SI Appendix

Materials and Methods

Culture of the Fission Yeast

S. pombe cells were cultured routinely in YES (yeast extract with supplements) or EMM (Edinburgh minimal media 2) with appropriate supplements (Moreno et al., 1991, Molecular genetic analysis of fission yeast *Schizosaccharomyces pombe*. *Methods in enzymology* 194:795-823). For dilution assays, cells in log-phase growth were harvested, counted, plated as a five-fold serial dilutions on YEs media with or without the indicated HU concentration, and photographed after three to five days of incubation at 30ºC. For the cell viability assay log-phase cells were cultured in the presence of 12.5 mM HU for 3 hours. Cells were harvested, washed to remove HU, and plated on YES plates. The number of colonies was counted after four or five days of incubation.

The Preparation of Whole Cell Extracts and Chromatin Extracts

Whole cell extracts were prepared from 5 ml of cell culture (OD_{600}=0.8~1.0). Cells were harvested, washed once with 1 ml of 20% TCA and resuspended in 500 µl 20% TCA. 500 µl of acid-washed glass beads (Sigma, G8772) were added and cells broken by vortexing (8 min). The supernatant was transferred to a fresh tube, centrifuged at 3000 rpm for 10 min and the precipitate resuspended in 1× SDS loading buffer (100 mM Tris pH=11, 2% SDS, 100 mM DTT), boiled at 95ºC for 5 min, separated by SDS-PAGE, and transferred to 0.45 µm PVDF membrane (Millipore, immobilon-P^{SQ}). Membranes were sequentially incubated with primary and

secondary antibodies and detected by SuperSignal West Pico chemiluminescence (Thermo, 34078).

Chromatin extracts were prepared from 1 liter of culture. Cells were harvested, washed with the stopping buffer (20 mM Tris-HCl pH 7.5, 150 mM NaCl, 50 mM NaF, 10 mM EDTA, 1 mM NaN_3), resuspended in 2 ml of 1.2 M sorbitol containing 10 mM DTT and 1.5 mg/ml lysing Enzyme (Sigma, L1412) plus 1KU lyticase (Sigma, L4025) and incubated at 30 ºC for 30~60 min (until ~90% cells became "ghost" cells upon addition of 1% Triton-X100). Spheroplasts were harvested by centrifugation (1000 g 5 min.), resuspended in buffer D_{100} (25 mM Tris-HCl pH 7.5, 100 mM NaCl, 10% glycerol, 1 mM PMSF, proteinase inhibitor cocktail (Amresco)) and lysed with 1% Triton X-100. Following centrifugation (3000 g, 7 min.) the supernatants (whole cell extracts: WCEs) were loaded onto 10x volume of 30% sucrose dissolved in buffer (25 mM Tris-HCl pH 7.5, 100 mM NaCl, 30% sucrose, 0.25% Triton X-100) and centrifuged (35000 g 30 min). Chromatin containing pellets were suspended, washed twice with buffer D_{100} and resuspended in buffer D_{600} (25 mM Tris-HCl pH 7.5, 600 mM NaCl, 10% glycerol, 1 mM PMSF, proteinase inhibitor cocktail) and incubated for 5 min on ice. Following centrifugation (35000 g 10 min.) the supernatants (chromatin extracts) were collected.

Isolation of Replication Forks

Cells at log phase with or without HU treatment of 3 hrs were crosslinked with 1% formaldehyde (Sigma, F8775) at 25°C for 8 min and stopped by adding 1/20 volume of 2.5 M glycine. Chromatin was obtained and sonicated with 6 bursts of 150 W for 10 s, with 1 min intervals

between pulses. The size of the sheared DNA fragments was between 300-1000 bp. The sheared chromatin sample was incubated with anti-Flag M2 beads (Sigma, A2220) for 3 h at 4 °C. Following extensive washing, with 1 x PBS buffer the immunoprecipitated chromatin was eluted with 200 ng/ul 3X Flag peptide， and the eluted fraction, supplemented with 1 x TES (50 mM Tris-HCl, pH 7.4, 1 mM EDTA, 2% SDS), was incubated at 65°C for 2 hrs for reversing crosslinking and then subjected to quantification of the indicated proteins.

Expression and Purification of *S. pombe* Cds1

cds1^{+} was cloned into plasmid pSSB-Y1 containing an 8his-T7SSB tag (C-terminal) for affinity purification. The Cds1-8his-T7SSB fusion protein was overexpressed in *S. pombe* using EMM medium without thiamine (13 hours) and cells were treated with 12.5 mM HU for a further 3 hr. Cells were broken by Lyticase treatment and resuspended in D_{100} buffer supplemented with 1% Triton X-100, 50 mM NaF and 1 mM Na_3VO_4. Insoluble material was removed by centrifugation (35000 g, 30 min, 4 ºC) and solid ammonium sulphate was added to the supernatant to 30% saturation then centrifuged (35000 g, 10 min, 4 ºC). The ammonium sulphate was adjusted to 40% in the supernatant fraction. Precipitated protein was collected by centrifugation (35000 g, 30 min, 4 ºC) and resuspended in D_{100} buffer + 50 mM NaF+1 mM Na_3VO_4. The soluble fraction was purified by gel filtration, a Ni-NTA (QIAGEN column) and ssDNA cellulose elution. Fractions containing Cds1 were pooled and dialyzed against buffer D_{100} and stored at -80ºC.

The Overexpression and Purification of *S. pombe* Cdc45

cdc45^{+} was cloned into plasmid pMAL-c2X containing MBP (N-terminus) and 6His-tag (C-terminal) tags. *E. coli* BL21 (DE3) cells harboring the plasmid were cultured in LB at 37 ºC to an A_{600nm}=0.3~0.4. 1 mM IPTG was added and the culture incubated for 14 hours at 18 ºC. Cells were harvested, washed with ddH_2O and resuspended in buffer D_{100}. After cell lysis by sonication, insoluble materials were removed by centrifugation (35000g, 30 min, 4 ºC). The supernatant was incubated with Amylose Resin (NEB, E8021L) for 1 hour and eluted with D_{100} buffer containing 10 mM maltose. Eluted fractions containing Cdc45 were pooled and mixed with Ni-NTA resin for 30 min at 4 ºC. The resin was washed with D_{100} supplemented with 20 mM imidazole, and Cdc45 eluted with a gradient of buffer D_{100}/20 mM imidazole to buffer D_{100}/250 mM imidazole. Fractions containing Cdc45 were pooled, dialyzed against buffer D_{100}, and stored at -80 ºC.

***In vitro* Phosphorylation of Cdc45 by Cds1**

5 pmol of purified MBP-Cdc45-6his was mixed with 20 pmol of purified Cds1-8his-3HA in 20µl of buffer (10 mM HEPES-NaOH pH 7.5, 75 mM KCl, 1 mM DTT, 4 mM $MgCl_2$, 0.5 mM EDTA, 0.1 mM PMSF, 20 µCi [γ-^{32}P]-ATP (Perkin Elmer) and 0.1 mM ATP) and incubated for 30 min at 30ºC. The reaction was stopped by adding SDS loading buffer and boiled at 95 ºC for 5 min. Cdc45 proteins were separated by 8% SDS-PAGE gel, fixed with 5% methanol and dried for autoradiography.

Isolation of Replicative Helicase CMG Complex from Chromatin

Five expression plasmids were constructed and integrated into specific genomic loci in one strain: *ars1::(nmt1-cdc45$^{WT/3A/3D/3E}$-Flag)*, *arg3::(nmt1-sld5, mcm2, mcm3)*, *ade6::(nmt1-mcm5, mcm6)*, *leu2::(nmt1-mcm7, psf2-8his-3HA)*, and *leu1::(nmt1-psf1, psf3, mcm4)*. The five plasmids are listed in Table S2. Cells expressing the CMG complex were grown in EMM medium at 26ºC for 24 hr. 100 liters of culture were harvested and chromatin isolated (see above). Chromatin was resuspended in buffer D_{100} and broken by sonication. Insoluble material was removed by centrifugation (35000 g, 30 min, 4ºC), 0.5 mM $CaCl_2$ +1 mM $MgCl_2$ +5000 units of DNase I + proteinase inhibitors + phosphatase inhibitors cocktail (Merck) were added to the supernatant and mixed with 500 µl of anti-Flag M2 beads (Sigma, A2220) for 2 hours at 4ºC. Beads were washed with buffer D_{200} (buffer D_{100} plus 100 mM NaCl). CMG complex was eluted in buffer D_{100} supplemented with 0.4 mg/ml 3×Flag peptide. The fractions containing CMG complex were pooled and mixed with 40 µl of anti-HA-Agarose beads (Sigma, A2095) at 4 ºC for 3 hours. Beads were washed with buffer D100 and subsequently used in the *in vitro* assay of CMG helicase activity.

Phos-tag SDS-PAGE

Chromatin extracts were separated using Phos-tag SDS-PAGE (6% w/v polyacrylamide, 50 µM Phos-tag acrylamide (Wako Chemicals),100 µM $MnCl_2$). The gel was dialyzed against transfer buffer + 10 mM EDTA for three times (60 min), and then immunoblotted.

Preparation of Specific Antibodies Against Phosphorylated Cdc45 at S275, S322, or S397 Site

Peptides used for generating antibodies against phosphorylated Cdc45 S275, S322, or S397 are listed in Table S3. Phosphorylated S275, S322, or S397 peptides (GL Biochem) were conjugated with keyhole limpet hemocyanin (KLH) and injected into rabbits. Crude serum was affinity purified by adding phosphorylated peptides crosslinked to CNBr-activated Sepharose resin, washing with D_{100} and eluting with 100 mM Glycine-HCl (pH 2.5) followed by immediate neutralization with 1 M Tris-HCl (pH 8.0). Fractions containing antibody were pooled and applied to an equivalent unmodified peptide column and the flow-through collected and dialyzed against phosphate buffered saline (PBS). 50% (v/v) glycerol was added into the purified antibodies and stored at -20 ºC.

EdU Incorporation and Detection

10 μM EdU was added to cultures for indicated the time periods. For EdU detection, cells were fixed with 70% ethanol and treated with the AlexaFluor 488, 555, or 647 Click-iT kit (Invitrogen) following the manufacturers' instructions. Treated cells were washed twice with PBS+3% bovine serum albumin (BSA) and sonicated softly in PBS +10 μg/ml RNase A+1 μg/ml RNase T1. Cells were the subject to FACS analysis.

Single Molecule-based DNA Combing

For single-color DNA combing assay, a concentration of 10 μM EdU was used. For IdU/CldU

dual-color labelling, 2.5 μM IdU and 50 μM CldU were used. For detection: 10 ml of cells were harvested and embedded in 0.65% low melting-point agarose plugs. Cell walls were removed using lyticase and proteins digested with Proteinase K and plugs were extensively washed in TE the MES buffer (pH 6.3) and agarose was digested with β-Agarase I. Genomic DNA fibers were stretched and adhered to silanized glass coverslips (Genomic Vision, COV-001). After heat crosslinking (60 ºC for 2 hours) labeled DNA fibers were detected as follows: EdU detection, coverslips were treated with PBS and labeled with AlexaFlour 555 using a Click-iT kit using the manufacturers' instructions. IdU/CldU, DNA fibers were denatured in 1 M NaOH for 20~22 min and neutralized by washing in PBS 5 times. Slides were blocked with PBS+3% BSA+0.02% Tween-20 for 35 min and incubated with anti-IdU (BD, 347580, 1:200) and anti-CldU antibodies (Lifespan Bioscience, C188215, 1:50) for 35 min. DNA Fibers were washed in PBS and incubated with secondary antibody (Molecular probes, A11030 and A11006, 1:200) for 35 min. After washing with PBS slides were mounted in SlowFade Gold with DAPI (Invitrogen, S36939). DNA fibers were imaged on a Delta Vision Elite microscope (Applied Precision) using a 1.4 NA 60× lens. 10 z axis sections were deconvolved and projected using softWoRx (Applied Precision). DNA fibers images were analyzed using Volocity (Perkin Elmer). A minimum of 500 DNA fibers per sample were scored. The data were evaluated for statistical significance using χ^2, 2-tailed Z statistics and Mann-Whitney Test.

Rad52 Foci

5 ml of cell culture either untreated or treated with HU at the indicated concentration was harvested, fixed with cold 70% ethanol (1 hour), washed extensively with PBS and mounted in

SlowFade Gold with DAPI. Rad52 foci were imaged on a Delta Vision Elite microscope with a 100× lens. 10 z axis sections were deconvolved and projected as described above. >3000 cells per sample from 2 to 4 independent experiments were analyzed.

In vitro CMG Helicase Activity

TTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTGATTAAGTTGG GTAACGCCAGGGTTTTCCCAGTCACGAC oligonucleotide was synthesized and labelled with ^{32}P at its 5'-end and annealed to M13mp18 ssDNA circular DNA (3'-39 nucleotides homology) to provide a substrate for the CMG helicase. 1 pmol of purified CMG complex plus 0.5 nM radiolabeled substrates were mixed in buffer (25 mM HEPES-NaOH, pH 7.5, 10 mM Magnesium acetate, 50 mM sodium acetate, 1 mM DTT, 40 μg/ml BSA, 4 mM ATP) and incubated at 30 ^{o}C for 30 min. The reaction was stopped by adding loading dye containing 0.1% SDS and 10 mM EDTA. Reaction products were separated by 8% native PAGE. The gel was washed with 0.5× TBE, mounted on Whatman 3MM paper and dried for autoradiography.

Table 1. *S. pombe* Strains Used in This Study

Strains	Genotype	Source
LD330	*h-, ura4-D18*	Lilin Du
LY01	*h-, ura4-D18, cdc45-R319I:ura4*	This Study
LY02	*h-, ura4-D18, ura4:cdc45-R319I*	This Study
LY03	*h-, ura4-D18, cdc45-S275A:ura4*	This Study
LY04	*h-, ura4-D18, cdc45-S275D:ura4*	This Study
LY05	*h-, ura4-D18, cdc45-S322A:ura4*	This Study
LY06	*h-, ura4-D18, cdc45-S322D:ura4*	This Study
LY07	*h-, ura4-D18, cdc45-S397A:ura4*	This Study
LY08	*h-, ura4-D18, cdc45-S397D:ura4*	This Study
LY09	*h-, ura4-D18, cdc45-S275A S322A S397A:ura4*	This Study
LY10	*h-, ura4-D18, cdc45-S275D S322D S397D:ura4*	This Study
LY11	*h-, ura4-D18, cdc45-S275E S322E S397E:ura4*	This Study
LY12	*h-, ura4-D18, cdc45-S275T S322T S397T:ura4*	This Study
LY13	*h-, ura4-D18, cdc45::cdc45-8his-3HA*	This Study
LY14	*h-, ura4-D18, cdc45::cdc45-S275A S322A S397A-8his-3HA*	This Study
Ets13	*h+, ade6-704, cds1::kanMX6, leu1-32, ura4-D18*	A. M. Carr
LY15	*h+, ade6-704, cds1::kanMX6, leu1-32, ura4-D18, cdc45-R319I:ura4*	This Study
LY16	*h+, ade6-704, cds1::kanMX6, leu1-32, ura4-D18, ura4:cdc45-R319I*	This Study
LY17	*h+, ade6-704, cds1::kanMX6, leu1-32, ura4-D18, cdc45::cdc45-8his-3HA*	This Study
LY18	*h+, ade6-704, cds1::kanMX6, leu1-32, ura4-D18, cdc45::cdc45-S275A S322A S397A-3HA*	This Study
LY24	*h+, ade6-704, cds1::kanMX6, leu1-32, ura4-D18, cdc45-S275A:ura4*	This Study
LY25	*h+, ade6-704, cds1::kanMX6, leu1-32, ura4-D18, cdc45-S275D:ura4*	This Study
LY26	*h+, ade6-704, cds1::kanMX6, leu1-32, ura4-D18, cdc45-S322A:ura4*	This Study
LY27	*h+, ade6-704, cds1::kanMX6, leu1-32, ura4-D18, cdc45-S322D:ura4*	This Study
LY28	*h+, ade6-704, cds1::kanMX6, leu1-32, ura4-D18, cdc45-S397A:ura4*	This Study
LY29	*h+, ade6-704, cds1::kanMX6, leu1-32, ura4-D18, cdc45-S397D:ura4*	This Study
LY30	*h+, ade6-704, cds1::kanMX6, leu1-32, ura4-D18, cdc45-S275A S322A S397A:ura4*	This Study
LY31	*h+, ade6-704, cds1::kanMX6, leu1-32, ura4-D18, cdc45-S275D S322D S397D:ura4*	This Study

LY32	*h+, ade6-704, cds1::kanMX6, leu1-32, ura4-D18, cdc45-S275E S322E S397E:ura4*	This Study
LY33	*h+, ade6-704, cds1::kanMX6, leu1-32, ura4-D18, cdc45-S275T S322T S397T:ura4*	This Study
LY34	*h-, ade6-704, chk1::kanMX6, leu1-32, ura4-D18, cdc45::cdc45-8his-3HA*	This Study
LY35	*h-, ura4-D18, rad3::kanMX6, cdc45::cdc45-8his-3HA*	This Study
J2172	*h-, smt0, leu1-32, ura4-D18, ade6-704, leu1::[leu1-adh:hENT1], his7+, adh1::hsv-TK, cdc10-m17*	A.M. Carr
LY36	*h-, smt0, leu1-32, ura4-D18, ade6-704, leu1::[leu1-adh:hENT1], his7+, adh1::hsv-TK, cdc10-m17, cdc45-R319I:ura4*	This Study
LY37	*h-, smt0, leu1-32, ura4-D18, ade6-704, leu1::[leu1-adh:hENT1], his7+, adh1::hsv-TK, cdc10-m17, cdc45-S275A S322A S397A:ura4*	This Study
LY38	*h-, smt0, leu1-32, ura4-D18, ade6-704, leu1::[leu1-adh:hENT1], his7+, adh1::hsv-TK, cdc10-m17, cdc45-S275D S322D S397D:ura4*	This Study
LY39	*h-, smt0, leu1-32, ura4-D18, ade6-704, leu1::[leu1-adh:hENT1], his7+, adh1::hsv-TK, cdc10-m17, cds1::hphMX*	This Study
LY40	*h-, smt0, leu1-32, ura4-D18, ade6-704, leu1::[leu1-adh:hENT1], his7+, adh1::hsv-TK, cdc10-m17, cds1::hphMX, cdc45-S275A S322A S397A:ura4*	This Study
LY41	*h-, smt0, leu1-32, ura4-D18, ade6-704, leu1::[leu1-adh:hENT1], his7+, adh1::hsv-TK, cdc10-m17, cds1::hphMX, cdc45-S275D S322D S397D:ura4*	This Study
LY42	*h-, ura4-D18, rad22-GFP:kanMX6*	This Study
LY43	*h-, ura4-D18,cdc45-S275A S322A S397A:ura4, rad22-GFP:kanMX6*	This Study
LY44	*h-, ura4-D18,cdc45-S275D S322D S397D:ura4,rad22-GFP:kanMX6*	This Study
LY45	*h-, ura4-D18, cds1::hphMX, rad22-GFP:kanMX6*	This Study
LY46	*h-, ura4-D18, cds1::hphMX, cdc45-S275A S322A S397A:ura4, rad22-GFP:kanMX6*	This Study
LY47	*h-, ura4-D18, cds1::hphMX, cdc45-S275D S322D S397D:ura4, rad22-GFP:kanMX6*	This Study
TK8	*h+, leu1-32, ura4-D18, ade6-M216*	Our stocks
LY48	*h+, leu1-32, ura4-D18, ade6-M216, ars1:(nmt1-cdc45-Flag), arg3:(nmt1-sld5, mcm2 ,mcm3), ade6:(nmt1-mcm5, mcm6), leu2:(nmt1-mcm7, psf2-8his-3HA), leu1:(nmt1-psf1, psf3, mcm4)*	This Study
LY49	*h+, leu1-32, ura4-D18, ade6-M216, cdc45::cdc45-8his-3HA, spb70::spb70-3Flag*	This Study

LY50	*h+, leu1-32, ura4-D18, ade6-M216, cdc45::cdc45-8his-3HA, cdc1::cdc1-3Flag*	This Study
LY51	*h+, leu1-32, ura4-D18, ade6-M216, cdc45::cdc45-8his-3HA, pol2::pol2-3Flag*	This Study
LY52	*h+, ade6-704, cds1::kanMX6, leu1-32, ura4-D18, cdc45::cdc45-8his-3HA, spb70::spb70-3Flag*	This Study
LY53	*h+, ade6-704, cds1::kanMX6, leu1-32, ura4-D18, cdc45::cdc45-8his-3HA, cdc1::cdc1-3Flag*	This Study
LY54	*h+, ade6-704, cds1::kanMX6, leu1-32, ura4-D18, cdc45::cdc45-8his-3HA, pol2::pol2-3Flag*	This Study
LY55	*h-, ura4-D18, ssb1-GFP:kanMX6*	This Study
LY56	*h-, ura4-D18,cdc45-S275A S322A S397A:ura4, ssb1-GFP:kanMX6*	This Study
LY57	*h-, ura4-D18,cdc45-S275D S322D S397D:ura4,ssb1-GFP:kanMX6*	This Study
LY58	*h-, ura4-D18, cds1::hphMX, ssb1-GFP:kanMX6*	This Study
LY59	*h-, ura4-D18, cds1::hphMX, cdc45-S275A S322A S397A:ura4, ssb1-GFP:kanMX6*	This Study
LY60	*h-, ura4-D18, cds1::hphMX, cdc45-S275D S322D S397D:ura4, ssb1-GFP:kanMX6*	This Study
LY61	*h-, ura4-D18, cdc45-S275C S322C S397C:ura4*	This Study
LY62	*h-, ura4-D18, cdc45-S275K S322K S397K:ura4*	This Study
LY63	*h-, ura4-D18, cdc45-S275N S322N S397N:ura4*	This Study
LY64	*h-, ura4-D18, cdc45-S275G S322G S397G:ura4*	This Study
LY65	*h-, ura4-D18, cdc45-S275V S322V S397V:ura4*	This Study
LY66	*h+, ade6-704, cds1::kanMX6, leu1-32, ura4-D18, cdc45-S275C S322C S397C:ura4*	This Study
LY67	*h+, ade6-704, cds1::kanMX6, leu1-32, ura4-D18, cdc45-S275K S322K S397K:ura4*	This Study
LY68	*h+, ade6-704, cds1::kanMX6, leu1-32, ura4-D18, cdc45-S275N S322N S397N:ura4*	This Study
LY69	*h+, ade6-704, cds1::kanMX6, leu1-32, ura4-D18, cdc45-S275G S322G S397G:ura4*	This Study
LY70	*h+, ade6-704, cds1::kanMX6, leu1-32, ura4-D18, cdc45-S275V S322V S397V:ura4*	This Study

Table 2. Plasmids Used in This Study

Plasmid	Description	Source
pFa6a	containing kanMX6	Susan Forsburg
pBluescript	vector for cloning	Stratagene
pSLF1072	vector for protein overexpression controlled by nmt1 promoter in *S. pombe*	Susan Forsburg
pSSB-Y1	vector for protein overexpression controlled by nmt1 promoter in *S. pombe*	Daochun Kong
pBlue-ura4	ura4 were cloned into pBlue at Hind III site	This study
pBlue-hph	hph were cloned into pBlue at EcoR V and Hind III site	This study
pBlue-nat	nat were cloned into pBlue at EcoR V and Hind III site	This study
pBlue-leu1	leu1 were cloned into pBlue at EcoR I and BamH I site	This study
pBlue-ura4-cdc45	cdc45 were cloned at Kpn I and SalI, for replacing cdc45 gene in genome	This study
pBlue-ura4-*cdc45-R319I*	containing *cdc45-R319I*, for replacing cdc45 gene in genome	This study
pBlue-ura4-*cdc45-S275A*	containing *cdc45-S275A*, for replacing cdc45 gene in genome	This study
pBlue-ura4-*cdc45-S275D*	containing *cdc45-S275D*, for replacing cdc45 gene in genome	This study
pBlue-ura4-*cdc45-S275T*	containing *cdc45-S275T*, for replacing cdc45 gene in genome	This study
pBlue-ura4-*cdc45-S322A*	containing *cdc45-S322A*, for replacing cdc45 gene in genome	This study
pBlue-ura4-*cdc45-S322D*	containing *cdc45-S322D*, for replacing cdc45 gene in genome	This study
pBlue-ura4-*cdc45-S322T*	containing *cdc45-S322T*, for replacing cdc45 gene in genome	This study
pBlue-ura4-*cdc45-S397A*	containing *cdc45-S397A*, for replacing cdc45 gene in genome	This study
pBlue-ura4-*cdc45-S397D*	containing *cdc45-S397D*, for replacing cdc45 gene in genome	This study
pBlue-ura4-*cdc45-S397T*	containing *cdc45-S397T*, for replacing cdc45 gene in genome	This study
pBlue-ura4-*cdc45-S275A S322A S397A*	containing *cdc45-S275A S322A S397A*, for replacing cdc45 gene in genome	This study

pBlue-ura4-*cdc45-S275D S322D S397D*	containing *cdc45-S275D S322D S397D*, for replacing cdc45 gene in genome	This study
pBlue-ura4-*cdc45-S275E S322E S397E*	containing *cdc45-S275E S322E S397E*, for replacing cdc45 gene in genome	This study
pBlue-ura4-*cdc45-S275T S322T S397T*	containing *cdc45-S275T S322T S397T*, for replacing cdc45 gene in genome	This study
pBlue-ura4-cdc45-8his-3HA	cdc45 tagged with -8his-3HA at C-terminal	This study
pBlue-ura4-*cdc45-S275A S322A S397A-8his-3HA*	*cdc45-S275A S322A S397A* tagged with -8his-3HA at C-terminal	This study
pSLF1072-cdc45-flag	cdc45 tagged with -flag at C-terminal, cloned at Xho I and Sma I	This study
pBlue-leu1-psf1-psf3-mcm4	psf1, psf3 and mcm4 were controlled by nmt1 promoter, psf1 were cloned at EcoR I, psf3 were at Kpn I and mcm4 were at Sac I	This study
pBlue-hph-sld5-arg3-mcm2-mcm3	sld5, mcm2 and mcm3 were controlled by nmt1 promoter, sld5 were cloned at Sal I, mcm2 were at Kpn I, mcm3 were at Not I and arg3 were at Sac I	This study
pBlue-nat-mcm5-mcm6-ade6	mcm5 and mcm6 were controlled by nmt1 promoter, mcm5 were cloned at Apa I, mcm6 were at Sal I and ade6 were at Sac I	This study
pFa6a-psf2-mcm7-leu2	psf2 and mcm7 were controlled by nmt1 promoter, psf2 were cloned at EcoR I and Pme I, mcm7 were at Bgl II and leu2 were at Asc I	This study
pBlue-ura4-*cdc45-S275C S322C S397C*	containing *cdc45-S275C S322C S397C*, for replacing cdc45 gene in genome	This study
pBlue-ura4-*cdc45-S275K S322K S397K*	containing *cdc45-S275K S322K S397K*, for replacing cdc45 gene in genome	This study
pBlue-ura4-*cdc45-S275N S322N S397N*	containing *cdc45-S275N S322N S397N*, for replacing cdc45 gene in genome	This study
pBlue-ura4-*cdc45-S275G S322G S397G*	containing *cdc45-S275G S322G S397G*, for replacing cdc45 gene in genome	This study
pBlue-ura4-*cdc45-S275V S322V S397V*	containing *cdc45-S275V S322V S397V*, for replacing cdc45 gene in genome	This study

Table 3. The Sequences of Peptides Used for the Generation of Specific Antibodies against Phosphorylated S275, S322 and S397 in Cdc45

Phosphorylation sites	Sequence (N- to C- terminal)	Purity	Note
S275	NRSYpSLLKDEVNRC-KLH	>95%	Immunizing Rabbit
	NRSYpSLLKDEVNRC	>95%	Antibody purification
	NRSYSLLKDEVNRC	>95%	Antibody purification
S322	RHWpSLYDSMLHSC-KLH	>95%	Immunizing Rabbit
	RHWpSLYDSMLHSC	>95%	Antibody purification
	RHWSLYDSMLHSC	>95%	Antibody purification
S397	DDVIFHpSFTRTYC-KLH	>95%	Immunizing Rabbit
	DDVIFHpSFTRTYC	>95%	Antibody purification
	DDVIFHSFTRTYC	>95%	Antibody purification

Table 4. Antibodies Used in This Study

Epitope	Manufacturer	Use	Note
HA	Sigma(H3663)	IB:1:1000; IP:1:200	Primary antibody; mouse
Flag	MBL Life Science(M185-3L)	IB:1:5000	Primary antibody; mouse
spCdc45-S275ph	In-lab	IB:1:500	Primary antibody; rabbit
spCdc45-S322ph	In-lab	IB:1:500	Primary antibody; rabbit
spCdc45-S397ph	In-lab	IB:1:300	Primary antibody; rabbit
spCdc45	In-lab	IB:1:1000	Primary antibody; rabbit
spMcm3	In-lab	IB:1:1000	Primary antibody; rabbit
spMcm4	In-lab	IB:1:1000	Primary antibody; rabbit
spMcm7	In-lab	IB:1:5000	Primary antibody; rabbit
spPsf1	In-lab	IB:1:1000	Primary antibody; rabbit
spH2B	In-lab	IB:1:5000	Primary antibody; rabbit
spPCNA	In-lab	IB:1:2000	Primary antibody; rabbit

spPol3	In-lab	IB:1:2000	Primary antibody; rabbit
spH3	Bioeasytech (BE3015)	IB:1:5000	Primary antibody; mouse
CldU	Lifespan Bioscience(C188215)	IF:1:50	Primary antibody; rat
IdU	BD(347580)	IF:1:200	Primary antibody; mouse
BrdU	Abcam (ab6326)	IF: 1:50	Primary antibody, rat
ssDNA	Millipore(MAB3868)	IF:1:50	Primary antibody; mouse
Alexa Fluor 546	Molecular probes(A11030)	IF:1:200	Secondary antibody; goat anti-mouse
Alexa Fluor 488	Molecular probes(A11006)	IF:1:200	Secondary antibody; goat anti-rat
Alexa Fluor 488 azide	Molecular probes(C10337)		EdU conjugates
IgG	Bioeasytech(BE0101)	IB:1:10000	Secondary antibody; goat anti-rabbit
IgG	Bioeasytech(BE0102)	IB:1:10000	Secondary antibody; goat anti-mouse
Anti-Flag beads	Sigma(A2220)	-	-
Anti-HA beads	Sigma(A2095)	-	-

Supplementary figure legends

Fig. S1. Screen for mutants that rescue Cds1 deficiency.

(A) Spontaneous genetic mutations were selected that render Cds1^{Chk2-} cells resistant to hydroxyurea. Top: wild type cells (WT) are resistant to HU. Bottom: *cds1*$^{-}$ cells are sensitive to 5 mM HU, but a few dozen (of 1×10^{6}) cells form colonies. (B) Schematic of screening strategy. (C) Five-fold serial dilution assays of representative mutants that grew on the 5mM HU. WT (*cds1*$^{Chk2+}$), *cds1*$^{-}$ (*cds1*$^{Chk2}\Delta$). Genomic DNA was isolated and subjected to deep sequencing to determine which genes were mutated. (D) Serial dilution assay of diploid heterozygous cells showing that *cdc45-R319I* is recessive. (E) Mass spectrometry traces of Cdc45 isolated from HU-treated cells showing phosphorylation on S275, S322 and S397. The phosphorylated peptides of Cdc45 protein were shown. (F) Western blotting against serine-phosphorylated peptides and control un-phosphorylated peptides to characterize the three antibodies that specifically recognize phosphorylated S275, S322 and S397. (G) Conservation of the Cdc45 S275, S322 and S397 residues from yeast to *H. sapiens*. Top: Domain structure of Cdc45. S275, S322 and S397 sites are located in the MCM and GINS interaction domain (CID). Bottom: Clustal Omega alignments S275, S322 and S397 are indicated by a solid arrow. Open arrow: nearby serine and threonine residues in human Cdc45. (H, I) Five-fold serial dilution assays of the mutants *cdc45-3A*, *cdc45-3D*, *cdc45-3E*, *cdc45-3C*, *cdc45-3K*, *cdc45-3V*, *cdc45-3N*, *cdc45-3G*, and *cdc45-3T* under the presence or absence of HU in the checkpoint-proficient or –deficient *cds1*$^{Chk2}\Delta$ cells.

Fig. S2. Cell cycle progression and Rad52 foci during HU treatment and after release.

(A) FACS analysis of cells from Fig. 3*A*. The concentration of HU is 12.5 mM. (B) Representative images of the Rad52 recombinant protein foci.

Fig. S3. The length distributions of DNA fibers.

(A) Representative DNA fibers from combing experiments using the indicated strains during unperturbed growth, under replication stress (HU presence), or after HU release. (B) The frequencies of DNA fiber lengths from the indicated strains.

Fig. S4. *cdc45*-3E cells grow poorly.

(A) Growth of the indicated stains on YE plates. *cdc45*-3E cells had a very poor growth. (B) FACS analysis showed *cdc45*-3E cells have an abnormal DNA content. (C) Bright field and fluorescent imaging showing an abnormal phenotype for *cdc45*-3E cells.

Fig. S5. *cdc45*-3A cells have a normal level of Cdc45 protein.

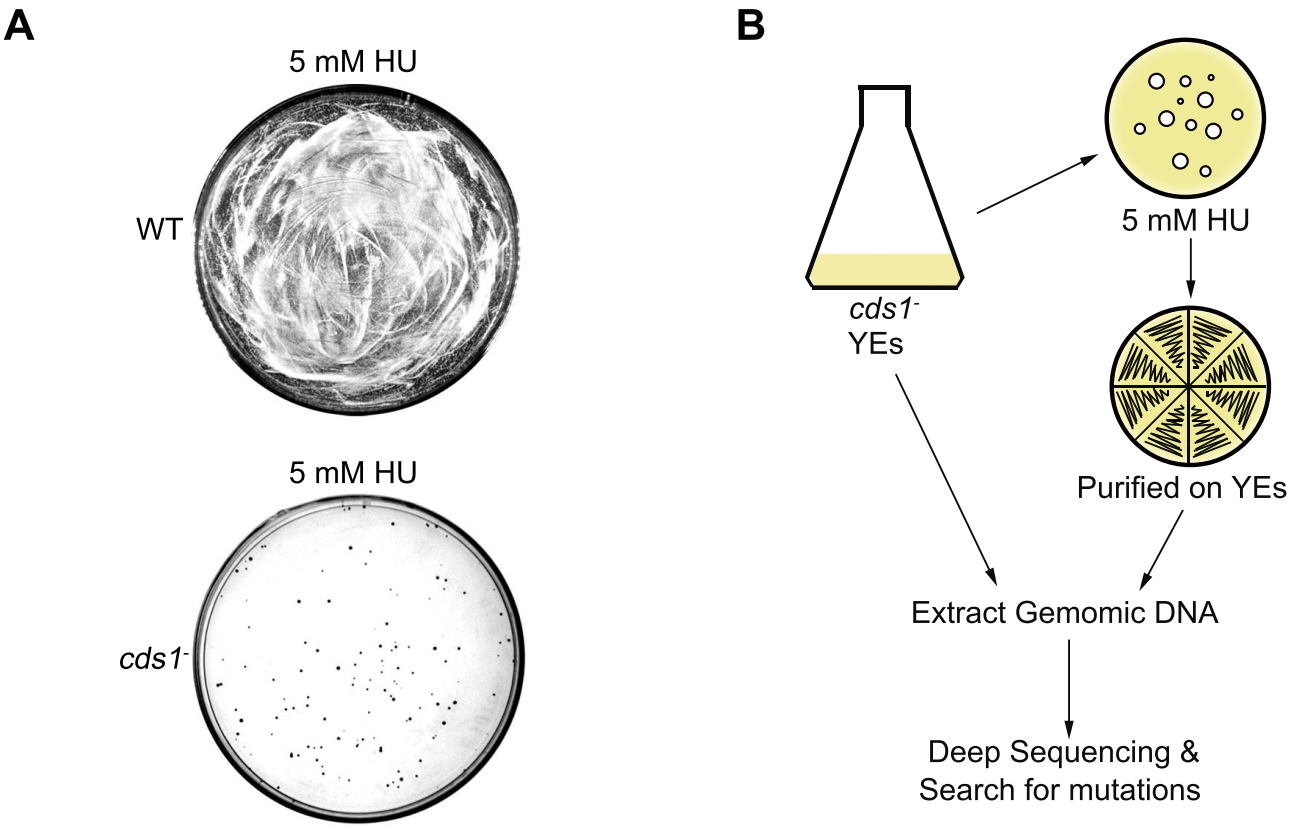
(A) Western blots of *cdc45*$^{+}$ (WT) and *cdc45*-3A cells demonstrating an equal amount of Cdc45 protein in whole cell and chromatin extracts under normal cell conditions or during replication stress (HU). (B) Western blot of chromatin extracts following extraction of chromatin proteins with increasing salt concentration shows that the affinity of Cdc45-3A for chromatin is not changed, either in presence or absence of HU. (C) Western blot assay examining the amounts of Mcm3, Mcm4 and Psf1 CMG subunits on chromatin in presence or absence of HU. The formation of the CMG complex was not altered in *cdc45*-3A cells. (D) A five-fold serial dilution assays show that *cdc45*-3T cells were very slightly more sensitive than *cdc45*$^{+}$ to 5.0 mM HU

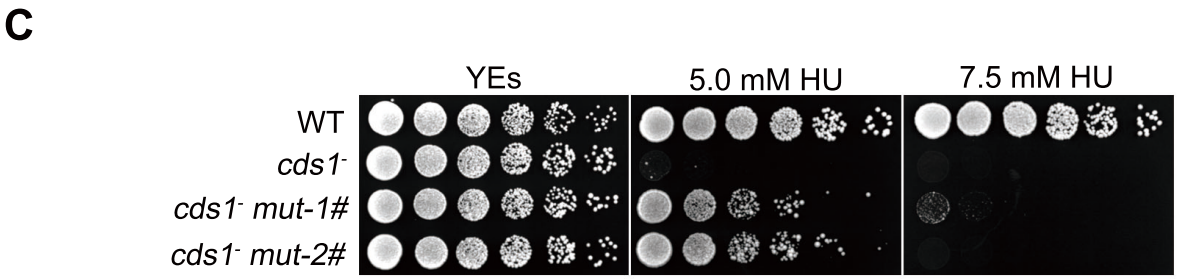
and moderately rescued the sensitivity of the *cds1*^{Chk2}△ cells to 1.5 mM HU.

Fig. S6. RPA foci in wt and *cds1*$^{-}$ cells during HU treatment.

The cultures were incubated at 30ºC to log phase, then 12.5 mM HU was added. The cell fractions were taken for the analysis of RPA foci at indicated time during HU treatment. For examining cellular level of broken DNA or vulnerability of chromatin DNA to ssDNA endonuclease P1, the cell cultures of 50 ml at log-phase were added with 12.5 mM HU and incubated for additional 3 hours. Then, the cells were collected and treated with lysing enzyme and lyticase until 95% cells could be lysed by 1% triton. The cells were collected, washed once with PBS and lysed in presence of 1% triton X-100. 200 U nuclease P1 was added, and the reactions were incubated at 37ºC for 12 min. DNA was prepared and subjected to 1% agarose gel electrophoresis. The nuclease P1 digests both ssDNA and RNA.

Figure S1


A
5 mM HU
WT
5 mM HU
cds1-
B
cds1-
YEs
5 mM HU
Purified on YEs
Extract Gemomic DNA
Deep Sequencing &
Search for mutations


C
YEs
5.0 mM HU
7.5 mM HU
WT
cds1-
cds1- mut-1#
cds1- mut-2#

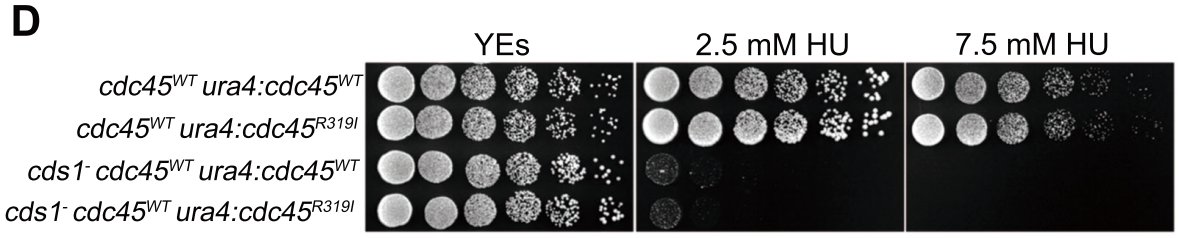
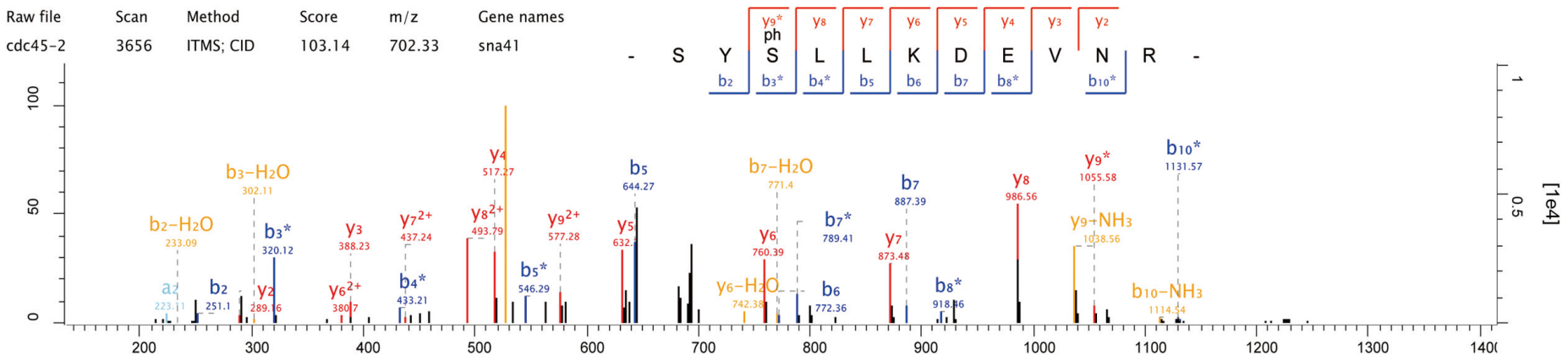

D
YEs
2.5 mM HU
7.5 mM HU
cdc45WT ura4:cdc45WT
cdc45WT ura4:cdc45R319I
cds1- cdc45WT ura4:cdc45WT
cds1- cdc45WT ura4:cdc45R319I

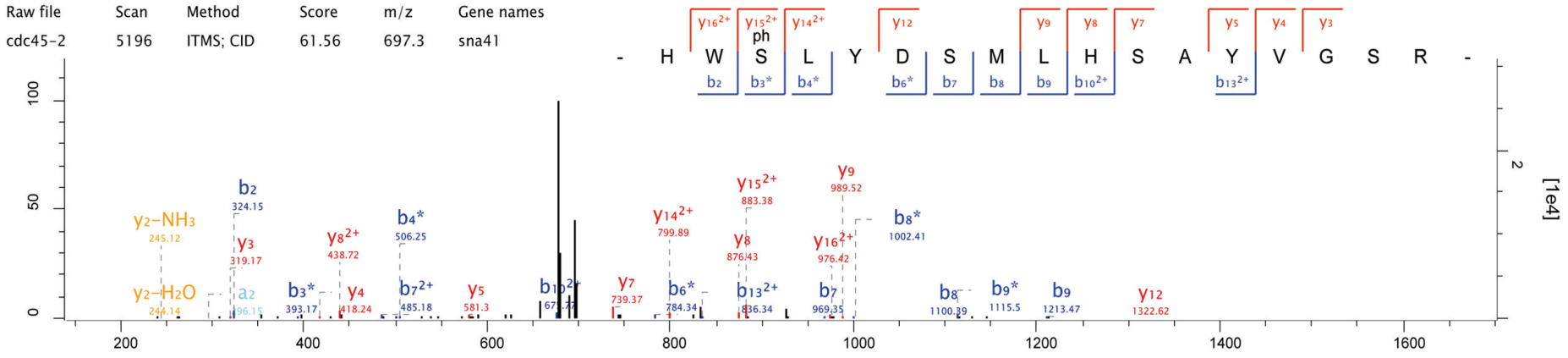
Figure S1

E

Cdc45-pS275



Cdc45-pS322



Cdc45-pS397

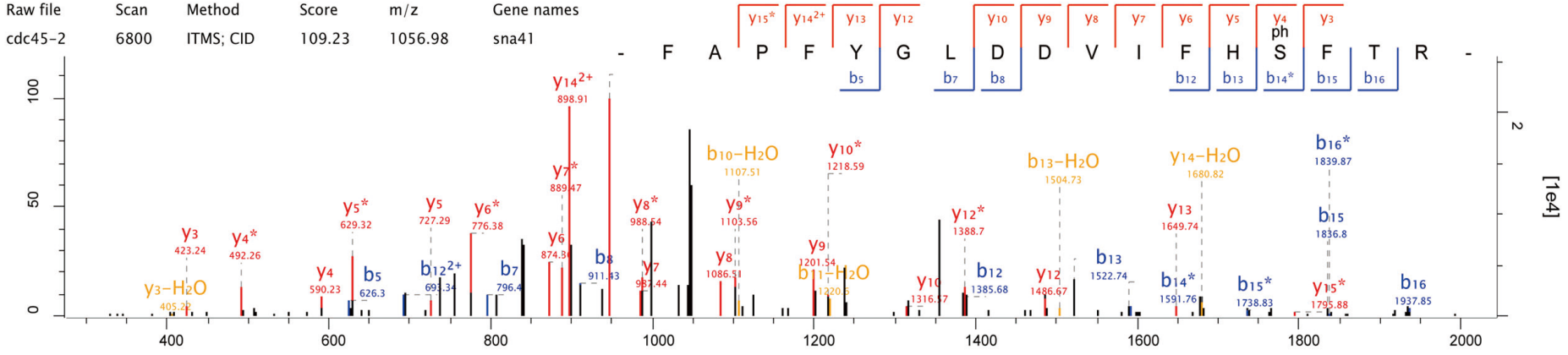
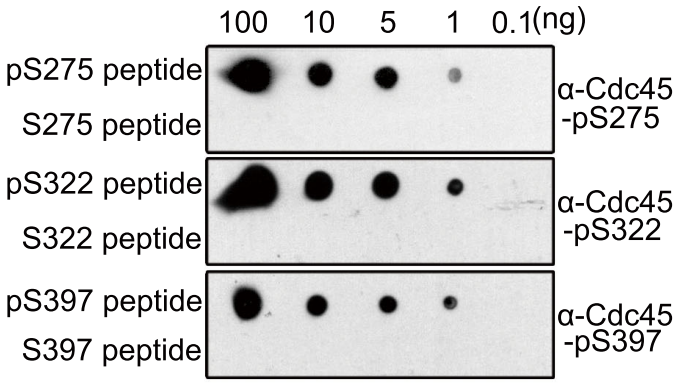
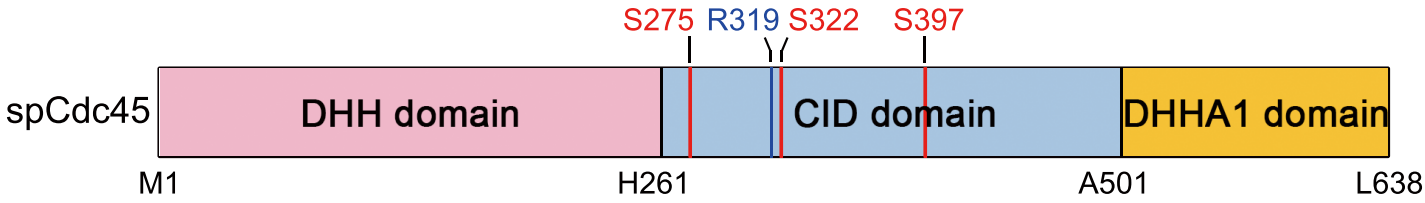


Figure S1

F


100 10 5 1 0.1(ng)
pS275 peptide
S275 peptide
α-Cdc45 -pS275
pS322 peptide
S322 peptide
α-Cdc45 -pS322
pS397 peptide
S397 peptide
α-Cdc45 -pS397

G


S275 R319 S322 S397
spCdc45
DHH domain
CID domain
DHHA1 domain
M1
H261
A501
L638

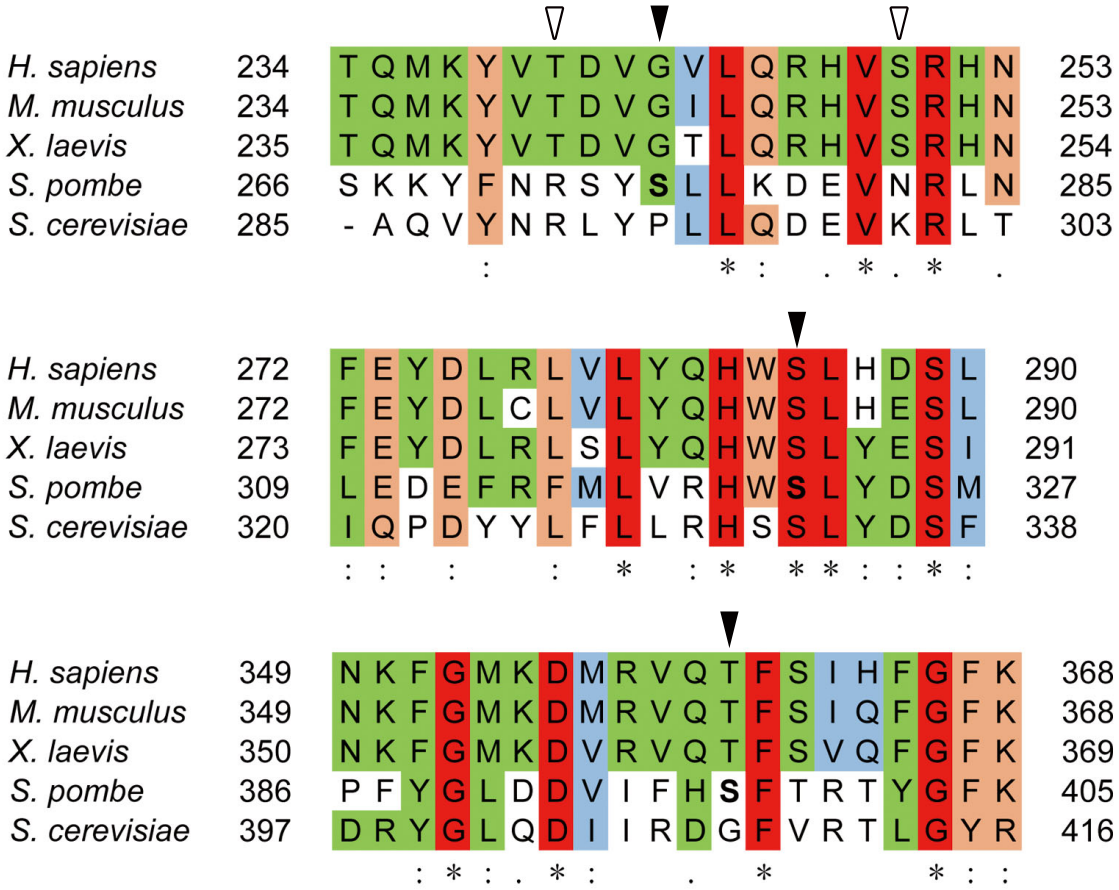

H. sapiens 234 TQMKYVTDVGVLQRHVSRHN 253
M. musculus 234 TQMKYVTDVGILQRHVSRHN 253
X. laevis 235 TQMKYVTDVGTLQRHVSRHN 254
S. pombe 266 SKKYFNRSYSLLKDEVNRLN 285
S. cerevisiae 285 -AQVYNRLYPLLQDEVKRLT 303
H. sapiens 272 FEYDLRLVLYQHWSLHDSL 290
M. musculus 272 FEYDLCLVLYQHWSLHESL 290
X. laevis 273 FEYDLRLSLYQHWSLYESI 291
S. pombe 309 LEDEFRFMLVRHWSLYDSM 327
S. cerevisiae 320 IQPDYYLFLLRHSSLYDSF 338
H. sapiens 349 NKFGMKDMRVQTFSIHFGFK 368
M. musculus 349 NKFGMKDMRVQTFSIQFGFK 368
X. laevis 350 NKFGMKDVRVQTFSVQFGFK 369
S. pombe 386 PFYGLDDVIFHSFTRTYGFK 405
S. cerevisiae 397 DRYGLQDIIRDGFVRTLGYR 416

Figure S1

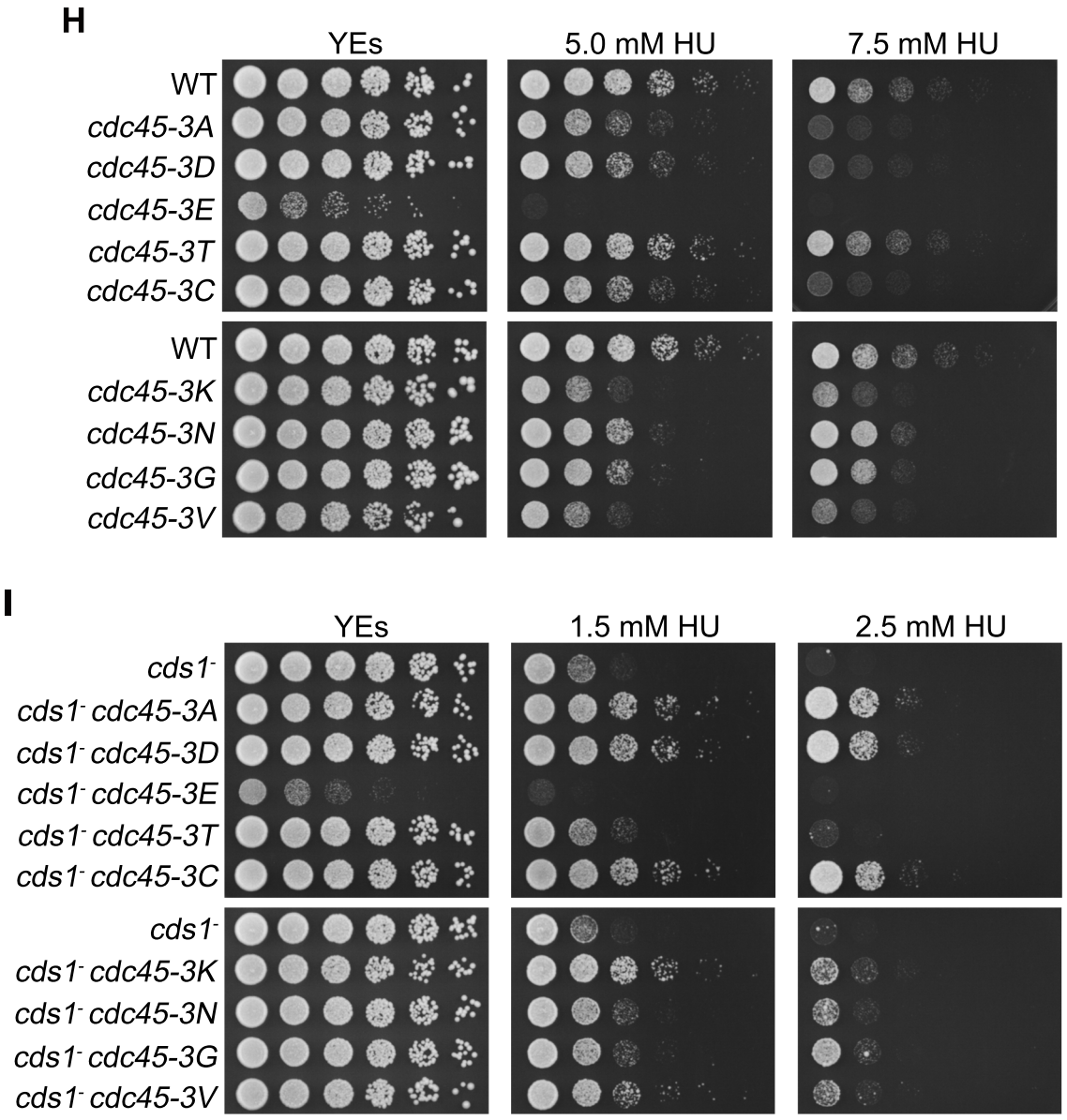

H
YEs
5.0 mM HU
7.5 mM HU
WT
cdc45-3A
cdc45-3D
cdc45-3E
cdc45-3T
cdc45-3C
WT
cdc45-3K
cdc45-3N
cdc45-3G
cdc45-3V
I
YEs
1.5 mM HU
2.5 mM HU
cds1-
cds1- cdc45-3A
cds1- cdc45-3D
cds1- cdc45-3E
cds1- cdc45-3T
cds1- cdc45-3C
cds1-
cds1- cdc45-3K
cds1- cdc45-3N
cds1- cdc45-3G
cds1- cdc45-3V

Figure S2

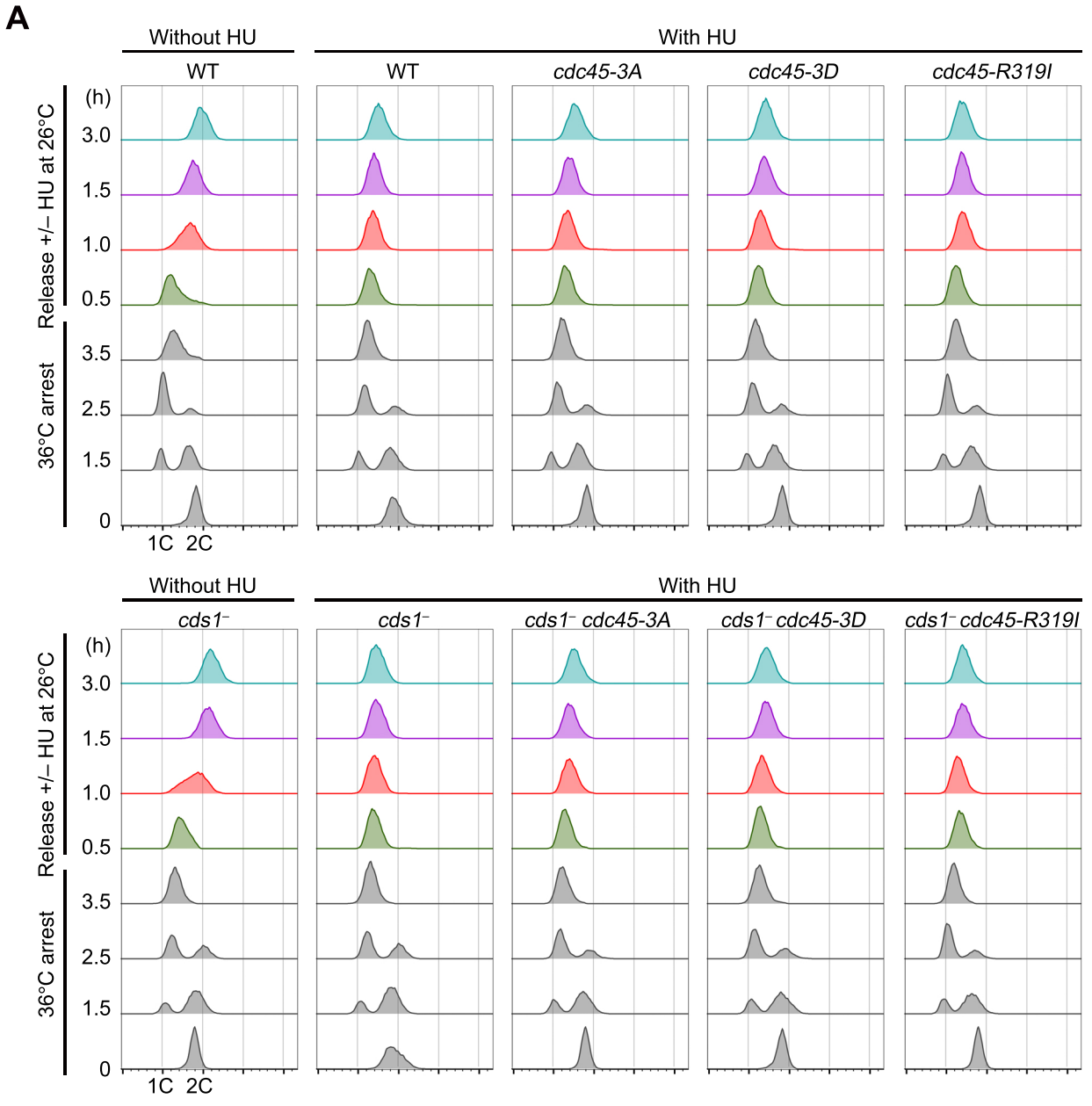

A
Without HU
With HU
WT
WT
cdc45-3A
cdc45-3D
cdc45-R319I
(h)
3.0
1.5
1.0
0.5
3.5
2.5
1.5
0
Release +/– HU at 26°C
36°C arrest
1C 2C
Without HU
With HU
cds1⁻
cds1⁻
cds1⁻ cdc45-3A
cds1⁻ cdc45-3D
cds1⁻ cdc45-R319I
(h)
3.0
1.5
1.0
0.5
3.5
2.5
1.5
0
Release +/– HU at 26°C
36°C arrest
1C 2C

Figure S2

B

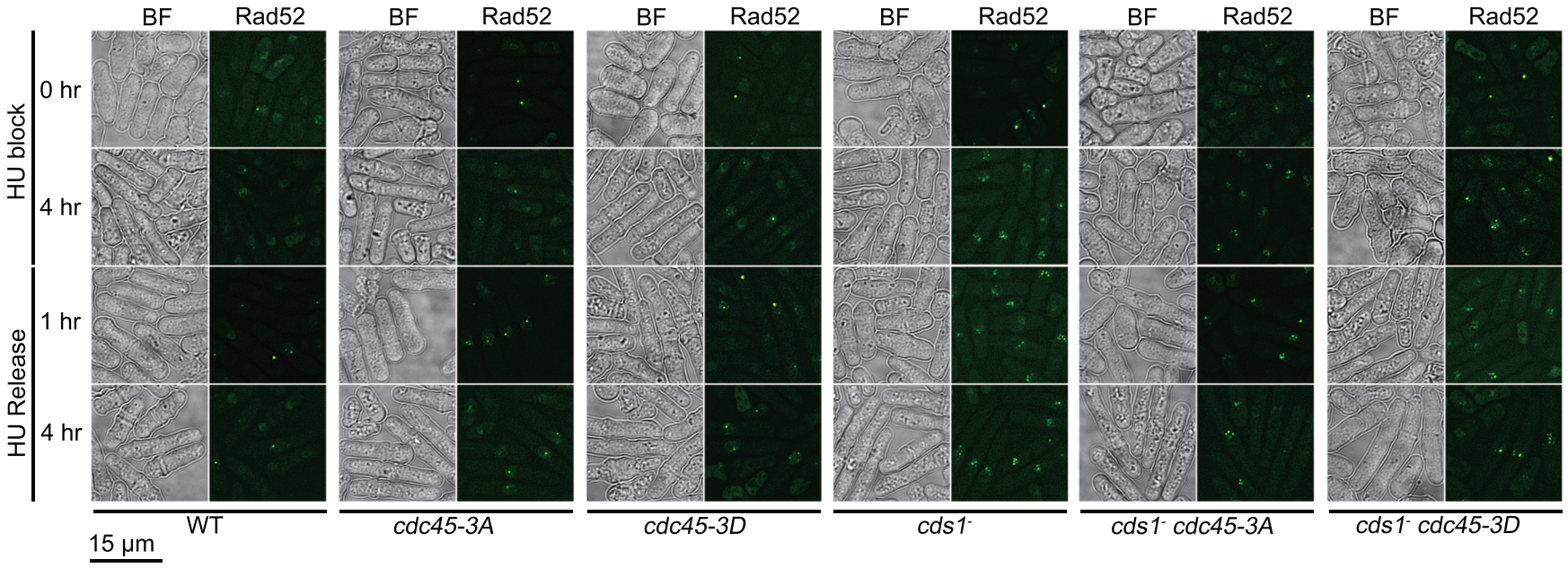

BF
Rad52
HU block
0 hr
4 hr
HU Release
1 hr
4 hr
WT
cdc45-3A
cdc45-3D
cds1-
cds1- cdc45-3A
cds1- cdc45-3D
15 µm

Figure S3

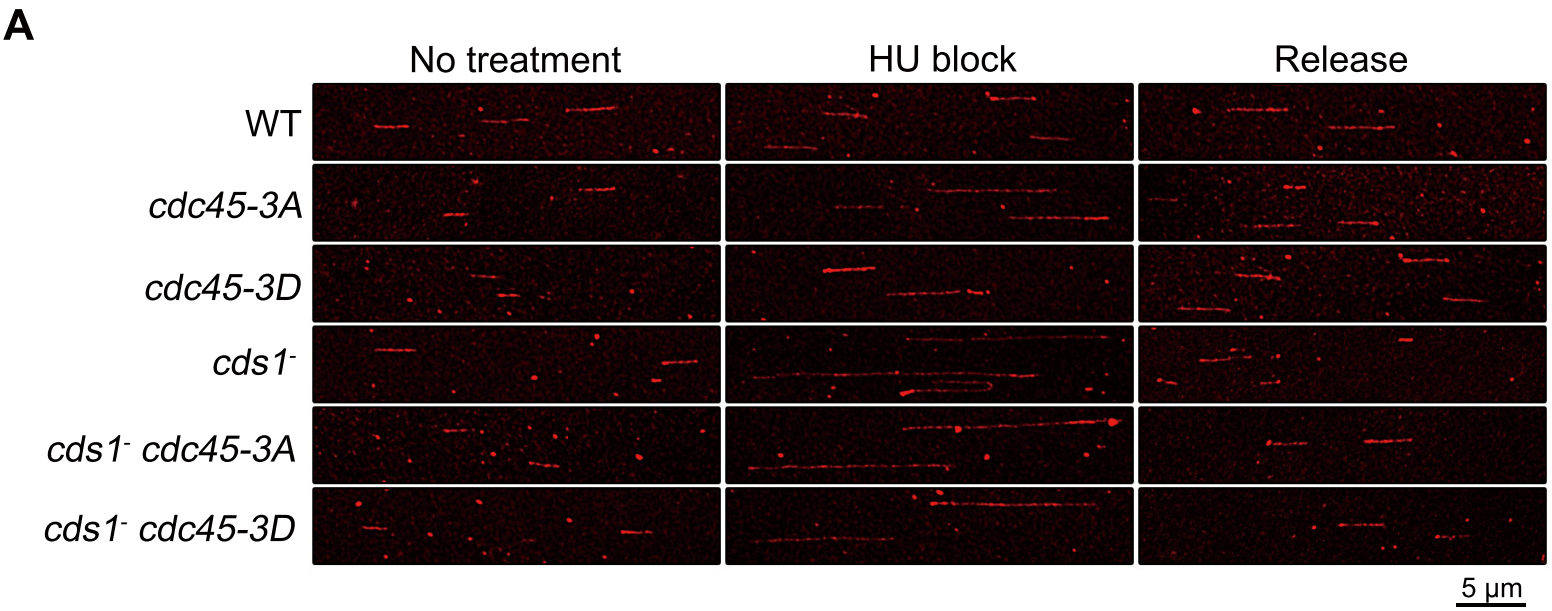

A
No treatment
HU block
Release
WT
cdc45-3A
cdc45-3D
cds1-
cds1- cdc45-3A
cds1- cdc45-3D
5 µm

Figure S3

B

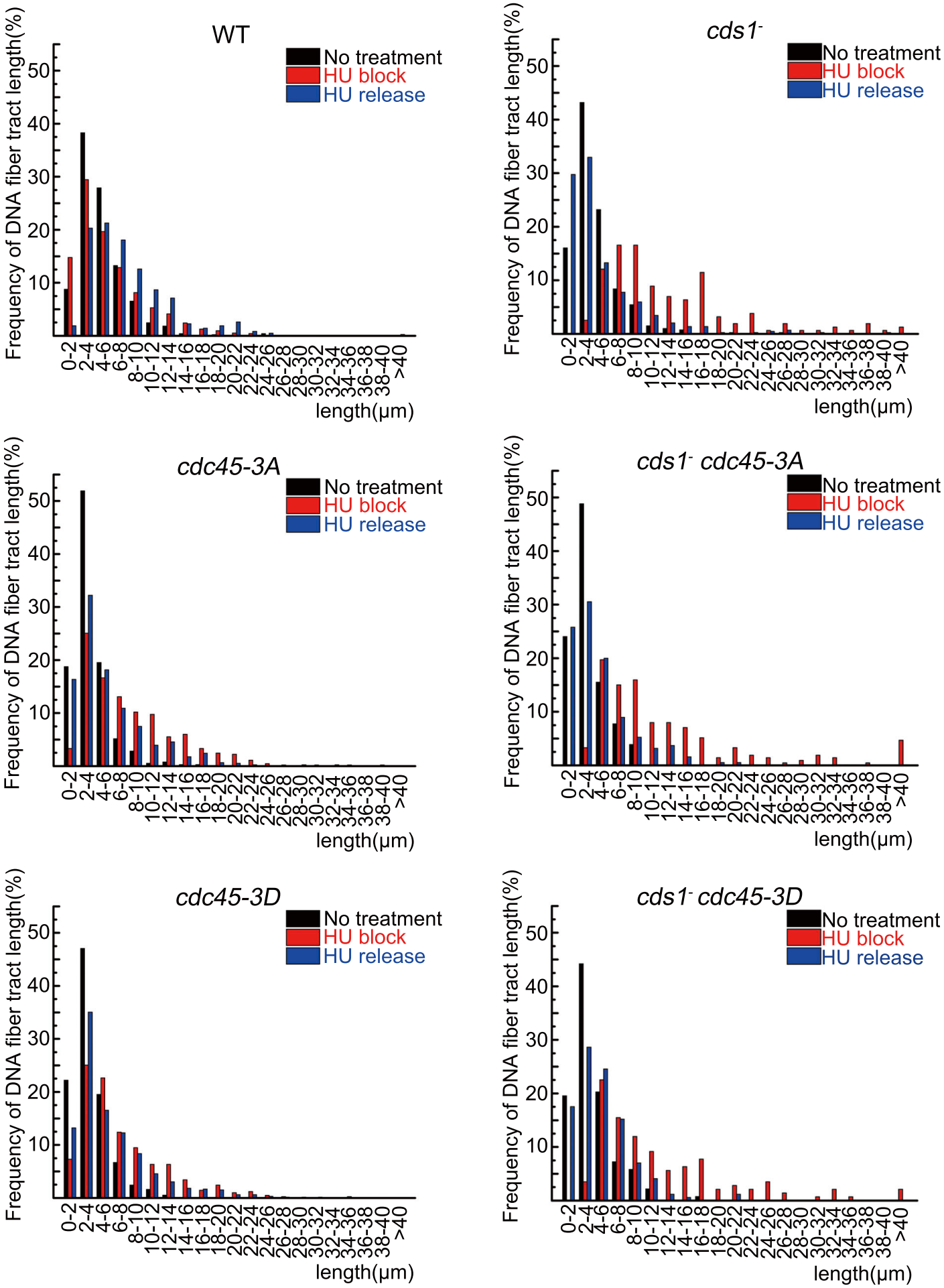
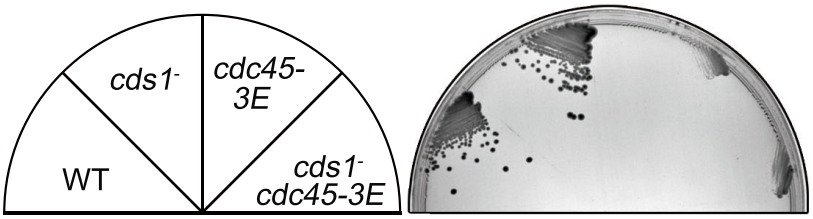
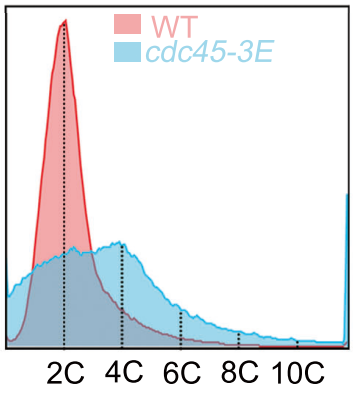

WT
cds1-
cdc45-3A
cds1- cdc45-3A
cdc45-3D
cds1- cdc45-3D
No treatment
HU block
HU release
Frequency of DNA fiber tract length(%)
length(μm)
0-2
2-4
4-6
6-8
8-10
10-12
12-14
14-16
16-18
18-20
20-22
22-24
24-26
26-28
28-30
30-32
32-34
34-36
36-38
38-40
>40

Figure S4

A



B



C

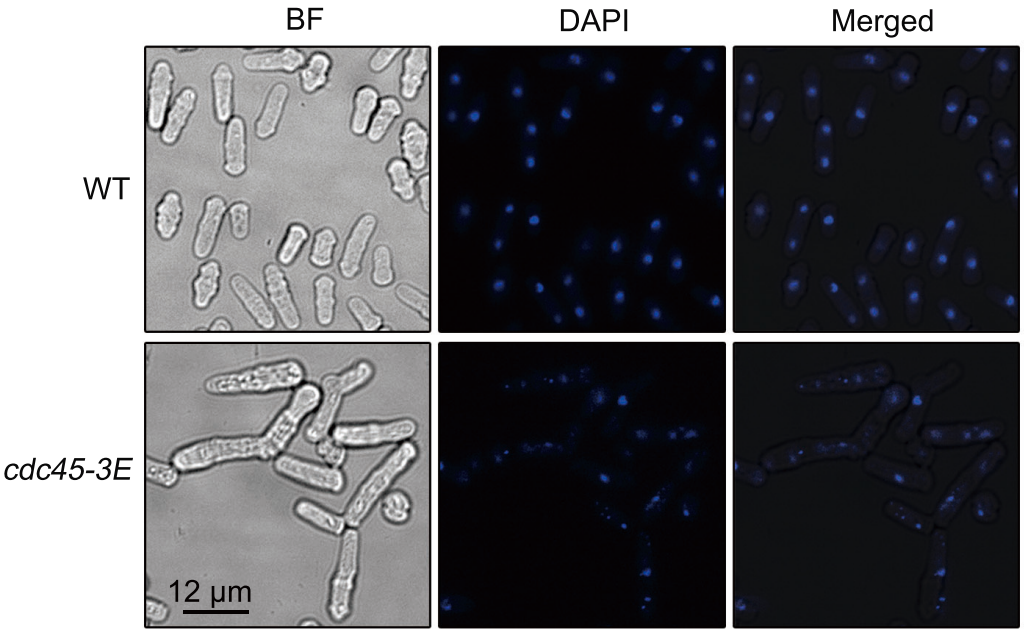


Figure S5

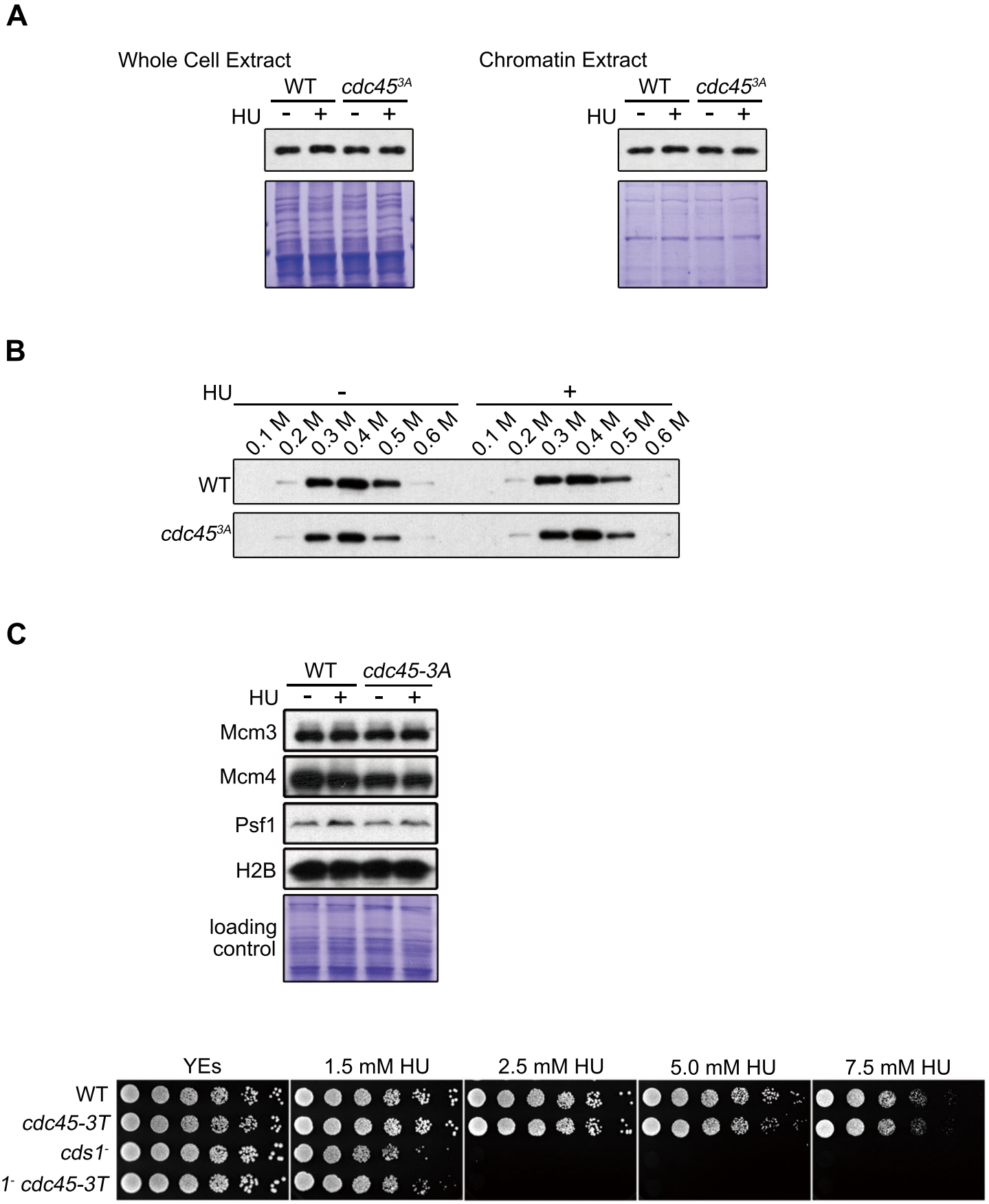

A
Whole Cell Extract
WT
cdc45^3A
HU - + - +
Chromatin Extract
WT
cdc45^3A
HU - + - +
B
HU - +
0.1 M 0.2 M 0.3 M 0.4 M 0.5 M 0.6 M 0.1 M 0.2 M 0.3 M 0.4 M 0.5 M 0.6 M
WT
cdc45^3A
C
WT cdc45-3A
HU - + - +
Mcm3
Mcm4
Psf1
H2B
loading control
D
YEs
1.5 mM HU
2.5 mM HU
5.0 mM HU
7.5 mM HU
WT
cdc45-3T
cds1-
cds1- cdc45-3T

Figure S6

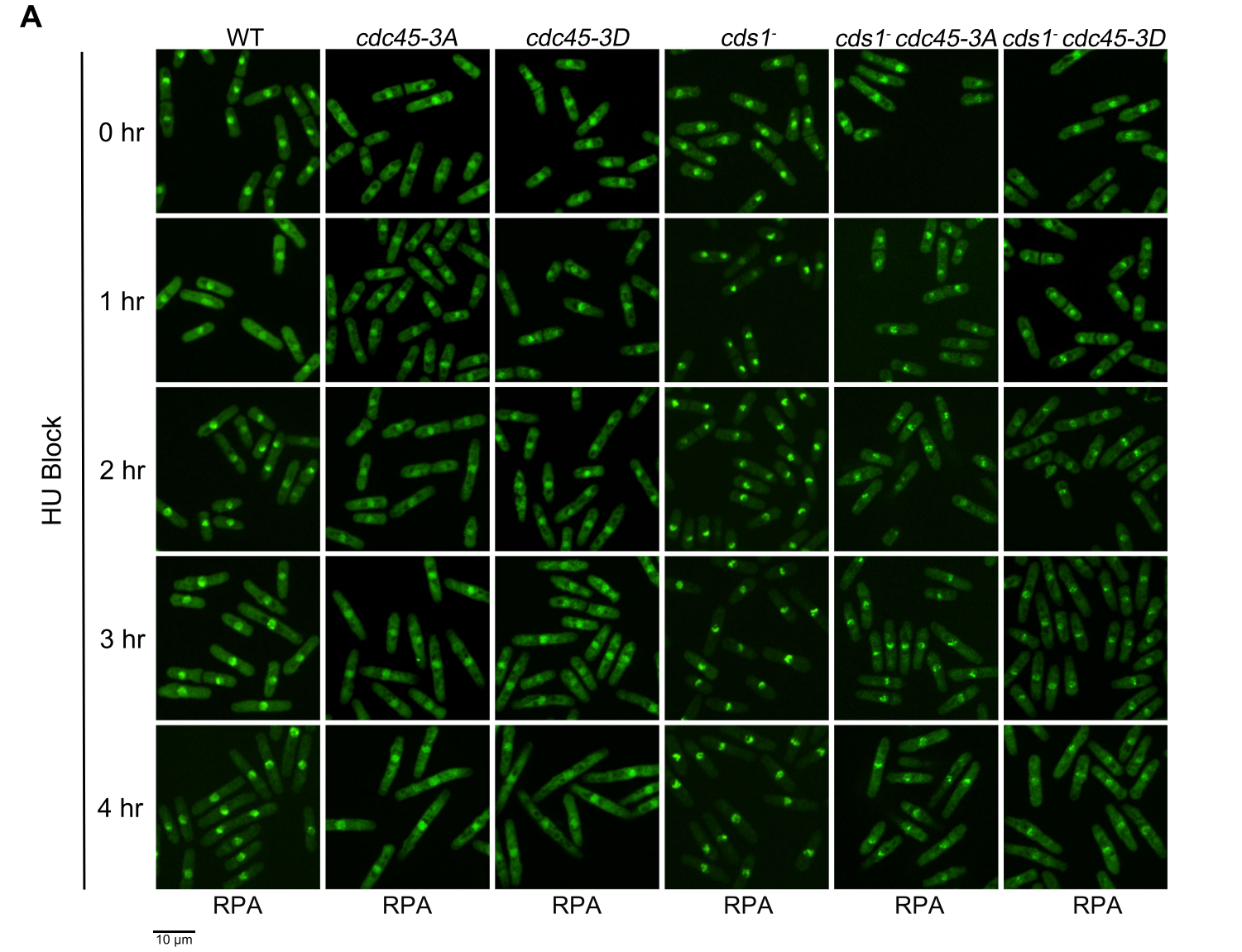
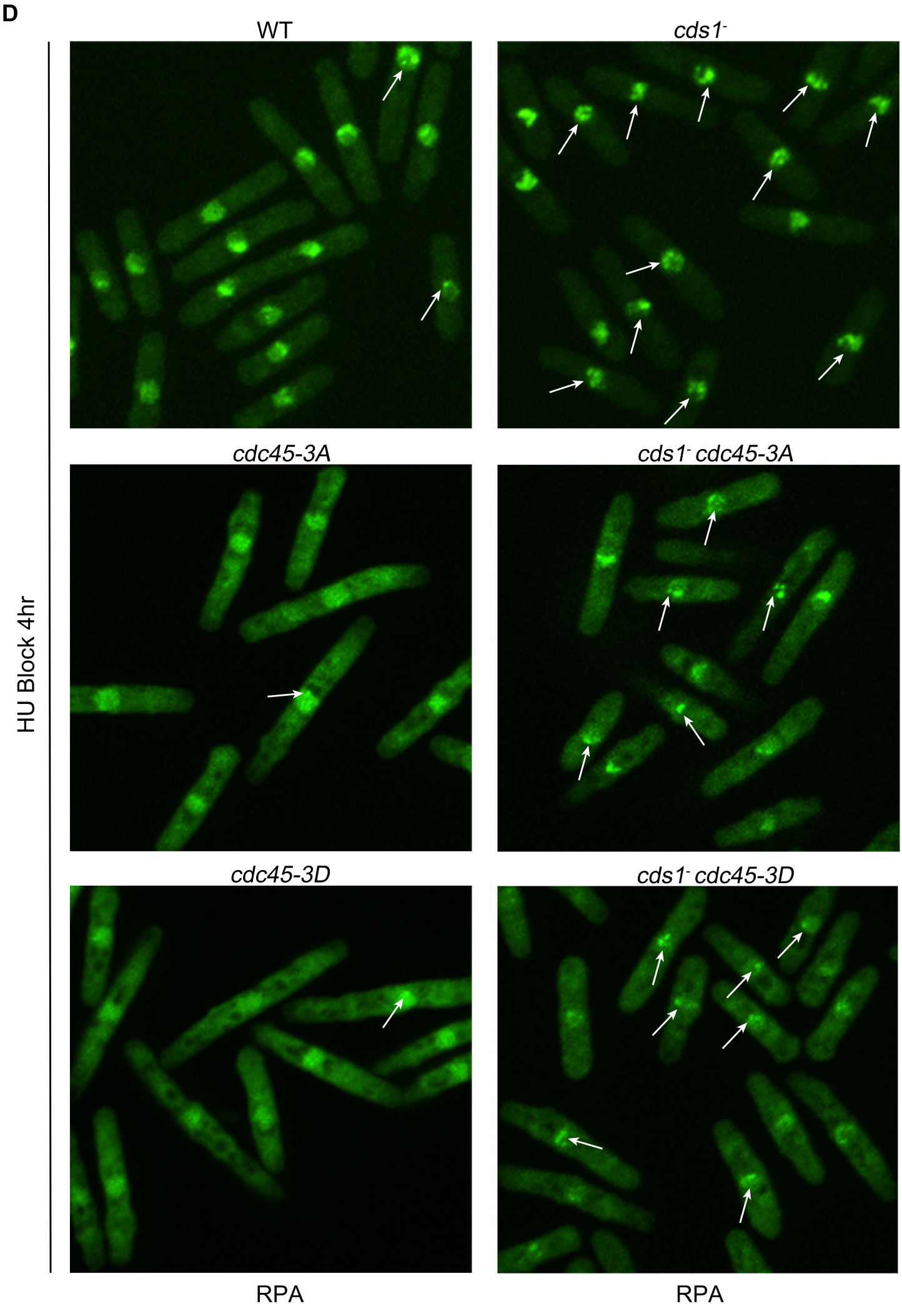

A
WT
cdc45-3A
cdc45-3D
cds1-
cds1- cdc45-3A
cds1- cdc45-3D
0 hr
1 hr
2 hr
3 hr
4 hr
HU Block
RPA
10 µm
B
WT
cdc35-3A
cdc45-3D
cds1-
cds1- cdc45-3A
cds1- cdc45-3D
% cells with RPA foci
Time (hr) in HU
C
WT
cds1-
Nuclease P1
Marker
bp
RNA

Figure S6


D
WT
cds1-
cdc45-3A
cds1- cdc45-3A
cdc45-3D
cds1- cdc45-3D
HU Block 4hr
RPA
RPA
10 μm