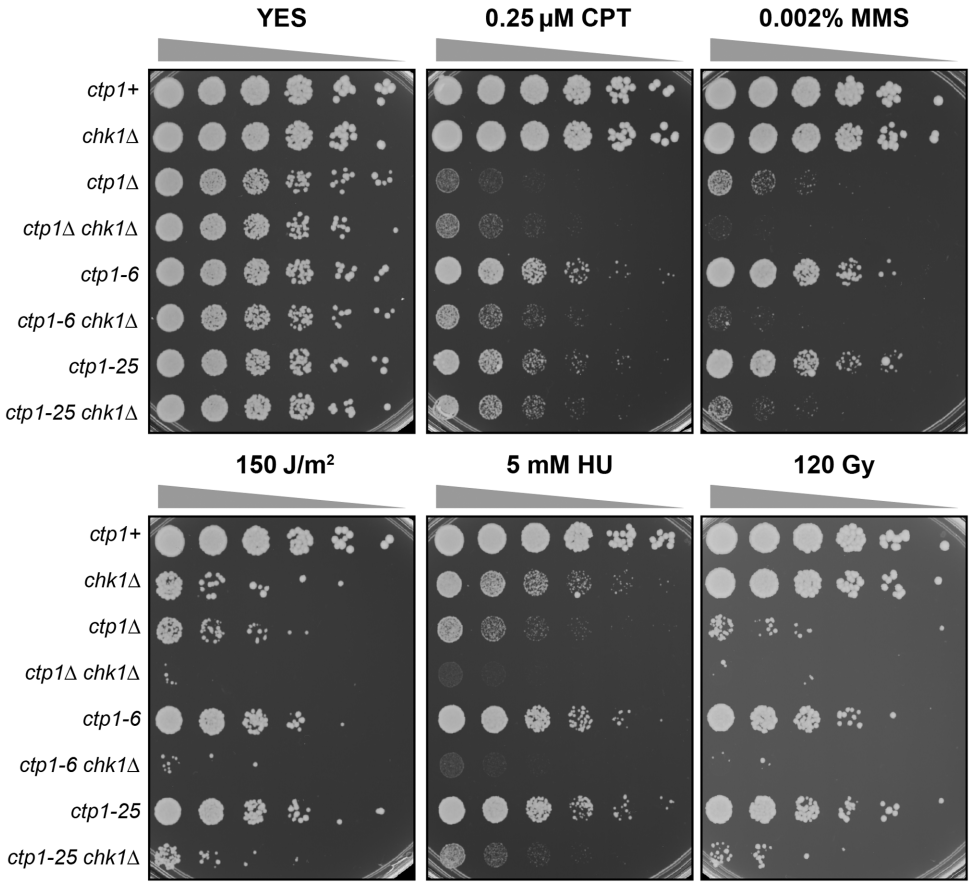

Figure 5. Checkpoint activation is critical for survival of DNA damage in *ctp1-CD* strains. In the absence of Chk1 the *ctp1-CD* mutants are nearly as sensitive as *ctp1Δ*. This effect is especially evident in cells treated with IR.

much weaker, as expected if DSB repair is nearly ablated in *ctp1Δ* cells.

DISCUSSION

The *ctp1-6* and *ctp1-25* mutations impair clipping and resection

MRN and Ctp1 endonucleolytically clip covalently bound Rec12 from 5′ DNA ends, displace non-covalently bound Ku from DSBs, and initiate 5′ to 3′ resection. To fully understand DSB repair, it is critical to know whether these activities are mechanistically distinct. Starting with a screen to identify Ctp1 mutants that were preferentially sensitive to CPT versus MMS, Ma *et al.* uncovered *ctp1* mutants that were unable to efficiently repair DSBs formed by Rec12 yet were competent to resect and repair a meganuclease-generated DSB in meiosis (16). These data strongly suggested that the clipping and resection activities of MRN-Ctp1 are genetically separable. In this study we found that these *ctp1-CD* mutants are sensitive to many DNA damaging agents. They are less sensitive than *ctp1Δ* mutants, but they fail to form colonies in concentrations of CPT or MMS that are expected to cause multiple DSBs each cell cycle, yet are well tolerated by wild type. We uncovered no evidence that *ctp1-CD* mutants are preferentially sensitive to CPT versus MMS. Their DNA damage sensitivities are suppressed by genetically eliminating Ku, indicating that removal of covalently bound Rec12 and non-covalently bound Ku from DNA ends occur by very similar mechanisms requiring endonucleolytic clipping by MRN-Ctp1. These results are consistent with our earlier findings that Ku remains attached to DSBs in *mre11-H134S* mutants that lack endonuclease activity, as well as in *ctp1Δ* cells (6). However, we found that genetic suppression of *ctp1-CD* mutants by eliminating Ku depends on Exo1, which is inconsistent with the notion that *ctp1-CD* mutants are competent for resection. On the other hand, we found that *ctp1-CD* mutants effectively repair a programmed fork break at the mating type locus, unlike *ctp1Δ* cells. The most parsimonious explanation of these findings is that the *ctp1-CD* alleles are non-specifically hypomorphic, impairing both clipping and resection, and they simply lower the threshold of DNA damage that is lethal. These mutants can usually repair a single DSB formed at the mating type locus in S-phase, but they can't cope with DNA ligase or Rad2/FEN-1 mutations that are expected to result in multiple DSBs each cell cycle. By the same token, *ctp1-CD* mutants can effectively repair a single DSB created by a meganuclease in *rec12Δ* cells, but are unable to complete the repair of all of the multiple DSBs formed by Rec12 in meiosis (16). Our findings do not exclude the possibility that removal of Rec12 poses a greater challenge to functionally impaired MRN-

Ctp1, but they argue against the suggestion that the endonucleolytic clipping required to remove Rec12, displace Ku, and initiate resection are genetically separable or mechanistically distinct.

Ctp1 hypomorphs combine severe spore inviability with modestly reduced Rec12-dependent meiotic recombination

The proposal that the *ctp1-CD* mutants are general hypomorphs can explain their apparently contradictory phenotypes of poor spore viability paired with relatively efficient meiotic recombination and resection of a meganuclease formed DSB (16). The overall spore viability of *ctp1-6 and ctp1-25* crosses was less than 0.1% that of *ctp1*$^{+}$(*ctp1Δ* spore viability was less than 0.001%). Of the spores that survived, the rate of recombination in an ∼1.1 megabase interval in *ctp1-CD* crosses was only decreased by a factor of about two compared to *ctp1*$^{+}$, whilst recombination in *ctp1Δ* crosses decreased by a factor of ∼45. These results indicate that there is only a moderately reduced probability of repairing any individual meiotic DSB in *ctp1-CD* crosses, but the probability of repairing all DSBs required for spore viability is very low. Note that approximately 50–150 DSBs are generated per *S. pombe* genome during meiosis (28). As mentioned above, these data are consistent with our observation that a single broken fork is efficiently repaired in the *ctp1-CD mat2,3Δ* background, but the probability of repairing all of the multiple broken forks in *ctp1-CD rad2Δ* or *ctp1-CD cdc17-K42* backgrounds is very low.

Threshold effects in *ctp1-CD* and *sae2Δ* mutants

Other studies have noted that certain DNA repair mutants appear to have stronger than expected clastogen sensitivities relative to their weak defects in resecting and repairing a meganuclease generated DSB. For example, one investigation noted that the resection defect of a budding yeast *sae2Δ* mutant was less pronounced than its IR sensitivity (29). This difference is typically ascribed to Sae2 being specifically required for processing dirty DNA ends that might be generated by IR. However, this explanation does not easily comport with suppressing *sae2Δ* IR sensitivity by genetically eliminating Ku (29), unless one supposes that Ku blocks processing of dirty but not clean DNA ends. There is no evidence to support this idea; indeed, Ku blocks repair of the single clean DNA end formed by fork collapse at the mating type locus in fission yeast *ctp1Δ* cells (6). If Mre11-mediated resection is unaffected by loss of Sae2, it is not obvious why Exo1 should be critical for suppression of *sae2Δ* IR sensitivity by genetically eliminating Ku (29). Nor does the dirty end model provide a straightforward explanation for Sae2 being essential for repairing clean DNA ends formed in the absence of the FEN-1 ortholog Rad27, or the complete suppression of this requirement by eliminating Ku (29).

It is interesting to note that Mre11-Rad50-Xrs2^{Nbs1} (MRX) complex is not required for HR-mediated mating type switching in budding yeast. Thus, even the complete loss of MRX does not abrogate repair of a single DSB. Loss of MRX can therefore be viewed as a situation that lowers the threshold for lethal levels of DSBs, as does loss of Sae2, albeit to a much higher threshold. As previously suggested by Mimitou and Symington (29), the resection properties and clastogen sensitivities of *sae2Δ* mutants are consistent with a model in which they can efficiently repair one or two DSBs formed by a meganuclease but are overwhelmed by the large number of DSBs generated by IR, which is a threshold effect. In this respect there are parallels between *sae2Δ* mutants in budding yeast and *ctp1-CD* mutants in fission yeast.

Checkpoint arrest is critical when HR is impaired

We found that activation of the Chk1-dependent DNA damage checkpoint is critical for survival of *ctp1-CD* mutants exposed to DNA damaging agents, especially when DNA damage is applied in a single acute dose, such as when cells are exposed to ionizing radiation. These observations are consistent with DSB repair being generally inefficient or slow in *ctp1-CD* cells, in that the checkpoint provides more time to complete repair before mitosis. When the checkpoint is eliminated there is only a small difference between *ctp1Δ* and *ctp1-CD* backgrounds in DNA damage sensitivity. This relationship parallels observations with meiosis in which *ctp1-CD* mutants display very poor spore viability that for some alleles nearly approaches the defect seen in *ctp1Δ* backgrounds (16). In this context it is interesting to note that a meiotic progression checkpoint activated by unrepaired DSBs is either very weak or absent in fission yeast (30). The lack of a robust DNA damage checkpoint response during meiosis can further explain why spore viability is so poor in *ctp1-CD* mutants.

Removing Top1-DNA adducts does not require MRN-Ctp1

The screen to isolate *ctp1-CD* mutants assumed that clipping activity provided by the MRN-Ctp1 complex would be required for removal of Top1-DNA adducts (Top1cc) that are stabilized on the 3′ end of DNA in the presence of CPT, analogous to removal of the Rec12 5′ DNA adducts (16). However, multiple pathways are capable of removing Top1 covalent complexes. The tyrosyl-DNA phosphodiesterase (Tdp1) breaks the covalent bond between Top1 and the DNA (31). Tdp1 together with the XPF-ERCC1 family nucleotide excision repair endonuclease (*S. pombe* Rad16-Swi10) initiate the major Top1cc repair pathways in fission yeast (32). Indeed, *ctp1Δ* mutants do not accumulate Top1cc in DNA when treated with CPT (33). Moreover, the suggestion that MRN-Ctp1 could be directly responsible for clipping Top1cc from 3′ DNA ends runs counter to its well-known biochemical activity in removing 5′ linked Rec12 and Top2 from DNA ends (33–36), and it would require MRN-Ctp1 to cut the DNA strand that it typically leaves intact. Finally, if MRN-Ctp1 were defective at removing Top1cc we would not expect genetic elimination of Ku to suppress *ctp1Δ* or *ctp1-CD* mutants, but as we have shown the *pku80Δ* mutation is a very effective suppressor of the CPT sensitivity of these mutants.

Functional domains of Ctp1

The fission yeast Ctp1 protein N-terminal domain has a well-conserved coiled-coil domain that is important for pro-

tein homo-tetramerization (7,14). Also located in the N-terminal region is the SXT motif, which interacts with MRN via the Nbs1 subunit (37). The C-terminal region contains two other conserved sequences, the RHR and CxxC motifs (7). The function of the CxxC motif remains elusive, but the RHR motif has been shown to promote intrinsic Ctp1 DNA binding activity (14). The MR complex has also been shown to dimerize and facilitate DNA binding and bridging of multiple DNA molecules (8). A motif within the coiled-coil domain of Ctp1, 'KKxR' (residues 41–44) and a nearby arginine at residue 32, cooperates with the RHR motif for efficient DNA binding *in vitro* (14). Both motifs are required for efficient DNA bridging. Ctp1 R32A K41A or individual mutation of the arginine residues of the RHR motif results in loss of DNA binding *in vitro*. *In vivo,* the *ctp1-R32A K41A* strain is as sensitive to DNA damage as *ctp1Δ*, while mutation of the arginine residues in RHR results in an intermediate level of sensitivity (14). We can predict that *ctp1-CD* alleles are able to form tetramers and interact with the MRN complex, however, their ability to bind DNA and bridge multiple DNA molecules together is likely impaired due to loss of the RHR motif. Furthermore, the RHR mutants were rescued by *pku80Δ* and this rescue depends on *exo1*$^{+}$, indicating that DNA binding provided by the C-terminal domain is required for proper resection and eviction of Ku from DSBs (14).

Cannavo and Cejka (15) reported that a C-terminal region of Sae2 stimulates a double-stranded DNA (dsDNA) endonuclease activity of MRX that is critical for DNA clipping and the short-range enzymatic resection catalyzed by MRX that is sufficient for repair of most DSBs. There is significant sequence homology between Sae2 and Ctp1 in this C-terminal region, suggesting that the *ctp1-CD* alleles may be specifically defective in this activity.

In summary, the C-terminal domain mutations of Ctp1 isolated by Ma *et al*. are substantially defective in clipping Rec12 from DNA ends (16), but our studies show that these mutants are sensitive to all conditions that create DSBs and they are highly reliant on Exo1 for completing HR repair of DSBs. This reliance on Exo1 is inconsistent with the idea that MRN-Ctp1 clipping and resection activities are mechanistically distinct, and therefore we propose they are one in the same.

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