Two separable functions of Ctp1 in the early steps of meiotic DNA double-strand break repair

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ABSTRACT

Meiotic programmed DNA double-strand break (DSB) repair is essential for crossing-over and viable gamete formation and requires removal of Spo11-oligonucleotide complexes from 5′ ends (clipping) and their resection to generate invasive 3′-end single-stranded DNA (resection). Ctp1 (Com1, Sae2, CtIP homolog) acting with the Mre11-Rad50-Nbs1 (MRN) complex is required in both steps. We isolated multiple *S. pombe ctp1* mutants deficient in clipping but proficient in resection during meiosis. Remarkably, all of the mutations clustered in or near the conserved CxxC or RHR motif in the C-terminal portion. The mutants tested, like *ctp1Δ*, were clipping-deficient by both genetic and physical assays. But, unlike *ctp1Δ*, these mutants were recombination-proficient for Rec12 (Spo11 homolog)-independent break-repair and resection-proficient by physical assay. We conclude that the intracellular Ctp1 C-terminal portion is essential for clipping, while the N-terminal portion is sufficient for DSB end-resection. This conclusion agrees with purified human CtIP resection and endonuclease activities being independent. Our mutants provide intracellular evidence for separable functions of Ctp1. Some mutations truncate Ctp1 in the same region as one of the CtIP mutations linked to the Seckel and Jawad severe developmental syndromes, suggesting that these syndromes are caused by a lack of clipping at DSB ends that require repair.

INTRODUCTION

DNA double-strand breaks (DSBs) are severe lesions that, when left unrepaired, can impair cell viability, genome stability and sexual reproduction. DSBs can occur spontaneously during any stage of the cell cycle by faulty DNA metabolism or by exposure to DNA damaging agents.

During meiosis, DSBs are generated in a programmed manner by an evolutionarily conserved protein Spo11 (called Rec12 in the fission yeast *Schizosaccharomyces pombe*), which has the active site for DSB formation and becomes covalently linked to the 5′ DNA ends at the DSB [1]. Protein bound to DNA ends must be removed to allow further DSB processing. The Spo11-oligonucleotides are released from the DSB ends by endonucleolytic cleavage, referred to as 'clipping.' After Spo11 removal from the ends, naked 5′ ends of DNA are further resected to generate ends with longer 3′ single-stranded (ss) overhangs, referred to as 'resection.' These ssDNA ends are then coated by Rad51 and invade homologous double-stranded DNA (dsDNA) to form joint molecules which are processed to complete recombination, including crossover formation. Therefore, generation of long tracts of ssDNA occurs in two steps: initiation by endonucleolytic removal of Spo11-oligonucleotides and elongation by exonucleolytic resection to produce ssDNA tracts.

In the clipping step, several proteins work in concert to remove Spo11-oligonucleotide complexes from DSB ends. The Mre11-Rad50-Xrs2 (MRX) complex in the budding yeast *Saccharomyces cerevisiae* or its homolog Mre11-Rad50-Nbs1 (MRN) complex in other species recognizes DSB ends [2,3]. Mre11 nuclease activity and Rad50 are required for Spo11-oligonucleotide removal ('clipping') [4–7]. Sae2 (Com1), whose homolog is named Ctp1 in *S. pombe* and CtIP in mammals, also plays an essential role in this process. *S. cerevisiae* Sae2 purified from *E. coli* has endonuclease activity [8], and purified human CtIP was recently reported to have intrinsic endonuclease activity [9,10]. In *S. cerevisiae* there are two size classes of Spo11-attached oligonucleotides [7], but in *S. pombe* there is only one size class [5–6,11]. Sae2, Ctp1 and CtIP physically interact with MRX or MRN via the N-terminal forkhead-associated (FHA) domain of Nbs1 [8,12–13]. The detailed mechanism of how these proteins work together to carry out clipping is still unclear.

Multiple nucleases participate in the resection step for long-tract ssDNA generation. dsDNA end-resection can occur by purified *S. cerevisiae* Sgs1, Dna2 and RPA; purified Top3-Rmi1 and MRX stimulate resection [14]. In human cells, depletion of CtIP, Mre11 or ExoI leads to a dramatic reduction in end-resection from site-specific DSBs [15]. Genetic evidence also suggests that *S. cerevisiae* ExoI,

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Dna2 and the helicase Sgs1 are involved in this process in mitotic cells, but Sgs1 does not play a detectable role in meiotic resection (16,17). Exo1 catalyzes $5' \rightarrow 3'$ exonucleolytic degradation of linear dsDNA (18) and is stimulated by MRX and Sae2 in *S. cerevisiae* (19). However, Sae2 does not appear to function in induced (non-Spo11) site-specific DSB end resection in meiosis, unlike the situation in *S. pombe* (20). In *S. pombe*, Ctp1 is required for the repair of I-*Sce*I-promoted site-specific DSBs to generate meiotic recombinants in the presence of the MRN complex; since I-*Sce*I makes DSBs without protein bound at the ends, Ctp1 is presumably necessary for resection (21).

The observations cited above indicate that during meiosis *S. pombe* Ctp1 participates in both the clipping and resection steps of ssDNA tract generation. Ctp1 shares conserved domains with mammalian CtIP (hence its name) (22) and was also identified as a suppressor of an *nbs1* FHA domain mutation (23). The Ctp1 N-terminal region contains a predicted coiled-coil domain and two casein kinase 2 phosphorylation sites (SXT, amino acids 74–94) within an Nbs1-interacting domain (13). The coiled-coil motif in human CtIP mediates homodimerization (24). *S. pombe* Ctp1 also strongly interacts with itself by yeast two-hybrid assay (22). Recently, the crystal structure of the N-terminal amino acids 5–60 of Ctp1 showed that this region forms a tetramer (dimer of dimers) via its coiled-coil domain, while the rest of the protein is largely intrinsically disordered (25). The C-terminal region is highly conserved from *S. pombe* to human and contains CxxC (amino acids 226–229) and RHR motifs (amino acids 273–275) (Figure 1A). The CxxC motif is potentially involved in zinc chelation, and RHR is part of a DNA-binding motif (25). Ctp1 action along with MRN is required for clipping and presumably for resection (5–6,21). However, there is limited information about which regions within Ctp1 play essential roles in both clipping and resection to promote meiotic recombination.

In this paper we address two questions: how does Ctp1 function in these two steps of meiotic DSBs repair (the initial clipping and the subsequent resection), and are these two functions separable? To answer these questions, we have designed a mutant screen that allowed us to isolate *ctp1* mutants (randomly generated) that were differentially altered in the two functions, clipping and resection. Our results show that the C-terminal region of Ctp1 is required for the clipping function, while the N-terminal region is sufficient for resection. Our separation-of-function study helps elucidate the mechanism of homologous recombination, and it also sheds light on certain rare human diseases, such as the dwarfism disorder Seckel and Jawad syndromes (26), probably caused by an effect on homologous recombination due to CtIP C-terminal truncation mutations similar to certain mutations reported here.

MATERIALS AND METHODS

S. pombe strains

S. pombe strains used in this study and their genotypes are listed in Supplementary Table S4. Media for growth were described (27,28). *ctp1* mutations on the chromosome were constructed as follows: the *ctp1::ura4+* strain was constructed with the method described (29). Primers OL3002 and OL3003 (Supplementary Table S5) with 80 bp of homology to DNA flanking the *ctp1* ORF (open reading frame) were used to amplify the *ura4+* ORF from plasmid pFY20 (30). Strain GP4915 was transformed to uracil-prototrophy with the polymerase chain reaction (PCR) product. Integration at the *ctp1* locus was verified by PCR using primers OL326 and OL3358 (Supplementary Table S5). Transformation of *S. pombe* was performed using the lithium acetate method (29). The *ctp1* mutated ORF with flanking DNA was released from the identified library plasmid by *Bam*HI cleavage, and integrated into the chromosome at the *ctp1* locus by substitution of *ctp1::ura4+* in strain GP7808. The transformants were selected on minimal medium (NBA+Ade+Ura+Arg) containing 1 mg/ml FOA (5-fluoroorotic acid monohydrate; Toronto Research Chemicals, catalog NO. F595000). The correct integration at *ctp1* was identified by PCR using primers OL2624 and OL2979 (Supplementary Table S5).

Analysis of meiotic recombination and viable spore yield

Meiotic recombination and viable spore yields were determined as described (31,32). I-*Sce*I induced meiotic recombination was assayed as described (21).

Construction of *ctp1* mutant library and mutant isolation

The wild-type *ctp1+* gene with 524 bp of 5′ and 504 bp of 3′ flanking DNA was cloned into the low-copy vector pFY20 at the *Bam*HI site to produce plasmid pNM01. Random mutations in the *ctp1* ORF and ~450 bp of flanking sequence were introduced by PCR from pNM01 using the Genemorph II random mutagenesis kit (Agilent catalog NO. 200550) and primers OL3355 and OL3356 (Supplementary Table S5) containing *Bam*HI sites. The mutagenized PCR product (~1.8 kb) was digested with *Bam*HI and ligated into *Bam*HI-digested pFY20. After *E. coli* transformation, ~1000 transformants were pooled; plasmid DNA was prepared and used to transform GP7287 (*ctp1Δ*) to uracil-prototrophy. Transformants were patched onto minimal medium plates to maintain selection for the plasmid and then replica-plated onto rich medium (YEA+Ade) with CPT (1.25 μM) or MMS (0.005%) or neither. DNA was extracted from CPT-sensitive and MMS-resistant yeast cells and used to transform *E. coli* to ampicillin-resistance; plasmid DNA was recovered and sequenced. The mutations were put onto the chromosome as described above.

Assay for Rec12-oligonucleotide clipping

Rec12-oligonucleotide complexes in *ctp1+* and *ctp1* mutants were assayed as described (5). Briefly, meiosis was induced and cells were harvested hourly for 6 h. DNA content was analyzed by flow cytometry (unpublished data similar to those in Supplementary Figure S2). The cells were lysed by beating with glass beads in ice-cold 10% trichloroacetic acid. The precipitated proteins were solubilized in sodium dodecyl sulfate (SDS) extraction buffer, diluted in 2x immunoprecipitation (IP) buffer and incubated overnight with monoclonal anti-FLAG antibody (clone M2, Sigma-Aldrich) pre-bound to magnetic protein
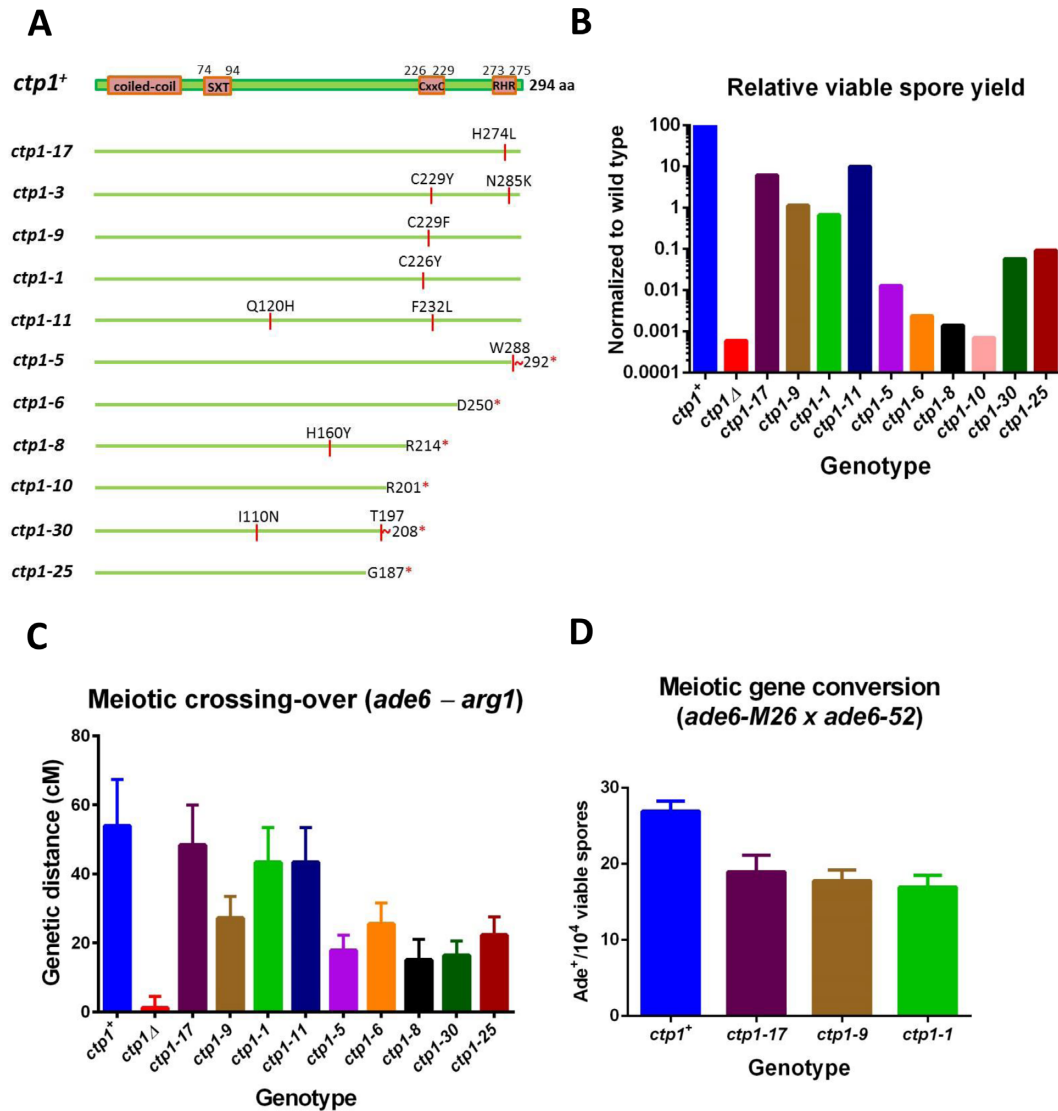
Figure 1. Sequences and phenotypes of new *ctp1* mutants. (**A**) Schematic representation of each of the *ctp1* mutations studied here, indicated by the amino acid change at the codon numbered. * indicates non-sense mutation; red wavy line indicates region of amino acids altered by a frameshift mutation and the ensuing out-of-frame non-sense codon. Data in Figures 1–4 are from strains with these alleles integrated at the endogenous chromosomal locus. (**B**) Relative viable spore yields of *ctp1* mutants. Data are from Supplementary Table S2, 'chr' column. (**C**) Meiotic crossing-over between *ade6* and *arg1* in *ctp1* mutants. Data are from Supplementary Table S2, 'chr' column. Error bars are the SD of observed frequencies converted to cM using Haldane's equation. (**D**) Meiotic recombination between *ade6-M26* and *ade5–52* in *ctp1* mutants. Data are from Supplementary Table S2. Error bars are SEM; *n* = 4 for each strain.

G-agarose beads (Dynabeads, Invitrogen). After washing of the beads, the bound oligonucleotides were labeled using terminal deoxynucleotidyltransferase (TdT) and [α-^{32}P] dCTP. The complexes were washed and eluted by boiling. The eluted Rec12-oligos were separated by SDS-PAGE and transferred to Immobilon-P membrane (Millipore). The DNA was detected by exposure to X-ray film, and the Rec12-FLAG protein was detected by immunoblotting using anti-FLAG antibody conjugated to horseradish peroxidase (clone M2, Sigma) and an enhanced chemiluminescence detection kit (Supersignal, WestPico-Pierce).

Assay for resection at I-*Ppo*I-induced DSBs

All strains used for the resection assay harbor a single I-*Ppo*I cleavage site near the 5′ end of *lys1*. Meiosis was induced and DNA content was analyzed by flow cytometry as described (33) (Supplementary Figure S2). Three h after induction of meiosis, 3 μM anhydrotetracycline (ahTet) (Acros 13803–65–1) was added to the culture, and samples were harvested hourly for 4 h after addition of ahTet. Cells were embedded in agarose plugs, and DNA was extracted as described (28). DNA in agarose plugs was digested with *Xba*I overnight at 37°C, electrophoresed and transferred to a nylon membrane (Zeta-probe GT membrane, Biorad). A 1-kb PCR product (primers OL3240 and OL3241) labeled with [α-^{32}P] dCTP (3000 Ci/mmol) was used as a probe

to detect resected DNA, which hybridized 3–4 kb away from the I-*Ppo*I cutting site (Figure 4A). Signals were detected using a Typhoon storage PhosphorImaging system (GE Healthcare).

RESULTS

A screen to isolate *ctp1* mutants differentially altered in clipping and resection

Our genetic screen to isolate *ctp1* mutants takes advantage of the sensitivity of *ctp1Δ* mutants to two DNA-damaging agents during mitotic growth: CPT (camptothecin) and MMS (methyl methanesulfonate) (22,23). CPT stabilizes topoisomerase I-DNA cleavage complexes (34), which must be removed from the DNA ends for DNA repair. This step may be similar to the removal of Rec12 during meiosis ('clipping'). These proteins are linked to the 3′ and 5′ ends, respectively, but purified Sae2 with MRX can cleave DNA with either type of linkage (35). Lesions caused by MMS are likely 'clean' DNA ends (i.e. without bound protein) (36), and hence the broken ends need only to be resected further to generate 3′ ssDNA overhangs for DNA repair. This step may be similar to the resection after Rec12 removal. Our screen used the relative difference in sensitivity to CPT and MMS as a guide to isolate interesting novel *ctp1* mutants. We presumed that a CPT-sensitive but MMS-resistant phenotype reflects deficiency of the clipping function but proficiency of the resection function. Although our screen used mitotic phenotypes as a way to isolate novel mutants, we draw our conclusions from assays in meiotic cells. Our meiotic physical and genetic assays verified these expectations of the mutants we isolated and describe here.

ctp1 mutations clustered in the C-terminus differentially alter sensitivity to CPT and MMS

With the genetic screen described above and in Materials and Methods, we isolated eleven novel *ctp1* mutants. As controls, we used the *mre11-D65N* mutant, which is nuclease-deficient, does not release Rec12 from DSB ends, and is CPT-sensitive but MMS-resistant (5,37). We also used *ctp1Δ*, which neither releases Rec12 from DSB ends nor promotes resection and is both CPT-sensitive and MMS-sensitive. We used a range of concentrations and found that 1.25 μM CPT was the lowest concentration at which neither *mre11-D65N* nor *ctp1Δ* grew and that 0.005% MMS was the highest concentration at which *mre11-D65N* grew but *ctp1Δ* did not grow (Supplementary Table S1). Therefore, the screening was carried out using 1.25 μM CPT and 0.005% MMS.

A library of *ctp1* mutant genes was prepared by PCR random mutagenesis of the *ctp1* open reading frame flanked by ~450 bp of non-coding DNA cloned into the low-copy plasmid pFY20 (30). The plasmid was transformed into an *S. pombe* strain bearing a *ctp1* complete deletion (*ctp1Δ*). The transformants were then tested for CPT and MMS resistance on solid rich media. Among the ~9400 colonies tested, 52 were differentially CPT-sensitive and MMS-resistant compared to *ctp1+*, which is resistant to both drugs, and to *ctp1Δ*, which is sensitive to both. The

ctp1 genes in these 52 isolates were sequenced, and eleven unique mutants were identified, along with multiple sisters.

All of the mutations clustered in the C-terminal region of the gene (Figure 1A). Three mutations (*ctp1-1*, *ctp1-3* and *ctp1-9*) fell in the conserved CxxC domain, and one (*ctp1-17*) in the conserved RHR domain. Moreover, three C-terminal truncation (nonsense) mutations with slight MMS-resistance but strong CPT-sensitivity (*ctp1-8, ctp1-10* and *ctp1-30*) were also identified. All of the mutated genes (except for *ctp1-3,* which had two mutations, one of which was at the same position as that in *ctp1-9*) were transferred to the *ctp1* chromosomal locus, and the sensitivity to CPT and MMS was tested (Supplementary Figure S1A). Eight of these ten mutants were more resistant to MMS than *ctp1Δ* and were strongly CPT-sensitive. Chromosomal versions of *ctp1-8, ctp1-10* and *ctp1-30* had a nearly null phenotype in these sensitivity assays, and *ctp1-11* was nearly wild-type, although the plasmid-borne versions had slightly different phenotypes (Supplementary Figure S1). All of the mutants described here were more proficient than *ctp1Δ* in other assays described below, indicating that they are not null mutants. Three mutants (*ctp1-8*, *ctp1-11* and *ctp1-30*), like *ctp1-3*, had two mutations each and were less thoroughly characterized below. The *ctp1-H274A* mutant protein, altered in the same amino acid as *ctp1-17* (H274L), and other missense and non-sense mutant proteins studied by Andres et al. (25), are expressed at the same level as the wild-type protein. We therefore expect that these mutant phenotypes are caused by alterations in Ctp1's activity (or activities), and not to altered levels of protein abundance. We cannot exclude, however, more complex scenarios in which Ctp1 protein levels are reduced in some mutants and resection is less sensitive than clipping to reductions in Ctp1 protein levels.

Comparison of three non-sense mutants suggested a curious feature of Ctp1. Truncation of the 44 C-terminal amino acids in *ctp1-6* produced partial MMS-resistance; truncation of the 83 C-terminal amino acids in *ctp1-10* produced strong MMS-sensitivity; but truncation of the 97 C-terminal amino acids in *ctp1-25* produced partial MMS-resistance (Supplementary Figure S1A). Perhaps the 15 amino acids (from 187 to 201) between the *ctp1-10* and *ctp1-25* truncation points affect protein folding and alter the function of the N-terminal portion required for MMS-resistance. We also noted that *ctp1-5*, a frameshift mutation at W288, removing only seven codons from the C-terminus, was fully CPT-sensitive, indicating a critical function of these C-terminal amino acids.

ctp1 C-terminal mutations severely reduce the yield of viable spores

To assess the overall function of Ctp1, the viable spore yields of the *ctp1* mutants were determined by crossing strains with the chromosomally integrated *ctp1* mutations with a *ctp1Δ* strain. Homozygous *ctp1Δ* produced less than 0.001% as many viable spores as wild-type *ctp1+* (reduction by a factor of 10^5). Most of the C-terminal truncation mutations (*ctp1-5*, *ctp1-6*, *ctp1-8*, *ctp1-25* and *ctp1-30*) reduced the viable spore yields to less than 0.1% of wild type, but the mutant yields were still 2- to 100-fold higher than that of *ctp1Δ*; the viable spore yield of *ctp1-10* was comparable

to that of *ctp1Δ* (Figure 1B, Supplementary Table S2). The viable spore yields decreased with increasing extent of the truncation, except for *ctp1-25 and ctp1-30.* All four missense mutations (*ctp1-1*, *ctp1-9*, *ctp1-11* and *ctp1-17*) reduced the viable spore yields to 0.5–10% of that of wild type. The low viable spore yields in *ctp1* mutants are consistent with the requirement of Ctp1 in meiosis (22).

The variable decrease of viable spore yield in these new *ctp1* mutants might be caused either by a deficiency in Rec12 clipping or a deficiency in resection after Rec12 removal. After Ctp1-mediated Rec12 clipping, at least 10–30 nucleotides of ssDNA overhang with a 3′ end are left at the DSB (11). This short resected DNA may be sufficient for some DSB repair (recombination) and viable spore formation by redundant factors other than Ctp1; for example, in addition to the Ctp1-MRN complex, Exo1 may resect the ends (21). These considerations suggest to us that the greater reduction of viable spore yield in some *ctp1* mutants reflects their greater deficiency in clipping, although both clipping and resection promoted by Ctp1 may play a role in viable spore formation.

ctp1 C-terminal mutants have only slightly reduced Rec12-dependent meiotic recombination

To assess meiotic recombination among the viable spores in *ctp1* mutants with sufficiently high viable spore yield, we measured nine mutants for crossing over (as *ade6–arg1* intergenic recombination) and three mutants for both crossing over and gene conversion (as *ade6* intragenic recombination). Crossing over between *ade6* and *arg1* was reduced, compared to wild type, by a factor of 45 in *ctp1Δ* but by a factor of three or less in the C-terminal *ctp1* mutants (Figure 1C). Four missense mutants altered in or near the CxxC motif or RHR motif had intergenic recombination levels not significantly different from that in wild type (*ctp1-17, ctp1-1* and *ctp1-11*) or about half (*ctp1-9*) that in wild type (Figure 1C and Supplementary Table S2). These mutants yielded enough viable spores to allow measurement of intragenic recombination, which for the three tested was ∼60–70% as high as that in wild type (Figure 1D). We infer that the Ctp1 C-terminal mutants are resection-proficient (from the meiotic recombination results) but clipping-deficient to varying degrees (from the viable spore yield results). This inference was confirmed by direct physical assays below.

ctp1 C-terminal mutants are clipping-deficient during meiosis

To assay the *ctp1* mutants' clipping function directly, we measured Rec12-oligonucleotides by immunoprecipitating Rec12 covalently linked to DNA, whose 3′ ends were radioactively labeled following extraction from meiotic cells (5). *S. pombe* Ctp1 is essential to release Rec12 from the DNA ends, since no Rec12-oligonucleotide complexes can be detected in *ctp1Δ* cells after DSB formation (5,6). We tested representative mutants with low (*ctp1-6*), medium (*ctp1-25*) and high (*ctp1-17*) viable spore yields. Abundant Rec12-oligonucleotides were detected at 4 h after meiotic induction in *ctp1*⁺ cells (Figure 2A). No detectable Rec12-oligonucleotides were observed even 6 h after meiotic induction in *ctp1Δ*, *ctp1-17* (RHR mutation) and *ctp1-6* (44

amino acid C-terminal truncation) (Figure 2B, C and D, upper panels). Similar results were observed with *ctp1-1* cells (CxxC mutation; unpublished data). There was a faint Rec12-oligo signal at the position of the principal signal from wild type detectable in *ctp1-25* (107 amino acid C-terminal truncation) (Figure 2E). There was also a weak signal of material with heterogeneous low mobility in *ctp1-6* and *ctp1-25;* this signal might reflect clipping farther from the DSB end than the length of the Rec12-oligos (10–30 nucleotides long) would predict, but in any case the amount of this material was much less than that of Rec12-oligos in wild type. Rec12-FLAG protein appeared after induction in both *ctp1*⁺ and *ctp1* mutant cells (Figure 2A–E, lower panels), indicating that the lack of Rec12-oligonucleotides was not caused by the failure to induce Rec12 protein. Thus, these three *ctp1* mutants, carrying a C-terminal CxxC or RHR motif mutation or a truncation mutation, like *ctp1Δ*, do not detectably release Rec12-oligonucleotide complexes.

Clipping-deficient *ctp1* C-terminal mutants are proficient for I-*Sce*I induced meiotic recombination

To study the intracellular resection function of *ctp1* mutants in meiotic DSB repair without the need for Rec12 removal from DSB ends, we introduced the I-*Sce*I cut site into *ade6* (*ade6–3061*) and replaced the *rec12* coding sequence with the I-*Sce*I coding sequence to supply meiotically induced I-*Sce*I endonuclease and to eliminate all other (Rec12-dependent) DSBs (21). Thus, the breakage at *ade6–3061* without protein attachment was the only break in the entire genome. In meiosis, DNA end resection must occur to allow repair of this I-*Sce*I-induced break and recombination. To investigate the resection function of Ctp1, we measured recombination between *ade6–3061* and *ade6–52* (772 bp apart) in the *ctp1* mutants with single codon changes.

In the *ctp1Δ* cells, I-*Sce*I-induced meiotic recombination was reduced by a factor of five (Figure 3 and Supplementary Table S3), a reflection of Ctp1's role in resection, as reported by Farah et al. (21). Two mutants [*ctp1-10* (R201*) and *ctp1-17* (H274L)] had recombination levels indistinguishable from that of the wild type (*P* > 0.3 by *t*-test). The other five tested C-terminal mutants (c*tp1–1*, *ctp1-5*, *ctp1-6*, *ctp1-9* and *ctp1-25*) had even higher recombination frequencies than the wild type (*P* < 0.03 by *t*-test) (Figure 3 and Supplementary Table S3). Overall, the *ctp1* C-terminal mutants were highly proficient in meiotic recombination initiated by Rec12-independent breaks. These results suggest that alteration or lack of the Ctp1 C-terminal region does not affect its resection function and that the Ctp1 N-terminal region is needed for resection.

Recombination-proficient *ctp1* C-terminal mutants are resection-proficient by physical assay

We wanted to directly assay meiotic DSB-end resection by a physical method. I-*Sce*I cuts only about 0.6% of the total intracellular DNA, however, and resected DNA is undetectable by Southern blot hybridization (21). The homing endonuclease I-*Ppo*I rapidly cuts >80% of the DNA containing its cognate site within 1 h after induction by anhydrotetracycline (ahTet) (38), which allowed the development of a convenient physical resection assay in *S. pombe*
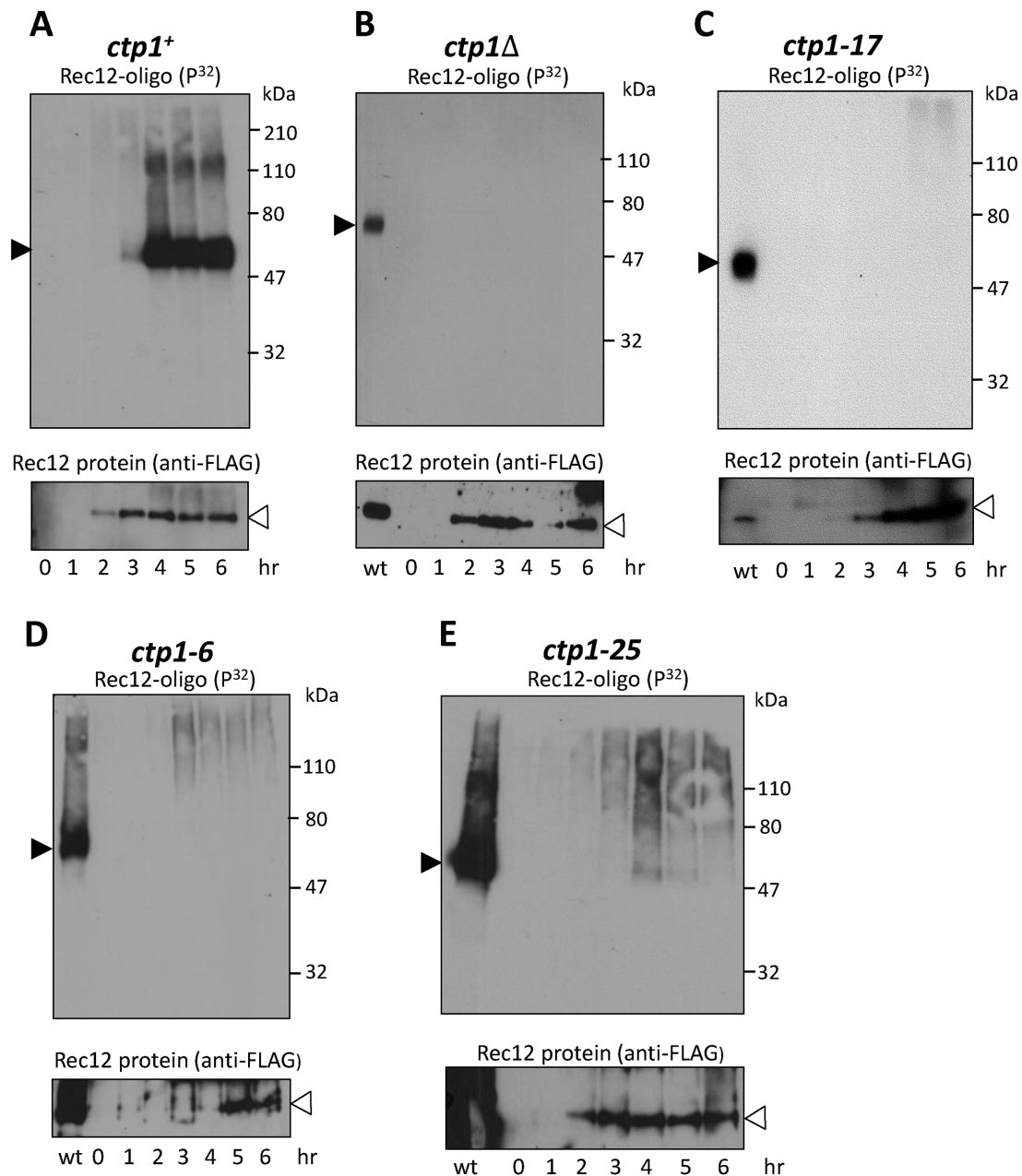
Figure 2. *ctp1* C-terminal mutants, like *ctp1Δ*, release little or no detectable Rec12-oligonucleotide complexes. Strains with the indicated chromosomal *ctp1* mutations, *ctp1+* (**A**), *ctp1Δ* (**B**), *ctp1-17* (**C**), *ctp1-6* (**D**) or *ctp1-25* (**E**), were induced for meiosis, and extracted DNA was assayed at the indicated times for Rec12-oligos by labeling with [α-^{32}P] dCTP and TdT, followed by gel electrophoresis. In panels (**B**) and (**C**), about four times more cell extract was loaded for the mutants than for *ctp1+*, to increase the sensitivity of detecting Rec12-oligos in the mutants. The membrane was exposed to X-ray film to detect ^{32}P (top panel) and then western blotted to detect Rec12-FLAG (bottom panel) using anti-FLAG antibody. Filled arrowhead, ^{32}P-labeled Rec12-oligonucleotide complexes; open arrowhead, total Rec12 protein. 'wt' in panels B, C, D and E indicates *ctp1+* cell extract assayed at 4 h after meiotic induction.

during meiosis. For this assay, Rec12 was deleted and a single I-*Ppo*I cut site was introduced near the *lys1* locus (Figure 4A). I-*Ppo*I expression was tightly controlled with an ahTet-inducible promoter. Cut DNA was detected by Southern blot hybridization as a 3.9 kb fragment after *Xba*I digestion. DNA that was resected, either on one or both strands, was detected as a smear of DNA migrating more rapidly than the cut DNA fragment (Figure 4A). We tested this system first in *ctp1+* cells and detected the resected DNA, but the

observed amount of resected DNA varied between different experiments (unpublished data). This variation may reflect a dynamic balance between breakage and repair, which may vary between experiments. *S. pombe* Mei4 is a meiotic transcription factor that regulates the induction of many meiotic middle genes, including *exo1* but not *ctp1* (39). Mei4 does not significantly influence meiotic DNA replication, an early meiotic event (31). In order to block DSB repair and allow accumulation of resected DNA, we assayed re-
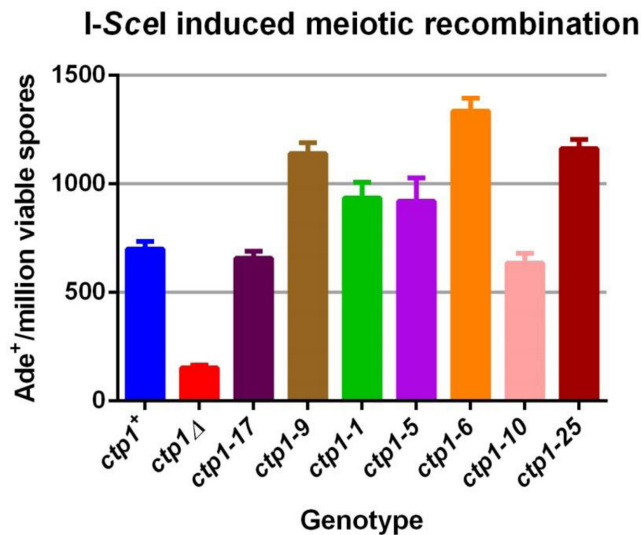
I-*Sce*I induced meiotic recombination

Figure 3. I-*Sce*I-dependent meiotic recombination of *ctp1* C-terminal region mutants is the same as or even higher than that of *ctp1⁺* cells. Ade⁺ recombinants were measured in crosses with the I-*Sce*I-cuttable allele *ade6–3061* and *ade6–52* in strains with the indicated *ctp1* mutations on the chromosome. Data are from Supplementary Table S3. Error bars are SEM; $n = 16$ for *ctp1⁺* and *ctp1Δ*, and 4 for the other mutants.

section from the I-*Ppo*I-induced DSB in *ctp1* mutant cells in a *mei4Δ* background, in which the DNA repair activity is subdued and in which the amount of resected DNA in *ctp1⁺* cells was more reproducible (unpublished data).

In these experiments ahTet was added 3 h after meiotic induction (i.e. about 1 h after DNA replication) (Supplementary Figure S2). In both *ctp1⁺* and *ctp1* mutant cells I-*Ppo*I cut DNA appeared at 1 h after ahTet induction (Figure 4B, C and E) and about 80% of total DNA was cut (left panels in Figure 4D and F). Resected DNA appeared with similar kinetics and extent in *ctp1⁺* and *ctp1* C-terminal missense mutants [*ctp1-1* (CxxC mutation) and *ctp1-17* (RHR mutation)] and nonsense mutants [*ctp1-6* (44 amino acid truncation), *ctp1-10* (93 amino acid truncation) and *ctp1-25* (107 amino acid truncation)]. All of these mutants were recombination-proficient in the I-*Sce*I recombination assay (Figure 3). In *ctp1Δ* cells the amount of resected DNA was about 50% of that in wild-type cells (mean of four time points after induction in three inductions of wild type and two inductions of *ctp1Δ*; right panels in Figure 4D). For comparison, resected DNA in mitotically growing *ctp1Δ* mutant cells, determined by a different assay at HO-induced site-specific DSBs, is about 60% of that in wild-type cells, when assayed either near or far from the break site (40). In our assay, quantification showed that the level of resected DNA among cut DNA in *ctp1* C-terminal mutant cells was 90–100% as much as that in wild-type cells (mean of four time points after induction; right panels in Figure 4D and F). From this physical resection assay, we conclude that the *ctp1* C-terminal mutants are proficient for resection, which is consistent with their proficiency for both Rec12-dependent and -independent recombination (Figures 1C, D, 3 and 4).

DISCUSSION

In this study, we isolated and investigated eleven *ctp1* randomly generated mutations to understand Ctp1 clipping and resection functions in meiotic DSB repair and to address the question of whether these two functions are separable. We used an established physical assay to investigate the clipping function by detecting Rec12-oligonuclotides and developed a physical assay to investigate resection by detecting the resected DNA directly. These two assays together with the genetic assays have allowed us to dissect molecularly the two functions of clipping and resection in these *ctp1* mutants within meiotic cells. The *ctp1* C-terminal mutants tested were clipping-deficient (Figure 2) but resection-proficient (Figure 4) and exhibited proficient recombination, both Rec12-dependent and independent (Figures 1C, D and 3). Our genetic and biochemical data are consistent with each other and indicate that the two functions of Ctp1 in meiotic DNA DSB repair are indeed separable. Our data indicate that the C-terminal region is required for clipping but not for resection, which requires the N-terminal region.

Conserved CxxC and RHR motifs in the Ctp1 C-terminal portion are required for clipping function

ctp1 mutants generated by random mutagenesis and showing differential CPT- and MMS-sensitivity, relative to *ctp1⁺* and *ctp1Δ*, had alterations clustered in the C-terminal portion of the protein (Figure 1A and Supplementary Figure S1). Among the eleven *ctp1* mutants studied, four had mutations in the highly conserved CxxC or RHR motif, indicating their importance in Ctp1 function. The ten *ctp1* C-terminal mutants tested further had reduced viable spore yields (Figure 1B), and all four mutants tested by physical assay were incapable of clipping (Figure 2; unpublished data). *ctp1Δ* cells had strongly reduced viable spore yields (0.001% of that of wild type) but a much lesser reduction in recombination (2% of that of wild type; Figure 1B and C; Supplementary Table S2). We infer from these differential results that Ctp1 may be, with the MRN complex, the most critical factor for clipping, in order to produce viable spores, while there are other redundant factors, such as Exo1 and Sgs1-Dna2 in *S. cerevisiae*, for resection and recombination (17,21,41). Since the viable spore yield in three of the five *ctp1* C-terminal mutants tested for resection was much higher (0.1–10% of wild type) than that in *ctp1Δ* (0.001% of wild type), there must be some residual clipping to allow viable spore formation, at least in these three mutants. Furthermore, the drastically low viable spore yield of *ctp1Δ* implies that there is no redundant factor responsible for clipping, and that there is residual clipping activity in the Ctp1 mutant proteins too low to be detected by our physical assay (Figure 2).

Removal of Rec12 bound to DNA ends (clipping) requires endonuclease activity, which could in principle be provided by either Ctp1 or the MRN complex. Purified *S. cerevisiae* Sae2 and human CtIP have been reported to possess endonuclease activity (8–10). Recently, Cannavo and Cejka (35) reported that purified Sae2 promotes MRX endonuclease activity to clip dsDNA with protein bound to
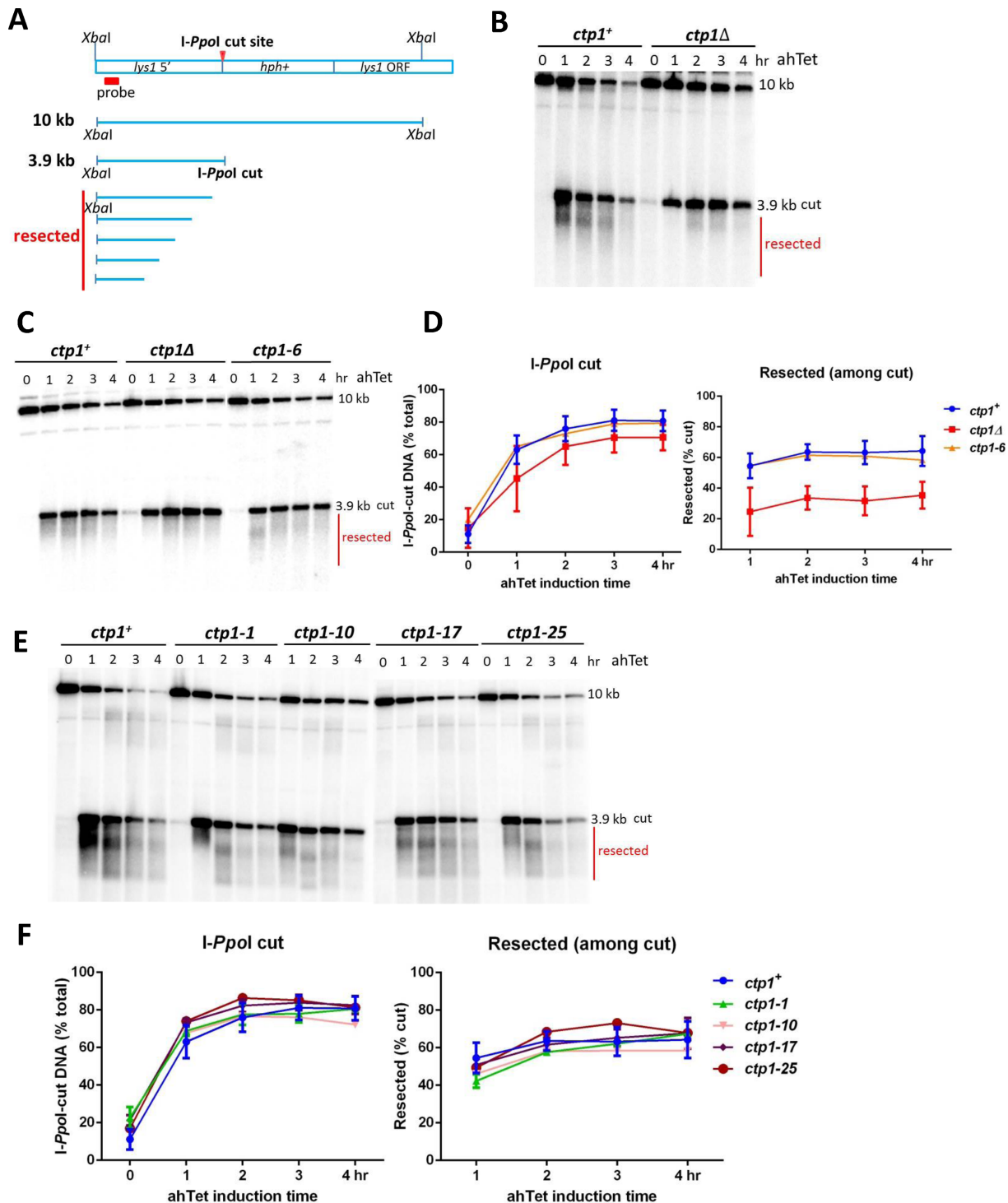
Figure 4. *ctp1* C-terminal mutants are proficient for DSB end resection. (**A**) Diagram of the 10 kb *Xba*I chromosomal DNA fragment containing the I-*Ppo*I cut site, the 3.9 kb I-*Ppo*I cut fragment, and the resected DNA fragments (smear). The red box is the location of the probe for Southern blots. (**B**, **C** and **E**) DNA was extracted from strains with the indicated chromosomal *ctp1* mutations at the indicated times after addition of 3 μM anhydrotetracycline (ahTet) added 3 h after meiotic induction, when DNA replication was complete (Supplementary Figure S2). DNA was digested with *Xba*I, electrophoresed, transferred to a membrane and hybridized with the probe indicated in panel (A). Resected DNA is indicated with a red line. (**D** and **F**) Quantification of I-*Ppo*I cut and resected DNA in parallel from panels (B), (C) and (E). The radioactivity on the blots in panels (B), (C) and (E) was quantified with a PhosphorImager. Data are the mean ± SEM of three (for wild-type cells) or the range for two (for mutant cells except *ctp1-6*, *ctp1-10* and *ctp1-25*) independent experiments. For each strain, I-*Ppo*I-cut data are the percent of the total I-*Ppo*I-cut DNA (3.9 kb cut band plus the resected fraction) at each time point relative to the sum of all *Xba*I released DNA (total of 10 kb *Xba*I cut band, 3.9 kb cut band and resected fraction). Resected data are the percentage of the resected DNA at each time point relative to the total I-*Ppo*I cut DNA. *ctp1⁺* DNA in panels (B), (C) and (E) was from three separate inductions. *ctp1Δ* DNA in panels (B) and (C) was from two separate inductions. In panel C, *ctp1⁺* and *ctp1Δ* were on one blot, and *ctp1-6* on another. In panel E, *ctp1⁺*, *ctp1-1* and *ctp1-10* were on one blot, and *ctp1-17* and *ctp1-25* on another.

the ends. A Sae2 C-terminal truncation or mutants altered in the conserved C-terminal residues R264 or R300, which corresponds to the second 'R' of the RHR motif in *S. pombe*, do not stimulate Mre11 endonuclease activity on protein-bound dsDNA. Moreover, the C-terminal portion of Sae2 alone can promote MRX endonuclease activity. The amino acids regulating human CtIP endonuclease activity are dispersed in the primary structure of the protein (9). Truncation of CtIP at position 790, which is equivalent to truncation at position 210 of Ctp1, deletes the CxxC and RHR motifs from the protein and reduces CtIP endonuclease activity (9). These data, like ours, demonstrate the importance of the C-terminal region of Ctp1 (Sae2, CtIP) for clipping.

It is still uncertain whether the active site(s) for the clipping and resection activities are in MRX (MRN) or Sae2 (Ctp1 or CtIP) and whether there is one catalytic site or two. Genetic assays indicate that both MRN and Ctp1 are required for both activities (4,21). The simplest view is that a catalytic site is in one protein and that the other protein is required for this activity as an allosteric effector. Sae2 purified from sf9 insect cells and Ctp1 purified from *E. coli* have no detectable nuclease activity (25,35), although Sae2 purified from *E. coli* does have endonuclease activity (8). It cannot be excluded, however, that the first two purified proteins have endonuclease activity but that it was not detectable under the conditions used. Our genetic evidence strongly indicates that the C-terminal region of Ctp1 is important for clipping, either for the intrinsic nuclease activity or to stimulate MRX's endonuclease activity. In the present study, we observed that a single amino acid change in the CxxC motif (*ctp1-1*) or in the RHR motif (*ctp1-17*) or eliminating RHR motif (*ctp1-6*) prevented cells from clipping off Rec12-oligonucleotides (Figure 2C and D; unpublished data). *ctp1-25*, with a 107 amino acid C-terminal truncation, eliminated both motifs and, as expected, was clipping-deficient (Figure 2E). In the absence of any direct evidence showing that Ctp1 in *S. pombe* has endonuclease activity, our results strongly indicate that the C-terminal part of Ctp1 is required for the nuclease activity involved in Rec12 removal, either by stimulating MRN or via its intrinsic endonuclease activity.

The most C-terminal seven amino acids of Ctp1 are crucial for its function

The *ctp1-5* mutation has a frameshift mutation at W288, which is expected to produce a Ctp1 protein lacking only the C-terminal seven amino acids. Interestingly, these cells showed severely reduced viable spore yields (∼0.01% of wild type, i.e. only about 10-fold above that of *ctp1Δ*; Figure 1B and Supplementary Table S2), and they were as CPT-sensitive as *ctp1Δ* (Supplementary Figure S1A). In sharp contrast, they were only partially sensitive to MMS (Supplementary Figure S1A) and only modestly reduced for Rec12-dependent meiotic crossing-over (3-fold down from that in wild type) and even slightly more proficient than wild type for I-*Sce*I induced meiotic recombination (Figures 1C and 3; Supplementary Table S2). Thus, these seven amino acids appear to be crucial for Ctp1's clipping function. One

of these amino acids, D291, is important for DNA binding (25) and presumably also for the clipping function.

Ctp1 N-terminal portion functions in resection

Among the six *ctp1* C-terminal truncation mutants we tested, three mutants (*ctp1-6*, *ctp1-10* and *ctp1-25*) lack one or both conserved motifs (CxxC and RHR); *ctp1-25* encodes only 186 amino acids of the Ctp1 N-terminal region. Interestingly, all of these three mutants retained proficient resection activity as tested by both genetic and physical assays (Figures 3, 4C and E). The Rec12-dependent meiotic recombination of the *ctp1-6* and *ctp1-25* mutants was only 2-fold lower (*P* < 0.02 by chi-square test) than that of wild type but >10-fold higher than that of *ctp1Δ* (*P* < 0.0001 by chi-square test) (Figure 1C, Supplementary Table S2). (*ctp1-10* was not assayed because of its low viable spore yield.) Furthermore, I-*Sce*I induced meiotic recombination of these mutants was the same as or even higher than that in wild type regardless of the distance between the two intragenic markers crossed (Figure 3 and Supplementary Figure S3; Supplementary Table S3). The resected DNA level in the three mutants (*ctp1-6, ctp1-10* and *ctp1-25*) tested by physical assay was also similar to the wild-type level, whereas *ctp1Δ* was about 2-fold lower than the wild type (Figure 4D and F). These results clearly indicate that the N-terminal portion of Ctp1 is sufficient for the resection function of I-*Ppo*I induced meiotic DSBs (i.e. in the absence of Rec12 adduct).

Ctp1 and Exo1 can both function in resection, but their interaction with two DNA end-binding proteins, MRN and Ku, differs in meiotic and mitotic cells. In mitotic cells, deleting *pku80* suppresses the DNA damaging-agent sensitivity of *ctp1Δ,* and this suppression is dependent on Exo1 (22,25). Thus, in mitotic cells Exo1 can apparently replace Ctp1 for resection, provided Ku is absent. In meiotic cells, however, Ku appears not to prevent Exo1 action; rather, MRN does. In the absence (but not presence) of MRN, I-*Sce*I-induced meiotic recombination is reduced in *exo1Δ* cells, even in a *pku*+ background (21). Furthermore, Ku may not be present at high levels during meiosis. In mouse and fruit fly nuclei, Ku protein is not detectable in early meiosis (42–44), and *S. pombe pku70* and *pku80,* encoding Ku70 and Ku80, are not induced during meiosis (45). These considerations explain why *ctp1Δ* reduced the frequency of I-*Sce*I-induced meiotic recombination even in *pku*+ cells. Because of these complexities, we draw our conclusions from our investigations in meiotic cells, although the mitotic screen we used suggests that the mutations reported here affect the mitotic functions of Ctp1 similarly.

Ctp1 interacts with Nbs1 via the Ctp1 N-terminal SXT domain at amino acids 74–94 when phosphorylated, and this interaction is critical for DSB repair (13,46). Wang et al. (47) reported that the CDK-dependent phosphorylation sites present in the N-terminal region of human CtIP are critical for its interaction with Nbs1 and for DSB repair. Phosphorylation of N-terminal CtIP-S276 and T315 is required for CtIP interaction with PIN1 prolyl-isomerase, and the CtIP-2A (S276A and T315A double) phosphomutant increased hyperphosphorylation of ssDNA-binding-protein RPA2 (a subunit of the RPA complex) and RPA

focus-formation after etoposide-induced DSB-formation (48), implying more DSB resection in CtIP-2A cells. While the detailed mechanism of Ctp1 N-terminal function in resection is unclear, one possibility is that Ctp1 interacts with MRN through its N-terminal region to resect DNA. Another possibility is that phosphorylation modulates Ctp1's structure, conformation or stability to alter resection.

Two functions of Ctp1 in DSB repair are separable

The genetic and physical analysis of *ctp1* mutants showed that the Ctp1 C-terminal region functions in clipping DSB ends to release Rec12-oligonucleotide complexes and that mutants lacking the C-terminal region are resection-proficient. These results mean that the resection function of Ctp1 is at least partially separable from its clipping function. CtIP endonuclease activity on branched DNA structures or hairpin structures was recently reported by two groups, and I-*Sce*I induced homologous recombination did not require its nuclease activity, suggesting that these two functions are also separable for human CtIP (9,10). Our results of *S. pombe* Ctp1 are consistent with their findings using purified human CtIP and provide genetic and intracellular evidence for this conserved protein function in meiotic DSB repair.

SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online.

REFERENCES

1. Keeney,S. (2007) In: Egel,R and Lankenau,D.-H. (eds). *Recombination and meiosis: crossing-over and disjunction.* Springer-Verlag, Berlin, pp. 81–123.
2. Williams,G.J., Lees-Miller,S.P. and Tainer,J.A. (2010) Mre11-Rad50-Nbs1 conformations and the control of sensing, signaling, and effector responses at DNA double-strand breaks. *DNA Repair*, **9**, 1299–1306.
3. Stracker,T.H. and Petrini,J.H. (2011) The MRE11 complex: starting from the ends. *Nat. Rev. Mol. Cell Biol.*, **12**, 90–103.
4. Hartsuiker,E., Mizuno,K., Molnar,M., Kohli,J., Ohta,K. and Carr,A.M. (2009) Ctp1^{CtIP} and the Rad32^{Mre11} nuclease activity are required for Rec12^{Spo11} removal but Rec12^{Spo11} removal is dispensable for other MRN-dependent meiotic functions. *Mol. Cell. Biol.*, **29**, 1671–1681.
5. Milman,N., Higuchi,E. and Smith,G.R. (2009) Meiotic DNA double-strand break repair requires two nucleases, MRN and Ctp1, to produce a single size class of Rec12 (Spo11)-oligonucleotide complexes. *Mol. Cell. Biol.*, **29**, 5998–6005.
6. Rothenberg,M., Kohli,J. and Ludin,K. (2009) Ctp1 and the MRN-complex are required for endonucleolytic Rec12 removal with release of a single class of oligonucleotides in fission yeast. *PLoS Genet.*, **5**, e1000722.
7. Neale,M.J., Pan,J. and Keeney,S. (2005) Endonucleolytic processing of covalent protein-linked DNA double-strand breaks. *Nature*, **436**, 1053–1057.
8. Lengsfeld,B.M., Rattray,A.J., Bhaskara,V., Ghirlando,R. and Paull,T.T. (2007) Sae2 is an endonuclease that processes hairpin DNA cooperatively with the Mre11/Rad50/Xrs2 complex. *Mol. Cell*, **28**, 638–651.
9. Makharashvili,N., Tubbs,A.T., Yang,S.H., Wang,H., Barton,O., Zhou,Y., Deshpande,R.A., Lee,J.H., Lobrich,M., Sleckman,B.P. *et al.* (2014) Catalytic and noncatalytic roles of the CtIP endonuclease in double-strand break end resection. *Mol. Cell*, **54**, 1022–1033.
10. Wang,H.L., Li,Y.J., Truong,L.N., Shi,L.D.Z., Hwang,P.Y.H., He,J., Do,J., Cho,M.J., Li,H.Z., Negrete,A. *et al.* (2014) CtIP maintains stability at common fragile sites and inverted repeats by end resection-independent endonuclease activity. *Mol. Cell*, **54**, 1012–1021.
11. Fowler,K.R., Sasaki,M., Milman,N., Keeney,S. and Smith,G.R. (2014) Evolutionarily diverse determinants of meiotic DNA break and recombination landscapes across the genome. *Genome Res.*, **24**, 1650–1664.
12. Sartori,A.A., Lukas,C., Coates,J., Mistrik,M., Fu,S., Bartek,J., Baer,R., Lukas,J. and Jackson,S.P. (2007) Human CtIP promotes DNA end resection. *Nature*, **450**, 509–514.
13. Williams,R.S., Dodson,G.E., Limbo,O., Yamada,Y., Williams,J.S., Guenther,G., Classen,S., Glover,J.N., Iwasaki,H., Russell,P. *et al.* (2009) Nbs1 flexibly tethers Ctp1 and Mre11-Rad50 to coordinate DNA double-strand break processing and repair. *Cell*, **139**, 87–99.
14. Cejka,P., Cannavo,E., Polaczek,P., Masuda-Sasa,T., Pokharel,S., Campbell,J.L. and Kowalczykowski,S.C. (2010) DNA end resection by Dna2-Sgs1-RPA and its stimulation by Top3-Rmi1 and Mre11-Rad50-Xrs2. *Nature*, **467**, 112–116.
15. Zhou,Y., Caron,P., Legube,G. and Paull,T.T. (2014) Quantitation of DNA double-strand break resection intermediates in human cells. *Nucleic Acids Res.*, **42**, e19.
16. Zakharyevich,K., Ma,Y., Tang,S., Hwang,P.Y., Boiteux,S. and Hunter,N. (2010) Temporally and biochemically distinct activities of Exo1 during meiosis: double-strand break resection and resolution of double Holliday junctions. *Mol. Cell*, **40**, 1001–1015.
17. Zhu,Z., Chung,W.-H., Shim,E.Y., Lee,S.E. and Ira,G. (2008) Sgs1 helicase and two nucleases Dna2 and Exo1 resect DNA double-strand break ends. *Cell*, **134**, 981–994.
18. Szankasi,P. and Smith,G.R. (1992) A DNA exonuclease induced during meiosis of *Schizosaccharomyces pombe*. *J. Biol. Chem.*, **267**, 3014–3023.
19. Nicolette,M.L., Lee,K., Guo,Z., Rani,M., Chow,J.M., Lee,S.E. and Paull,T.T. (2010) Mre11-Rad50-Xrs2 and Sae2 promote 5′ strand resection of DNA double-strand breaks. *Nat. Struct. Mol. Biol.*, **17**, 1478–1485.
20. Neale,M.J., Ramachandran,M., Trelles-Sticken,E., Scherthan,H. and Goldman,A.S. (2002) Wild-type levels of Spo11-induced DSBs are required for normal single-strand resection during meiosis. *Mol. Cell*, **9**, 835–846.
21. Farah,J.A., Cromie,G.A. and Smith,G.R. (2009) Ctp1 and Exonuclease 1, alternative nucleases regulated by the MRN complex, are required for efficient meiotic DNA repair and recombination. *Proc. Natl. Acad. Sci. U.S.A.*, **106**, 9356–9361.
22. Limbo,O., Chahwan,C., Yamada,Y., de Bruin,R.A., Wittenberg,C. and Russell,P. (2007) Ctp1 is a cell-cycle-regulated protein that functions with Mre11 complex to control double-strand break repair by homologous recombination. *Mol. Cell*, **28**, 134–146.
23. Akamatsu,Y., Murayama,Y., Yamada,T., Nakazaki,T., Tsutsui,Y., Ohta,K. and Iwasaki,H. (2008) Molecular characterization of the role of the *Schizosaccharomyces pombe nip1*$^+$/*ctp1*$^+$ gene in DNA double-strand break repair in association with the Mre11-Rad50-Nbs1 complex. *Mol. Cell. Biol.*, **28**, 3639–3651.
24. Dubin,M.J., Stokes,P.H., Sum,E.Y.M., Williams,R.S., Valova,V.A., Robinson,P.J., Lindeman,G.J., Glover,J.N.M., Visvader,J.E. and Matthews,J.M. (2004) Dimerization of CtIP, a BRCA1- and

CtBP-interacting protein, is mediated by an N-terminal coiled-coil motif. *J. Biol. Chem.*, **279**, 26932–26938.

25. Andres,S.N., Appel,C.D., Westmoreland,J.W., Williams,J.S., Nguyen,Y., Robertson,P.D., Resnick,M.A. and Williams,R.S. (2015) Tetrameric Ctp1 coordinates DNA binding and DNA bridging in DNA double-strand-break repair. *Nat. Struct. Mol. Biol.*, **22**, 158–166.

26. Qvist,P., Huertas,P., Jimeno,S., Nyegaard,M., Hassan,M.J., Jackson,S.P. and Borglum,A.D. (2011) CtIP mutations cause Seckel and Jawad syndromes. *PLoS Genet.*, **7**, e1002310.

27. Smith,G.R. (2009) In: Keeney,S (ed). *Meiosis*. Humana Press, Totowa, NJ, pp. 65–76.

28. Hyppa,R.W. and Smith,G.R. (2009) In: Keeney,S (ed). *Meiosis*. Humana Press, Totowa, NJ, pp. 235–252.

29. Bahler,J., Wu,J.-Q., Longtine,M.S., Shah,N.G., McKenzie,A. III, Steever,A.B., Wach,A., Philippsen,P. and Pringle,J.R. (1998) Heterologous modules for efficient and versatile PCR-based gene targeting in *Schizosaccharomyces pombe*. *Yeast*, **14**, 943–951.

30. Li,Y.F., Numata,M., Wahls,W.P. and Smith,G.R. (1997) Region-specific meiotic recombination in *S. pombe*: the *rec11* gene. *Mol. Microbiol.*, **23**, 869–878.

31. Young,J.A., Hyppa,R.W. and Smith,G.R. (2004) Conserved and nonconserved proteins for meiotic DNA breakage and repair in yeasts. *Genetics*, **167**, 593–605.

32. Ellermeier,C., Schmidt,H. and Smith,G.R. (2004) Swi5 acts in meiotic DNA joint molecule formation in *Schizosaccharomyces pombe*. *Genetics*, **168**, 1891–1898.

33. Cervantes,M.D., Farah,J.A. and Smith,G.R. (2000) Meiotic DNA breaks associated with recombination in *S. pombe*. *Mol. Cell*, **5**, 883–888.

34. Pommier,Y. (2006) Topoisomerase I inhibitors: camptothecins and beyond. *Nat. Rev. Cancer*, **6**, 789–802.

35. Cannavo,E. and Cejka,P. (2014) Sae2 promotes dsDNA endonuclease activity within Mre11-Rad50-Xrs2 to resect DNA breaks. *Nature*, **514**, 122–125.

36. Ma,W.J., Westmoreland,J.W., Gordenin,D.A. and Resnick,M.A. (2011) Alkylation base damage is converted into repairable double-strand breaks and complex intermediates in G2 cells lacking AP endonuclease. *PLoS Genet.*, **7**, e1002059.

37. Hartsuiker,E., Neale,M.J. and Carr,A.M. (2009) Distinct requirements for the Rad32(Mre11) nuclease and Ctp1(CtIP) in the removal of covalently bound topoisomerase I and II from DNA. *Mol. Cell*, **33**, 117–123.

38. Sunder,S., Greeson-Lott,N.T., Runge,K.W. and Sanders,S.L. (2012) A new method to efficiently induce a site-specific double-strand break in the fission yeast *Schizosaccharomyces pombe*. *Yeast*, **29**, 275–291.

39. Mata,J., Wilbrey,A. and Bähler,J. (2007) Transcriptional regulatory network for sexual differentiation in fission yeast. *Genome Biol*, **8**, R217.

40. Langerak,P., Mejia-Ramirez,E., Limbo,O. and Russell,P. (2011) Release of Ku and MRN from DNA ends by Mre11 nuclease activity and Ctp1 is required for homologous recombination repair of double-strand breaks. *PLoS Genet.*, **7**, e1002271.

41. Mimitou,E.P. and Symington,L.S. (2008) Sae2, Exo1 and Sgs1 collaborate in DNA double-strand break processing. *Nature*, **455**, 770–774.

42. Goedecke,W., Eijpe,M., Offenberg,H.H., van Aalderen,M. and Heyting,C. (1999) Mre11 and Ku70 interact in somatic cells, but are differentially expressed in early meiosis. *Nat. Genet.*, **23**, 194–198.

43. Hamer,G., Roepers-Gajadien,H.L., van Duyn-Goedhart,A., Gademan,I.S., Kal,H.B., van Buul,P.P.W., Ashley,T. and de Rooij,D.G. (2003) Function of DNA-protein kinase catalytic subunit during the early meiotic prophase without Ku70 and Ku86. *Biol. Reprod.*, **68**, 717–721.

44. Boutanaev,A.M., Mikhaylova,L.M. and Nurminsky,D.I. (2007) Up-regulation of the Ku heterodimer in Drosophila testicular cyst cells. *FEBS Lett.*, **581**, 1707–1715.

45. Wilhelm,B.T., Marguerat,S., Watt,S., Schubert,F., Wood,V., Goodhead,I., Penkett,C.J., Rogers,J. and Bahler,J. (2008) Dynamic repertoire of a eukaryotic transcriptome surveyed at single-nucleotide resolution. *Nature*, **453**, 1239–1243.

46. Dodson,G.E., Limbo,O., Nieto,D. and Russell,P. (2010) Phosphorylation-regulated binding of Ctp1 to Nbs1 is critical for repair of DNA double-strand breaks. *Cell Cycle*, **9**, 1516–1522.

47. Wang,H., Shi,L.Z., Wong,C.C., Han,X., Hwang,P.Y., Truong,L.N., Zhu,Q., Shao,Z., Chen,D.J., Berns,M.W. *et al.* (2013) The interaction of CtIP and Nbs1 connects CDK and ATM to regulate HR-mediated double-strand break repair. *PLoS Genet.*, **9**, e1003277.

48. Steger,M., Murina,O., Huhn,D., Ferretti,L.P., Walser,R., Hanggi,K., Lafranchi,L., Neugebauer,C., Paliwal,S., Janscak,P. *et al.* (2013) Prolyl isomerase PIN1 regulates DNA double-strand break repair by counteracting DNA end resection. *Mol. Cell*, **50**, 333–343.