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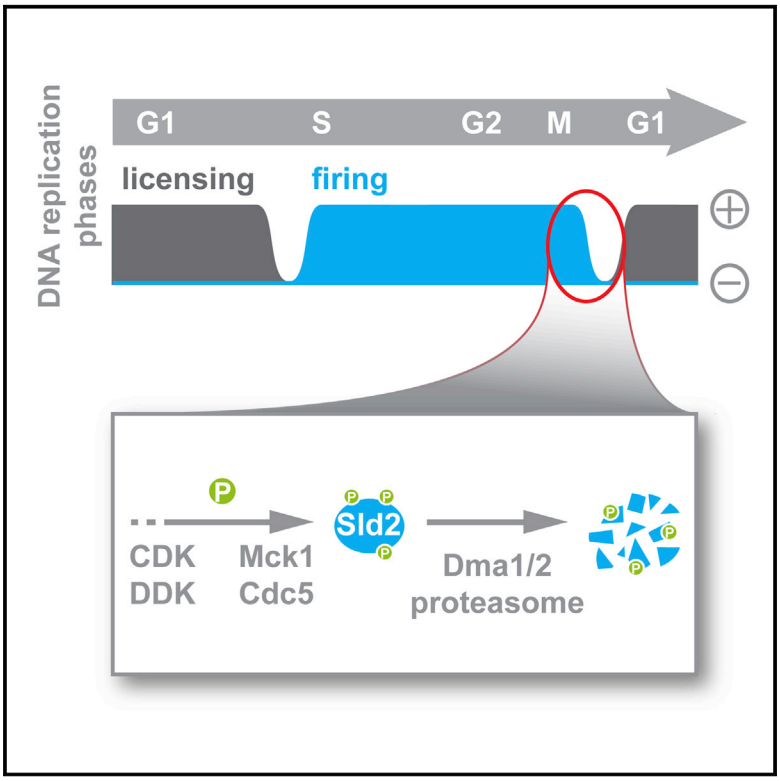
Robust Replication Control Is Generated by Temporal Gaps between Licensing and Firing Phases and Depends on Degradation of Firing Factor Sld2

Graphical Abstract



Authors

Karl-Uwe Reusswig, Fabian Zimmermann, Lorenzo Galanti, Boris Pfander

Correspondence

bpfander@biochem.mpg.de

In Brief

Reusswig et al. reveal that activation and inactivation of replication factors during the cell cycle underlies a temporal order that includes gaps/buffer zones between the two replication initiation phases (licensing and firing). They find that cell-cycle-regulated degradation of Sld2 controls the gap in mitosis and thereby contributes to genome stability.

Highlights

- Temporal gaps separate origin licensing and firing phases during the cell cycle
- In mitosis, the firing factor Sld2 is inactivated by rapid degradation
- Dma1/2 mediate rapid Sld2 degradation by recognizing a complex phospho-degron
- Stable Sld2 variants impede robust separation of firing and licensing in mitosis

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Robust Replication Control Is Generated by Temporal Gaps between Licensing and Firing Phases and Depends on Degradation of Firing Factor Sld2

Karl-Uwe Reusswig,[1] Fabian Zimmermann,[1] Lorenzo Galanti,[1] and Boris Pfander[1,2,*]
[1]Max Planck Institute of Biochemistry, DNA Replication and Genome Integrity, 82152 Martinsried, Germany
[2]Lead Contact
*Correspondence: bpfander@biochem.mpg.de
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SUMMARY

Temporal separation of DNA replication initiation into licensing and firing phases ensures the precise duplication of the genome during each cell cycle. Cyclin-dependent kinase (CDK) is known to generate this separation by activating firing factors and at the same time inhibiting licensing factors but may not be sufficient to ensure robust separation at transitions between both phases. Here, we show that a temporal gap separates the inactivation of firing factors from the re-activation of licensing factors during mitosis in budding yeast. We find that gap size critically depends on phosphorylation-dependent degradation of the firing factor Sld2 mediated by CDK, DDK, Mck1, and Cdc5 kinases and the ubiquitin-ligases Dma1/2. Stable mutants of Sld2 minimize the gap and cause increased genome instability in an origin-dependent manner when combined with deregulation of other replication regulators or checkpoint mechanisms. Robust separation of licensing and firing phases therefore appears indispensable to safeguard genome stability.

INTRODUCTION

To maintain a stable genome, eukaryotic cells tightly couple DNA replication to cell division but furthermore must ensure that each part of the genome is duplicated exactly once during each cell cycle. Particularly, as eukaryotic cells replicate their genome from multiple autonomous replication origins, this control has to be implemented at the level of each individual replication origin. Eukaryotic cells have therefore evolved a replication control, which temporally separates replication initiation at each individual replication origin into two phases called licensing and firing (reviewed in Blow and Dutta, 2005; Siddiqui et al., 2013; Tanaka and Araki, 2010).

Two principal aspects of replication control ensure exact duplication of the genome. First, licensing and firing are interdependent, as origins can only fire after they have been licensed and conversely firing or passive replication of origins removes their license. Second, licensing and firing are mutually exclusive, as both reactions are coupled to specific cell-cycle stages by the oscillating activity of cyclin-dependent kinases (CDKs), which promotes firing and at the same time inhibits licensing (Siddiqui et al., 2013; Tanaka and Araki, 2010; Zegerman, 2015). Licensing can occur from late mitosis to the end of G1 (Diffley et al., 1994; Seki and Diffley, 2000) and mechanistically corresponds to the loading of the replicative DNA helicase Mcm2–7 at replication origins as inactive precursors (Evrin et al., 2009; Remus et al., 2009). In contrast, firing can occur from the start of S phase until late mitosis (Heller et al., 2011) and mechanistically corresponds to the activation of the Mcm2–7 precursors by the association of the additional Cdc45 and GINS subunits and the subsequent formation of replisomes (Gambus et al., 2006; Gros et al., 2014; Heller et al., 2011; Muramatsu et al., 2010; On et al., 2014; Yeeles et al., 2015). Specific sets of licensing and firing factors mediate both the licensing and the firing reaction (see below) and are thus differentially regulated during the cell cycle (Drury et al., 2000; Elsasser et al., 1999; Labib et al., 1999; Nguyen et al., 2000, 2001; Tanaka et al., 2007; Zegerman and Diffley, 2007).

The licensing reaction depends on the origin recognition complex (ORC), Cdc6, Mcm2–7, and Cdt1, which together form pre-replicative complexes (pre-RCs) at replication origins (Cocker et al., 1996; Evrin et al., 2009; Remus et al., 2009). In budding yeast, all licensing factors are individually inhibited through phosphorylation by CDK, thereby restricting the licensing reaction to late mitosis and G1. CDK phosphorylation of the soluble Mcm2–7-Cdt1 complex leads to its nuclear export, CDK phosphorylation of ORC leads to its inhibition, and CDK phosphorylation of Cdc6 triggers its degradation (Chen and Bell, 2011; Drury et al., 2000; Elsasser et al., 1999; Labib et al., 1999; Liku et al., 2005; Nguyen et al., 2000, 2001; Tanaka and Diffley, 2002). Additionally, CDK inhibits ORC and Cdc6 by direct binding (Mimura et al., 2004; Wilmes et al., 2004).

The firing reaction depends on the specific firing factors Sld2, Sld3, and Dpb11 (Kamimura et al., 1998, 2001; Masumoto et al., 2000, 2002; Tanaka et al., 2007; Zegerman and Diffley, 2007). CDK facilitates firing by phosphorylating Sld2 and Sld3 and thereby enabling these proteins to interact with Dpb11 (Kamimura et al., 1998; Masumoto et al., 2002; Tanaka et al., 2007; Zegerman and Diffley, 2007). This complex appears to cause activation of the Mcm2–7 helicase by promoting the association of the additional subunits Cdc45 and GINS (Gambus et al., 2006;

Heller et al., 2011; Moyer et al., 2006; Muramatsu et al., 2010; Yeeles et al., 2015). Furthermore, firing also requires the Dbf4-dependent kinase (DDK), which phosphorylates the Mcm2–7 helicase to alleviate an inhibitory mechanism within the Mcm2–7 complex (Bousset and Diffley, 1998; Donaldson et al., 1998; Sheu and Stillman, 2010).

Previous studies have investigated the consequences of replication initiation deregulation. First, uncoupling of the licensing reaction from its cell-cycle regulation was achieved in budding yeast by combination of CDK-inhibition-resistant versions of the licensing factors ORC, Cdc6, and Mcm2-7 (Nguyen et al., 2001). Deregulation of two or more of the licensing factors induces re-licensing, over-replication, gene amplification, and cell death. Moreover, these conditions also lead to the occurrence of DNA damage, presumably by breakdown of replication forks during over-replication (Green et al., 2006, 2010; Green and Li, 2005; Nguyen et al., 2001; Tanny et al., 2006). These findings therefore suggest that over-replication induces genome instability by at least two mechanisms: DNA damage and copy-number increase. Second, deregulated origin firing was induced by uncoupling the firing factors Sld2 and Sld3 from CDK control together with a bypass of DDK control and resulted in cell death as well (Tanaka et al., 2007; Zegerman and Diffley, 2007). Taken together, these findings strongly suggest that mutual exclusivity of licensing and firing reactions is an essential feature of eukaryotic DNA replication.

Another important feature of the replication control system appears to be robustness, providing the system with a high tolerance to perturbations (Diffley, 2011). Robustness can be seen, for example, in the fact that CDK promotes several overlapping mechanisms to regulate licensing and firing and has several targets in both reactions (Nguyen et al., 2001; Tanaka et al., 2007; Zegerman and Diffley, 2007). Importantly, if single licensing or firing factors are uncoupled from CDK regulation, no overt over-replication is observed over a cell population, but chromosomal rearrangements still occur with enhanced rates (Tanaka and Araki, 2011). This suggests that under conditions where replication control is less robust but apparently still functional in most cells, sporadic over-replication can occur in a subset of cells and lead to genome instability.

It is unknown, how cells ensure a robust separation of the origin licensing and firing phase at the cell-cycle transitions when CDK is turned on or off and whether special mechanisms, in addition to the general CDK regulation of replication initiation, are required to achieve this separation.

Here, we demonstrate that a temporal gap separates firing inactivation and licensing activation at the firing-to-licensing transition during mitosis, as CDK phosphorylation marks on firing factors disappear before CDK phosphorylation marks on licensing factors. Strikingly, the regulation of the firing factor Sld2 is a critical determinant of the gap size, as CDK phosphorylation marks on Sld2 disappear before the marks on other replication factors. Our study reveals that the early inactivation of Sld2 is mediated by mitotic degradation of Sld2 by the ubiquitin-proteasome system. This degradation depends on a complex phosphorylation-dependent degradation motif (phospho-degron) on Sld2, which is phosphorylated by the four cell-cycle kinases CDK, DDK, Mck1, and Cdc5 and recognized by the FHA-domain-containing ubiquitin ligases Dma1/2. Stable variants of Sld2 fully support origin firing but reduce the temporal gap between firing inactivation and licensing activation. Importantly, stable variants of Sld2 increase the occurrence of chromosomal rearrangements in the context of different replication initiation or checkpoint mutants. These findings are consistent with a model whereby a robust separation of firing and licensing phases at cell-cycle transitions is necessary to avoid sporadic over-replication as a source of genome instability.

RESULTS

Licensing and Firing Phases Are Separated by Temporal Gaps in G1 and Mitosis

Previous studies collectively showed that the CDK phosphorylation status of licensing and firing factors allows to predict the phase of replication initiation (Heller et al., 2011; Liku et al., 2005; Masumoto et al., 2002; Nguyen et al., 2001; Tanaka et al., 2007; Yeeles et al., 2015; Zegerman and Diffley, 2007). The current model of the replication control system proposes a strict separation of licensing and firing phases. However, it is unclear whether specialized mechanisms exist to ensure a robust separation at transitions between both phases. To address this question, we have developed an experimental system, which allows us to measure in a single yeast strain the CDK-dependent phosphorylation of the firing factors Sld2 and Sld3 as well as the CDK-dependent phosphorylation of the licensing factors Orc6, Mcm3, and Cdc6 (Figure S1A).

Using this system, we monitored protein phosphorylation/dephosphorylation at both the G1-S transition and the mitotic transition. In the case of the G1-S transition, it has been suggested that licensing inactivation and firing activation may occur at different times, as some licensing factors (Cdc6 and Mcm2–7) are targeted by G1-Cln-CDK (CDK in complex with G1 cyclins), whereas the firing factors Sld2 and Sld3 are targeted by S-Clb-CDK (CDK in complex with S phase cyclins) (Brümmer et al., 2010; Drury et al., 2000; Labib et al., 1999; Tanaka et al., 2007; Zegerman and Diffley, 2007). Our data support this model, as cells that are blocked at the G1-S transition by overexpression of a non-destructible version of the Clb-CDK inhibitor Sic1 display hyper-phosphorylation of Mcm3 and low levels of Cdc6, while at the same time, the firing factors Sld2 and Sld3 are in a hypo-phosphorylated state (Figure S1B). Furthermore, when we artificially delayed phosphorylation of S-Clb-CDK-specific substrates by deletion of the S phase cyclins Clb5 and Clb6, we observed a temporal gap between licensing inactivation and firing activation, as hyper-phosphorylation of Mcm3 and a drop in Cdc6 levels occurred 15–20 min earlier than hyper-phosphorylation of the firing factors Sld2 and Sld3 (Figure S1C). We therefore conclude that licensing and firing phases are separated by a temporal gap at the G1-S transition.

Next, we tested whether a similar temporal gap would separate firing and licensing phases at the mitotic transition. We therefore measured the phosphorylation status of licensing and firing factors in a synchronous cell cycle following either a G1 release (Figures 1A–1C and S1D) or an S release (using hydroxyurea; Figures 1D–1F and S1E). Quantification of hyper- and

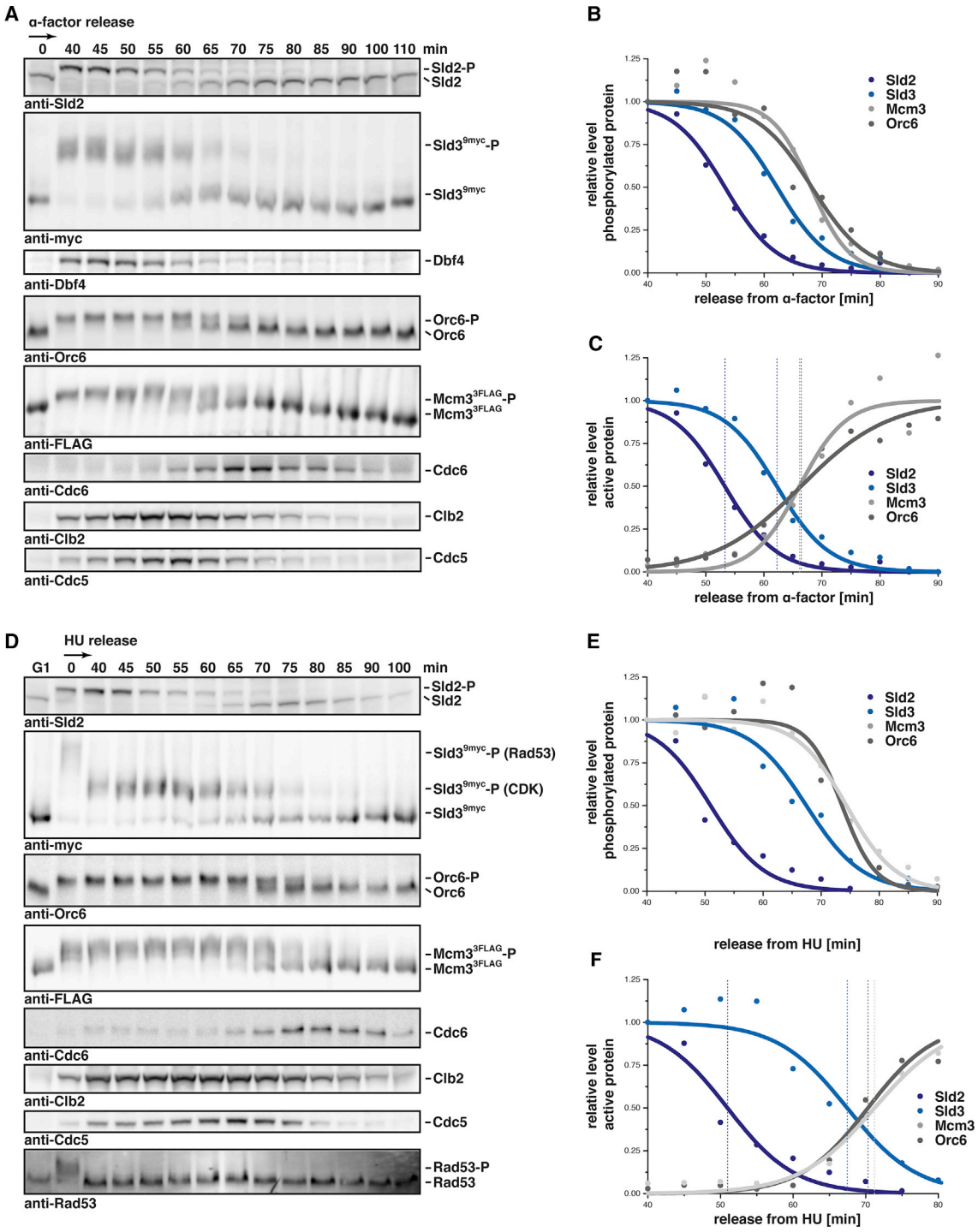

Figure 1. A Temporal Gap Separates the Inactivation of Firing Factors from the Re-activation of Licensing Factors in Mitosis

(A and D) Western-blot- and Phostag-based visualization of CDK phosphorylation (see Figure S1A) of licensing (Orc6, Mcm3, and Cdc6) and firing factors (Sld2 and Sld3) after (A) α-factor arrest/release or (D) hydroxyurea (HU) arrest/release. Clb2, Cdc5, Dbf4, and Rad53 serve as markers of cell-cycle progression and checkpoint activity (see Figures S1D and S1E for DNA content measurements by flow cytometry).

(B and E) Quantification of CDK-phosphorylated species of Sld2, Sld3, Orc6, and Mcm3 as in (A) and (D). Data were approximated with a logistic function (two parameters, $f(t) = 1/(1 + \exp(-k(t - t_{1/2})))$.

(C and F) Quantification of active (hypo-phosphorylated Mcm3 and Orc6, hyper-phosphorylated Sld2 and Sld3) protein species in (A) and (D) as in (B) and (E). Dotted lines indicate the time points when the active form of the respective replication factor reached 50%.

See also Figure S1.

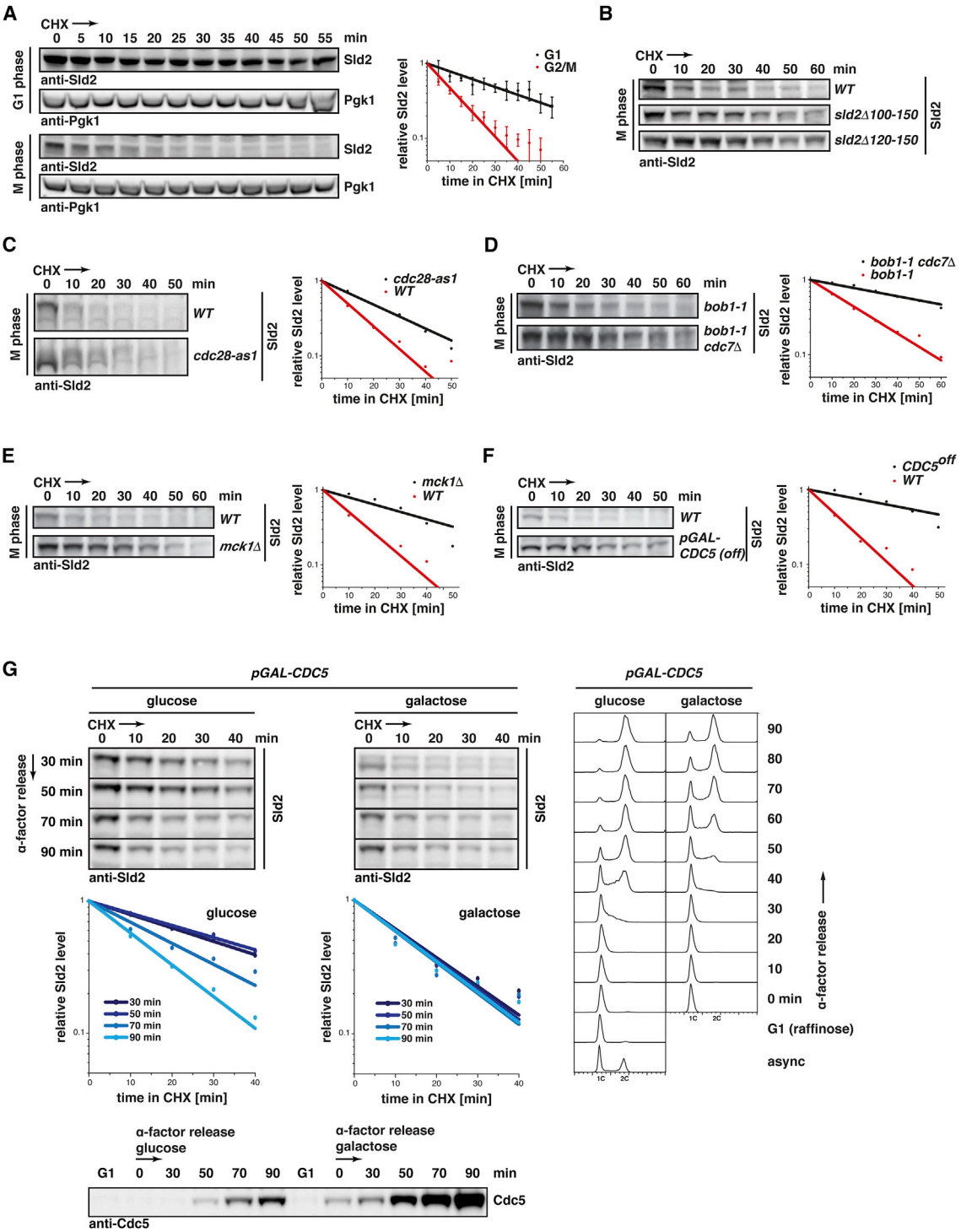

Figure 2. The Firing Factor Sld2 Is Rapidly Degraded in a Cell-Cycle-Regulated and CDK-, DDK-, Mck1-, and Cdc5-Dependent Manner

(A) Measurement of Sld2 stability using protein translation shutoff with cycloheximide (CHX) in cells arrested in G1 (α-factor) or M (nocodazole). Quantification of protein levels (mean ± SD) from two or three independent experiments is shown in the right inset. Larger cutouts of the gels are shown in Figure S2I.

(B) Stability of Sld2 and Sld2 truncations (*sld2Δ100–150* and *sld2Δ120–150*) was measured as in (A).

(C–F) Influence of cell-cycle kinases on Sld2 degradation in CHX shutoff experiments as in (A). (C) CDK (Cdc28) was inhibited in mitotically arrested cells using *cdc28-as1* and 1-NM-PP1. (D) *CDC7* was deleted in a *bob1-1* background. (E) *MCK1* was deleted. (F) Cdc5 was inactivated by transcription shutoff using *pGALL-CDC5* and glucose addition in G1 prior to release to a mitotic arrest.

(legend continued on next page)

hypo-phosphorylated protein species allowed us to observe that CDK-phosphorylated protein species of the firing factors Sld2 and Sld3 disappeared earlier compared to CDK-phosphorylated protein species of the licensing factors (Figures 1B and 1E). Conversely, the active versions of the firing factors disappeared before the active versions of the licensing factors re-appeared (Figures 1C and 1F). These experiments therefore suggest that also in mitosis licensing and firing phases are separated by a gap.

While CDK phosphorylation on Sld3 disappeared 5 min before CDK phosphorylation on licensing factors, hyper-phosphorylated Sld2 disappeared even earlier (15–20 min before Orc6), and we therefore investigated the underlying mechanism. Sld2 as well as some licensing factors have been shown to be substrates of the mitotic phosphatase Cdc14 (Bloom and Cross, 2007; Zhai et al., 2010). It is therefore possible that different affinities of Cdc14 for firing and licensing factors may generate the observed temporal order of substrate dephosphorylation in mitosis (Bouchoux and Uhlmann, 2011). However, we observed that even at the restrictive conditions of the *cdc14-3* mutant hyper-phosphorylated Sld2 disappeared quickly (within 3–5 min), while hyper-phosphorylated Orc6 was stabilized (Figure S1I). We therefore conclude that additional mechanisms must exist that lead to the disappearance of hyper-phosphorylated Sld2.

Notably, in our release experiments (Figures 1C and 1F), we observed a decrease in the steady-state levels of Sld2 at 45–55 min after release (Figures S1F and S1G). This timing coincided with mitotic onset (indicated by the rise of Cdc5 levels; Figures 1A and 1D) and with the disappearance of hyper-phosphorylated Sld2, indicating that hyper-phosphorylated Sld2 may be a target of cell-cycle-specific degradation.

Rapid Sld2 Degradation Depends on Cell-Cycle Kinases and Is Restricted to Mitosis by Cdc5

To investigate if the drop in Sld2 levels in mitosis is due to cell-cycle-specific degradation, we performed translation shutoff experiments by adding cycloheximide (CHX) to cells that were arrested either in G1 by α-factor or in mitosis by nocodazole. Strikingly, Sld2 was rapidly degraded in mitosis (half-life of <10 min), whereas Sld2 was relatively stable in G1 cells (half-life of 40 min; Figure 2A). Furthermore, Sld2 was found to be unstable in mitosis but stable in G1 and S, when CHX was added at different times to cells synchronously progressing through the cell cycle after G1 arrest/release (Figure S2A).

The mitotic degradation of Sld2 was dependent on the ubiquitin-proteasome system, as the proteasome mutant *cim3-1* increased Sld2 steady-state levels and allowed that ubiquitylated species of Sld2 could be detected (Figure S2B). Using truncations and internal deletions, we found that a region close to the N terminus of Sld2 (aa102–152) was necessary for the mitotic degradation (Figure S2C). In particular *sld2Δ120–150*, a mutant lacking 30 amino acids, was stable in mitosis (Figure 2B). This part of the protein contains several phosphorylation sites (Swaney et al., 2013), suggesting that aa120–150 may contain a phospho-degron. Therefore, we tested whether specific candidate kinases may play a role in the degradation of Sld2. We compared Sld2 stability in wild-type (WT) cells with cells lacking specific kinase activities (Figures 2C–2F). These experiments revealed that CDK, DDK, the GSK3-related kinase Mck1, and the yeast polo-like kinase Cdc5 were all required for the rapid degradation of Sld2 in mitosis (Figures 2C–2F and S2D–S2G).

We therefore sought to determine whether any of these kinases would be responsible for restricting Sld2 degradation to mitosis. Given its role as central regulator of mitosis, Cdc5 was the prime candidate. We deregulated Cdc5 by cell-cycle-independent expression from a galactose-inducible promoter. Induction of Cdc5 in S phase (Figure 2G, right, "galactose") triggered fast degradation of Sld2, which in control cells (Figure 2G, left, "glucose") occurred only upon natural Cdc5 accumulation in mitosis (Figure 2G). In contrast, overexpression of Dbf4 did not induce premature degradation of Sld2 (Figure S2H). These data therefore suggest that Cdc5 accumulation restricts Sld2 degradation to mitosis.

Cell-Cycle Kinases Directly Target the Sld2 Degron

We aimed to ascertain whether the four cell cycle kinases would directly target Sld2 to trigger its degradation or indirectly contribute to the stability of Sld2. First, we tested in vitro whether these kinases would directly phosphorylate Sld2 using a purified truncated version (^{GST}Sld2$^{1–150}$; see Figure S3A and Supplemental Experimental Procedures for detailed information). Sld2 is a well-known CDK target (Loog and Morgan, 2005; Masumoto et al., 2002; Tak et al., 2006), and consistently, we observed that a model CDK was able to phosphorylate Sld2$^{1–150}$ (Figure 3A). Similarly, we observed that Sld2$^{1–150}$ was a direct Cdc5 substrate (Figure 3A). Strikingly, we found that neither Mck1 nor DDK was able to substantially phosphorylate unmodified Sld2$^{1–150}$. However, both kinases were able to target Sld2$^{1–150}$ after it had been phosphorylated by CDK in a priming step (Figure 3B). This priming effect was restricted to CDK, since we did not observe any strong positive or negative effect in all other combinations of priming and downstream kinases (Figures S3B–S3D). Therefore, these experiments suggest first that all four kinases phosphorylate a non-overlapping set of phosphorylation sites of Sld2$^{1–150}$ and second that CDK acts as a priming kinase for Mck1 and DDK phosphorylation of Sld2.

Furthermore, we analyzed the in vitro kinase reactions by mass spectrometry. Here, we could detect phosphorylated Sld2 sites in the degron region when Sld2$^{1–150}$ was incubated with CDK or Cdc5, but not with DDK or Mck1 alone (Figure 3C), consistent with the radioactive assay. For CDK, we observed phosphorylation of two SSP motifs (S128 and S138); for Cdc5, we observed phosphorylation of T122 and T143 (Figure 3C).

Mass-spectrometric analysis did not allow a clear assignment of DDK and Mck1 target sites after priming phosphorylation with CDK, possibly due to complex multiply phosphorylated

(G) Analysis of Sld2 stability in cells overexpressing *CDC5* from a galactose-inducible promoter. Cdc5 expression was induced for 2 hr before G1 release, and CHX was added at indicated time points after release. Right: flow cytometric measurements of DNA content. Middle left: quantifications of the Sld2 levels are shown below the Sld2 western blots. Bottom: Cdc5 levels before CHX addition.
See also Figure S2.

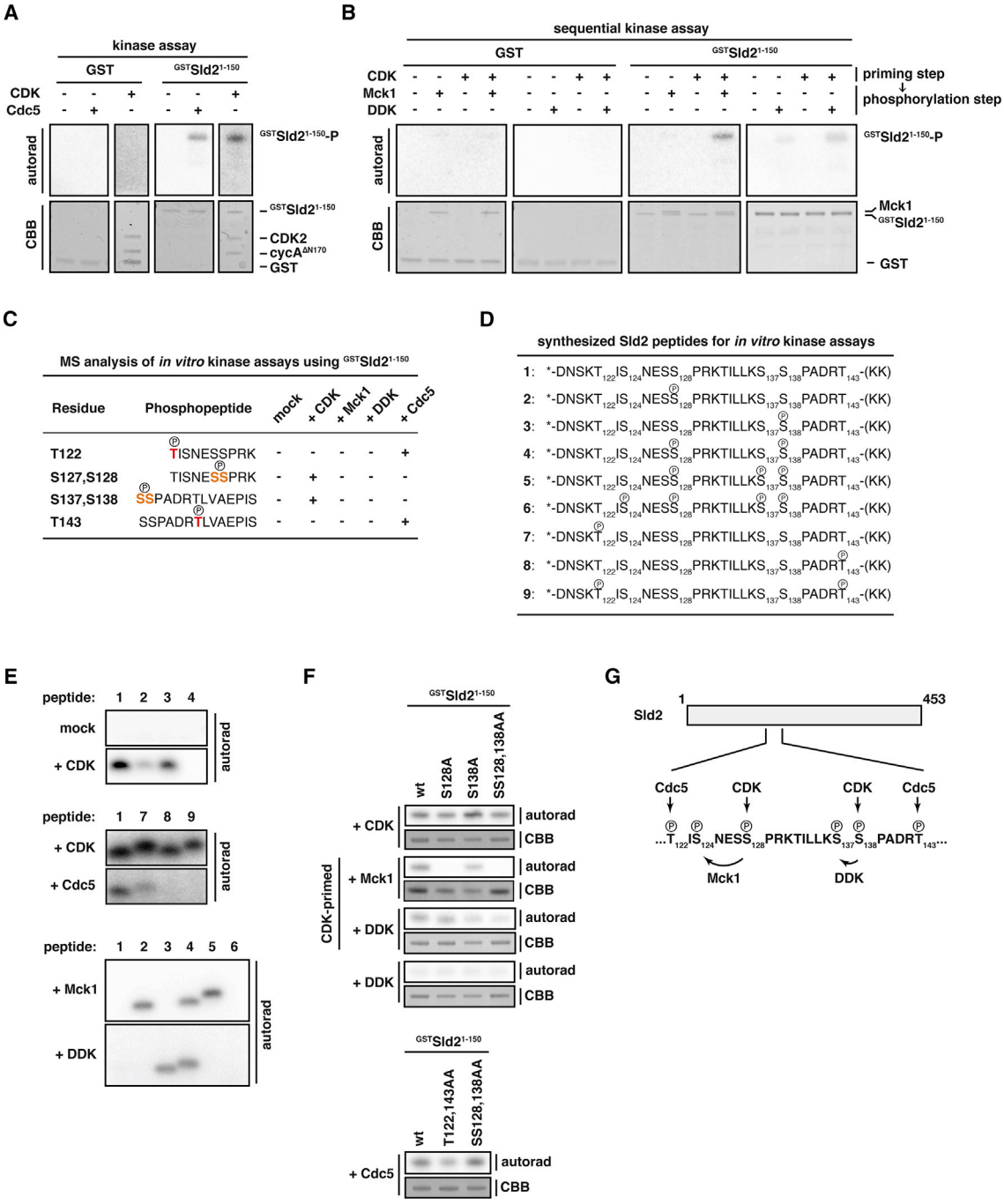

Figure 3. Purified CDK, DDK, Mck1, and Cdc5 Phosphorylate Sld2 Directly and within the Degron Sequence

(A) $^{GST}Sld2^{1–150}$ or GST were incubated with CDK2/cycA$^{\Delta N170}$ (CDK), Cdc5 or mock treated in the presence of [γ^{32}P]-ATP.

(B) $^{GST}Sld2^{1–150}$ was phosphorylated with CDK and non-radioactive ATP in a priming step before treatment with either Mck1 or DDK in a separate reaction in the presence of [γ^{32}P]-ATP.

(C) Mass spectrometric identification of in vitro phosphorylation sites in the Sld2 degron sequence. Color-coding indicates phosphate localization probability (red, ≥0.98; orange, 0.5). S128 and S138 match the (minimal) CDK consensus (S/TP), phospho-S128 generates a putative Mck1 consensus phosphorylation site at S124 (S-XXX-pS), and phospho-S138 generates a DDK consensus site at S137 (S-pS).

(D) Desthiobiotin-labeled synthetic Sld2 peptides reconstituting differentially phosphorylated states of Sld2$^{118–143}$.

(E) Sld2 peptides (numbers as in (D)) were incubated with the indicated kinases or mock treated in the presence of [γ^{32}P]-ATP.

(F) $^{GST}Sld2^{1–150}$ and phospho-mutant variants were treated in single-step or sequential phosphorylation reactions as in (A) and (B).

(G) Summary of the identified phosphorylation sites in the Sld2 degron and their kinase dependencies.

See also Figure S3.

peptides. To circumvent these limitations, we conducted kinase assays with synthetic peptides spanning the Sld2 degron sequence. Specifically, we used an unphosphorylated 26-mer peptide covering all putative phosphorylation sites of the Sld2 degron as well as phosphorylated derivatives (Figure 3D). In this assay, CDK and Cdc5 were able to target the unphosphorylated Sld2 degron peptide, while Mck1 and DDK again strictly required priming phosphorylation (Figures 3E and S3E). Moreover, when we blocked putative CDK- and Cdc5-phosphorylation sites in the synthetic phospho-peptides (phospho-S128 and phospho-S138 for CDK, phospho-T122 and phospho-T143 for Cdc5), we observed reduced or no phosphorylation by the corresponding kinase, confirming S128 and S138 as target sites for CDK and T122 and T143 as target sites for Cdc5 (Figure 3E). Notably, using synthetic phospho-peptides we were also able to identify the priming sites for Mck1 and DDK. We observed that a phospho-S128 peptide was efficiently phosphorylated by Mck1 and that a phospho-S138 peptide was phosphorylated by DDK (Figure 3E), suggesting that CDK-phosphorylation of two sites in the degron (S128 and S138) will differentially act as priming factor for Mck1 and DDK phosphorylation. We were then able to map S124 as Mck1 target site and S137 as DDK target site (Figure 3E) by introducing additional phospho-residues.

Lastly, using mutant versions of $Sld2^{1-150}$ in in vitro kinase assays, we found that an S128A mutant, even after priming phosphorylation with CDK, was a poor substrate for Mck1, and conversely, an S138A mutant was a poor DDK substrate (Figure 3F). In case of Cdc5, we observed that a TT122,143AA double mutant showed reduced phosphorylation (Figure 3F), while CDK could still efficiently phosphorylate the SS128,138AA double mutant (Figure 3F), consistent with previous results showing multiple CDK target sites in the N terminus of Sld2 in addition to S128 and S138 (Masumoto et al., 2002; Tak et al., 2006).

Next, we employed quantitative mass spectrometry to provide evidence for the phosphorylation of these sites in vivo. Using a mutant condition that inhibited Sld2 degradation (see below), we found that the Sld2 degron sequence of endogenous Sld2 is phosphorylated in mitosis (Table S1). Particularly, when using increased expression of Sld2, we observed higher-order phosphorylated peptides containing phosphorylated T122, S124, S128, S137, S138, and T143 (Tables S1 and S2; Data S1). These data thus indicate a concerted action of the kinases targeting the Sld2 degron. Importantly, label-free quantification of Sld2 phosphorylation from cells arrested in G1 or mitosis revealed that all Sld2 degron sites were cell-cycle controlled and upregulated in mitosis (Figure S3F).

Overall, our data suggest that four kinases target the Sld2 degron at six sites in a stepwise reaction with CDK priming for phosphorylation by Mck1 and DDK (see Figure 3G for a summary).

Dma1 and Dma2 Recognize the Sld2 Phospho-degron and Mediate Rapid Sld2 Degradation

Our finding that the Sld2 degron is multiply phosphorylated raised the question whether this phosphorylation signal is directly recognized by an E3 ubiquitin ligase. While testing candidate E3s (known mitotic regulators or enzymes with phospho-protein binding propensity) for their ability to mediate rapid degradation of Sld2 in mitosis, we observed stabilization of mitotic Sld2 in a translation shutoff experiment when we introduced deletions of *DMA1* and *DMA2* (Figure 4A). Already the *dma1Δ* single mutant showed moderate stabilization of Sld2, whereas the *dma2Δ* single mutant showed little effect on its own (Figure 4A). Importantly, in the *dma1Δ dma2Δ* double-mutant mitotic Sld2 became even further stabilized and to a degree, which is similar to Sld2 outside of mitosis (Figure 4A; half-life of 50 min). These results therefore suggest that Dma1 and Dma2 have overlapping functions in regulating Sld2 levels, consistent with the high sequence similarity of the two paralogs.

Dma1 and Dma2 both harbor a forkhead-associated (FHA) domain, a known phospho-protein binding domain (Durocher et al., 2000). We therefore tested whether Dma1 and Dma2 could act as readers of the Sld2 phospho-degron in a modified two-hybrid system, where Sld2 degradation is prevented by deletion of endogenous *DMA1* and *DMA2* and additional RING-finger mutations in the Gal4-AD-Dma1/2 two-hybrid constructs (CH345,350SA in Dma1 and CH451,456SA in Dma2). Using this system, we indeed observed a robust interaction between the RING-finger mutants of Dma1 and Dma2 and LexA-BD-Sld2 (Figures 4B, S4A, and S4B), while we only detected a weak interaction between Sld2 and WT Dma1 (Figure 4B). This experiment not only provides evidence for a physical interaction between Sld2 and Dma1/2 but also suggests that RING finger domains of Dma1 and Dma2 are involved in degradation of Sld2.

Next, we tested whether Dma1 binding to Sld2 was dependent on phosphorylation of the Sld2 phospho-degron. Different versions of Sld2 (SS128,138AA [2SA] lacking the CDK sites, TT122,143AA [2TA] lacking the Cdc5 sites, a 4A mutant lacking all CDK and Cdc5 sites, and a 6A mutant lacking all characterized phospho-sites in the degron) all showed strongly reduced binding to Dma1 (Figures 4C and S4C). Furthermore, individual mutation of any of the six Sld2 phosphorylation sites within the degron caused a reduction in the two-hybrid interaction with Dma1 (Figures S4D and S4E). From these results, we thus infer that Dma1 acts as a reader of the Sld2 phospho-degron and that all phosphorylation sites of the Sld2 phospho-degron contribute to this interaction in vivo.

We also tested whether the FHA domain of Dma1 was required for Sld2 binding and introduced previously characterized FHA mutations into the Dma1 construct (G192E and SH220,223AL [Bieganowski et al., 2004; Loring et al., 2008]). Both FHA mutants of Dma1 showed strongly reduced interaction with Sld2 (Figures 4D and S4F), suggesting that the FHA domain contributes to Sld2 binding, potentially by binding to phosphorylated residues in the phospho-degron.

Moreover, we assessed whether Dma1 bound directly and in a phosphorylation-dependent manner to the Sld2 degron. To this end, we used three different assays to test whether recombinant, purified Dma1 or an N-terminal fragment containing the FHA domain could bind to synthetic Sld2 degron phospho-peptides (Figures 4E, 4F, and S4G). Fluorescence anisotropy and microscale thermophoresis measurements suggested that T143-phosphorylated peptides can bind to a FHA fragment with a $K_d < 10\mu M$, while we did not observe binding with peptides that lack T143 phosphorylation (Figures 4E and 4F). Peptide

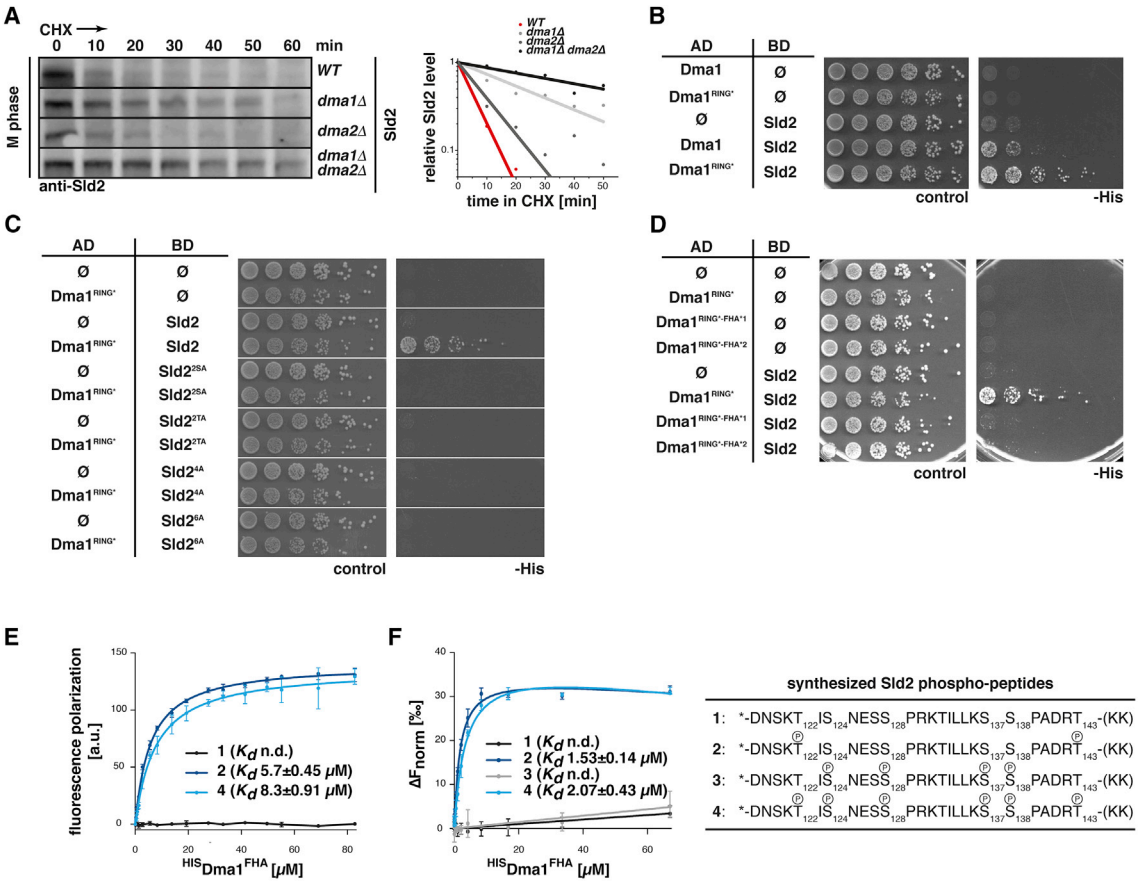

Figure 4. Dma1/2 Ubiquitin Ligases Recognize the Phospho-degron of Sld2 and Facilitate Sld2 Degradation during Mitosis

(A) Sld2 protein stability in WT, *dma1Δ*, *dma2Δ*, and *dma1Δ dma2Δ* cells arrested in mitosis. Left: anti-Sld2 western blots. Right: quantification of Sld2 protein levels.

(B–D) Two-hybrid interaction between Sld2 and Dma1. Cells are expressing lexA-BD and lexA-BD-Sld2 and derivative phosphorylation-site mutant constructs (C) as well as Gal4-AD, Gal4-AD-Dma1, Gal4-AD-Dma1-CH345,350SA ($Dma1^{RING^*}$), Gal4-AD-Dma1-GCH192,345,350ESA ($Dma1^{RING^*FHA^*1}$), and Gal4-AD-Dma1-SHCH220,223,345,350ALSA ($Dma1^{RING^*FHA^*2}$) (D) constructs.

(E and F) Fluorescence anisotropy (E) and microscale thermophoresis (F) measurements to determine binding affinities of His-$Dma1^{FHA}$ and fluorescein-labeled phospho-peptides of the Sld2 degron (see right inset in F for peptide sequences). Curves represent the mean of three independent experiments; error bars indicate SD.

See also Figure S4.

pull-downs using a purified FHA fragment or full-length glutathione S-transferase (GST)-Dma1 confirmed this specificity. Overall, all assays therefore show a crucial requirement of Cdc5-dependent phosphorylation at T143 for Dma1 binding, suggesting that T143 is the primary binding site (Figures 4E, 4F, and S4G) and consistent with the general view of FHA domains as phospho-threonine binding motifs (Mahajan et al., 2008; Byeon et al., 2005; Lee et al., 2008). While we observed a slight increase in Dma1-binding when using a peptide phosphorylated at T122, S124, S128, S137, S138, and T143 in the peptide pull-down, this effect was not observed in the biophysical binding assays (Figures S4G, 4E, and 4F). Therefore, it seems most likely that phosphorylation of the non-Cdc5 sites has an indirect influence on Dma1 binding in vivo (Figure S4D), perhaps by promoting T143 phosphorylation, for example via a conformational switch (Tak et al., 2006). Overall, these data provide strong evidence for a direct recognition of the Sld2 phospho-degron by Dma1/2, which likely triggers ubiquitylation and degradation.

Alterations in the Phospho-degron Stabilize Sld2 in Mitosis without Affecting Its Origin Firing Function

Since deletion of the degron (*sld2Δ120–150*) abolished the rapid degradation of Sld2 in mitosis, we tested whether mutation of phosphorylation sites within the degron would stabilize Sld2 as well. We used the Dma1-interaction-deficient variants *sld2-2SA* (*sld2-SS128,138AA*, deficient in degron phosphorylation by CDK), *sld2-2TA* (*sld2-TT122,143AA*, deficient in degron phosphorylation by Cdc5), *sld2-4A* (*sld2-TSST122,128,138,143AAAA*, deficient in degron phosphorylation by CDK and Cdc5), and *sld2-6A* (*sld2-TSSSST122,124,128,137,138,143AAAAAA* deficient in degron phosphorylation by all four kinases) (Figure 4C). Compared to WT Sld2, all four mutant versions of Sld2 were strongly stabilized in mitosis (Figure 5A). Additionally, we observed

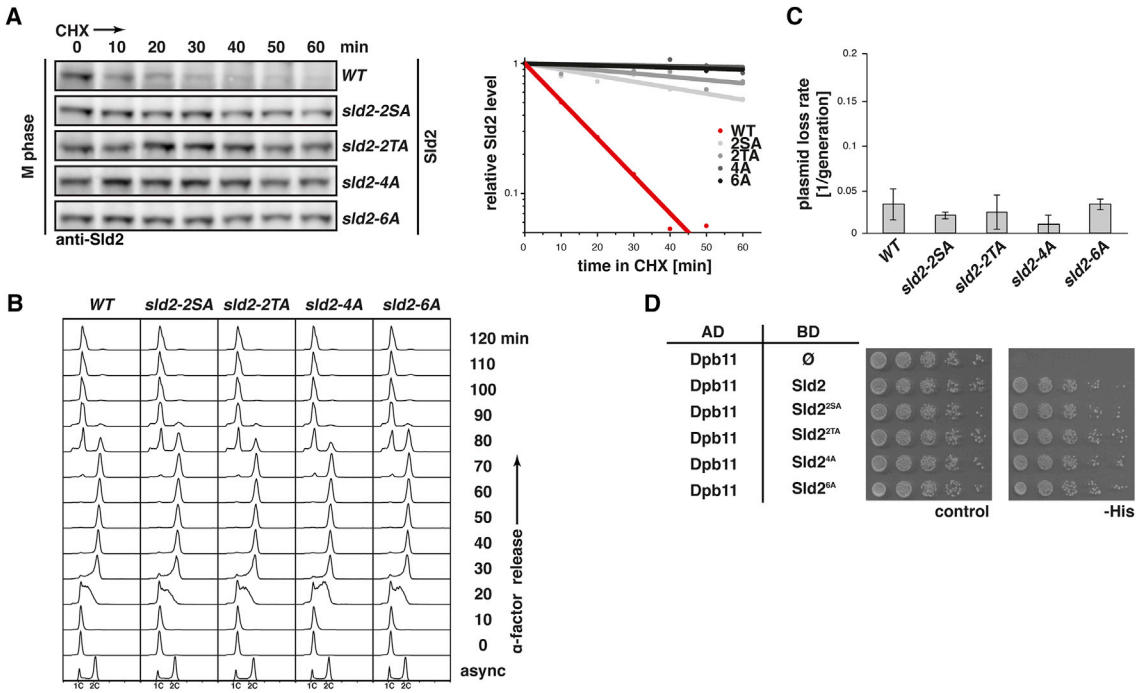

Figure 5. Mutations of Phosphorylation Sites in the Sld2 Degron Stabilize Sld2 in Mitosis, but Do Not Affect its S Phase Role in Promoting Origin Firing

(A) CHX translation shutoff to determine Sld2 protein stability in *sld2* phospho-mutant cells (*sld2-2SA, sld2-SS128,138AA; sld2-2TA, sld2-TT122,143AA; sld2-4A, sld2-TSST122,128,138,143AAAA; sld2-6A, sld2-TSSSST122,124,128,137,138,143AAAAAA*). Left: anti-Sld2 western blots. Right: quantification of Sld2 protein levels.

(B) DNA content of WT and *sld2* phospho-mutant cells (as in A) synchronously progressing through the cell cycle after α-factor arrest/release.

(C) Plasmid loss rates of an ARS/CEN plasmid (YCplac33) in WT and *sld2*-degron mutant strains (as in A). Values represent the mean of three independent experiments, error bars indicate SDs.

(D) Two-hybrid interaction of Gal4-AD-Dpb11 with lexA-BD-Sld2 and phospho-mutant derivatives (as in A).

See also Figure S5.

that *sld2-S124A* and *sld2-S137A* mutants (deficient in degron phosphorylation by Mck1 or DDK, respectively) were stabilized in mitosis as well, albeit to a lesser extent (Figure S5A). Overall, these data suggest that the target sites within the degron sequence are required for efficient Sld2 degradation in mitosis, consistent with a stepwise mechanism of Sld2 degradation that involves phosphorylation of the Sld2 degron, recognition of these phosphorylation marks by Dma1/2, and ubiquitin-proteasome-dependent degradation.

We also tested if the essential S phase function of Sld2 was affected by the mutation of the phosphorylation sites. These control experiments are particularly important, given that several CDK sites in the N-terminal half of Sld2 have been shown to regulate the essential phosphorylation of T84, which is required for Dpb11 binding (Tak et al., 2006). However, we found that all four fully stabilizing *sld2*-degron mutants (*2SA*, *2TA*, *4A*, and *6A*) were viable, progressed through the cell cycle with kinetics that are indistinguishable from WT strains, and did not show any defects in replication kinetics based on DNA content measurements by flow cytometry (Figure 5B). Using a plasmid loss assay, which is a sensitive test for defects in licensing or firing (Hogan and Koshland, 1992), we observed that *sld2*-degron mutants behaved as WT strains even in the background of deregulation of the firing factor Sld3 (Figures 5C and S5E). Finally, using a two-hybrid assay, we observed normal interaction with Dpb11 for all *sld2*-degron mutants (Figures 5D and S5B). Collectively, these data suggest that the phosphorylation sites in the Sld2 degron are not required to facilitate origin firing during S phase but that they specifically influence Sld2 degradation during mitosis.

Stabilizing Mutations of the Sld2 Phospho-degron Narrow the Gap between Firing Inactivation and Licensing Activation

The temporal correlation of Sld2 degradation and the disappearance of phosphorylated Sld2 in mitosis suggested that Sld2 degradation could be a critical mechanism to generate the temporal gap between firing and licensing phases. We therefore measured the disappearance of CDK-phosphorylated WT Sld2 as well as of stable versions of Sld2 (2SA, 2TA, 4A, and 6A) in cells that were synchronously progressing through the cell cycle (released from S phase arrest [Figures 6A, 6B, and S6D] or released from G1 [Figures S6A–S6C]). We observed that the disappearance of CDK-phosphorylated Sld2 was delayed in the *sld2*-degron mutants compared to WT (Figures 6A, 6B, S6A, and S6B). Interestingly, the disappearance of CDK-phosphorylated Sld2 in the *sld2*-degron mutants was transiently

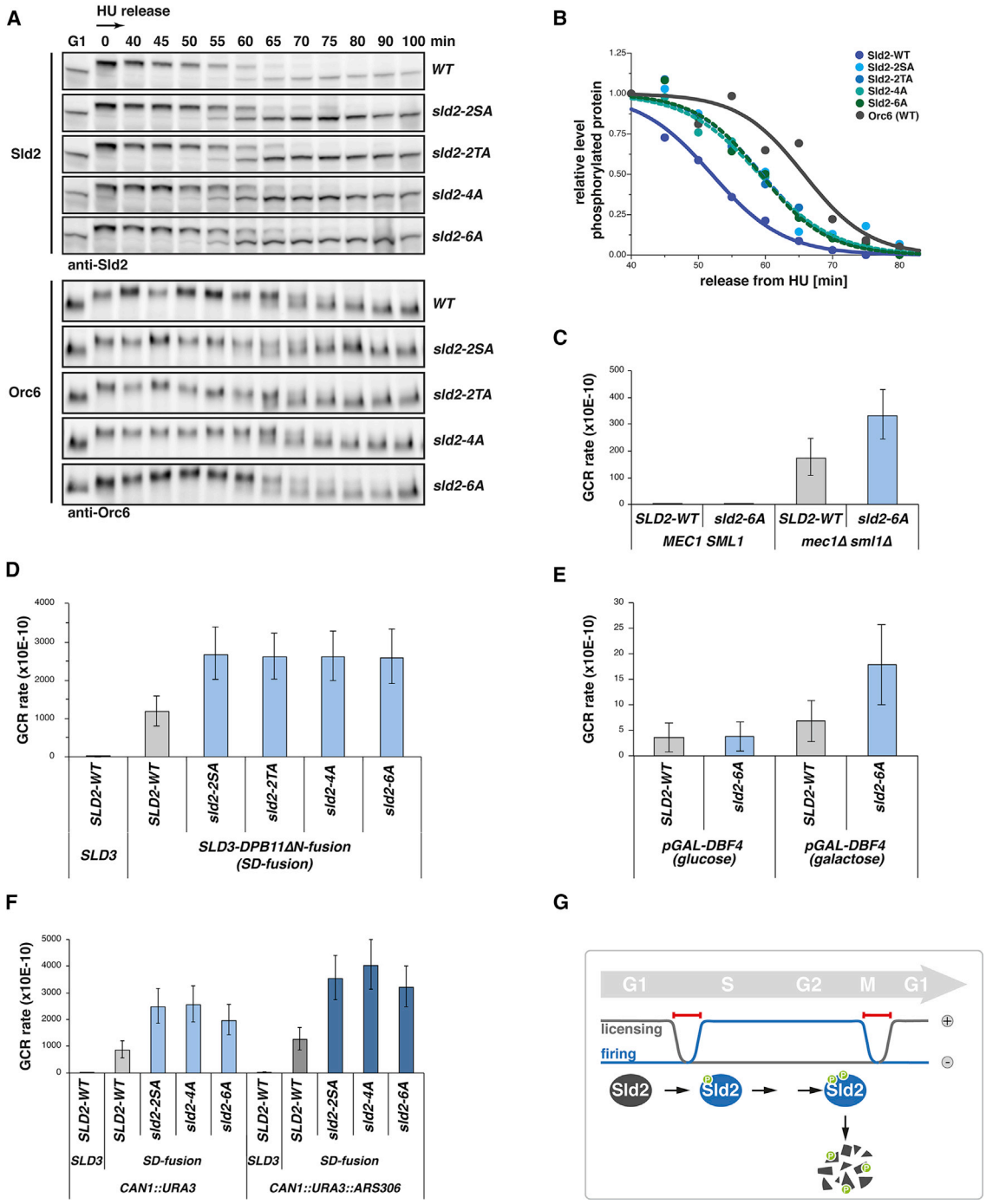

Figure 6. Mitotically Stabilized Sld2 Narrows the Gap between Firing and Licensing Phases and Leads to Increased Chromosomal Rearrangements Rates in Combination with Replication or Checkpoint Mutants

(A) Measurement of the gap between disappearance of the CDK-phosphorylated licensing factor Orc6 and CDK-phosphorylated firing factor Sld2 in WT and *sld2*-degron mutant cells synchronously released from HU arrest (S phase).

(B) Quantification of the CDK-phosphorylated protein species from western blots in (A).

(C–E) Rates of gross chromosomal rearrangement (GCR) events per cell per division using the *CAN1::URA3* (Chr V) reporter in WT (gray) and *sld2*-degron mutant (blue) in the indicated mutant backgrounds: (C) *mec1Δ sml1Δ*, (D) *SLD3-DPB11ΔN-fusion* as a constitutively active version of Sld3, and (E) *pGAL-DBF4* to achieve constitutive expression of Dbf4 in the presence of galactose. Eight fluctuations were used per condition to calculate the rates; error bars indicate a 95% confidence interval.

(F) GCR rates as in (C)–(E) but using a modified reporter that harbors the efficiently firing origin ARS306 ~500 bp downstream of *CAN1::URA3*.

(G) Model of the replication control (top) and Sld2 regulation (bottom) throughout the cell cycle. Gaps separating licensing and firing phases are indicated in red. See also Figure S6.

delayed for 5–10 min, but not completely abolished, and was now reset with similar timing as Sld3 (compare Figure 6B with Figure 1E and Figure S6B with Figure 1B). In these experiments, we also measured the disappearance of CDK-phosphorylated Orc6 as a marker for the activation of the next licensing phase and did not observe any change in the tested strains (Figures 6A, 6B, S6A, and S6B). We thus conclude that the temporal gap between firing inactivation and licensing activation is narrowed, but not completely abolished, in *sld2*-degron mutants.

Inherently, defects in protein degradation will lead to increased protein levels. The stable versions of Sld2 could therefore elicit phenotypes simply by an increased Sld2 level. However, when we followed Sld2 levels through the cell cycle in WT and *sld2-2TA* and *sld2-6A* mutants in cells that were released from G1 arrest, we observed that all Sld2 variants showed a similar level in S phase. Specifically upon entry into mitosis, the stable Sld2 versions became more abundant compared to WT Sld2, and this difference started to level out in the next G1 phase (Figures S5C and S5D). Hence, stable versions of Sld2 appear to affect mitotic Sld2 in particular.

Stable Sld2 Increases Incidents of Chromosomal Rearrangements When Combined with Deregulation of Replication Initiation or the Checkpoint

In the absence of Sld2 degradation in mitosis, the temporal gap between firing inactivation and licensing activation is narrowed but still present. We therefore reasoned that under these conditions the separation of firing and licensing reactions would still be largely functional but less robust. Also, we did not observe evidence for overt over-replication in strains with stable Sld2, which showed normal viability, DNA content (Figures S6C and S6D), and no signs of DNA damage checkpoint activation. However, a less robust replication control system may be prone to sporadic incidents of over-replication. As a readout that is more sensitive to such sporadic over-replication events, we used a gross chromosomal rearrangements assay that measures the loss of a non-essential chromosomal region spanning at least 7.5 kb at the end of the left arm of chromosome 5 (Chen and Kolodner, 1999). We did not observe increased gross chromosomal rearrangement (GCR) rates in any of the *sld2*-degron mutants in an otherwise WT background (Figure S6E). However, when *sld2-6A* was combined with the *mec1Δ sml1Δ* mutation, which is defective in the checkpoint response to replication problems, we observed a synthetic increase in GCR rates (Figure 6C). As over-replication-prone cells are particularly dependent on a functional checkpoint (Archambault et al., 2005), these data are therefore consistent with Sld2 misregulation causing over-replication.

Next, we tested whether stable versions of Sld2 would trigger GCRs in checkpoint-proficient cells, when combined with additional misregulation of origin firing. We combined the *sld2*-degron mutants with an allele of Sld3, where Sld3 is covalently fused to Dpb11 and constitutively active throughout the cell cycle (*SLD3-DPB11ΔN-fusion* [Zegerman and Diffley, 2007]), and observed highly increased GCR rates (2,500–3,000 × 10E−10; Figure 6D). Notably, the *SLD3-DPB11ΔN-fusion* mutant showed by itself a highly increased GCR rate (1,000 × 10E−10; Figure 6D). The later phenotype could potentially arise from Sld3 being uncoupled from cell cycle regulation but more likely from the pronounced S phase defect in *SLD3-DPB11ΔN-fusion* cells (Figure S6F) or a combination of both. Thus, we used an alternative means of bypassing Sld3 cell cycle regulation, the *cdc45*$^{JET1-1}$ allele (Tanaka et al., 2007). Also in the background of this mutant, a stable Sld2 version induced increased GCR rates (Figure S6G). Thus, we conclude that a combined deregulation of Sld2 and Sld3 causes genome instability.

We also tested effects of combined misregulation of Sld2 and licensing factors or Sld2 and DDK, the second principal kinase regulator of replication control (Bousset and Diffley, 1998; Donaldson et al., 1998; Sheu and Stillman, 2010). When we used the *cdc6ΔNT* mutant, which renders the licensing factor Cdc6 insensitive to CDK-dependent degradation (Drury et al., 2000; Drury et al., 1997), the double mutant combination with the *sld2-6A* mutant showed an increased GCR rate compared to both single mutants (Figure S6H). When we induced constitutive DDK activity by overexpression of Dbf4 from a galactose-inducible promoter, we observed a synthetic increase of GCR rates when combined with the *sld2-6A* mutant as well (Figure 6E). This further strengthens the idea that the GCR phenotype arises from sporadic incidents of over-replication when firing and licensing phases are not robustly separated. Indeed, a contribution of DDK to temporal separation of firing and licensing phases is consistent with the finding that in mitosis Dbf4 levels decrease before reactivation of licensing factors (Figures 1D and S1G; Ferreira et al., 2000).

Furthermore, we investigated whether the increase in GCR events upon deregulation of Sld2 and Sld3 was replication-origin dependent (and thus likely due to over-replication). We inserted the highly efficient, early-firing replication origin ARS306 in close proximity to the GCR reporter (using a similar strategy as (Tanaka and Araki, 2011)). In this setup, we observed substantial further enhancements of the GCR rates when we tested strains that contained stable variants of Sld2 and the *SLD3-DPB11ΔN-fusion* (Figure 6F), consistent with the genome instability phenotype being origin dependent.

Lastly, we tested whether the strength of the GCR phenotype would correlate with the size of the gap that separates firing factor inactivation and licensing factor reactivation. Therefore, we used the partially stabilized *sld2-S124A* variant (Figure S5A), which showed only slightly delayed inactivation in mitosis and reduced the size of the gap by <5 min. Consistently, this mutant showed only a slight increase in GCR rates in the background of the *SLD3-DPB11ΔN-fusion* (Figures S6I and S6J). Both phenotypes were less pronounced than in the *sld2-6A* mutant. Therefore, degradation rates, gap size, and genome instability phenotypes correlate in different Sld2 mutants. Overall, we conclude that robust separation of licensing and firing phases by mechanisms such as timely Sld2 degradation constitutes an additional layer of replication control to safeguard genome stability.

DISCUSSION

Division of the replication initiation reaction into two phases, licensing and firing, has been confirmed for all eukaryotic systems tested (Blow and Dutta, 2005; Siddiqui et al., 2013). The master-regulator that ensures the exclusiveness of licensing

and firing phases is CDK. However, it has been an open question, whether CDK itself is sufficient to robustly separate both phases or whether additional regulators are involved.

Our study supports a model in which the replication control system contains temporal gaps, which can be viewed as buffer zones that provide a robust separation of licensing and firing phases at cell-cycle transitions (see Figure 6G for a model). The first gap separates licensing inactivation (specifically of Mcm3 and Cdc6) and firing activation at the G1-S transition, consistent with a model collectively derived from previous studies (Brümmer et al., 2010; Drury et al., 2000; Labib et al., 1999; Tanaka et al., 2007; Zegerman and Diffley, 2007). Conversely, the second gap separates the inactivation of firing factors from the activation of licensing factors (Figure 6G). Overall, these findings strongly suggest that such gaps are inherent features of replication control in budding yeast.

While a temporal order in the phosphorylation or de-phosphorylation of licensing and firing factors could be achieved by differences in the affinities of substrate-kinase or substrate-phosphatase interactions (see Loog and Morgan, 2005; Bouchoux and Uhlmann, 2011) and thus CDK alone, we find that additional regulators promote the early disappearance of CDK-phosphorylated Sld2 in mitosis and thus enforce the temporal separation of firing and licensing phases (Figure 6G). Indeed, our characterization of the phosphorylation-dependent degradation of Sld2 revealed a mechanism of remarkable complexity: Sld2 is directly targeted by four different cell-cycle kinases, which act in a partially interdependent manner, resulting in the phosphorylation of the Sld2-degron on at least six positions. These phosphorylation signals are then read out by the ubiquitin-ligases Dma1/2, which target Sld2 for proteasomal degradation. Our in vitro experiments suggest that Cdc5 phosphorylation of the Sld2 degron creates a binding site for the FHA domain of Dma1, but the mechanism by which phosphorylation of the other kinases influences Sld2 degradation is less clear. Inducing a conformational change and thereby exposing specific target sites is a mechanism that has already been proposed for CDK-dependent regulation of Sld2-T84 (Tak et al., 2006) and could also be at play here.

We can envision two possible rationales for why Sld2 degradation depends on phosphorylation by several kinases at several sites. First, multi-site phosphorylation allows a very sharp (switch-like) response upon changes of kinase activity (Kapuy et al., 2009), and indeed, Sld2 degradation rates appear to change in a switch-like manner during the cell cycle (Figure S2A). Second, the four cell-cycle kinases that mediate Sld2 degradation could be viewed as independent regulators and the Sld2 degron as a coincidence detector, which integrates over several cellular signals. In this regard, it is interesting to note that CDK, DDK, and Cdc5 are all regulated in response to DNA damage by the DNA damage checkpoint (Gritenaite et al., 2014; Palou et al., 2015; Pasero et al., 1999; Weinreich and Stillman, 1999; Zhang et al., 2009) and that DNA damage is an important cellular signal for regulation of origin firing (Lopez-Mosqueda et al., 2010; Santocanale and Diffley, 1998; Shirahige et al., 1998; Zegerman and Diffley, 2010). It will therefore be interesting to test if Sld2 degradation plays an additional role in the inhibition of origin firing in response to DNA damage or replication stress.

Our results show that *sld2*-degron mutations narrow the firing-to-licensing gap, but even stable Sld2 is inactivated before licensing factors are activated. This finding highlights that several overlapping mechanisms collaborate to tightly control even a single replication factor such as Sld2. The existence of such overlapping mechanisms emphasizes the importance of robustly separating licensing and firing phases and safeguarding cells from over-replication. We therefore propose that a replication initiation program without gaps or with smaller gaps would be prone to the sporadic incidents of over-replication, which in turn will cause genome instability (Green et al., 2010; Green and Li, 2005; Tanaka and Araki, 2011).

Indeed, we find that decreasing the gap size between firing and licensing phases leads to increased rates of gross chromosomal rearrangements in the background of a deficient checkpoint. Additionally, even in the context of a proficient checkpoint, the stable Sld2 variants lead to increased GCR rates when combined with different factors that deregulate firing or licensing. While GCRs can arise from different causes, we show that in the case of misregulated firing factors the GCR phenotype is further exacerbated by transplanting an efficiently firing origin next to the GCR reporter, suggesting that it arises from misregulated replication initiation, probably due to sporadic over-replication events. Our results therefore strongly suggest that the presence of a gap between firing and licensing phases is critical and that its size matters, too.

The principles of replication control have been shown to be conserved among eukaryotes, whereas specific regulatory mechanisms differ among species (exemplified by additional vertebrate-specific mechanisms such as geminin and Cdt2 [reviewed in Blow and Dutta, 2005; Siddiqui et al., 2013]). It therefore appears likely that higher eukaryotes also need to robustly separate origin licensing and firing phases to prevent over-replication at cell-cycle transitions. Future research will therefore need to address whether replication control in metazoans utilizes similar gaps between licensing and firing phases, particularly given the importance of replication control as a barrier to cancer formation (Halazonetis et al., 2008).

EXPERIMENTAL PROCEDURES

All yeast strains are based on W303 and were constructed using standard methods. Plasmids were constructed using the In-Fusion HD cloning kit (Clontech Laboratories), and mutations were introduced by oligonucleotide-directed site-specific mutagenesis. A summary of all yeast strains and plasmids used in this study can be found in Supplemental Experimental Procedures.

Cell-cycle synchronization was achieved using α-factor (G1), hydroxyurea (S), or nocodazole (mitosis). DNA content was measured by flow cytometry with a MACSquant Analyzer 10 (Miltenyi Biotec) using SYTOX green to stain DNA. Protein stability was measured in translation shutoff experiments using cycloheximide. Western blot data were quantified using the ImageJ-based software package Fiji. Yeast two-hybrid, protein interaction, in vitro kinase, and peptide binding assays were performed as described elsewhere (Gritenaite et al., 2014; Pfander and Diffley, 2011). Mass spectrometry data were analyzed using MaxQuant.

GCR events were measured using a *CAN1::URA3* reporter on chromosome V (Chen and Kolodner, 1999). GCR rates and the respective 95% confidence intervals were determined by fluctuation analysis using the Ma-Sandri-Sarkar maximum likelihood estimator (MSS-MLE) method in the online tool FALCOR (Hall et al., 2009). The use of the MSS-MLE allows comparing

GCR event estimators in two-tailed Student's t tests, and the calculated p values are given in the Supplemental Information.

Detailed experimental procedures are available in Supplemental Experimental Procedures.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures, six figures, two tables, and one data file and can be found with this article online at http://dx.doi.org/10.1016/j.celrep.2016.09.013.

AUTHOR CONTRIBUTIONS

K.-U.R. and B.P. designed the study. K.-U.R., F.Z., L.G., and B.P. conducted the experiments and analyzed the data. B.P. wrote the paper. All authors commented on and revised the paper.

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