assess dominance. Capital **S** and **D** represent wild-type *SLD3* and *DBF4*, whereas lowercase **s** and **d** represent *SLD3-m25* and *dbf4-m25* respectively.

Figure 4. **Inappropriate firing of late origins elicits DNA damage. a**, Flow cytometry of wild-type or mutant strains synchronized in G1 with α-factor, released into a 200 mM HU block for 2 hours, then released into medium with α-factor. **b**, Immunoblots of samples from **a**, taken at indicated time points and probed for Rad53. **c**, Quantification of cells with one or more Rad52-GFP foci after incubating for 90 minutes in the presence of nocodazole or 200 mM HU. Error bars represent s.e.m.; *n* = 3. **d**, Survival assay of strains synchronized in G1 and released into 200 mM HU for 2, 4 or 6 hours. Error bars represent s.e.m; *n* is indicated on graph.

Supplementary Methods

All yeast strains used in this study are of s288c strain background unless otherwise noted and were created using standard yeast techniques. All constructs were sequenced verified.

Epitope tagging of Sld3 at the endogenous locus was done by integration as follows: 3FLAG::HygroR cassette[30] was targeted to the *SLD3* ORF to yield *SLD3-3Flag*::*HygroR (yJLO18). SLD3-3Flag::HygroR* was PCR amplified from yJLO18 and cloned into pGEM-T-easy (Promega) to generate pJLO2. Mutagenesis of Sld3 at the endogenous locus was done by integration as follows: pJLO2 was mutated using quik-change site-directed mutagenesis (Stratagene) following manufacturer's instructions. Resultant plasmids were then digested to release constructs used to transform diploid strains. (eg. (yNLM133) *SLD3-m25-3Flag::HygroR*; pNM23 was digested to release a StuI/BstZ17I fragment used to target SLD3). T607A T609A T631A T638A, were not identified by mass spec but were mutated because these sites are present in a peptide shown to bind Dpb11 in vitro[8,9]. To create sld3-m21D-3Flag, a DNA fragment including amino acids corresponding to the last 21 mutations in the *SLD3-m25-3Flag* allele was synthesized, such that each of these 21 phosphorylation sites were now mutated to aspartic acids, and cloned into pJ201 (DNA 2.0). An EcoN1/BamH1 fragment (857bp) was subcloned into pNM14 digested with EcoN1/BamH1. The resulting plasmid pJLO18, was then digested with EcoN1 and Bstz17I to integrate it at the endogenous *SLD3* locus in diploid cells. Diploids were subsequently sporulated to obtain haploid cells.

Dbf4 was cloned into pGEM-T-easy to generate pJLO12. Mutations identified by mass spec were mutated using quik-change mutagenesis as described above to generate pJLO15. Mutations in the Dbf4 N-terminal domain were synthesized and cloned into pUC57 (Bio-Basic). A SacII/BsaBI fragment from pUC57 was subcloned into pJLO15 to generate pJLO16. pJLO16 was modified by quik-change mutagenesis to revert mutations (A3S A11S) to generate pJLO17.

pJLO17 was digested with SacII to integrate into yJLO110 at the *dbf4Δ::KanMx* locus.

Rad53-R70A,R605A (FHA1 FHA2 mutants). A heterozygous diploid *SLD3/SLD3-m25 RAD53/rad53Δ DBF4/dbf4-m25 SML1/sml1-1* was transformed with a CEN plasmid harboring *rad53-R70A,R605A::URA3.* Resultant transformants were sporulated and dissected. Previous work has shown that Rad53 has an additional direct role in DNA replication leading to synthetic lethal interactions with *cdc7* or *dbf4* alleles[31]. This interaction is checkpoint-independent, since *RAD53* alleles with mutations in both FHA domains, which lack all checkpoint functions[32,33], are fully competent for this checkpoint-independent function. To avoid complications arising from this, we have used this rad53 allele.

MAT a/a diploids were created by transforming a/αdiploid cells were a CEN plasmid containing p*Gal-HO::URA3*. Mating type switching was induced with 2% galactose for 30 minutes. Diploids were then plated on 5-FOA to lose the plasmid. Resulting diploids were then tested for the ability to arrest in G1 with α-factor.

Phosphatase Assay
Sld3-3Flag or Dbf4-TAP was over-expressed and immuno-precipitated using anti-M2 Flag sepharose (Sigma) or IgG sepharose (GE Healthcare) from cells treated with 0.05% MMS or 2 μg/mL 4NQO. Immuno-precipitates were washed twice in λ-phosphatase buffer and treated with 100 units of λ-phosphatase (NEB) for 30 minutes at 30°C in the presence or absence of phosphatase inhibitors ($ZnCl_2$, 1 mM NaF, 1 mM Na-orthovanadate).

Flow cytometry
Yeast cells were fixed with 70% ethanol and stored at 4°C. Cells were then sonicated, treated with 0.25 mg/mL RNase A for 1 hour at 50°C, followed by digestion with 0.125 mg/mL Proteinase K for 1 hour at 50°C and labeling with 1 μM Sytox Green (Invitrogen). Data was collected using a FACSCalibur (Becton Dickinson) and analyzed with FlowJo software (Tree Star, Inc.).

Two-dimensional Gel Electrophoresis
Asynchronous cultures were harvested at an OD600 ~1. Synchronized cultures were held in G1 with α-factor until 90% of cells were unbudded. α-factor was washed out and cells were resuspended in media pre-warmed to 30°C containing 200 mM HU. At the indicated time-points, aliquots were collected and all samples were processed as previously described in Friedman et al[20],. DNA was digested with Pst1 for *ARS607;* Xba1 for *ARS501*; EcoR1 for *ARS305*, *ARS609.* DNA probes were labeled using Prime-it II kit (Stratagene).

Spot assays

Mid-log cultures were diluted to OD600=0.5. Cultures were subjected to 5-fold serial dilution and 5 μL were spotted on plates containing various HU concentrations. Plates were subsequently incubated at 30°C for 18-36 hours.

HU Survival Assay

Overnight cultures were diluted to OD600=0.3 and incubated at 30°C for 2 hours before synchronizing in G1 with α-factor for an additional 2 hours. α-factor was washed out and cells were re-suspended in pre-warmed media containing 200 mM HU. At the indicated time points, 200 μL of each culture was diluted 5000-fold in media and 200 μL of cells were plated and subsequently grown at 30°C for 36 hours. For asynchronous cultures, cells were diluted back to OD600=0.3 for 2 hours before treating with 0 mM, 12.5 mM, 25 mM, or 50 mM HU for 3 hours. Cells were counted, diluted to 200 cells/mL, plated and grown at 30°C for 36 hours. Colonies were scored as percent of untreated.

GFP microscopy

Images of Rad52-GFP foci were obtained on a Leica DMRXA fluorescence microscope with 100x, 1.4 NA PlanApo Olympus Oil Immersion objective. GFP fluorescence was detected using a Chroma FITC filter set (excit. 485/20 nm, emiss. 515/30 nm). Images were taken with a Hamamatsu C4742-95 CCD camera. Data was analyzed with OpenLab software (Improvision). Black and white images were all captured for the same exposure times, and contrast enhancement was performed equivalently on all panels.

Western Blotting

An equivalent of 6 OD600 cells were pelleted, washed with cold water, and resuspended in pre-heated SDS sample buffer (50 mM Tris pH 7.5, 5 mM EDTA, 5% SDS, 10% glycerol, 0.5% β-mercaptoethanol, 0.05% bromophenol blue, 1 μg ml^{-1} leupeptin, 1 μg ml^{-1} pepstatin A, 1 mM benzamidine, 17 μg ml^{-1} PMSF, 5 mM sodium fluoride, 80 mM β-glycerophosphate and 1 mM sodium orthovanadate). Cells were lysed by bead beating for 3 min in a Mini BeadBeater (Biospec, Bartlesville, OK) using 100 μL glass beads. Samples were then centrifuged at 15,000 rpm and heated to 95°C for 5 minutes and clarified by centrifugation. Extracts were analyzed by SDS–polyacrylamide gel electrophoresis (SDS–PAGE) and probed with antibodies against Flag (Clone M2, Sigma, St Louis, MO), rad53 (rabbit anti-Rad53 provided by D. Durocher, Univ. Toronto), myc (9E10) and the TAP tag (rabbit anti-CBP, provided by J. Weissman, University of California, San Francisco, CA).

Protein preps

18 L of yeast cells were grown to an OD600=1.0 and Sld3-3Flag was induced for 6 hours with 2% galactose and treated with 0.05% MMS or left untreated. Cells were resuspended in 50 mL lysis buffer (25 mM Hepes-KOH pH 7.5, 150 mM KCl, 0.1 mM EDTA, 0.5 mM EGTA, 2 mM $MgCl_2$, 20% glycerol, 0.02% NP40, 1 mM DTT, 1 μg ml^{-1} leupeptin, 1 μg ml^{-1} pepstatin A, 1 mM benzamidine, 17 μg ml^{-1} PMSF, 1 mM NaF, 1 mM Na-orthovanadate, 80 mM β-glycerolphosphate).

Cells were lysed by 5 sequential passes through a microfluidizer (Microfluidics) with coil on iced-water. Crude extracts were clarified by centrifugation and clarified extracts were incubated with 500 μL M2-FLAG resin (Sigma) for 4 hours at 4°C. Beads were washed 3x with 10 mL lysis buffer supplemented with 300 mM KCl. Sld3 was eluted from beads with five-500 μL elutions using 150 ng/mL 3xFlag peptide.

Dbf4-MORF was over-expressed from 6 L of yeast in synthetic complete-Uracil drop-out medium + 2% galactose for 6 hours and treated with 0.05% MMS. Cells were lysed by bead beating in lysis buffer (25 mM Hepes-HCl pH 8.0, 300 mM NaCl, 0.1% NP40, 1 mM EDTA, 33 mM EGTA, 1 mM PMSF, 50 mM NaF, 80 mM β-glycerolphosphate, 1 mM Na-orthovanadate, 1 μg ml^{-1} leupeptin, 1 μg ml^{-1} pepstatin A, 1 mM benzamidine). Crude extracts were clarified by centrifugation and clarified extracts were incubated with 200 μL IgG sepharose (GE Healthcare) for 2 hours at 4°C. Beads were washed 3x in 10 mL wash buffer (25 mM Hepes-HCl pH 8.0, 300 mM NaCl, 0.1% NP40). Dbf4 was eluted from beads in sample buffer and separated by SDS-PAGE. Dbf4 was excised from silver-stained gel for mass spectrometry.

GST-SLD3-3Flag and GST-SLD3-m25-3Flag were expressed and purified from *Rosetta (DE3) pLysS cells* (Novagen). Expression was induced with 0.5 mM IPTG at 19°C for 15 hours and lysed in 1x PBS pH 7.4 supplemented with 1% triton-X100, 1 mM EDTA, 1 μg ml^{-1} leupeptin, 1 μg ml^{-1} pepstatin A, 1 mM benzamidine, 17 μg ml^{-1} PMSF, 10 μg/mL RNAse A, 20 U Dnase. Crude extracts were centrifuged at 10,000 g for 30 minutes at 4°C (Beckman JA25.50). Clarified extracts were then incubated with 500 μL M2-FLAG resin (Sigma) for 2.5 hours at 4°C. Resin was washed 5 times in 10 mL 1x PBS 1 mM EDTA. Proteins were eluted 5 times with 500 μL 3xFLAG peptide [150ng/mL] in 1x PBS. Proteins were then dialyzed overnight against 1xPBS 10% glycerol 4°C before being frozen.

CDK Clb5-TAP was over-expressed in 3 L of yeast for 6 hours in synthetic complete-Uracil drop-out medium + 2% galactose. Cells were harvested and washed 1x with cold water and frozen in liquid nitrogen. Cells were lysed by bead beating in 40 mL lysis buffer (25 mM Hepes-HCl pH 8.0, 300 mM NaCl, 0.1% NP40, 1 mM EDTA, 33 mM EGTA, 1 mM PMSF, 50 mM NaF, 80 mM β-glycerolphosphate, 1 mM Na-orthovanadate, 1 μg ml^{-1} leupeptin, 1 μg ml^{-1} pepstatin A, 1 mM benzamidine). Crude extracts were centrifuged at 15,000 RPM for 30 minutes at 4°C. Cleared extracts were then incubated with 200 μL IgG-sepharose (50% slurry: GE Healthcare) for 2 hours at 4°C. Resin was washed 5 times with wash buffer (25 mM Hepes-HCl pH 8.0, 300 mM NaCl, 0.1% NP40) followed by two washes with TEV cleavage buffer (25 mM Hepes-HCl pH 8.0, 300 mM NaCl, 0.1% NP40, 0.5 mM EDTA, 1 mM DTT, 10% glycerol). CDK-Clb5 was cleaved off resin in 200 μL TEV buffer supplemented with 10 μL TEV protease.

6xHis-Rad53 and 6x-His-Rad53-KD(K227A) (plasmids were generously provided by D. Durocher) were induced in 1 L *Rosetta(DE3)pLysS cells* (Novagen) with 1 mM IPTG for 2 hours. Cells were harvested and lysed in 20 mL lysis buffer (50 mM Hepes-HCl pH 7.4, 300 mM NaCl, 5 mM Imidazole, 1 μg ml^{-1} leupeptin, 1 μg ml^{-1} pepstatin A, 1 mM benzamidine, 17 μg ml^{-1} PMSF). Clarified extract was incubated with 200 μL Ni-NTA resin (50% slurry: Qiagen) for 2 hours at 4^{o}C. Resin was washed 3 times in 10 mL wash buffer (50 mM Hepes-HCl pH 7.4, 300 mM NaCl, 20 mM Imidazole). Proteins were eluted twice with two-column volumes elution buffer (50 mM Hepes-HCl pH 7.4, 300 mM NaCl, 250 mM Imidazole). Eluted proteins were dialyzed overnight against (20 mM Hepes-HCl pH 7.5 10% glycerol), aliquoted and frozen.

In vitro kinase and binding assay

Kinase reactions were done in 15 μL kinase buffer (20 mM Tris-HCl pH 7.5, 20 mM $MgCl_2$, 2 mM $MnCl_2$, 1 mM DTT, 1 mM ATP) for 30 minutes at room temperature using ~ 150 nM substrate and ~ 40 nM Rad53 or 25 nM Clb5-CDK. Reactions were stopped by adding sample buffer and reactions were separated by SDS-PAGE. Binding reactions were done essentially as described in Tanaka, S. et al[8].

Mass Spectrometry

TCA precipitates were resuspended in digestion buffer (100 mM Tris-HCl, pH 8.5, 8 M urea), digested by the sequential addition of lys-C and trypsin proteases, and analyzed using shotgun proteomics methods on a LTQ-Orbitrap mass spectrometer (Thermofisher) as previously described[34-37]. SEQUEST and DTASelect algorithms were used to identify peptides sequences from tandem mass spectra[38,39]. Phosphopeptides were identified using a differential modification search that considered a mass shift of +79.9663 on serines, threonines, and tyrosines. If a phosphopeptide contained multiple phosphorylatable residues that prevented the confident identification of the exact residue that was phosphorylated, all potential phosphoacceptor residues in the peptide were considered as candidates for mutagenesis.

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