Supplemental Information

Supplemental Table 1a and b. **Damage-induced phosphorylation sites.** **a,** Identification of damage-induced Sld3 and Dbf4 phosphorylation sites *in vivo* and *in vitro* Rad53 phosphorylation sites on recombinant Sld3 and Sld3-m25 by mass spectrometry. Sld3 sites T607, T609, T631 and T638 were not mapped. In **b**, sites in bold were mapped *in vivo*.

Supplemental Figure 1. **Molecular model of Rad53-dependent regulation of late origin firing. a,** Origin firing requires the CDK-dependent phosphorylation of Sld3 and Sld2, as well as the DDK-dependent phosphorylation of the MCMs. CDK-phosphorylated Sld3 and Sld2 bind Dpb11 and collectively promote recruitment of pre-initiation complex (pre-IC) to facilitate origin firing. **b**, Upon intra-S-phase checkpoint activation, Rad53 prevents late origin firing by phosphorylating Sld3 and Dbf4. Rad53 inhibits an Sld3-Dpb11 interaction and possibly prevents recruitment of other pre-IC components.

Supplemental Figure 2. **Damage-induced phosphorylation sites.** **a**, Sld3 illustration indicating mutated sites (set 3: T451A, S456A, S463A, S466A; set 4: S493A, T495A, S497A, S505A, T507A; Set 5: T582A, S591A; Set 6: S639A, T642A, T646A; *sld3-m20*: set 1-6 + S521A S534A; *SLD3-m25: sld3-m20* + T607A T609A T618A T613A T638A).**b,** Immunoblot of Sld3 and Rad53 from cells expressing wild-type or mutant Sld3-3Flag in the presence or absence of 0.05% MMS for 90 minutes.

Supplemental Figure 3. ***SLD3-m25 dbf4-m25* cells are wild-type for growth. a**, Representative tetrads from an *SLD3/SLD3-m25 DBF4/dbf4-m25 MCM5/mcm5-bob1* heterozygote. **b**, Flow cytometry of wild-type, *SLD3-m25, dbf4-m25, and SLD3-m25 dbf4-m25* cells synchronized in G1 with α-factor and released into medium without α-factor at 30^{o}C.

Supplemental Figure 4. **Growth assay of *SLD3-m25 dbf4-m25* cells. a**, 5-fold serial dilutions of wild-type or mutant cells in two strain backgrounds (s288c and w303) grown at 20^{o}, 23^{o}, 30^{o}, 32^{o}, and 37^{o}C. **b**, 5-fold serial dilutions of wild-type or mutant strains grown on 0 mM, 100 mM, or 200 mM HU, as in Fig. 3c, but in w303 cells. In this strain background, *SLD3-m25* cells are partially HU-sensitive.

Supplemental Figure 5. ***sld3-m20* Δ*109N-dbf4* cells are intra-S-phase checkpoint deficient**. **a,** Flow cytometry of wild-type, *rad53*Δ, *SLD3-m25 dbf4-m25, sld3-m20 dbf4-m25, and sld3-m20* Δ*109N-dbf4* cells synchronized in G1 with α-factor and released into 0.033% MMS at 30^{o}C. **b,** 5-fold serial dilutions of wild-type or mutant strains grown on 0 mM and 100 mM.

Supplemental Figure 6. **Inappropriate firing of a late origin**. **a**, Schematic illustrating relevant replication structures and their corresponding migration pattern after two-dimensional gel electrophoresis. **b** and **c**, Time-course analysis

of replication intermediates from *SLD3 DBF4*, *SLD3-m25 DBF4*, *SLD3 dbf4-m25*, *SLD3-m25 dbf4-m25*, and *rad53* cells (w303) synchronously released into 200 mM HU. DNA was prepared from each time-point and probed for **b**, ARS305 (early origin) or **c**, ARS609 (late origin).

Supplemental Figure 7. **An Sld3 phosphorylation mimic has a slow S-phase. a.** Flow cytometry of *SLD3 DBF4, rad53*Δ*, SLD3-m25, SLD3-m25 dbf4-m25* cells or the phosphorylation mimic *sld3-m21D* synchronously released into medium containing 0.033% MMS. **b**, Immunoblot of samples from Fig. 3b, probed for Rad53. "MMS" are control samples from cells treated with 0.05% MMS for 90 minutes. **c**, Two-dimensional gel electrophoresis of asynchronous cells expressing either *SLD3-3Flag* or the phosphorylation mimic, *sld3-m21D.* DNA was digested with EcoR1 and probed for the early origin ARS305.

Supplemental Figure 8**. Sld3-Dpb11 fusion cells are intra-S-phase checkpoint competent.** Flow cytometry of wild-type, *rad53*Δ, *SLD3-m25*, and *sld3-dpb11* fusion cells synchronized in G1 with α-factor and released into 0.033% MMS at 30^{o}C .

Supplemental Figure 9. ***SLD3-m25*** **is epistatic to** ***rad53*** **mutant cells. a,** 5-fold serial dilutions of wild-type or mutant strains grown on 0 mM, 7 mM, 10 mM HU for epistasis analysis. The *rad53-R70,R605* allele has mutations in the FHA1 and FHA2 domains, respectively, and lacks all checkpoint function, but is proficient for Rad53's checkpoint-independent role in DNA replication[30]. (See Supplemental methods) **b,** Growth curves of wild-type or mutant strains grown asynchronously in the presence of 0 mM, 12.5 mM, 25 mM, or 50 mM HU for three hours at 30^{o}C. For each concentration, ~200 cells were plated and grown for 3 days at 30^{o}C. **c,** Immunoblots from asynchronous cells grown in the presence or absence of 0.05% MMS for 90 minutes.

Supplemental Figure 10, 11, 12. **Rad52-GFP Foci.** Representative cells from *in vivo* fluorescent microscopy of Rad52-GFP foci in wild-type (Supplemental Fig. 10), *SLD3-m25 dbf4-m25* (Supplemental Fig. 11) and *rad53*Δ (Supplemental Fig. 12) strains arrest in G1 with α-factor and released into 200 mM HU for 2 hours.

Supplemental Table S2

strain	genotype	source
yJLO18	*SLD3-3Flag::HygroR his3Δ1 leu2Δ0 met15Δ0 ura3Δ*	this study
yJLO23	*pGAL1-10-URA3-SLD3-3Flag::HygroR his3Δ1 leu2Δ0 met15Δ0 ura3Δ*	this study
yJLO20	*SLD3-3Flag::HygroR rad53Δ::KanMx sml1Δ::LEU2his3Δ1 leu2Δ0 met15Δ0 ura3Δ*	this study
yNLM133	*SLD3-m25-3Flag::HygroR his3Δ1 leu2Δ0 met15Δ0 ura3Δ*	this study
yJLO92	*mcm5-bob1 his3Δ1 leu2Δ0 met15Δ0 ura3Δ*	this study
yJLO110	*SLD3/SLD3-m25-3Flag::HygroR DBF4/dbf4Δ::KanMx MCM5/mcm5-bob1*	this study
yJLO119	*SLD3/SLD3-m25-3Flag::HygroR DBF4/dbf4-m25::LEU2::dbf4Δ::KanMx MCM5/mcm5-bob1*	this study
yJLO123	*SLD3-m25-3Flag::HygroR dbf4-m25::LEU2::dbf4Δ::KanMx*	this study
yJLO124	*dbf4-m25::LEU2::dbf4Δ::KanMx*	this study
yJLO155	*Rad52-GFP::HIS3 SLD3-m25-3Flag::HygroR dbf4-m25::LEU2::dbf4Δ::KanMx his3Δ1 leu2Δ0 met15Δ0 ura3Δ*	this study
yJLO156	*Rad52-GFP::HIS3 SLD3-m25-3Flag::HygroR his3Δ1 leu2Δ0 met15Δ0 ura3Δ*	this study
yJLO157	*Rad52-GFP::HIS3 dbf4-m25::LEU2::dbf4Δ::KanMx his3Δ1 leu2Δ0 met15Δ0 ura3Δ*	this study
yJLO158	*Rad52-GFP::HIS3 rad53Δ::KanMx sml1Δ::LEU2 his3Δ1 leu2Δ0 met15Δ0 ura3Δ*	this study
yJLO159	*SLD3-m25-3Flag::HygroR dbf4-m25::LEU2::dbf4Δ::KanMx rad52Δ::KanMx his3Δ1 leu2Δ0 met15Δ0 ura3Δ*	this study
yJLO163	*SLD3-m25-3Flag::HygroR/SLD3-m25-3Flag::HygroR dbf4-m25::LEU2::dbf4Δ::KanMx/dbf4-m25::LEU2::dbf4Δ::KanMx*	this study
yJLO164	*SLD3-3xFlag::Hyg/SLD3-3xFlag::Hyg dbf4-25A::LEU2::dbf4Δ::KanMx/dbf4-25A::LEU2::dbf4Δ::KanMx*	this study
yJLO165	*SLD3-3xFLAG/SLD3-m25-3xFlag::HyG dbf4-25A::LEU2::dbf4Δ::KanMx/dbf4-25A::LEU2::dbf4Δ::KanMx*	this study
yJLO70	*HygroR::pTEF-sld3dpb11::DPB11 his3Δ1 leu2Δ0 met15Δ0 ura3Δ*	this study
yNLM91	*sld3-m4-3Flag::HygroR (set3: T451A S456A S463A S466)*	this study
yNLM93	*sld3-m5-3Flag::HygroR (set4: S493A T495A S497A S505A T507A*	this study
yNLM95	*sld3-m2-3Flag::HygroR (set5: T582A S591A)*	this study
yNLM97	*sld3-m4-3Flag::HygroR (set6: S639A T642A T646A)*	this study
yNLM99	*sld3-m7-3Flag::HygroR (set 4&5: S493A T495A S497A S505A T507A T582A S591A)*	this study
yNLM101	*sld3-m10-3Flag::HygroR (Set4-6: S493A T495A S497A S505A T507A T582A S591A S639A T642A T646A*	this study
yNLM107	*sld3-m18-3Flag::HygroR (set1-6: Y153F Y154F T370A T373A T451A S456A S463A S466A S493A T495A S497A S505A T507A T582A S591A S639A T642A T646A*	this study
yNLM147	*sld3-m20-3Flag::HygroR (Y153F Y154F T370A T373A T451A S456A S463A S466A S493A T495A S497A S505A T507A S521A S534A T582A S591A S639A T642A T646A)*	this study
yNLM133	*SLD3-m25-3Flag::HygroR (Y153F Y154F T370A T373A T451A S456A S463A S466A S493A T495A S497A S505A T507A S521A S534A T582A S591A T607A T609A S618A T631A T638A S639A T642A T646A)*	this study
yJLO180	*SLD3-3Flag::HygroR DUN1-TAP::HISMx*	this study
yJLO181	*SLD3-3Flag::HygroR rad53Δ::KanMx sml1Δ::LEU2 DUN1-TAP::HISMx*	this study
yJLO182	*SLD3-m25::HygroR dbf4-m25::LEU2::dbf4Δ::KanMx DUN1-TAP::HISMx*	this study
yJLO187	*SLD3-3Flag::HygroR DBF4-9Myc::TRP*	this study
yJLO188	*SLD3-m25-3Flag::HygroR dbf4-m25-9Myc::TRP*	this study
yJLO189	*SLD3-3Flag::HygroR DBF4-9Myc::TRP rad53-K227A*	this study
yJLO190	*sld3-m21D3FLAG::HygroR (T451D S456D S463D S466D S493D T495D S497D S505D T507D S521D S534D T582D S591D T607D T609D S618D T631D T638D S639D T642D T646D)*	this study

	SLD3-m25-3Flag::HygroR dbf4-m25::LEU2::dbf4Δ::KanMx	
yJLO192	*rad53Δ::KanMx sml1::LEU2*	this study
yJLO193	*CDC7-TAP::HIS3 SLD3-3Flag::HygroR DBF4-9Myc::TRP*	this study
yJLO194	*CDC7-TAP::HIS3 SLD3-3Flag::HygroR dbf4-m25-9Myc::TRP*	this study
yJLO260	*SLD3-m25-3Flag::HygroR dbf4-m25::LEU2 rad53Δ::HIS3 [rad53-R70A,R605A::URA3]*	this study
yJLO261	*SLD3-m25-3Flag::HygroR dbf4-m25::LEU2 rad53Δ::HIS3 [RAD53::URA3]*	this study
yJLO262	*SLD3-3Flag::HygroR DBF4 rad53Δ::HIS3 [rad53-R70A,R605A::URA3]*	this study
yJLO263	*SLD3-3Flag::HygroR DBF4 rad53Δ::HIS3 [RAD53::URA3]*	this study
yJLO264	*SLD3-m25-3Flag::HygroR DBF4 rad53Δ::HIS3 [rad53-R70A,R605A::URA3]*	this study
yJLO265	*SLD3-3Flag::HygroR dbf4-m25::LEU2 rad53Δ::HIS3 [rad53-R70A,R605A::URA3]*	this study
	rad52Δ::KanMx	Deletion Library
	pGal1-10-URA3-DBF4-Morf::URA3 [2μ]	Open Biosystems
	DPB11-TAP::HIS3Mx	TAP Library (Weissman&O'Shea)

Supplemental Table S3

Plasmid name	Relevant genotype
pJLO2	*SLD3-3Flag::HygroR (tagging construct)*
pJLO3	*sld3-3Flag::HygroR (set3: T451A S456A S463A S466A)*
pJLO4	*sld3-3Flag::HygroR (set4: S493A T495A S497A S505A T507A)*
pJLO5	*sld3-3Flag::HygroR (set6: S639A T642A T646A)*
pNM10	*sld3-m2::HygroR (set5: T582A S591A)*
pNM11	*sld3-m7::HygroR (set4&5: S493A T495A S497A S505A T507A T582A S591A)*
pNM12	*sld3-m10::HygroR (set4-6: S493A T495A S497A S505A T507A T582A S591A S639A T642A T646A)*
pNM13	*sld3-m14::HygroR (set3-6: T451A S456A S463A S466A S493A T495A S497A S505A T507A T582A S591A S639A T642A T646A)*
pNM15	*sld3-m16::HygroR (set2-6: T370A T373A T451A S456A S463A S466A S493A T495A S497A S505A T507A T582A S591A S639A T642A T646A)*
pNM16	*sld3-m18::HygroR (set1-6: Y153F Y154F T370A T373A T451A S456A S463A S466A S493A T495A S497A S505A T507A T582A S591A S639A T642A T646A)*
pNM19	*sld3-m21::HygroR (Y153F Y154F T370A T373A T451A S456A S463A S466A S493A T495A S497A S505A T507A T582A S591A T607A T609A S618A S639A T642A T646A)*
pNM21	*sld3-m20::HygroR (Y153F Y154F T370A T373A T451A S456A S463A S466A S493A T495A S497A S505A T507A S521A S534A T582A S591A S639A T642A T646A)*
pNM22	*sld3-m23::HygroR (Y153F Y154F T370A T373A T451A S456A S463A S466A S493A T495A S497A S505A T507A T582A S591A T607A T609A S618A T631A T638A S639A T642A T646A)*
pNM23	*SLD3-m25::HygroR (Y153F Y154F T370A T373A T451A S456A S463A S466A S493A T495A S497A S505A T507A S521A S534A T582A S591A T607A T609A S618A T631A T638A S639A T642A T646A)*
pNM25	*∆109N-Dbf4::LEU2 (pRS305)*
pJLO12	*DBF4::LEU2 (pRS305)*
pJLO13	*dbf4-m2::LEU2 (T171A T175A)*
pNM28	*dbf4-m1::LEU2 (S84A)*
pNM29	*dbf4-m2::LEU2 (S84A S235A)*
pNM30	*dbf4-m3::LEU2 (S84A S235A T473A)*
pNM33	*dbf4-m4::LEU2 (S84A S235A S356A T473A)*
pJLO15	*dbf4-m6::LEU2 (S84A Y139F S235A S356A T473A S632A)*
pJLO16	*dbf4-m27::LEU2 (S3A T5A S11A T16A T18A S29A T30A T31A S40A T50A T51A S53A S59A S84A S92A T95A T105A T114A S124A T131A Y139F T171A T175A S235A S356A T473A S632A)SacII/BsaBI fragment from pUC57 subcloned into pJLO15 SacII/BsaBI*
pJLO17	*dbf4-m25::LEU2 (T5A T16A T18A S29A T30A T31A S40A T50A T51A S53A S59A S84A S92A T95A T105A T114A S124A T131A Y139F T171A T175A S235A S356A T473A S632A)*
pJLO28	*GST-Sld3-3Flag pDEST15*
pJLO29	*GST-Sld3-m25-3Flag pDEST15*
pJLO31	*GST-dbf4-6xHis pDEST15*
pJLO32	*GST-dbf4-m25-6xHis pDEST15*
pJL033	*sld3-m21D-3Flag::HygroR (T451D S456D S463D S466D S493D T495D S497D S505D T507D S521D S534D T582D S591D T607D T609D S618D T631D T638D S639D T642D T646D)*
pGal-HO	*pGal-HO::URA3 (cen)*
rad53-FHA1FHA2	*rad53 R70A R605A:URA3 (cen)*