Cell cycle regulation of DNA double-strand break end resection by Cdk1-dependent Dna2 phosphorylation

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Supplementary information

Supplemental Table 1. Yeast strains used in this study

Strain name	Parental strain	Genotype	Source
JKM139		*MATa hoΔ hml::ADE1 hmr::ADE1 ade1-100 leu2-3,112 trp1::hisG lys5 ura3-52 ade3::GAL::HO*	[1]
JKM179		*MATalpha hoΔ hml::ADE1 hmr::ADE1 ade1-100 leu2-3,112 trp1::hisG lys5 ura3-52 ade3::GAL::HO*	[1]
yGI198	JKM139	*exo1::TRP1*	[2]
yGI200	JKM139	*sgs1::KanMX*	[2]
ySL88	JKM139	*mre11::hisG*	[2]
yWH054	JKM179	*pif-m2 dna2::KanMX*	[2]
yZZ046	JKM139	*cdk1-as1*	This study
yZZ119	JKM139	*cdk1-as1 exo1::TRP1*	This study
yZZ120	JKM139	*cdk1-as1 yku70::KanMX*	This study
yZZ157	JKM139	*cdk1-as1 yku70::KanMX sgs1::NatMX*	This study
yZZ355	JKM139	*cdk1-as1 pif1-m2 dna2::TRP1 yku70::KanMX*	This study
yZZ483	JKM139	*cdk1-as1 sae2::KanMX +pSAE2*	This study
yZZ485	JKM139	*cdk1-as1 sae2::KanMX +psae2-S267E*	This study
yWH526	JKM139	*cdk1-as1 DNA2-9Myc-TRP1*	This study
yZZ357	JKM139	*cdk1-as1 pif1-m2 dna2::TRP1*	This study
yXC2	JKM139	*DNA2-9Myc-TRP1*	This study

yXC5	yXC2	*chlk1::KanMX DNA2-9Myc-TRP1*	This study
yXC14	yXC2	*mec1::KanMX sml1::TRP1 DNA2-9Myc-TRP1*	This study
yXC93	yZZ357	*cdk1-as1 pif1-m2 dna2::TRP1 RFA1-3HA-KanMX*	This study
yXC96	JKM139	*cdk1-as1 DNA2-TAP-NatMX*	This study
yXC108	yXC93	*yXC93 + pRS316-DNA2-3FLAG*	This study
yXC110	yXC93	*yXC93 +pRS316-dna2ΔN248-3FLAG*	This study
yXC111	yXC93	*yXC93 +pRS316-dna2* $^{T4A\ S17A\ S237A}$*-3FLAG*	This study
yXC112	yXC93	*yXC93 +pRS316- dna2* ^{S237A} *-3FLAG*	This study
yXC116	yXC93	*yXC93 +pRS316- dna2* ^{T4A} *-3FLAG*	This study
yXC118	yXC93	*yXC93 +pRS316- dna2* $^{S17A\ S237A}$*-3FLAG*	This study
yXC122	yXC93	*yXC93 +pRS316- dna2* ^{S17A}*-3FLAG*	This study
yXC354	JKM139	*dna2::KanMX + pRS316-DNA2-GFP*	This study
yXC357	JKM139	*dna2::KanMX + pRS316- dna2* ^{T4A}*-GFP*	This study
yXC363	JKM139	*dna2::KanMX + pRS316- dna2* $^{S17A\ S237A}$*-GFP*	This study
yXC366	JKM139	*dna2::KanMX + pRS316- dna2* ^{S237A}*-GFP*	This study
yXC369	JKM139	*dna2::KanMX + pRS316- dna2* ^{S17A}*-GFP*	This study
yXC374	JKM139	*dna2::KanMX + pRS316- dna2* $^{T4A\ S17A\ S237A}$*-GFP*	This study
yXC378	JKM139	*dna2::KanMX + pRS316-dna2 ΔN248-GFP*	This study
yXC385	JKM139	*dna2::KanMX + pRS316-dna2* ^{S17D}*-GFP*	This study
yXC388	JKM139	*dna2::KanMX + pRS316-dna2* $^{S17D\ S237D}$*-GFP*	This study
yXC391	JKM139	*dna2::KanMX + pRS316-dna2* ^{S237D}*-GFP*	This study
yXC215	JKM139	*dna2::KanMX +pRS316-DNA2-3FLAG*	This study
yXC245	JKM139	*dna2::KanMX +pRS314-DNA2-3FLAG*	This study
yXC246	JKM139	*dna2::KanMX +pRS314-- dna2* $^{T4A\ S17A\ S237A}$*-3FLAG*	This study
yXC284	JKM139	*exo1::NatMX dna2::KanMX + pRS316-DNA2-3FLAG*	This study

yXC329	JKM139	*exo1::NatMX dna2::KanMX + pRS314-DNA2-3FLAG*	This study
yXC330	JKM139	*exo1::NatMX dna2::KanMX + pRS314--dna2* $^{T4A\ S17A\ S237A}$*-3FLAG*	This study
yXC544	JKM139	*dna2::KanMX +pRS316-dna2 ΔN248-3FLAG*	This study
yXC545	JKM139	*exo1::NatMX dna2::KanMX + pRS316-dna2 ΔN248-3FLAG*	This study
yXC587	JKM139	*cdk1-as1 yku70::KanMX dna2::NatMX +pRS316- dna2* $^{S17D\ S237D}$*-GFP*	This study
yXC795	yXC330	*yXC330 +Yep24- dna2* $^{T4A\ S17A\ S237A}$	This study
yXC796	yXC330	*yXC330 + Yep24 vector*	This study
BJ5464		*MATα ura3-52 trp1 leu2Δ1his3Δ200 pep4::HIS3 prb1Δ16R can1*	[3]
yHY1	BJ5464	*BJ5464 + pESC-URA-DNA2-6HIS*	This study
yHY2	BJ5464	*BJ5464 +pESC-URA- dna2* ^{S17A}*-6HIS*	This study
yHY3	BJ5464	*BJ5464 +pESC-URA- dna2* ^{S237A}*-6HIS*	This study
yHY4	BJ5464	*BJ5464 +pESC-URA- dna2* $^{S17A\ S237A}$*-HIS*	This study
yHY5	BJ5464	*BJ5464 +pESC-URA- dna2* $^{T4A\ S17A\ S237A}$*-6HIS*	This study

Supplementary Methods

Plasmid Construction

To construct plasmids expressing *DNA2*, the *DNA2* gene including 580 bp upstream and 430 bp downstream sequences along with a 3xFLAG tag inserted before the stop codon was subcloned into the pRS316 vector to make pRS316-*DNA2-3FLAG*. A *dna2-ΔN248* derivative of this plasmid was constructed. To make point mutations in Cdk1 consensus phosphorylation sites, a *Sac*II and *Kpn*I fragment of the *DNA2* gene was subcloned into *pUC18*. All point mutations were first created in this plasmid (*PL165*) using site directed mutagenesis (QuickChange, Stratagene). The *Cla*I and *Sna*BI fragment containing the

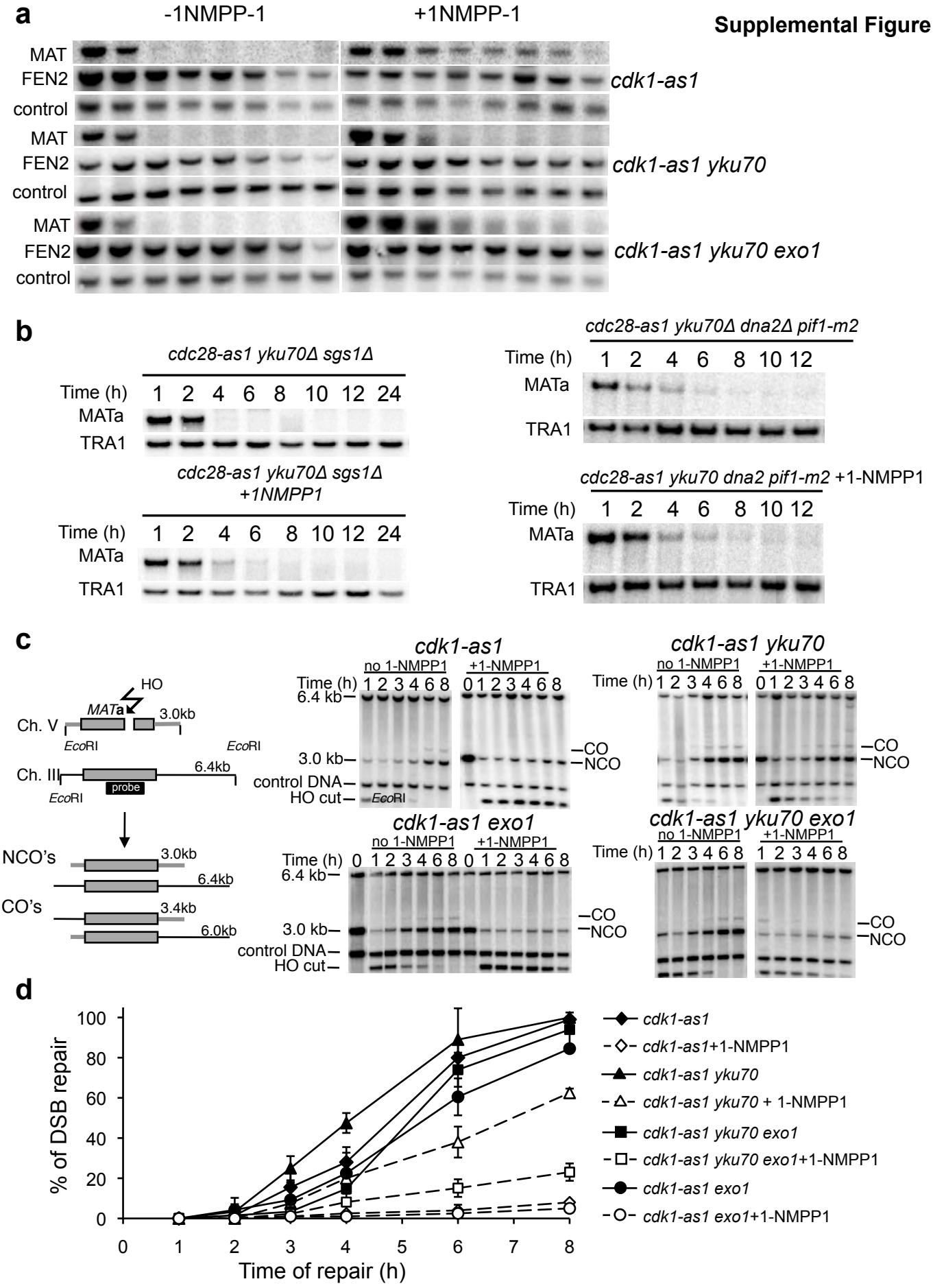
T4A, S17A, S237A, S237D, or *S17D* single point mutation or a combination of these mutations was excised and cloned into pRS316-*DNA2-3FLAG* in the *ClaI* and *Sna*BI sites. An identical set of plasmids was created in pRS314. To follow Dna2 protein localization in cells, the EGFP sequence was amplified by PCR and replaced 3xFLAG in the aforementioned set of plasmids in the pRS316 vector. To construct plasmids for overexpressing *DNA2* for the *in vitro* phosphorylation assay, full-length *DNA2* was amplified by PCR and the 6xHis tag coding sequence was added before the stop codon and subcloned into the pESC-URA vector. All plasmids used in this study were confirmed by sequencing. The sequences of all oligonucleotide primers used are available upon request.

Protein phosphatase treatment

Phleomycin was added to the culture at a final concentration of 50 μg ml^{-1} to induce double-strand breaks. Cells were grown for 3 h after treatment. The untreated or phleomycin-treated cell culture (250 ml) was harvested, and cells were washed once with lysis buffer (10 mM Tris-Cl, pH 8.0, 150 mM NaCl, 1 mM $MgAc_2$, 6 mM $CaCl_2$, 10 mM Na_4VO_3, 10 mM $Na_4P_2O_7$, 10 mM NaF, 0.1% NP-40, 0.2 mM phenylmethylsulfonyl fluoride, 10 mM β-mercaptoethanol and Roche EDTA-free protease inhibitors). The cell pellets were resuspended in 2 ml lysis buffer and disrupted by acid-washed glass beads on a Mini BeadBeater. Lysed mixtures were transferred to Eppendorf tubes followed by centrifugation at 20,000 x g for 10 min at 4°C. The supernatant from the samples was mixed with 100 μl IgG Sepharose beads at 4°C for 2 h. The beads were collected and washed four times with lysis buffer containing protease and phosphatase inhibitors. The phleomycin-treated samples were divided into two aliquots, one of which was resuspended in 100 μl of 1x Lambda phosphatase buffer supplemented with 2 mM $MnCl_2$ and Roche EDTA-free protease inhibitors and incubated with 4 μl of Lambda protein phosphatase (New England Biolabs, P0753S) at 30°C for 30 min. One volume of 2x SDS-PAGE gel loading buffer was added to stop the reaction and to elute proteins.

Supplemental Figures

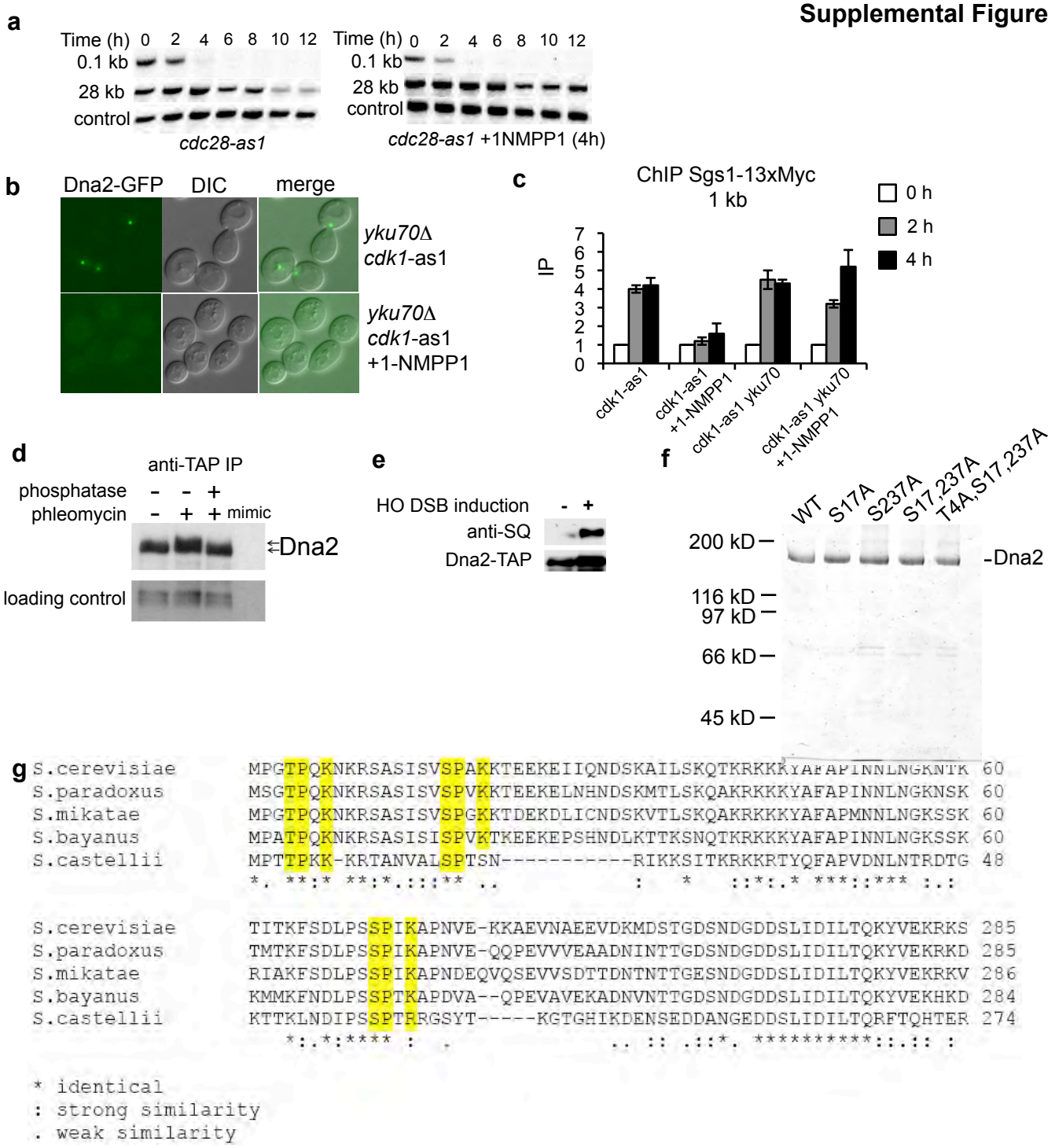
Supplemental Figure 1



Supplemental Figure S1. Exo1 promotes recombination in *yku70*Δ Cdk1-deficient cells.

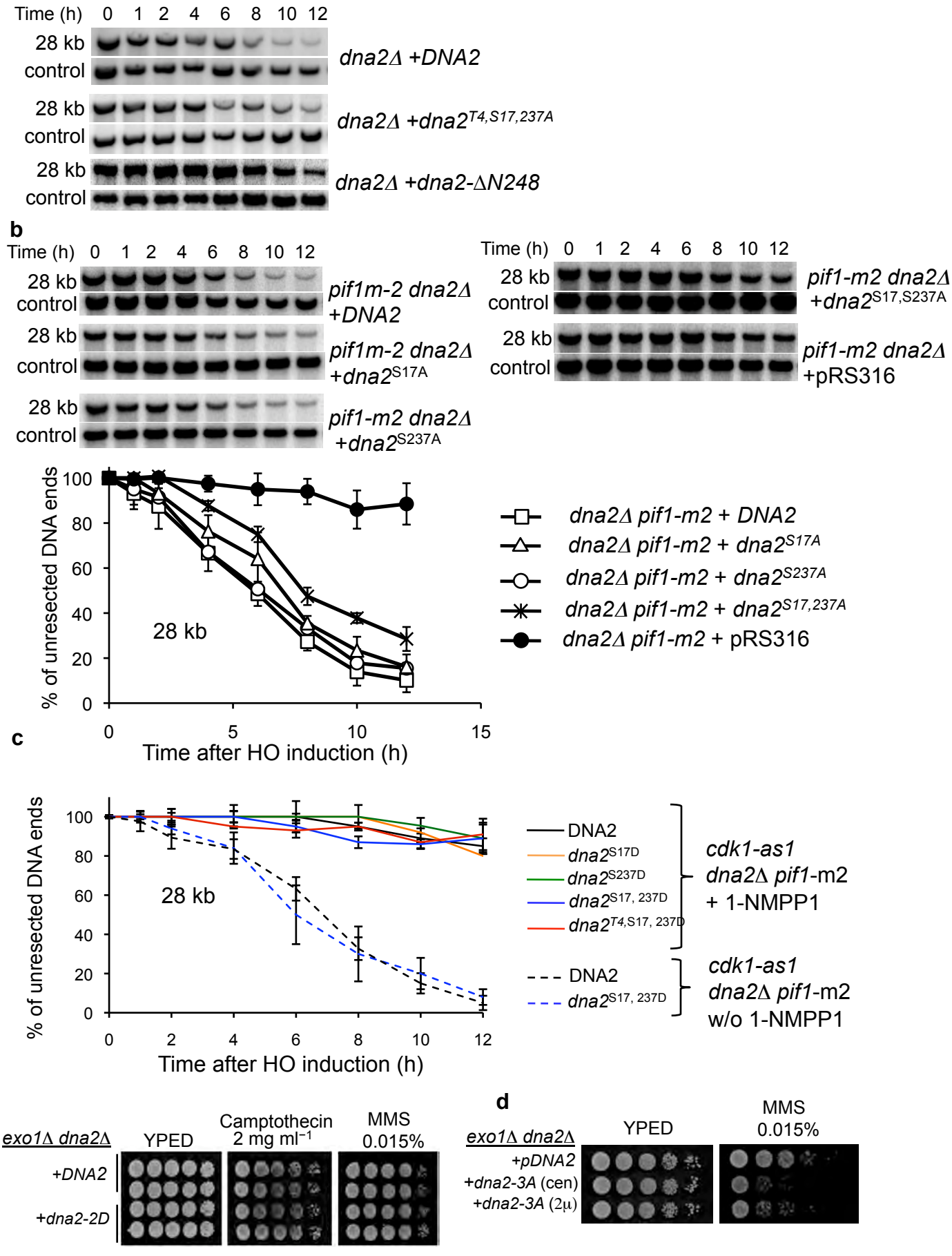
(a) Southern blot analysis of resection in mutants. Calculation of normalized band intensities over time as a measure of resection is shown in Figure 1a. **(b)** Southern blot analysis of initial DSB end resection. **(c)** Schematic representation of ectopic gene conversion and Southern blot analysis of DSB repair *via* ectopic gene conversion. CO – crossing-over product, NCO – noncrossover product. **(d)** Kinetics of product formation in ectopic recombination assay. Error bars correspond to s.d.

Supplemental Figure 2



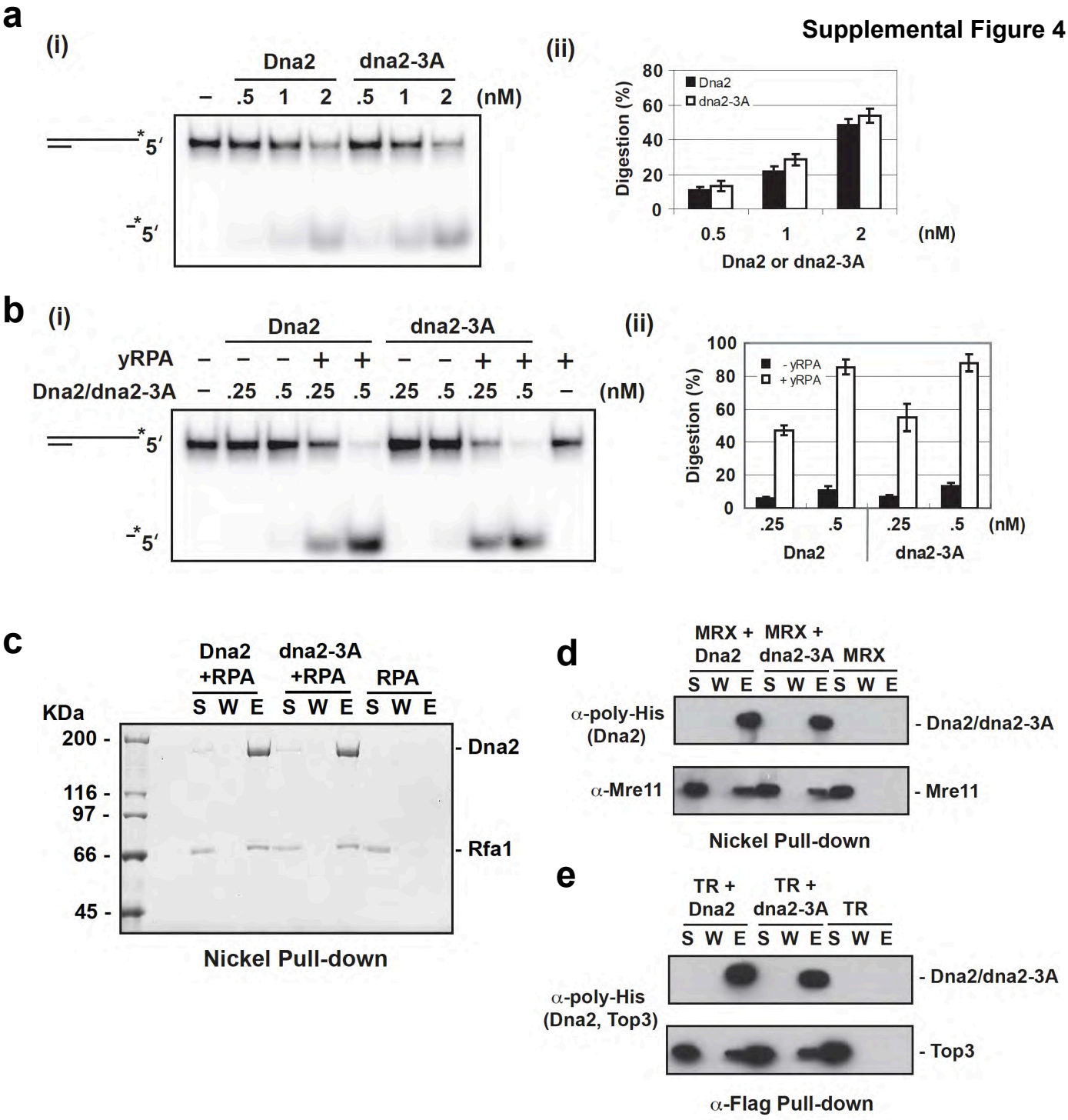
Supplemental Figure S2. Analysis of DSB recruitment of Dna2 and Sgs1.
(a) Southern blot analysis of resection in *cdk1-as1* cells where *cdk1-as1* kinase activity was blocked 4 hours after break induction. Calculation of normalized band intensities over time as a measure of resection is shown in Figure 1c. **(b)** Analysis of Dna2-GFP foci formation in response to DSBs in Ku and Cdk1 kinase deficient cells by fluorescence microscopy. **(c)** Analysis of Sgs1-13×Myc recruitment to DSBs by ChIP 1 kb upstream from the HO cut site (fold increase). Error bars correspond to s.d. **(d)** Phosphatase treatment of immunoprecipitated Dna2-TAP. **(e)** Immunoprecipitated Dna2-TAP probed with antibody against proteins phosphorylated by Mec1 and/or Tel1. **(f)** Coomassie stain of purified wild type and mutant Dna2 proteins. **(g)** Alignment of the N-terminal part of Dna2 sequences from *S. cerevisiae* and closely related species.

Supplemental Figure 3



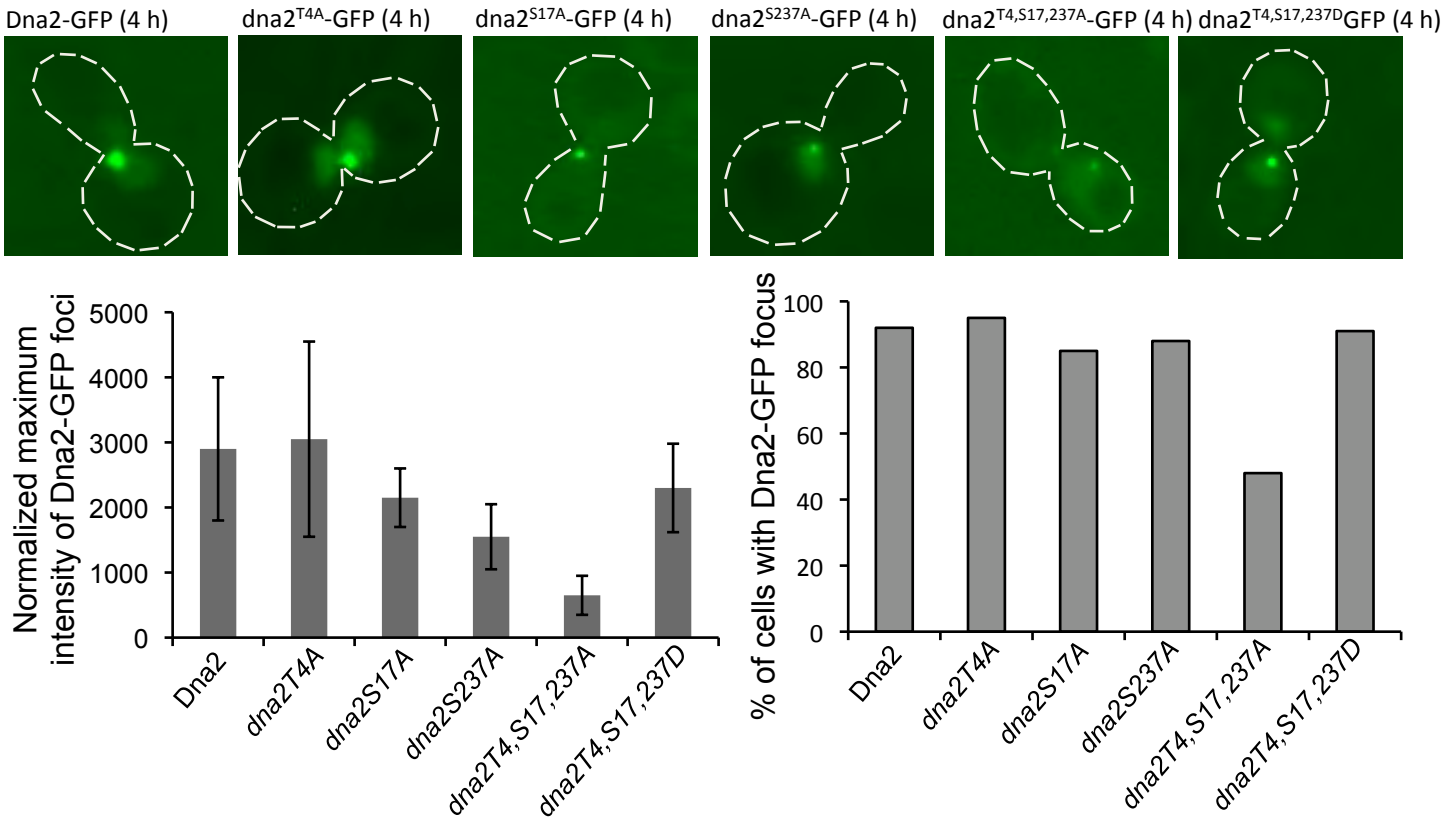
Supplemental Figure S3. Analysis of resection and DNA damage sensitivity of strains harboring *dna2* mutations.

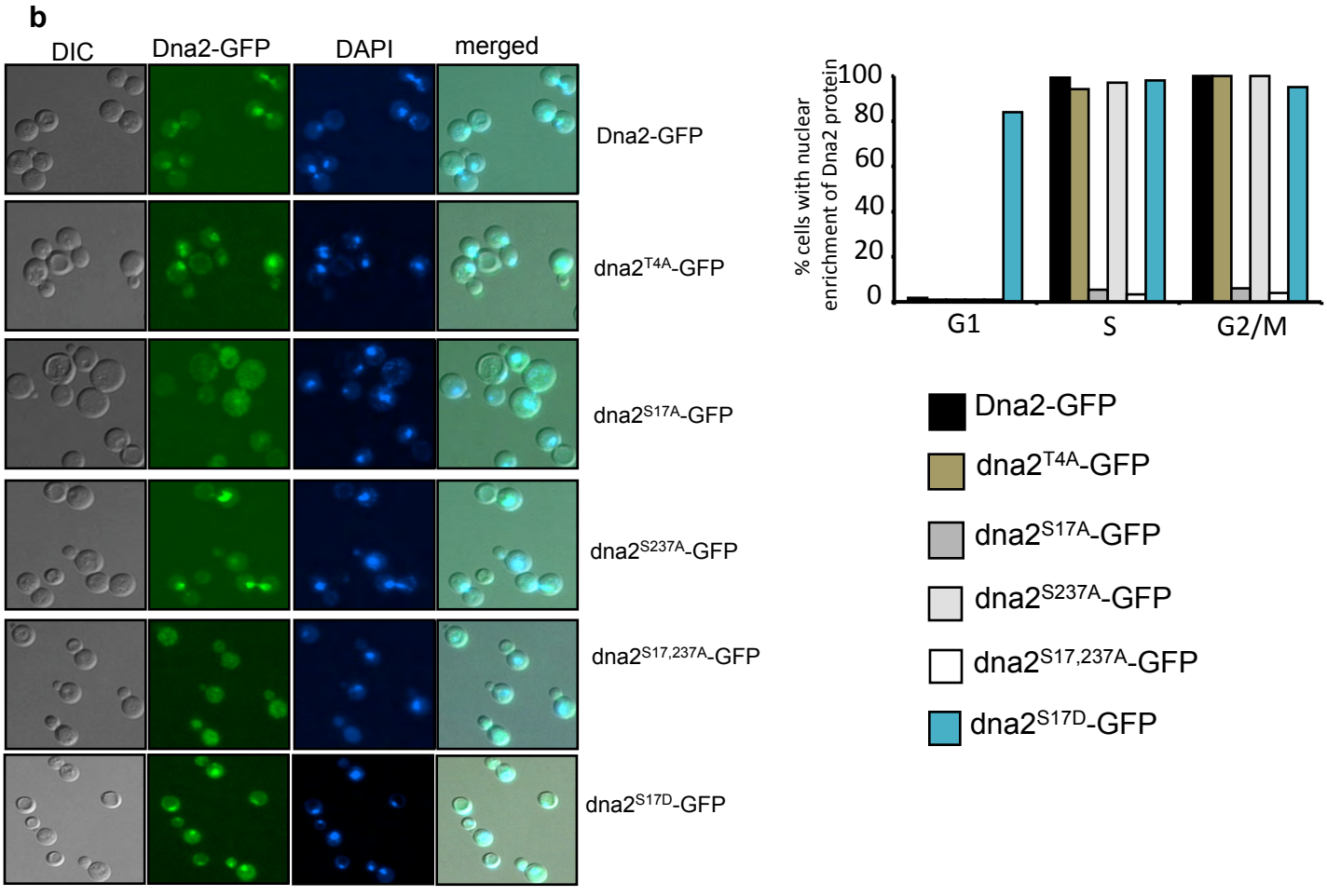
(a) Southern blot analysis of resection in *dna2*Δ cells complemented with a plasmid carrying either wild-type *DNA2* or the indicated *dna2* mutants. **(b)** Analysis of resection in *pif1*-m2 *dna2* mutants lacking one or two of the full consensus Cdk1 phosphorylation sites. Plotted values are the mean values ± SD from three independent experiments. Error bars correspond to s.d. **(c)** Analysis of extensive resection and DNA damage sensitivity in strains that harbor *dna2* phosphomimetic mutations. **(d)** DNA damage sensitivity analysis in *exo1*Δ *dna2*Δ strains complemented with a centromeric plasmid carrying either wild type *DNA2* or the *dna2-3A* allele or a multicopy plasmid carrying *dna2-3A*.



Supplemental Figure S4. Biochemical characterization of the dna2-3A mutant.
(a) Dna2 or dna2-3A was tested for 5′ end digestion. The results from (i) were quantified and plotted in (ii). **(b)** RPA stimulates 5′ end digestion by Dna2 or dna2-3A. The 5′ end labeled substrate was incubated with Dna2 or dna-3A in the absence or presence of RPA. The results from (i) are quantified and plotted in (ii). **(c, d, e)** Nickel affinity pulldown through the $(His)_6$ tag on FLAG-$(His)_6$-tagged Dna2 or dna2-3A to analyze their interaction with RPA in "c" and MRX in "d". Anti-FLAG affinity pulldown through the FLAG tag on FLAG-$(His)_6$-tagged Dna2 or dna2-3A to test for their interaction with the $(His)_6$-tagged Top3-Rmi1 complex in "e". The supernatant (S), wash (W) and SDS eluate (E) fractions were analyzed by SDS-PAGE and either stained by Coomassie Blue or immunoblotted with the indicated antibodies.

Supplemental Figure 5

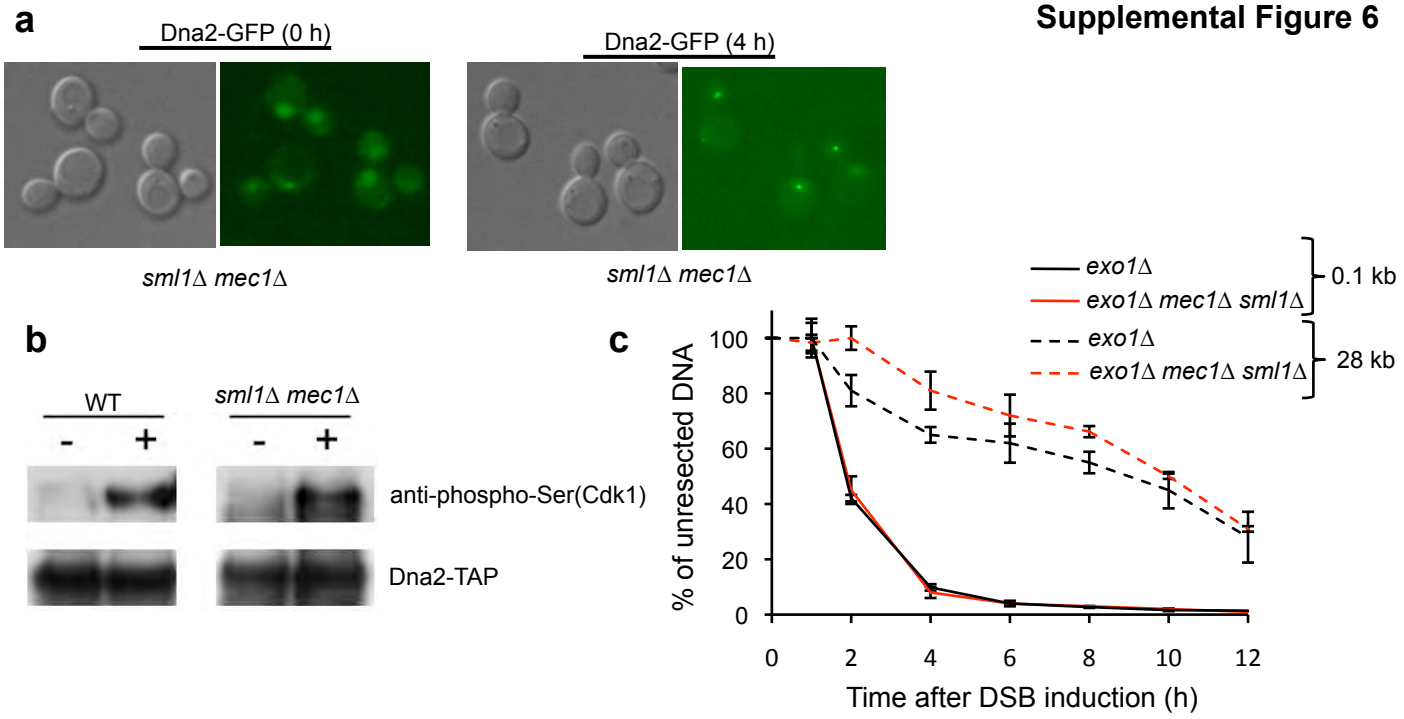




Supplemental Figure S5. Regulation of the nuclear localization and DSB recruitment of Dna2 by Cdk1 kinase.

(a) Measurement of the quantity and intensity of DSB-induced Dna2-GFP foci and indicated mutant dna2-GFP foci. Error bars correspond to s.d. **(b)** Analysis of nuclear localization of Dna2-GFP or mutant dna2-GFP in G1, S and G2/M phases of cells.

Supplemental Figure 6



Supplemental Figure S6. Mec1-dependent phosphorylation of Dna2 is dispensable for Dna2 recruitment to DSB, Cdk1-dependent phosphorylation and resection.
(a) Nuclear localization and damage dependent foci formation of Dna2 analyzed by fluorescence microscopy in *sml1Δ mec1Δ* cells. **(b)** Immunoblot showing TAP purified Dna2 probed with the antibody that specifically recognizes phospho serine residues targeted by Cdk1. **(c)** Analysis of long-range resection in the indicated mutants. Error bars correspond to s.d.

1. Lee, S.E. et al. Saccharomyces Ku70, mre11/rad50 and RPA proteins regulate adaptation to G2/M arrest after DNA damage. *Cell* **94**, 399-409 (1998).
2. Zhu, Z., Chung, W.H., Shim, E.Y., Lee, S.E. & Ira, G. Sgs1 helicase and two nucleases dna2 and exo1 resect DNA double-strand break ends. *Cell* **134**, 981-94 (2008).
3. Jones, E.W. Tackling the protease problem in Saccharomyces cerevisiae. *Methods Enzymol* **194**, 428-53 (1991).