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

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Abstract

DNA recombination pathways are cell cycle regulated to coordinate with replication. Cyclin-dependent kinase (Cdk1) promotes efficient 5'-strand resection at DNA double strand breaks (DSBs), the initial step of homologous recombination and damage checkpoint activation. The Mre11–Rad50–Xrs2 complex with Sae2 initiates resection, whereas two nucleases, Exo1 and Dna2, and the DNA helicase/topoisomerase complex Sgs1–Top3–Rmi1 generate longer ssDNA at DSBs. Using *Saccharomyces cerevisiae* we provide evidence for Cdk1-dependent phosphorylation of the resection nuclease Dna2 at Thr4, Ser17 and Ser237 that stimulates its recruitment to DSBs, resection and subsequent Mec1-dependent phosphorylation. Poorly recruited dna2$^{T4A\ S17A\ S237A}$ and dna2ΔN248 mutant proteins promote resection only in the presence of Exo1, suggesting crosstalk between Dna2- and Exo1-dependent resection pathways.

Cyclin dependent kinases (Cdks) drive the cell cycle to coordinate processes such as DNA replication and chromosome segregation. Dysfunction of these kinases in mammals is associated with increased proliferation and genome instability of cancer cells[1]. Recently, several proteins involved in the DNA damage response were shown to be phosphorylated by Cdk1, revealing its role in co-ordinating DNA repair with replication[2]. The activities of the budding yeast DNA helicase Srs2[3], checkpoint proteins Rad53 and Rad9, and the Rad9 homologue Crb2 in fission yeast are regulated by Cdk-mediated phosphorylation[4–7]. In human cells, phosphorylation of the tumor suppressor protein BRCA2 by Cdk in M phase

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Author contributions X.C. constructed most of the strains and plasmids, analyzed Dna2 phosphorylation in cells, performed fluorescence microscopy and ChIP. W-H.C. and A.P. analyzed resection in mutants and H.N. purified proteins, performed all the *in vitro* experiments. Z.Z. constructed initial plasmids carrying *dna2* mutant alleles. X.C., H.N., P.S. and G.I. designed the experiments, analyzed the data, and wrote the manuscript. E.Y.S. and S.E.L performed the ChIP assay for Sgs1-13×Myc.

inhibits its interaction with RAD51, which likely minimizes unscheduled recombination when chromosomes segregate[8].

Cdk1 in yeast controls the initial step of DSB-induced homologous recombination (HR), 5' strand resection. In G1 cells, DSB ends are poorly resected, thus enabling efficient repair by non-homologous DNA end-joining (NHEJ). In the S and G2 cells when sister chromatids are available, DSBs are resected promptly to generate a ssDNA substrate for HR[9,10]. Similarly in fission yeast, NHEJ and HR are cell cycle-regulated[11] and Cdk activity is essential for the recruitment of the Rad51 recombinase to DSBs induced by ionizing radiation (IR)[4]. Finally, in human cells Cdk is also required for early steps of HR[12]. Consistent with decreased DSB resection, Cdk1-kinase deficient yeast cells also fail to activate the DNA damage checkpoint in response to a single DSB, even though the upstream checkpoint kinase, Mec1, remains at least partially active[10,13,14]. These results have stimulated a search for targets of Cdk1 that help control early HR steps. Sae2 protein and its vertebrate orthologue CtIP, both involved in the initiation of resection together with Mre11-Rad50-Xrs2 [MRX, (MRE11-RAD50-NBS1 or MRN in human)], were found to be substrates of Cdk1 and key regulators of DSB repair pathway choice[15–17]. The expression of the fission yeast Sae2 orthologue, Ctp1, is also regulated during the cell cycle[18]. Besides Sae2 there are likely additional targets of Cdk1 needed for resection because a *SAE2* phospho-mimic allele does not efficiently bypass the need for Cdk1 in resection[15,19]. Evidence for the existence of additional targets comes from studies of resection in Cdk1 kinase deficient cells that lack also the Ku70–Ku80 complex, a central component of the NHEJ pathway. Several studies demonstrated that deletion of Ku proteins restores resection in Cdk1 deficient cells but extensive resection further from the break remains impaired[13,20–22]. Because Sae2 together with MRX likely act during the initial stages of resection, this result indicates that extensive resection is dependent on Cdk1 as well. We aimed to understand how Cdk1 controls extensive resection in budding yeast. Here we present our genetic and biochemical studies that reveal the role of Cdk1-mediated phosphorylation of Dna2, whose nuclease activity is important for extensive DSB resection in cells.

Results

Dependence of Dna2-mediated long-range resection on Cdk1

First, we examined which of the protein components involved in the DNA motor driven path of resection[23–25] - Exo1, Dna2, Sgs1 or MRX complex - remain active in *yku70*Δ Cdk1 kinase deficient cells. Resection was analyzed by Southern blots at an HO break located at *MAT* or 28 kb away from the break (*FEN2* locus), to follow the initial removal of 5' strands and long-range resection, respectively[26]. We constructed *exo1*Δ, *sgs1*Δ and *dna2*Δ *pif1-m2* derivatives of *yku70*Δ *cdk1-as1* cells and tested resection in these cells either with or without the ATP analogue, 1-NMPP1, that inhibits cdk1-as1 kinase activity[27]. The *pif1*-m2 mutation suppresses the lethality of *DNA2* deletion[28]. As previously noted[22], in Cdk1 kinase-deficient cells, deletion of *YKU70* restores resection only of sequences adjacent to the DSB, as evidenced by the lack of resection 28 kb away (Fig. 1a and Supplemental Fig. 1). We found that Exo1 but not Dna2 or Sgs1 was required for resection in *yku70*Δ cells where Cdk1

kinase is inactive. Importantly, in Cdk1 kinase deficient *exo1*Δ *yku70*Δ cells additional bands and smearing beneath the HO break intermediates that are typical for cells that lack both Exo1 and Dna2 accumulate. This confirms that the Dna2-dependent long range resection pathway is inactive in these conditions (Fig. 1b, Supplemental Fig. 1 and ref.[26]). As previously determined, MRX is responsible for the limited 5' strand cleavage (Fig. 1b). Similar to resection, DSB repair by ectopic recombination depends on Exo1 in Ku and Cdk1 kinase deficient cells (Supplemental Fig. 1). Consistent with this view, Ku blocks Exo1- and MRX-dependent resection in cycling cells[29,30] but not as significantly as in Cdk1 kinase deficient cells. Our results suggest that Exo1 and MRX with Sae2 are active, at least partially so, in the absence of both Ku and Cdk1 kinase, and that the Dna2-dependent resection pathway is likely regulated by Cdk1.

To directly test whether Cdk1 activity is needed to sustain normal resection rate further from the break, we measured resection in cells where for the first four hours after DSB induction Cdk1 is active and only then *cdk*-as1 kinase is blocked by 1-NMPP1. As shown in Figure 1c, long-range resection measured 28 kb from the DSB was still delayed when compared to cells that retained active Cdk1. Therefore Cdk1 controls both initial and long-range resection and, besides Sae2, additional targets of Cdk1 kinase likely exist.

One candidate protein is Dna2, previously shown to be a likely target of Cdk1 in genome wide screens[31]. First, we investigated whether Cdk1 activity is needed for Dna2 recruitment to DSBs. Recruitment of Dna2-9×Myc was followed using chromatin immunoprecipitation (ChIP) and qPCR using primers specific for sequences 1 kb upstream of the HO break. Recruitment of Dna2-9×Myc occurred efficiently in *yku70*Δ *cdk-as1* cells only when Cdk1 remains active (Fig. 1d). Accordingly, fluorescence microscopy revealed that Dna2-GFP foci are formed in *yku70*Δ *cdk-as1* cells only in presence of active Cdk1 (Supplemental Fig. 2). In contrast, the Sgs1 helicase, which works together with Dna2 in resection[23,24,26], is recruited normally to the DSB in *yku70*Δ Cdk1-kinase deficient cells (Supplemental Fig. 2).

Mec1- and Cdk1-dependent phosphorylation of Dna2

To verify whether Dna2 is phosphorylated, we examined the electrophoretic mobility of Dna2-9×Myc by Western blot. A mobility shift of Dna2-9×Myc was observed following DNA damage (Fig. 2a). The shift was due to phosphorylation as verified by treatment of immunoprecipitated Dna2 with lambda phosphatase (Supplemental Fig. 2). Dna2 phosphorylation is Cdk1-dependent, as it did not occur in kinase deficient cells (Fig. 2a). The maintenance of Dna2 phosphorylation also depends on Cdk1 activity, as revealed by adding 1-NMPP1 four hours after DSB induction (Fig. 2a). To investigate whether Dna2 phosphorylation requires damage checkpoint proteins we tested its phosphorylation in the *mec1*Δ *sml1*Δ mutant. Dna2 was not phosphorylated in *mec1*Δ *sml1*Δ mutant but remained phosphorylated in the absence of the signaling kinases Rad53, Chk1, or Dun1, suggesting that Dna2 is a direct target of Mec1 (Fig. 2a; data not shown). Immunoblotting of Dna2-TAP from cells 4 hours after HO break induction with a phospho-specific antibody confirmed that Dna2 is phosphorylated by Mec1 or its homolog Tel1 (Supplemental Fig. 2). Together, the results show that Cdk1 and Mec1 are required for Dna2 phosphorylation upon DNA damage.

Dna2 harbors 3 full S/T-P-x- K/R (Thr4, Ser17, Ser237) consensus sequences and 5 minimal S/T-P consensus sites for Cdk1 phosphorylation. The full consensus sites are conserved in related yeast species even though most of the N-terminal part of Dna2 is not (Supplemental Fig. 2). We purified wild-type Dna2 and dna2 mutants in which the serine or threonine within the full Cdk1 consensus sites had been replaced with alanine (Supplemental Fig. 2) and performed an *in vitro* phosphorylation assay with purified Cdk1–Clb2 as described[31] (Fig. 2b). We found reduced phosphorylation of dna2^{S17A} and dna2^{S237A}, minimal phosphorylation of dna2$^{S17A\ S237A}$, and absence of phosphorylation of dna2$^{T4A\ S17A\ S237A}$ (hereafter dna2-3A). These results demonstrate that Ser17, Ser237, and to a lesser degree Thr4, of Dna2 are phosphorylated by Cdk1. The five minimal S/T-P sequences are likely not targeted by Cdk1. The dna2-3A mutant retains biological activity, as it grows normally, whereas a complete deletion of *dna2* is lethal[28].

To examine whether the Cdk1 consensus sites in Dna2 are important for its phosphorylation in response to DSB induction, plasmid borne *DNA2*, *dna2*^{T4A}, *dna2*^{S17A}, *dna2*^{S237A} and *dna2*-3A genes tagged with FLAG were introduced into *dna2*Δ *pif1-m2* cells. The levels of wild-type and all the mutated proteins were similar, but a decrease in phosphorylation upon DSB induction was observed in *dna2*^{S17A} and *dna2*^{S237A} mutant cells and particularly in *dna2*$^{S17A\ S237A}$ double mutant and *dna2*-3A mutant cells (Fig. 2c). These results suggest an important role for Ser17 and Ser237 in Dna2 phosphorylation by Mec1 in response to DNA damage.

Role of Dna2 phosphorylation in the DNA damage response

To test the role of phosphorylation of the Cdk1 target residues in resection, we compared the rates of 5' resection of the HO break in *dna2*Δ cells harboring a centromeric plasmid carrying either wild-type *DNA2* or the *dna2*-3A mutant allele. As control, *dna2*Δ *pif1-m2* cells that show a dramatic defect in resection were included. Initiation of resection was identical in all the mutants, but the rate of resection further from the break site was decreased in the *dna2*-3A mutant strain (Fig. 3a, Supplemental Fig. 3). Similar resection rates were observed in the *dna2*Δ *pif1-m2* strain background complemented with plasmids carrying the *dna2* mutant alleles (Supplemental Fig. 3). A short delay in resection observed in *dna2*-3A cells suggests that phosphorylation of Ser17 and Ser237 is important for timely resection by Dna2. A similar partial defect was observed in mutants bearing a truncation of the N-terminal 248 residues of Dna2 (*dna2*Δ*N248*) where all three full Cdk1 consensus sites are eliminated. The growth rate of *dna2*-3A and N-terminal truncation mutants is comparable to that of the wild type strain. Importantly, the resection defect exhibited by the dna2 mutant harboring 8 point mutations eliminating all possible Cdk1 consensus sites was no more severe than that in the *dna2*-3A strain (data not shown).

Exol defines a second pathway of long range resection, thus it is possible that the dna2 mutants are compensated for by Exo1. To test this possibility we constructed an *exo1*Δ *dna2*Δ strain expressing either wild-type *DNA2*, *dna2-3A* or *dna2*Δ*N248* and tested resection rates. The growth rate of *exo1*Δ *exo1*Δ *dna2*-3A and *exo1*Δ *dna2*Δ*N248* is comparable. As shown in Figure 3b, the *exo1*Δ *dna2*-3A strain exhibited a very significant defect in extensive resection. An even more severe defect was observed in *exo1*Δ *dna2*Δ cells

harboring *dna2ΔN248*. Accordingly, *exo1Δ* mutants bearing Dna2 lacking the Cdk1 consensus sites or with the *N248* deletion are much more sensitive to DNA damage than *exo1Δ* cells (Fig. 3c). Overexpression of *dna2-3A* is able to slightly restore resistance to DNA damage indicating that a higher level of mutant protein can partially bypass the need for Cdk1 dependent phosphorylation. Importantly, the dna2$^{S17D\ S237D}$ mutant that mimics phosphorylation showed normal resection and resistance to DNA damage identical to wild type Dna2 in presence of Cdk1 (Supplemental Fig. 3). We concluded that first, Dna2 phosphorylation by Cdk1 stimulates its role in resection and DNA damage resistance and second, the *dna2-3A* or *dna2ΔN248* mutant can contribute to extensive resection mostly in an Exo1-dependent manner, suggesting crosstalk between the Exo1- and Dna2-dependent resection pathways.

Dna2 phosphorylation stimulates its recruitment to DSBs

To understand the role of Cdk1-dependent Dna2 phosphorylation we compared nuclease activity and protein-protein interactions of wild-type Dna2 and mutant dna2-3A proteins purified from yeast. We showed that dna2-3A possesses normal nuclease activity and interacts with other resection proteins as well as wild type Dna2 (Supplemental Fig. 4). We therefore looked for clues about the function of Dna2 phosphorylation in cells. Since Dna2 is not recruited to DSBs in Cdk1-kinase deficient cells (Fig. 1d), we asked whether Cdk1-dependent phosphorylation at Ser17 and Ser237 is required for Dna2 recruitment. To compare recruitment of wild-type and mutants, we constructed *dna2Δ* strains carrying GFP-tagged *DNA2* or mutant *dna2*^{S17A}, *dna2*^{S237A}, *dna2*$^{S17A\ S237A}$, *dna2-3A* and *dna2ΔN248* on a centromeric plasmid. We tested the recruitment of the GFP-tagged proteins to an HO-induced DSB 4 hours after break induction using fluorescence microscopy. The dna2^{S237A}-GFP and dna2^{S17A}-GFP mutant protein showed decreased intensity of foci compared to wild type, and the dna2$^{S17A\ S237A}$-GFP and dna2-3A-GFP mutants formed only barely visible foci. An even greater defect in focus formation was observed for dna2ΔN248-GFP (Fig. 4a and Supplemental Fig. 5). As noted previously[32], nuclear localization was diminished in the dna2^{S17A}-GFP mutant. These data suggest that serines 17 and 237 of Dna2 are important for its nuclear localization and recruitment to DSBs. Consistent with the microscopy data, ChIP analysis to examine for protein association 1 kb from the DSB ends showed decreased recruitment of the single mutants dna2^{S17A}-FLAG and dna2^{S237A}-FLAG, and severely impaired recruitment of dna2$^{S17A\ S237A}$-FLAG, dna2-3A-FLAG, and dna2ΔN248-FLAG (Fig. 4b).

When Ser17 and Ser237 were replaced with aspartic acid to mimic phosphorylation, Dna2 nuclear localization and DSB recruitment were largely restored (Fig. 4a–b, Supplemental Fig. 5). We therefore tested whether dna2$^{S17D\ S237D}$-GFP is normally recruited to DSBs in *yku70Δ* Cdk1-kinase deficient cells where resection is close to DSB ends is normal. However, even nuclear localization occurred normally, DNA damage focus formation of dna2$^{S17D\ S237D}$-GFP was impaired when Cdk1 kinase activity was blocked (Fig. 4c). Consistent with the poor DSB recruitment, the phosphomimetic *dna2*$^{S17D\ S237D}$ mutation does not restore long range resection in Cdk1 and Ku deficient cells (data not shown). These results suggest that another Cdk1-controlled event is needed for efficient Dna2 recruitment to DSBs. An upstream protein that may be important for Dna2 recruitment is the trimeric

replication protein A (RPA), the Rfa2 subunit of which is phosphorylated in a cell cycle-dependent manner[33]. As described above we have also demonstrated that Dna2 is phosphorylated by the checkpoint kinase Mec1. This phosphorylation is not needed for Dna2 phoshorylation by Cdk1, Dna2 nuclear localization, recruitment to DSBs, or Dna2-dependent resection as analyzed in Exo1 and Mec1 deficient cells (Supplemental Fig. 6). Once the Mec1-specific phosphorylation sites in Dna2 are identified it will be possible to define their significance.

Discussion

Cdk1 kinase is needed for proper 5' strands resection. Previously, Sae2 was reported to be a target of Cdk1[15] but the molecular function of Sae2 phosphorylation was not determined. Here we have provided evidence that in addition to Sae2, Dna2 is also phosphorylated by Cdk1. Two serines within Dna2 are targeted by Cdk1, an important event for the DSB recruitment of Dna2. A decrease in Dna2 recruitment results in impaired resection that is further aggravated by the deletion of *EXO1*. Clearly, the cell cycle control of resection by Cdk1 is more complex than previously anticipated, and additional Cdk1 target(s) relevant for DSB resection must exist, as Dna2 and Sae2 pseudo-phosphorylation is insufficient for normal resection in Cdk1 deficient cells.

The budding yeast *S. cerevisiae* has two resection pathways, controlled by the Exo1 and Sgs1 with Dna2, that can generate long ssDNA at DSBs[23,24,26,34]. These two pathways appear to be conserved in human cells[35]. Interestingly, BLM, the Sgs1 orthologue in humans, seems to stimulate both nucleases[36]. The precise reason for the existence of two resection pathways is not yet known. The Exo1-dependent pathway plays the major role in the resection of Spo11 induced DSB's during meiosis[37–39], in processing UV damage[40] or within the context of stalled replication forks in checkpoint deficient cells[41]. Why the Sgs1 with Dna2 pathway does not contribute in these circumstances remains to be established. In mitotic cells, elimination of Exo1 and particularly Dna2 slows down the rate of resection, suggesting that one pathway cannot completely substitute for the other, whereas elimination of both Dna2 and Exo1 eliminates long range resection[26]. It is possible that, while the two pathways can act independently, they normally collaborate during resection. Indeed, Exo1 and Dna2 may be present simultaneously at DSB ends, since a Dna2-GFP focus is present in all cells with an induced DSB and we have been able to show by ChIP that both Exo1 and Dna2 are associated with regions proximal to and farther away from the DSB site[26,30]. We have shown here that the *dna2*-3A and *dna2*ΔN248 mutants exhibit a modest defect in resection when compared to complete elimination of Dna2. However, Exo1 becomes indispensible for resection in these mutants, suggesting that dna2 mutant proteins that are recruited poorly to DSBs contribute to resection in conjunction with Exo1. One possibility is that the two nucleases are needed for resection because each may be impeded by unique DNA sequences or structures that are less inhibitory to the other.

Based on our results, we propose that Cdk1-mediated phosphorylation of Dna2 ensures an optimal response to DNA damage in S-phase when most of the spontaneous DNA damage occurs. However, it is likely that Cdk1-dependent phosphorylation of Dna2 is also important for other well documented functions of Dna2, most notably in Okazaki fragment

processing[42]. Our study makes a significant contribution toward delineating the cell cycle dependence and regulation of DNA damage repair and replication.

Methods

Strains and plasmids

All strains used in this study are derivates of JKM139 (*ho*Δ *MAT**a*** *hml*::*ADE1 hmr*::*ADE1 ade1-100 leu2-3,112 trp1*::*hisG' lys5 ura3-52 ade3*::*GAL*::*HO*). Strains used are listed in Supplemental Table 1. All plasmids were constructed using standard PCR and cloning techniques as described in Supplementary Methods.

Analysis of resection at DSB ends

Resection was analyzed at an HO endonuclease-induced DSB at the *MAT* locus on chromosome III using Southern blots as previously described[26]. Briefly, Genomic DNA was isolated using a standard phenol extraction method. Purified DNA was digested with EcoRI and separated on a 0.8% (w/v) agarose gel. Southern blotting and hybridization were performed as described previously[43]. Intensities of target bands were analyzed with ImageQuant TL (Amersham Biosciences) and normalized to the TRA1 probe. Resection efficiency was measured with probes located 0.1 kb (*MAT*), 3.8 kb (*BUD5*), 10 kb (*SNT1*) or 28 kb (*FEN2*) upstream of the DSB. Analysis was performed at least three times in each mutant strain.

Ectopic recombination assay

DSB repair kinetics and efficiency of DSB repair 8 hr after HO endonuclease induction was measure using ectopic recombination between chromosome III and chromosome V as described previously[44].

Fluorescence microscopy

Dna2-GFP foci were photographed using an EM-CCD digital camera (Hamamatsu) connected to an Axiovert 200M microscope (Zeiss). The images were analyzed using the AxioVision Software (Zeiss). To measure Dna2-GFP foci intensity eleven pictures were taken along the Z-axis at 0.3 mm intervals with an acquisition time of 750 ms. Foci intensity was measured in 20 cells in each strain. For DAPI staining we used mounting medium with DAPI H-1200 from Vector Laboratories, Inc. Burlingame. Nuclear localization of Dna2-GFP was verified in at least 100 cells per strain.

Chromatin immunoprecipitation

ChIP assays were performed as described by Sugawara *et al*. (2003)[45]. Briefly, cultures were grown to a density of ~1 × 10^7 cells per ml in pre-induction medium YEP-Raffinose (1% yeast extract, 2% peptone, 2% (w/v) raffinose) and the HO endonuclease was induced by adding 2% (w/v) galactose. Proteins were crosslinked for 10 min at room temperature by the addition of 1% (w/v) (final concentration) formaldehyde, followed by quenching with glycine (125 mM final concentration) for 5 min. Cells were lysed with glass beads, and the extracts were sonicated to shear the DNA to an average size of 0.5 kb. IP samples were

incubated with affinity-purified anti-Myc (Sigma M4439) or anti-FLAG (Sigma M2) antibody for overnight at 4°C and bound to protein-G agarose beads for 4 hr at 4°C. The protein bound beads were carried through a series of washes, followed by elution of the proteins and reversal of crosslinking (overnight at 65°C). Samples were treated with proteinase K followed by phenol extraction and ethanol precipitation. Purified DNA was analyzed by real-time quantitative PCR using primers that anneal ~1.1 kb from the DSB.

Whole cell extract preparation

Yeast cells were grown overnight in YEP raffinose medium to a density of ~1×10^7 cells per ml. The HO break was induced by adding 2% (w/v) galactose. Cells from 5 ml of culture were washed with water and resuspended in 10% trichloroacetic acid. The cells were lysed by vortexing with glass beads and the protein lysates were pelleted by centrifugation at 20,000 × *g* for 15 min. The pellet was washed with 80% acetone and proteins were dissolved in 2× SDS sample loading buffer by boiling samples for 5 min. Samples were centrifuged for 5 min at top speed in a microcentrifuge and the supernatant was retained as the protein extract.

Protein phosphatase treatment

To examine that Dna2 is phosphorylated, yeast cells carrying chromosomal tagged *DNA2*-TAP were cultured in YEPD to early log phase. Dna2 phosphorylation was induced by adding DSB-inducing agent phleomycin to culture. The TAP tagged Dna2 was immunoprecipitated and treated with Lambda protein phosphatase (New England Biolabs, P0753S). Drug treatment, cell lysis and protein phosphatase treatment were performed as described in Supplementary Methods.

Western blot analysis

Proteins samples were resolved on a 6.5% SDS-PAGE gel and transferred onto a PVDF membrane (Immobilon-P; Millipore) using semi-dry method following manufacturer's protocol. Mouse monoclonal antibodies anti-Myc (M4439) and anti-FLAG (M2) were bought from Sigma, anti-TAP antibody was obtained from GenScript, and the antibodies against phospho-(Ser/Thr) ATM and ATR substrates and phospho-Ser Cdk1 substrate were purchased from Cell Signaling Technology. The blots were developed using the ECL Western Blotting substrate (GE Healthcare).

Protein isolation and *in vitro* phosphorylation analysis

For *in vitro* kinase reaction, 500 ng of Dna2 or dna2 mutant was incubated with 4 ng of purified Cdk1-Clb2 in 10 μl of kinase reaction buffer (40 mM Tris-HCl pH 7.4, 2 mM $MgCl_2$, 2 mM ATP, 100 μg ml^{-1} BSA, 1 mM DTT, 50 mM KCl and 5 μCi [γ-^{32}P]-ATP) at 30°C for 20 min. The reaction was stopped by the addition of 2× SDS loading buffer and heating at 95°C for 3 min. The samples were split and fractionated by 7.5 % SDS-PAGE. The gels were dried and analyzed by phosphorimaging or stained with Coomassie blue.

Protein purification, Dna2 nuclease assay and Affinity pulldown assay

Dna2, dna2 mutants, the Mre11-Rad50-Xrs2 (MRX) complex, the Top3-Rmi1 (TR) complex and yeast RPA were purified to near homogeneity form yeast or *E. coli* cells tailored to express them, as previously described[24]. The Cdk1-Clb2 complex was purified from yeast cells, as described before[46]. Dna2 nuclease activity assay was conducted using a ^{32}P-labeled partial duplex harboring a 5' single-strand overhang with or without RPA exactly as described[24]. Affinity pulldown to check for interaction of the dna2-3A mutant with the MRX or TR complex was conducted exactly as described[24].

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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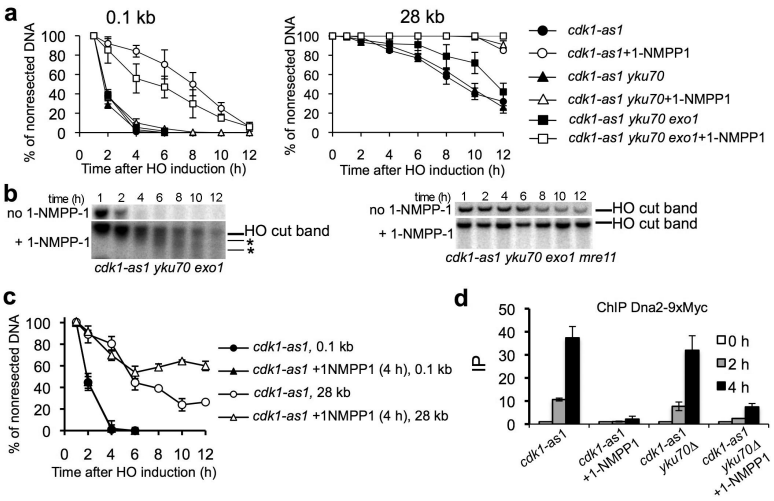

Figure 1.

Cdk1 regulates long range DSB resection by Dna2.

(a) Analysis of initial and extensive resection in mutants with either active or inactive Cdk1 kinase. The Southern blots corresponding to these experiments are presented in Supplemental Figure 1. Error bars correspond to s.d. **(b)** Southern blot analysis of initial resection in mutants. Smearing and additional bands below the HO cut band typical for mutants that lack extensive resection are indicated by asterisks. **(c)** Analysis of resection in cells where Cdk1 activity is blocked at 4 hours after break induction when all cells have initiated resection. **(d)** Recruitment of Dna2 to DSBs was monitored in indicated mutants by ChIP. Error bars correspond to s.d.

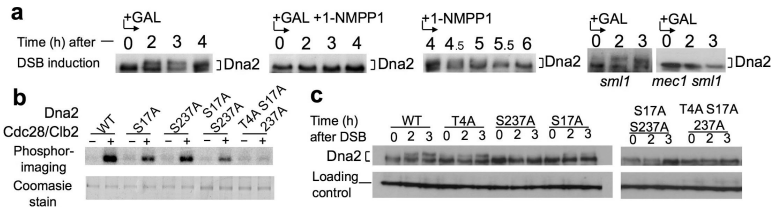

Figure 2.

Dna2 is phosphorylated by Cdk1 and Mec1.

(a) Western blot analysis of Dna2-9×Myc phosphorylation in *cdk1-as1* with or without Cdk1 inhibitor and in checkpoint deficient cells in response to a single DSB. **(b)** *In vitro* phosphorylation of wild-type Dna2 or dna2 mutant proteins lacking single or multiple Cdk1 phosphorylation consensus sites. **(c)** Western blot analysis of Dna2 phosphorylation in wild type cells and indicated *dna2* mutants cells.

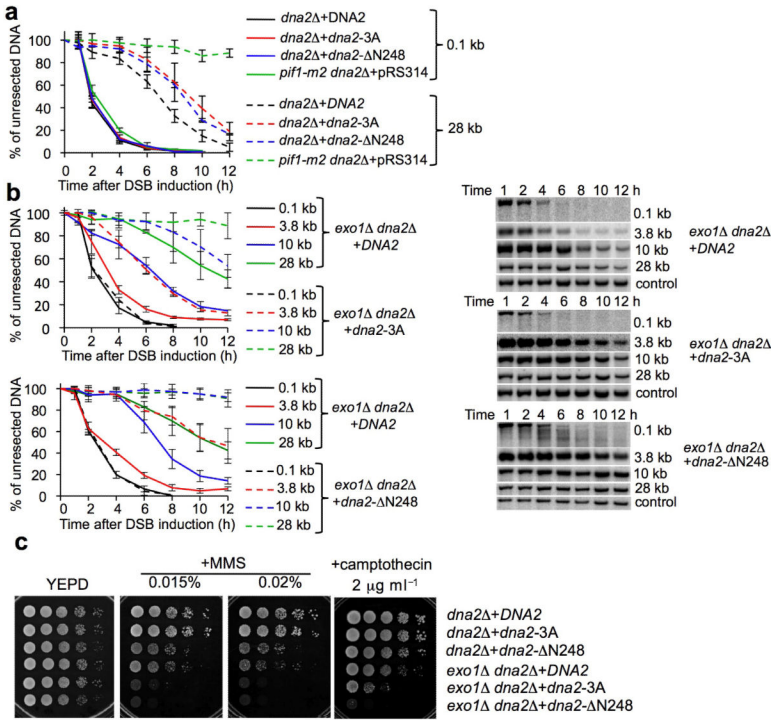

Figure 3.

Dna2 phosphorylation by Cdk1 stimulates resection.

(a) Analysis of 5' strand resection in *dna2*Δ cells complemented with plasmids carrying either wild-type or a mutant *DNA2* allele. Error bars correspond to s.d. The Southern blots corresponding to these experiments are presented in Supplemental Figure 3. **(b)** Analysis of resection in *exo1*Δ *dna2*Δ cells complemented with plasmids carrying either wild-type or a mutant *DNA2* allele. **(c)** Analysis of sensitivity to MMS and camptothecin in the indicated mutants.

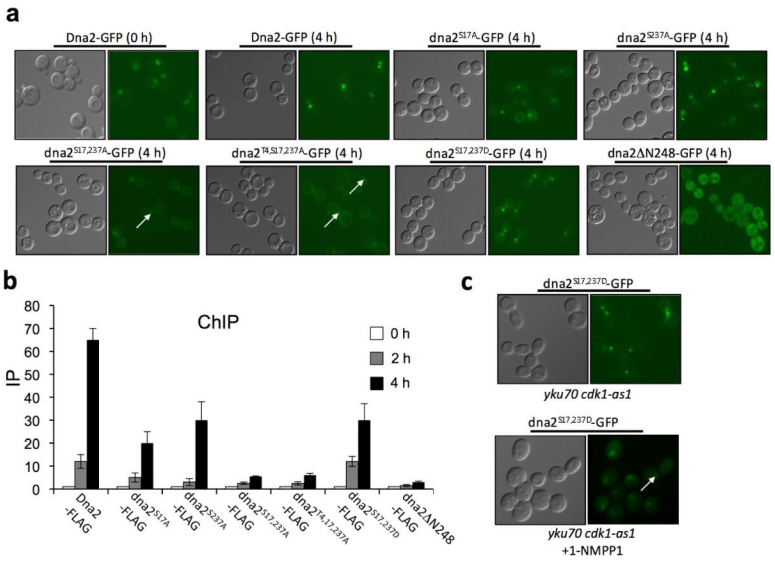

Figure 4.

Dna2 phosphorylation by Cdk1 is needed for its recruitment to DSBs.

(a) Analysis of DSB recruitment of GFP-tagged wild-type Dna2 and indicated mutant dna2 proteins. **(b)** Analysis of recruitment of FLAG-tagged wild-type Dna2 and indicated mutant dna2 proteins to DSB ends by ChIP using primers specific for sequences located 1 kb upstream of the DSB. Error bars correspond to s.d. **(c)** Analysis of recruitment of GFP-tagged phosphomimic dna2$^{S17D\ S237D}$ protein to DSB ends in Cdk1 and Ku deficient cells.