Supplementary Information for

A Conserved Ctp1/CtIP C-Terminal Peptide Stimulates Mre11 Endonuclease Activity

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Schizosaccharomyces pombe strain manipulation

Standard procedures were used for culturing and genetic manipulation of strains (1). The *S. pombe* strains used in this study are shown in SI Appendix, Table S2.

Viable spore yield assay

Viable spore yield assays were performed as described previously (2). Briefly, 1.2 x 10^6 cells of strain *h*$^-$ cells carrying *ctp1* variants (spAZ_114, spAZ_120, spAZ_123, spAZ_128, spAZ_0132, or spAZ_134) was crossed with an excess of *h*$^+$ *ctp1*Δ cells (YA1097: *h*$^+$, *his3-D1*, *leu1-32*, *ura4-D18*, *ade6-M210*, *ctp1*Δ::*ura4*$^+$) (3) on SPA sporulation agar. The mating mixtures were allowed to undergo meiosis at 25°C. Cells were harvested after 2–3 days and treated with glusulase and ethanol to kill vegetative cells. The cell mixture was then plated on yeast extract agar (YEA) and the number of colonies were counted after 2-3 days of incubation at 30°C. Viable spore yield was expressed as a percentage for each *h*$^-$ query strain, with 100% equal to the number of colonies obtained with the *h*$^-$ *ctp1*$^+$ control strain (YA119).

Oligo primers for PCR

Primers used in this study are listed in SI Appendix, Table S3.

Expression and purification of the Mre11-Rad50 (MR) complex

The *mre11*$^+$ cDNA on pBluescript II SK (+) (4) was subcloned to enable expression of the protein from the *GAL1/10* promoter in the pESC-Ura plasmid (Agilent Technologies), giving pESC-Mre11. The PSOL10009 primer introduced a 6×His tag at the C-terminus of Mre11. The *rad50*$^+$ cDNA was amplified using PSOL10010 and PSOL10011 and cloned into pESC-Trp (Agilent Technologies), giving pESC-Rad50. The two resultant plasmids were introduced into the protease-deficient *Saccharomyces cerevisiae* strain YRP654 (5). Cultures were grown overnight to stationary phase in SC medium lacking Ura and Trp (SC Ura$^-$ Trp$^-$) and diluted eightfold into SC Ura$^-$ Trp$^-$ media with 3% glycerol, 3% lactic acid, and 2% galactose. After 24 h of growth at 30°C, cells were harvested by centrifugation, immediately snap-frozen as small pellets in liquid nitrogen, and then ground to a fine powder under liquid nitrogen using a 6875 Freezer/Mill Cryogenic Grinder (SPEX, NJ, USA). The cell powder was stored at –80°C until required. About 20 g frozen cell powder was then suspended in 100 ml crude-

extract buffer (20 mM potassium phosphate [pH 7.5], 300 mM KCl, 10% glycerol, 0.05% polysorbate 20, 1 mM 2-mercaptoethanol, 0.5 mM phenylmethylsulfonyl fluoride [PMSF], and protease inhibitor cocktail [Roche]) at room temperature. As soon as the cell powder was suspended, the suspension was immediately put on ice. Subsequent steps were performed at 0–4°C. The cell suspension was centrifuged at 186,000 × *g* for 2 h in a Ti45 rotor (Beckman). The cleared supernatant was mixed with 4 ml Ni-NTA Superflow resin (Qiagen) in a capped test tube, which was gently rotated for 3 h. The slurry was poured into a 1 cm diameter disposable column, and the packed resin was slowly washed with a series of four different buffers in the following order: 300 ml buffer A (20 mM potassium phosphate [pH 7.5], 500 mM KCl, 1 mM ATP, 8 mM $MgCl_2$, 10% glycerol, 0.05% polysorbate 20, and 1 mM 2-mercaptoethanol, 30 mM imidazole), 100 ml buffer B (20 mM Tris-Cl [pH 8.0], 300 mM KCl, 1 mM ATP, 8 mM $MgCl_2$, 10% glycerol, 0.05% polysorbate 20, and 1 mM 2-mercaptoethanol, 30 mM imidazole), 100 ml buffer C (20 mM potassium phosphate [pH 7.0], 300 mM KCl, 1 mM ATP, 8 mM $MgCl_2$, 10% glycerol, 0.05% polysorbate 20, and 1 mM 2-mercaptoethanol, 30 mM imidazole), and 10ml buffer D (20 mM potassium phosphate [pH 7.5], 100 mM KCl, 10% glycerol, 0.05% polysorbate 20, and 1 mM 2-mercaptoethanol). The resin was resuspended in 15 ml elution buffer (20 mM potassium phosphate [pH 7.5], 100 mM KCl, 300 mM imidazole, 10% glycerol, 0.05% polysorbate 20, and 1 mM 2-mercaptoethanol) in the same column, which was capped to prevent leakage, and rotated continuously for ~15 h. The flow-through fraction, obtained by passing through the column, was diluted with 45 ml buffer E (20 mM potassium phosphate [pH 7.5], 10% glycerol, 0.05% polysorbate 20, 1 mM 2-mercaptoethanol, and 0.5 mM EDTA). The diluted sample was applied to a 5 ml Heparin HP column (GE Healthcare), pre-equilibrated with buffer E containing 100 mM KCl. The column was washed with 30 ml buffer E containing 200 mM KCl, and the proteins that were bound to the resin were eluted with a linear gradient of 200 mM to 1 M KCl in 20 ml buffer E. Peak fractions containing MR complex, as determined by western blotting with anti-His-tag antibody, were loaded onto a HiLoad 26/600 Superdex 200 pg (GE Healthcare) size-exclusion column pre-equilibrated with buffer F (20 mM Tris-Cl [pH 7.5], 10% glycerol, 0.05% polysorbate 20, 1 mM 2-mercaptoethanol, and 0.5 mM EDTA) containing 300 mM NaCl, and proteins were eluted with the same buffer. MR complex eluted at ~110–120 ml, separately from free Mre11, which eluted at ~130–150 ml. Fractions containing MR complex were diluted with two volumes of buffer F and applied to a 1 ml RESOURCE Q anion-exchange column (GE Healthcare). Proteins were eluted with a linear gradient of 50 mM to 1 M NaCl in 9 ml buffer F. Peak fractions containing MR complex were diluted to a final concentration of 100 mM NaCl with buffer F and concentrated by Amicon Ultra-4 10K Centrifugal Filters (Merck Millipore) to 0.3–1 mg/ml MR. The concentrated sample was dispensed into small aliquots,

snap-frozen in liquid nitrogen, and stored at –80°C until required.

cDNA corresponding to the nuclease-deficient $Mre11^{H134R}$ was made with the QuikChange II kit (Agilent) with two primers, oAZ_94 and oAZ_95, and pESC-Mre11 as the template. Expression and purification of the $M^{H134R}R$ complex was carried out by the same procedure as for the wild-type MR complex. The $M^{H134R}R$ complex behaved identically to wild-type MR during purification.

Expression and purification of Nbs1

For the purification of Nbs1 from insect cells, the *nbs1*$^{+}$ cDNA on pNT140 (3) was subcloned into a pFastBac vector, giving pFastBac-Nbs1. The PSOL9975 primer introduced a FLAG tag at the C-terminus of Nbs1. Bacmid, transfected Sf9 cells, and viruses were prepared according to the manufacturer's instructions (Bac-to-Bac, Invitrogen). High-titer virus was transfected into Sf9 cells (2×10^{6} cells/ml) in 800 ml cell culture. After incubation for 45 h at 27°C, cells were harvested by centrifugation (700 × *g* for 10 min), washed in phosphate-buffered saline, snap-frozen in liquid nitrogen, and stored at –80°C until further use.

All purification steps were performed at 4°C. Cells were resuspended and stirred gently in 8 ml swelling buffer (50 mM Tris-HCl [pH 7.5], 2 mM DTT) for 30 min. The following were added to the cell suspension: protease inhibitors (aprotinin, chymostatin, leupeptin, and pepstatin at final concentrations of 5 μg/ml each, and PMSF at a final concentration of 0.5 mM), 1 ml of 50% glycerol, and 4 M NaCl up to a final NaCl concentration of 300 mM. The suspension was stirred for 30 min, followed by centrifugation at 186,000 × *g* in a Ti45 rotor (Beckman) for 1 h. The supernatant was collected and mixed with 3 ml settled Anti-FLAG M2 Affinity Gel (Sigma) in a bottle, which was rotated for 2.5 h. The slurry was poured into a 1 cm diameter disposable column, and the packed resin was slowly washed with 200 ml T buffer (25 mM Tris-HCl [pH 7.5], 10% glycerol, 0.01% IGEPAL CA-630 [MP medical], and 1mM DTT) containing 500 mM KCl. The resin was incubated with 6 ml of 0.2 mg/ml 3×FLAG peptide (APExBIO) in 50 mM NaCl T buffer for 20 min to elute the proteins that were bound to the resin. The elution procedure was repeated twice. Eluted fractions were loaded onto a 5 ml HiTrap SP column (GE Healthcare). Proteins were eluted with a linear gradient of 100–500 mM NaCl in 50 ml buffer T. Nbs1-FLAG eluted with a peak corresponding to 360 mM NaCl. The peak fractions containing Nbs1-FLAG were diluted to 100 mM NaCl concentration with buffer T and concentrated using an Amicon Ultra-4 10K Centrifugal Filter (Merck Millipore).

For the purification of Nbs1-FLAG from *Escherichia coli*, the Nbs1-FLAG coding sequence in pFastBac-Nbs1 was introduced into the pBKN220 vector (a high-copy-number pET21a derivative) using the In-Fusion HD cloning kit (Takara) as described by the

manufacturer (with primers oAZ_203, oAZ_204, oAZ_205, and oAZ_206), giving pBKN-Nbs1-FLAG. The pBKN-Nbs1-FLAG plasmid was linearized by PCR with oAZ_265 and oAZ_266 primers, and a human rhinovirus 3C protease site and a 6×His tag were added at the C-terminus of Nbs1-FLAG. The resultant plasmid was named pBKN220-Nbs1-FLAG-3C-His and was introduced into the T7-expression host *E. coli* BL21-CodonPlus (DE3). Bacterial expression of Nbs1-FLAG-3C-His was performed by the auto-induction method at 30°C overnight (6).

E. coli cells were harvested by centrifugation (6,500 × *g* for 15 min), snap-frozen in liquid nitrogen, and stored at –80°C until required. All purification steps were performed at 4°C. A frozen pellet (~1 g) was resuspended in 10 ml extraction buffer (40 mM Tris [pH 8.0], 300 mM NaCl, 10% glycerol, 0.01% IGEPAL CA-630 [MP medical], 1 mM DTT, protease inhibitor cocktail [Roche], and 0.5 mM PMSF), followed by sonication (BRANSON Digital Sonifier 250, 20 kHz, 12.5 mm flat tip, 30% amplitude, 15 min on time, 0.5 s on / 1 s off pulse, 5°C temperature limit) and ultra-centrifugation (72,000 × *g* for 1 h). Ni-NTA Superflow resin (5 ml of slurry, Qiagen) was added to the crude extract in a bottle, which was rotated for 2.5 h. The resin was poured into a 1 cm diameter disposable column and slowly washed with 200 ml T buffer containing 25 mM imidazole and 500 mM KCl. Nbs1-FLAG-3C-His was eluted with 15 ml T buffer containing 360 mM imidazole and 100 mM NaCl. PreScission protease (GE Healthcare) was added to the eluted fraction, following the manufacturer's guidelines. The sample was then dialyzed against T buffer containing 90 mM NaCl, and the dialyzate was loaded onto a HiLoad 26/600 Superdex 200 prep-grade column (GE) that was pre-equilibrated with T buffer containing 100 mM NaCl. Proteins were eluted with T buffer containing 100 mM NaCl; fractions corresponding to 155–165 ml elution volume contained Nbs1-FLAG, and were pooled, concentrated with an Amicon Ultra-4 3K Centrifugal Filter (Merck Millipore), snap-frozen, and stored at –80°C until use.

Nbs1-FLAG proteins purified from insect cells and bacterial cells were judged to have the same properties.

Expression and purification of GST-Cka1

The *cka1*$^{+}$ cDNA was cloned into a pGEX-6P-1N plasmid, giving pGST-Cka1. GST-Cka1 fusion protein was expressed in *E. coli* BL21-CodonPlus (DE3) cells using the auto-induction method at 30°C for ~15 h (6). *E. coli* cells were harvested by centrifugation (6,500 × *g* for 15 min), snap-frozen in liquid nitrogen, and stored at –80°C until use. All purification steps were performed at 4°C. A frozen pellet (~1 g) was resuspended in lysis buffer (40 mM Tris-Cl [pH 7.5], 300 mM KCl, 1 mM DTT, 0.05% polysorbate 20 (MP Biomedical), protease-inhibitor cocktail (Roche), and 0.5 mM PMSF), followed by sonication (BRANSON Digital Sonifier 250,

20 kHz, 12.5 mm flat tip, 30% amplitude, 15min on time, 0.5 s on / 1 s off pulse, 5°C temperature limit) and ultra-centrifugation (75,000 × *g* for 1 h). Glutathione Sepharose 4B resin (5 ml slurry, GE Healthcare) was added to the crude extract in a bottle, which was rotated for 2 h. The resin was applied to a disposable 1 cm diameter column and slowly washed with 100 ml buffer T containing 25 mM imidazole and 500 mM KCl. The GST-Cka1 was eluted with 15 ml T buffer containing 50 mM glutathione and 50 mM KCl. The eluate was loaded directly onto a 1 ml HiTrap Heparin HP column. Proteins were eluted with a linear gradient of 200–700 mM KCl in 10 ml T buffer. Peak fractions containing GST-Cka1 (corresponding to ~475 mM KCl) were concentrated in an Amicon 30k unit (Millipore), snap-frozen, and stored at –80°C.

Expression and purification of Ctp1 and its derivatives

ctp1^{+} cDNA was cloned into pBKN220, giving pBKN-Ctp1(original). The first 11 codons of *ctp1* were corrected to the following sequence ATGAACGAAGAAGAACACAACAAATCTGTTCAC using site-directed mutagenesis. This sequence has a higher *E. coli* usage frequency while preserving the same amino-acid sequence. The resultant codon corrected plasmid was named pBKN-Ctp1. BL21-CodonPlus (DE3) *E. coli* cells transformed with pBKN-Ctp1 were grown to OD_{600} ≈ 0.3 in LB broth at 37°C. Expression of Ctp1 was induced by addition of isopropyl-β-D-thiogalactoside (final concentration, 1 mM). After a 3 h incubation, cells were harvested by centrifugation (6,500 × *g* for 15 min), snap-frozen in liquid nitrogen, and stored at –80°C until required.

All subsequent purification steps were performed at 4°C. A frozen pellet was resuspended in 20 ml R buffer (20 mM Tris-HCl [pH 8.0], 1 mM EDTA, 10% glycerol, and 1 mM DTT) containing 300 mM NaCl, 1 mM PMSF, protease-inhibitor cocktail (Roche), with DTT added to a final concentration of 5 mM. Cells were disrupted by sonication (BRANSON Digital Sonifier 250, 20 kHz, 12.5 mm flat tip, 30% amplitude, 15min on time, 0.5 s on / 1 s off pulse, 4°C temperature limit), and cleared extract was obtained by ultra-centrifugation (67,000 × *g* for 1 h). The sample was diluted with three volumes of R buffer and loaded onto two sequentially connected HiTrap SP HP 5 ml columns (GE Healthcare). Proteins were eluted with a linear gradient of 0–1 M NaCl over the volume of 100 ml R buffer. Peak fractions containing Ctp1 were pooled, adjusted to 2 mM ATP and 10 mM $MgCl_2$, and stirred gently for 1 h. The mixture was diluted with two volumes of R buffer and loaded onto a HiTrap Heparin 5 ml column (GE Healthcare) pre-equilibrated with R buffer containing 100 mM NaCl. Proteins were eluted with a linear gradient of 100–700 mM NaCl over the volume of 60 ml R buffer. Pooled peak fractions containing Ctp1 were diluted by addition of three volumes of R buffer and loaded onto a 1 ml RESOURCE Q column (GE Healthcare). Proteins were eluted

with a linear gradient of 50–500 mM NaCl over the volume of 9 ml R buffer. Peak fractions were diluted with R buffer to adjust the final concentration to 150 mM NaCl, and then were snap-frozen in small aliquots and stored at –80°C until required.

Codon changes within the conserved CxxC motif of Ctp1 resulting in the SxxS mutant were introduced by PCR linearizing the pBKN-Ctp1 plasmid using oAZ_235 an oAZ_236 primers and circularizing the generated fragment by In-Fusion HD cloning kit (Takara). Codons within the Ctp1 RHR motif were changed to RAA by site-directed mutagenesis using the oAZ_087 and oAZ_088 primers and pBKN-Ctp1 as a template. N-terminal truncation of Ctp1 was generated by running a PCR using oAZ_458 and T7 terminator primers on pBKN-Ctp1 as template, with the resulting fragment being ligated into the pBKN backbone following NdeI and BamHI digestion. These Ctp1 variants were purified by the same procedure as for wild-type Ctp1 protein.

C-terminal truncation of Ctp1 was generated by introducing Strep-Tag II via PCR using oAZ_153 & oAZ_158 primers and pBKN-Ctp1 as template followed by circularization of the generated fragment with the In-Fusion HD cloning kit. This protein was expressed in *E. coli*, and crude cell extract was obtained as described for wild-type Ctp1. To the crude extract of Ctp1(1-250)-strep, 1 ml Strep-Tactin Superflow resin (IBA) was added in a bottle, which was mixed slowly at 4°C for 1.5 h. Resin was washed with 100 ml R buffer containing 300 mM NaCl. Elution was performed by addition of 10 ml of 10 mM d-desthiobiotin. The eluate was further purified with a RESOURCE Q column and treated as described in the final step for the wild-type Ctp1 protein.

Preparation and dephosphorylation of *in vitro*-phosphorylated Ctp1 and its derivatives

Phosphorylation reaction mix containing 1 mg/ml Ctp1, 0.004 mg/ml GST-Cka1, 2 mM ATP, 20 mM $MgCl_2$, 50 mM NaCl, and 20 mM Tris-HCl (pH 7.5) was incubated at 30°C for 16 h. Phosphorylated protein was purified by HiTrap SP HP and HiTrap Heparin columns, as described for unphosphorylated Ctp1. Pooled fractions were adjusted to a final concentration of 100 mM NaCl in R buffer, concentrated using Amicon 10k (Millipore) filters, snap-frozen, and stored at –80°C. The same procedure was used for preparation of phosphorylated Ctp1(SXT-5A). Ctp1(1–120) was phosphorylated in the same way but used without the purification procedure. For dephosphorylation, the phosphorylated proteins were treated with Lambda Protein Phosphatase (NEB), as instructed by the manufacturer.

Human MRN purification

Untagged Human MRE11 (hMRE11) and hRAD50 with a C-terminal 6xHis tag were cloned into the ClaI and SmaI sites of pESC-Ura, respectively, to create pJD178. The hMR complex

was expressed by galactose induction in 20 L of the *S. cerevisiae* strain YRP654 (5). Flag-hNBS1 was cloned into pFastBac to create pJD83 and was used to create a baculovirus by transformation into DH10Bac cells. hNBS1 was expressed in High 5 insect cells by infection with this baculovirus and 1 L of cells were harvested after 48 hr. Both the hMR and hNBS1 pellets were broken in K buffer (20 mM KH_2PO_4, 10% glycerol, 0.5 mM EDTA, 0.5% Tween 20, 1 mM β-mercaptoethanol, and protease inhibitors: 1 mM PMSF and 5 mg/mL each of aprotinin, chymostatin, leupeptin, and pepstatin) with 300 mM KCl and spun down in a Beckman ultracentrifuge at 100,000 x *g* for 45 min. The clarified hMR and hNBS1 lysates were incubated with 2 ml of nickel or flag affinity resin, respectively, for 1 hr at 4°C. Each resin was washed sequentially with 15 ml K buffer with 300 mM KCl; 500 ml K buffer with 500 mM KCl, 1 mM ATP, and 8 mM $MgCl_2$; and 15 ml K buffer with 150 mM KCl. Then, the resin was treated five times with 2 ml of K buffer containing 150 mM KCl and, either 200 mM imidazole for hMR or 250 ng/ml of FLAG peptide (Sigma) for hNBS1, for 10 min to elute proteins. The hMR and hNBS1 eluates were mixed and incubated overnight at 4°C to form the hMRN complex. The resulting complex was then fractionated in a 1 ml heparin column (Amersham) with a 50-mL 150–650 mM KCl gradient. The peak was collected, concentrated in an Ultracel-100K device (Amicon), and loaded onto a 24-mL Superose 6 column (GE Healthcare) in K buffer with 250 mM KCl. Peak fractions were concentrated again, frozen in liquid nitrogen in small aliquots, and stored at -80°C.

Mass spectrometry

For mass spectrometry, Ctp1 was partially phosphorylated by incubating 0.33 mg/ml Ctp1 and 0.02 mg/ml GST-Cka1 in reaction buffer (20 mM Tris-HCl [pH 7.5], 20 mM $MgCl_2$, 2 mM ATP, 20 mM NaCl, and 80 mM KCl) for 5 min at 30°C. The phosphorylation reaction was stopped by addition of SDS-PAGE sample-loading buffer. Phosphorylated samples were reduced by addition of 1/10 volume of 250 mM Tris(2-carboxyethyl)phosphine (TCEP), and the samples were incubated for 15 min at room temperature. The samples were then alkylated by addition of a 1/10 volume of 500 mM freshly-made iodoacetamide and incubated at room temperature for 15 min. Samples were separated by 8% SDS-PAGE and stained with Coomassie brilliant blue G-250. Stained protein bands were excised, destained with 30% acetonitrile and 50 mM ammonium bicarbonate (ABC) solution, and dehydrated with 60% acetonitrile and 50 mM ABC solution. Dehydrated gel slices were dried further in a centrifugal evaporator, and then were rehydrated in 50 mM ABC solution containing 12.5 μg/ml Trypsin Gold (Promega) and incubated at 37°C overnight for digestion. The digested peptides were extracted in 50% acetonitrile, dried in a centrifugal evaporator, dissolved in 2% acetonitrile and 0.1% TFA solution, and desalted with a C18 Stage Tip (Nikkyo Technos).

After desalting, the peptide solution was dried in a centrifugal evaporator. The peptides were re-dissolved in 2% acetonitrile and 0.1% TFA solution, and subjected to nanoLC–MS/MS measurement on an Easy-nLC 1000 nanoLC system and a Q-Exactive mass spectrometer (ThermoFisher Scientific). The settings used have been described previously (7), except that the gradient condition of nanoLC was set as 10–40% at 30 min. MS/MS spectra were searched against the amino-acid sequences of *S. pombe* proteome obtained from UniProt Database (UP000002485) by using the Sequest HT algorithm within Proteome Discoverer 2.4 (Thermo Fisher Scientific). To detect phosphorylation sites, +79.966 Da dynamic modification was set at Ser, Thr and Tyr. The measurement was performed three times per each sample, and the three raw files were merged into one analysis data on Proteome Discoverer.

Co-immunoprecipitation

Mre11(6×His)-Rad50 (2.25 *μ*g), Nbs1-FLAG (0.7 *μ*g), and Ctp1 (1.5 *μ*g) were mixed in 15 *μ*l IP buffer (20 mM Tris-HCl [pH 7.5], 150 mM NaCl, 3 mM $MgCl_2$, 2.5% glycerol, 0.5% IGEPAL CA-630 [MP Biomedical]), and then incubated on ice for 20 min. The reaction was then mixed with 135 *μ*l Surebeads Protein G (Biorad), pre-treated with anti-6×His monoclonal antibody 28-75 (Wako, 014-23221) or with anti-FLAG M2 antibody (Sigma, F3165), in IP-BSA buffer (IP buffer containing 0.1mg/ml BSA). The mixtures were gently agitated at 4°C for 3 h, washed three times with IP buffer. and the proteins bound to the beads were eluted by boiling in SDS loading buffer and then analyzed by 8% SDS-PAGE.

DNA substrates

Double-stranded DNA made by annealing with oAZ_294 (5'-G**T**AAG TGCCG CGGTG CGGGT GCCAG GGCGT GCCCT TGGGC TCCCC GGGCG CGTAC TCCAC CTCAT GCA**TC**-3') and oAZ_295 (5'-GATGC ATGAG GTGGA GTACG CGCCC GGGGA GCCCA AGGGC ACGCC CTGGC ACCCG CACCG CGGCA CTTAC-3') (Eurofins Genomics, USA), where bold "**T**" and bold "**C**" represents the position of conjugation of biotin and TAMRA, respectively, were used for the endonuclease assay. oAZ_294 and oAZ_295 correspond to the sequence of PC210 and PC211 as described previously by Pinto et al (8), respectively, except for the addition of the TAMRA conjugated "**C**" as a single nucleotide overhang. The two oligonucleotides were annealed in a thermal cycler (~0.3°C/min linear temperature gradient starting from 95°C). For exonuclease assays, dsDNA was used, which was made by annealing of oAZ_135 (**A**AATGAACATAAAGTAAATAAGTC) and oAZ_136 (GACTTATTTACTTTATGTTCATTT) (Integrated DNA Technologies, USA), bold "**A**" denotes the attachment of TAMRA dye to the 5' phosphate.

Exonuclease assay

Standard exonuclease assays were performed in reaction mixture containing 50 mM NaCl, 20 mM Tris-HCl [pH 7.5], 10% glycerol, 1 mM DTT, 0.5 mM $MnCl_2$, 5mM $MgCl_2$, 50 nM DNA, 50 nM $(Mre11)_2(Rad50)_2$, 100 nM Nbs1, and 100 nM Ctp1 variants in terms of monomers. The assays followed the same procedures as the endonuclease assays.

ATPase assay

DNA used in ATPase assays was prepared by annealing oAZ_454 (5'-GTAAG TGCCG CGGTG CGGGT GCCAG GGCGT GCCCT TGGGC TCCCC GGGCG CGTAC TCCAC CTCAT GCATC-3') and oAZ_455 (5'-GATGC ATGAG GTGGA GTACG CGCCC GGGGA GCCCA AGGGC ACGCC CTGGC ACCCG CACCG CGGCA CTTAC-3') (Eurofins Genomics, Japan). Assays were performed in reaction mixture containing 150 mM NaCl, 20 mM Tris-Cl [pH 7.5], 10% glycerol, 1 mM DTT, 0.5 mM $MnCl_2$, 5 mM $MgCl_2$, 150 nM DNA, 0.2 mM ATP, 150 nM $(Mre11)_2(Rad50)_2$, 300 nM Nbs1, and 300 nM Ctp1. Aliquots (2 μl) were taken at designated timepoints and mixed with equal volumes of ice-cold 25 mM EDTA [pH 8.0] (stop reagent) and kept on ice until all aliquots were collected. ATPase activity was determined with a malachite-green assay kit (POMG-25H, Funakoshi), as instructed by the manufacturer.

SEC-MALS analysis

Wild-type (67 μg), phosphorylated wild-type (50 μg) and SXT-5A (30 μg) Ctp1 variants were loaded onto the preequilibrated Superose 6 Increase 10/300 gel filtration column (GE) and eluted isocratically with the same buffer (100mM NaCl, 20mM TRIS, pH 7) at 0.5 ml / min. The column was connected in-line with a DAWN EOS light-scattering and Optilab rex differential refractive index detectors (Wyatt Technology). Data was analyzed by using the ASTRA 5.3.4.20 software (Wyatt Technology).

Synthetic peptides

All peptides were ordered from Toray Research Center (Japan).

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Competing interests: The authors declare no competing interests.

Author contributions: A.Z., P.S., and H.I. conceived and designed the study. J.M.D. and A.D. purified the human MRN complex. K.I. and T.M. helped with all other protein purifications. T.N. performed MS experiments. A.Z. performed all other experiments. A.Z. and T.N. analyzed MS data. A.Z., J.M.D., A.D., Y.M., S.K., K.I., T.M., B.A., M.T., H.T., P.S., and H.I. analyzed all other data. Y.M., S.K., K.I., T.M., B.A., M.T., H.T., P.S., and H.I. provided expertise and reagents. Y.M., S.K., M.T., H.T., P.S., and H.I. supervised the study. A.Z., T.N., Y.M., B.A., H.T., P.S., and H.I. contributed to writing the manuscript.

Data and materials availability: All data are available in the main text or supplementary materials.

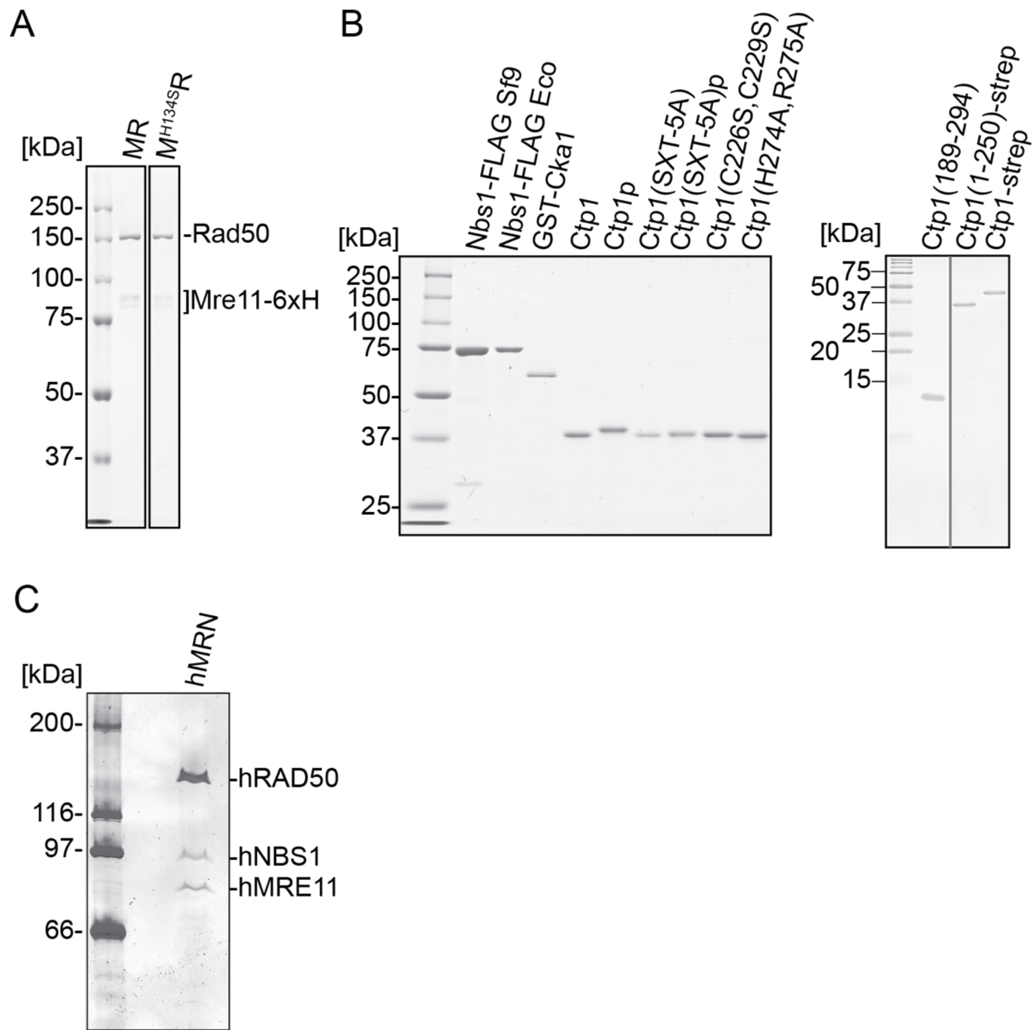

Fig. S1. SDS-PAGE gel image of purified proteins used in this study.

(A) MR complex and nuclease dead Mre11-Rad50 complex. (B) Nbs1, Cka1 and Ctp1 derivatives. (C) Human MRN complex.

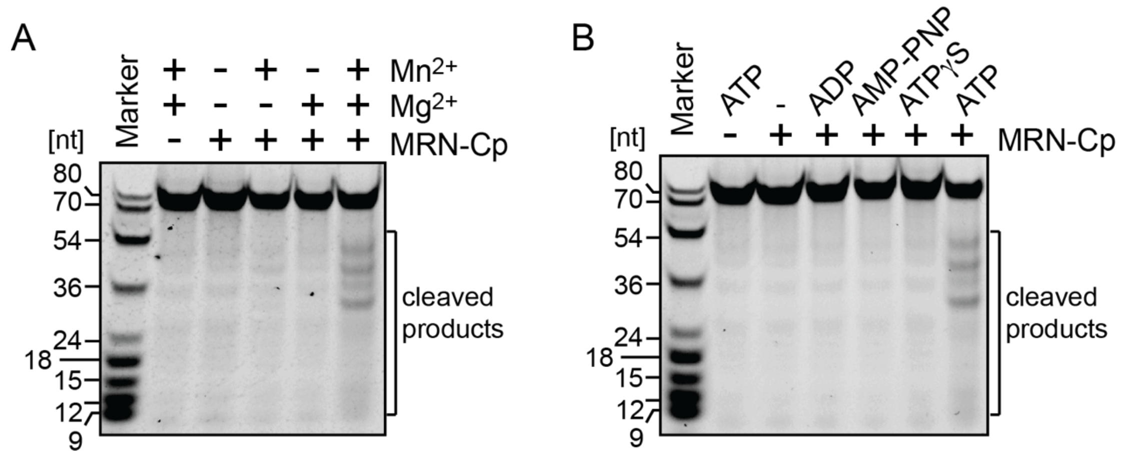

Fig. S2. Requirement for Mre11 endonuclease activity. (A) Both Mg^{2+} and Mn^{2+} are required for Mre11 endonuclease activity in the phosphorylated Ctp1-MRN complex. (B) ATP hydrolysis is required for Mre11 endonuclease in the phosphorylated Ctp1-MRN complex.

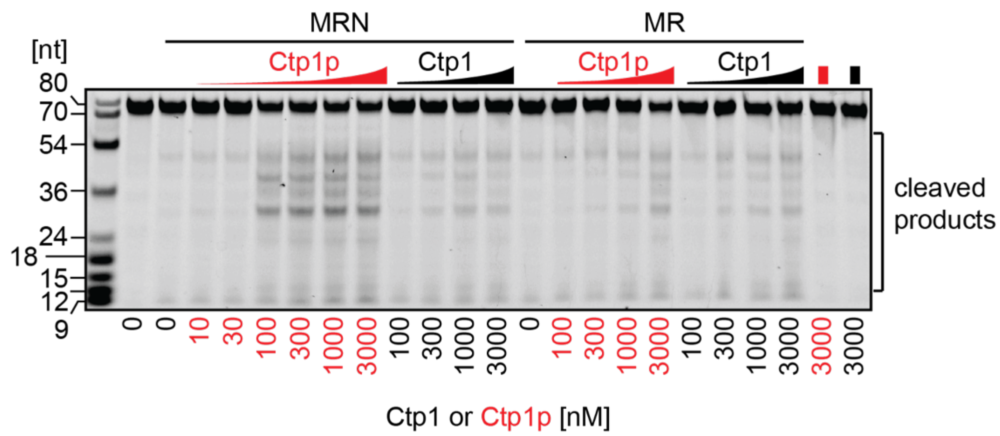

Fig. S3. Titration of Ctp1 in the Mre11 endonuclease assay.

Various concentrations of Ctp1 were included in the Mre11 endonuclease assay in reactions containing Mre11-Rad50-Nbs1 (MRN) or Mre11-Rad50 (MR). Representative gel image used for the quantification shown in Fig. 2C.

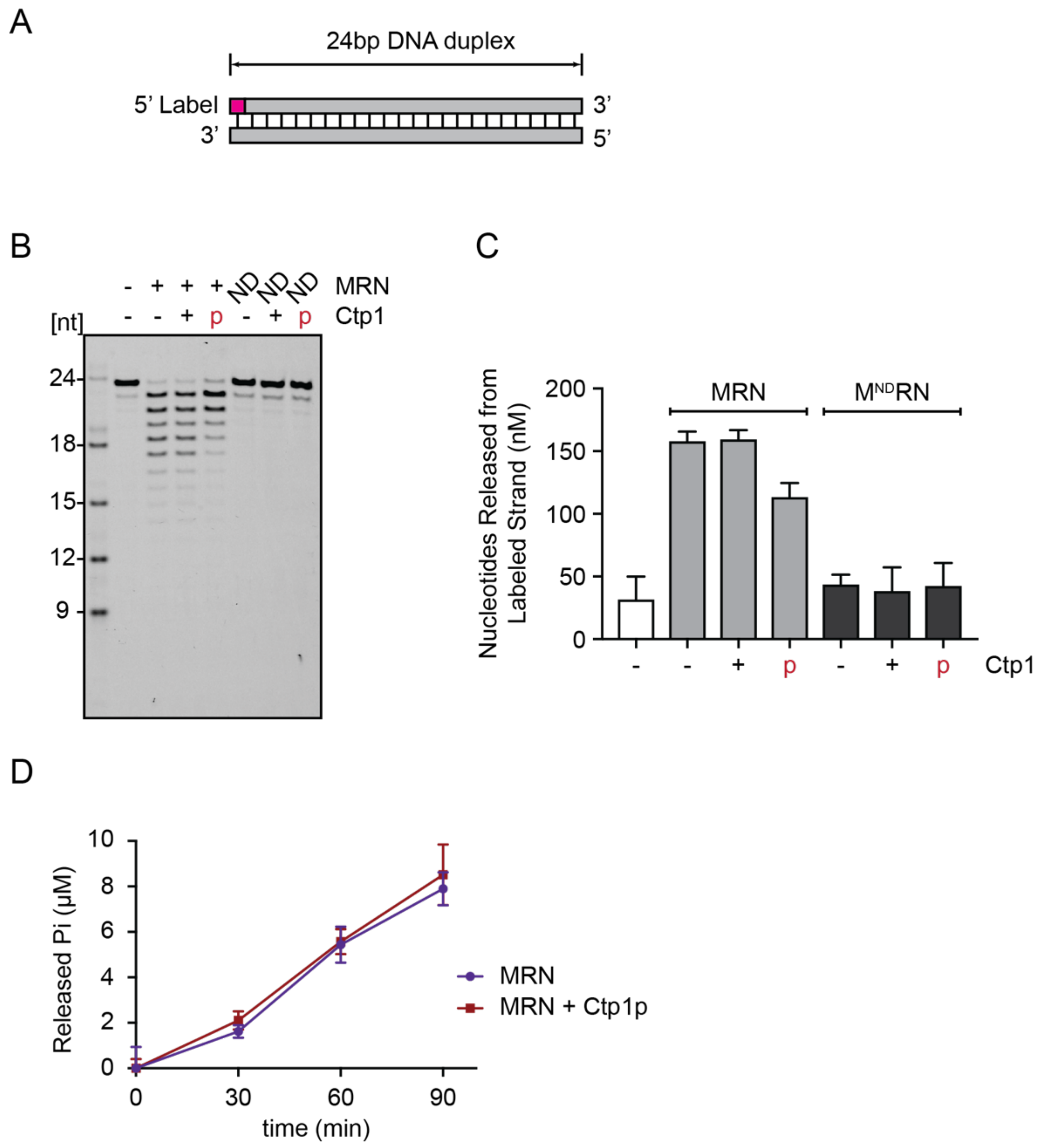

Fig. S4. The effect of phosphorylated Ctp1 (Ctp1-P) on the exonuclease and ATPase activities of MRN.

(A) Illustration of the substrate employed to assay Mre11 exonuclease activity. (B) Mre11 exonuclease activity is not stimulated by Ctp1 and is slightly inhibited by phosphorylated Ctp1. Representative gel image. Proteins were added at equimolar protein-monomer concentrations (100 nM) and incubated at 30°C for 60 min. Red "p" indicates phosphorylated Ctp1. ND indicates MRN complex containing nuclease-deficient Mre11^{H134S}, Rad50, and Nbs1. (C) Quantification of three independent sets of the exonuclease assay shown in (B). Error bars indicate standard deviation, n = 3. (D) Time course of the ATPase activity of MRN demonstrates that phosphorylation of Ctp1 does not affect ATPase activity.

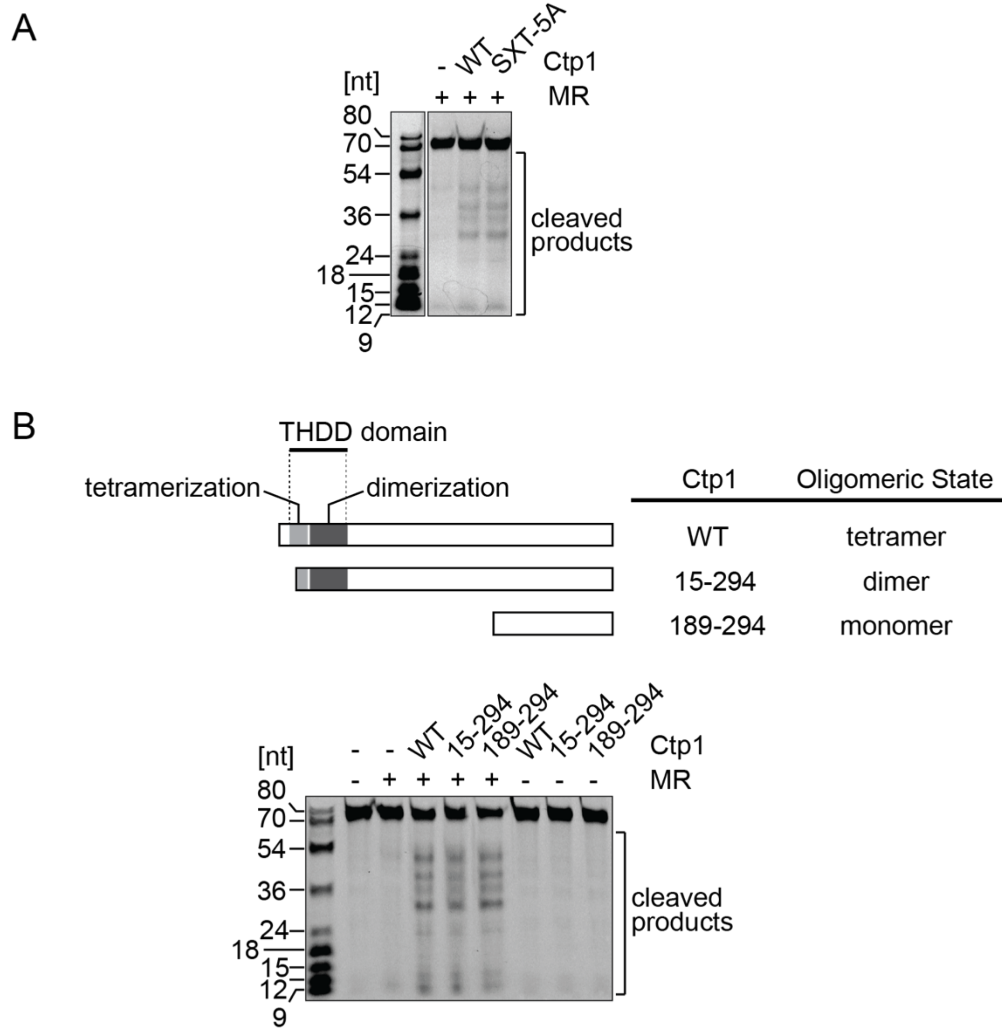

Fig. S5. SXT-5A and mutations that change the oligomeric status of Ctp1 can stimulate MR endonuclease activity.

SXT-5A (A), dimer (15-294) and monomer (189-94) mutants (B) were examined for their ability to stimulate MR endonuclease activity. Ctp1, wild type or variants, at 3 μM.

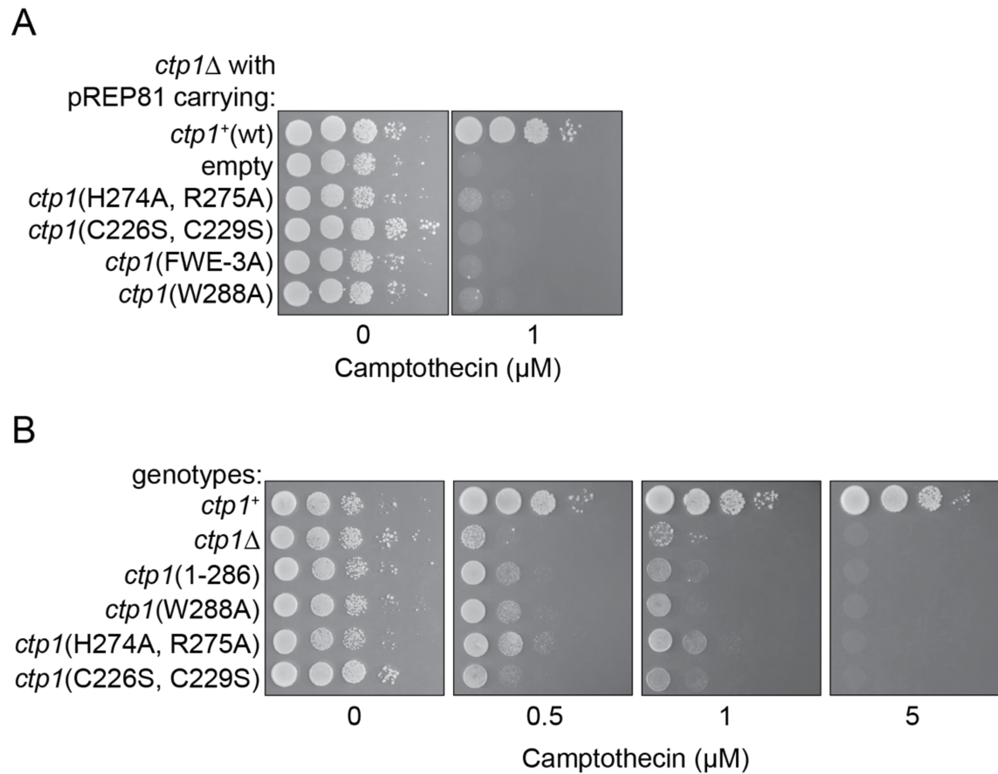

Fig. S6. CxxC and RHR mutants show severe sensitivity to camptothecin.
(A) Complementation of *ctp1Δ* camptothecin sensitivity with plasmids expressing Ctp1 variants. (B) Camptothecin sensitivity of strains with mutations in CxxC or RHR motifs at the endogenous *ctp1* locus. Serially diluted cells were spotted onto YES plates containing the indicated concentration of camptothecin.

Table S1. Ctp1 phosphorylation site analysis

Peptide Sequence	Position in Amino acid sequence	Sample	Number of PSMs	Modifications in Proteins	Theo MHplus in Da	Confidence by Search Engine Sequest HT	Percolator q-Value by Search Engine Sequest HT	Percolator PEP by Search Engine Sequest HT	XCorr by Search Engine Sequest HT
VSSNTIQELDSTTDEDEIPGSD TVDEEDPSLNAPFSEK	67-104	Ctp1	156	(no modification)	4110.8157	High	0.000211	1.61E-08	3.61
		Ctp1p (5min)	66	(no modification)	4110.8157	High	0.0002365	1.60E-09	3.58
			36	1xPhospho [T89(100)]	4190.78203	High	0.0002365	2.52E-08	3.68
			8	2xPhospho [T89(96.3); S/T]	4270.74836	High	0.0002365	1.71E-07	4.16
			2	3xPhospho [T89(97.9); S/T]	4350.7147	High	0.0002365	1.44E-06	4.29
		Ctp1p (full)	28	(no modification)	4110.8157	High	0.0002508	4.78E-08	4.42
			15	1xPhospho [T89(100)]	4190.78203	High	0.0002508	2.76E-07	3.11
			4	2xPhospho [S/T]	4270.74836	High	0.0002508	5.27E-09	4.92
			1	3xPhospho [T89(97.9); S/T]	4350.7147	High	0.0002508	1.77E-07	3.5
FLDTNPIGAESFESSDGEMHLR	141-162	Ctp1	241	(no modification)	2452.10853	High	0.000211	1.99E-14	6.6
			1	1xPhospho [S155(99.3)]	2532.07486	High	0.000211	2.72E-05	4.64
			8	2xPhospho [S154(100); S155(100)]	2612.04119	High	0.000211	0.004061	2.25
		Ctp1p (5min)	142	(no modification)	2452.10853	High	0.0002365	8.37E-13	6.55
			56	1xPhospho [S155(99.6)]	2532.07486	High	0.0002365	2.82E-07	5.43
			52	2xPhospho [S154(100); S155(100)]	2612.04119	High	0.0002365	4.16E-05	4.08
		Ctp1p (full)	64	(no modification)	2452.10853	High	0.0002508	2.01E-15	6.84
			30	1xPhospho [S155(99.4)]	2532.07486	High	0.0002508	1.54E-07	5.46
			144	2xPhospho [S154(100); S155(100)]	2612.04119	High	0.0002508	3.07E-06	5.1
			19	3xPhospho [S151(100); S154(100); S155(100)]	2692.00752	High	0.0002508	7.63E-06	2.4

Table S2. Strains used in this study

Strain name	Related Genotype	Plasmid	Source or reference
YA119	*h^- Msmt-0 ura4-D18 leu1-32 his3-D1 arg3-D1*		Akamatsu et al., 2008
YST046	*Same as YA119 but ctp1::ura4$^+$*		Akamatsu et al., 2008
YA1097	*h^+ his3-D1 leu1-32 ura4-D18 ade6-M210 ctp1::ura4$^+$*		Akamatsu et al., 2008
spAZ_063	Same as YST046	pREP81-Ctp1	This study
spAZ_064	Same as YST046	pREP81	This study
spAZ_090	Same as YST046	pREP81-Ctp1(F287A)	This study
spAZ_091	Same as YST046	pREP81-Ctp1(E289A)	This study
spAZ_092	Same as YST046	pREP81-Ctp1(F292A)	This study
spAZ_093	Same as YST046	pREP81-Ctp1(P284A)	This study
spAZ_094	Same as YST046	pREP81-Ctp1(P280A)	This study
spAZ_095	Same as YST046	pREP81-Ctp1(FWE-5A)	This study
spAZ_096	Same as YST046	pREP81-Ctp1(FWE-3A)	This study
spAZ_099	Same as YST046	pREP81-Ctp1(W288A)	This study
spAZ_114	Same as YA119 but *ctp1-F287A-KanMx6$^+$*		This study
spAZ_120	Same as YA119 but *ctp1-W288A-KanMx6$^+$*		This study
spAZ_123	Same as YA119 but *ctp1-F292A-KanMx6$^+$*		This study
spAZ_128	Same as YA119 but *ctp1-(1-286)-KanMx6$^+$*		This study

spAZ_132	Same as YA119 but *ctp1-(1-279)-KanMx6*$^{+}$		This study
spAZ_134	Same as YA119 but *ctp1-KanMx6*$^{+}$		This study
spAZ_156	Same as YA119 but *ctp1::KanMx6*$^{+}$		This study
spAZ_178	Same as YST046	pREP81-Ctp1(H274A, R275A)	This study
spAZ_184	Same as YST046	pREP81-Ctp1(C226S, C229S)	This study
spAZ_191	Same as YA119 but *ctp1-(H274A, R275A)-KanMx6*$^{+}$		This study
spAZ_196	Same as YA119 but *ctp1-(C226S, C229S)-KanMx6*$^{+}$		This study

Table S3. Primers used in this study

Designation	Sequence
oAZ_087	CAAAAAGTAGGCCGGGCTGCAAAATTAAACATCCCCAAAC
oAZ_088	GTTTGGGGATGTTTAATTTTGCAGCCCGGCCTACTTTTTG
oAZ_094	TCTCAATTCATGGTAATTCCGATGACCCTTCTGGT
oAZ_095	ACCAGAAGGGTCATCGGAATTACCATGAATTGAGA
oAZ_153	TGGAGCCACCCGCAGTTC
oAZ_158	GAACTGCGGGTGGCTCCAGTCATTCCAAGTAGGCGCAACG
oAZ_203	AGGTAAATCGCGCATTCTGTTCCCGGGCACCTATATCGTTGGTCGAAATGTATCT GACGATTCGTCCCACATTC
oAZ_204	GGATCCTTACTTATCGTCGTCATCCTTGTAATCAAAGTG
oAZ_205	GACGATAAGTAAGGATCCGAATTCGAGCTCCGTC
oAZ_206	AGAATGCGCGATTTACCTTTCAGGATATCGCCTTCCGCTTCAATAATCCACATATG TATATCTCCTTCTTAAAGTTAAACAAAATTATTTCTAGAG
oAZ_235	GAAAGCCCGGATAGCCAAAAGTTCTATGAGTTACATGGTCCAGTCAAAG
oAZ_236	TTGGCTATCCGGGCTTTCGTAAGCAGGAAGTGCTTTACGTTTATTAC
oAZ_265	CTGGAAGTTCTGTTCCAGGGGCCCGGTGGTAGCGGTGGTAGCCATCATCATCAT CATCACTGATAAGGATCCGAATTCGAGCTCCGTC
oAZ_266	CTGGAACAGAACTTCCAGCTTATCGTCGTCATCCTTGTAATCAAAGTGAAAC
oAZ_458	TCACATATGTCTGACAACCGCCAGAAAAAG
oAZ_514	GCTTACGAATCTCCGGATTCTCAAAAGGTAAGAGTATTTTTGGCTTTTTTCCCTAC
oAZ_515	TACCTTTTGAGAATCCGGAGATTCGTAAGCAGGAAGTGCTTTACGTTTATTACC
oAZ_516	TAGGCCGGGCTGCTAAATTAAACATCCCCAAACCTATTCCAAATGG
oAZ_517	TGGGGATGTTTAATTTAGCAGCCCGGCCTACTTTTTGCACCAAAGG
oAZ_522	GCTTACGAATCTCCGGATTCTCAAAAGTTCTATGAGTTACATGGTCCAGTCAA
oAZ_523	GAACTTTTGAGAATCCGGAGATTCGTAAGCAGGAAGTGCTTTACGT
PSOL10009	GAATTGTTAATTAAGTCAGTGATGATGATGATGATGATCATCTAAAATTTCGTCATC C

PSOL10010	TTGAAAATTCGAATTAAAAAAATGTCGTGCATTGACAGAATGTCCATCATG
PSOL10011	GAATTGTTAATTAAGTTAAAGAGGTTCTTTAACAATCATGCTCTTCTGATTCGTGTC
PSOL9975	TTATCCACTTCCAATGTTATTATCACTTATCGTCGTCATCCTTGTAATCAAAGTGAA ACTTGAGATCATTAAATTCATCG
T7ter	GCTAGTTATTGCTCAGCGG

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