

# CDK1-dependent Inhibition of the E3 Ubiquitin Ligase CRL4<sup>CDT2</sup> Ensures Robust Transition from S Phase to Mitosis\*

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Lindsay F. Rizzardi<sup>†1</sup>, Kate E. Coleman<sup>‡2</sup>, Dileep Varma<sup>§2</sup>, Jacob P. Matson<sup>§</sup>, Seun Oh<sup>§</sup>,  
and Jeanette Gowen Cook<sup>†§3</sup>

From the <sup>†</sup>Curriculum in Genetics and Molecular Biology and the <sup>§</sup>Department of Biochemistry and Biophysics, University of North Carolina, Chapel Hill, North Carolina 27599

**Background:** CRL4<sup>CDT2</sup> mediates replication-coupled destruction during S phase. CRL4<sup>CDT2</sup> substrates reaccumulate by an unexplored mechanism.

**Results:** CDK1 activity blocks CRL4<sup>CDT2</sup> by preventing chromatin recruitment of the substrate receptor, CDT2.

**Conclusion:** CDK1 activity facilitates CRL4<sup>CDT2</sup> substrate reaccumulation upon S phase exit; several of these substrates are then required for normal mitotic progression.

**Significance:** We provide the first evidence that CDK1 regulates the activity of CRL4<sup>CDT2</sup>.

Replication-coupled destruction of a cohort of cell cycle proteins ensures efficient and precise genome duplication. Three proteins destroyed during replication via the CRL4<sup>CDT2</sup> ubiquitin E3 ligase, CDT1, p21, and SET8 (PR-SET7), are also essential or important during mitosis, making their reaccumulation after S phase a critical cell cycle event. During early and mid-S phase and during DNA repair, proliferating cell nuclear antigen (PCNA) loading onto DNA (PCNA<sup>DNA</sup>) triggers the interaction between CRL4<sup>CDT2</sup> and its substrates, resulting in their degradation. We have discovered that, beginning in late S phase, PCNA<sup>DNA</sup> is no longer sufficient to trigger CRL4<sup>CDT2</sup>-mediated degradation. A CDK1-dependent mechanism that blocks CRL4<sup>CDT2</sup> activity by interfering with CDT2 recruitment to chromatin actively protects CRL4<sup>CDT2</sup> substrates. We postulate that deliberate override of replication-coupled destruction allows anticipatory accumulation in late S phase. We further show that (as for CDT1) *de novo* SET8 reaccumulation is important for normal mitotic progression. In this manner, CDK1-dependent CRL4<sup>CDT2</sup> inactivation contributes to efficient transition from S phase to mitosis.

Ubiquitin-mediated degradation of cell cycle proteins is essential to ensure timely cell cycle transitions that maintain genome integrity. Conversely, cell cycle-dependent protein stabilization by preventing ubiquitination allows rapid protein accumulation at the right time to bring about robust cell cycle

transitions. Replication-coupled destruction is a particularly important protein control mechanism that coordinates the degradation of a cohort of proteins with the process of DNA synthesis. Much has been learned about how replication-coupled destruction is initiated in S phase (1–5), but less is known about how it is inhibited as S phase ends.

Among the cohort of human proteins reported to be subject to replication-coupled destruction are CDT1, SET8, p12, the CDK inhibitor p21, and, most recently, thymine DNA glycosylase (6, 7). Their destruction in S phase is particularly critical to ensure precise and efficient genome duplication (1–7). CDT1 is required in G<sub>1</sub> for rendering DNA replication origins competent for initiation in S phase (a process termed “origin licensing”) (8, 9). SET8 is the sole enzyme responsible for histone H4 lysine 20 monomethylation (H4K20me1)<sup>4</sup> and, like CDT1, is also required for origin licensing (10). Degradation of CDT1 and SET8 at the onset of S phase restricts DNA replication to no more than once per cell cycle by preventing relicensing of replicated origins. Failure to degrade either CDT1 or SET8 results in multiple rounds of origin firing, leading to DNA rereplication and ultimately significant DNA damage and genome instability (1, 10–17). Likewise, persistence of human thymine DNA glycosylase in S phase slows proliferation (6). Degradation of p21 in early S phase stimulates CDK2 activity, which, in turn, triggers key S phase events, including DNA replication initiation and origin licensing inhibition (18, 19). During S phase, p12 levels are 35% of their G<sub>2</sub>/M and G<sub>1</sub> levels, resulting in the presence of both Polδ3 (Polδ lacking p12) and Polδ4 (Polδ containing p12) during S phase (5). Because the two forms of Polδ have complementary biochemical properties (5, 20), CRL4<sup>CDT2</sup>-mediated degradation of p12 during S phase may be important for DNA replication fidelity.

Each of the proteins known to be subject to replication-coupled destruction follows a pattern of low abundance during S phase and then rapid reaccumulation prior to mitosis (illus-

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<sup>1</sup> Present address: Johns Hopkins University School of Medicine, Center for Epigenetics, Baltimore, MD 21205.

<sup>2</sup> Both authors contributed equally to this work.

<sup>3</sup> To whom correspondence should be addressed: Dept. of Biochemistry and Biophysics, University of North Carolina at Chapel Hill, 120 Mason Farm Rd., Campus Box 7260, Chapel Hill, NC 27599-7260. Tel.: 919-843-3867; Fax: 919-966-2852; E-mail: jgcook@email.unc.edu.

<sup>4</sup> The abbreviations used are: H4K20me1 and H4K20me2, histone H4 lysine 20 monomethylation and dimethylation, respectively; PCNA, proliferating cell nuclear antigen; PCNA<sup>DNA</sup>, DNA-loaded PCNA; CDK, cyclin-dependent kinase; PIP, PCNA interaction protein.

trated in Fig. 1). Importantly, CDT1, p21, and SET8 also function during mitosis, making their reaccumulation critical for normal mitotic progression (21–23). Replication-coupled destruction is triggered by the ubiquitin E3 ligase, CRL4<sup>CDT2</sup>. The mechanism of CRL4<sup>CDT2</sup> substrate recognition is unique in that the substrates must first interact with DNA-loaded PCNA (PCNA<sup>DNA</sup>), and PCNA is DNA-loaded during both S phase and DNA repair (24–27). Given the robust reaccumulation of CRL4<sup>CDT2</sup> substrates well in advance of mitosis, we sought to determine the relationship between PCNA unloading in late S phase and reaccumulation of CRL4<sup>CDT2</sup> substrates.

We have discovered that, surprisingly, CRL4<sup>CDT2</sup> substrates accumulate *prior* to PCNA unloading and the completion of replication. Moreover, we demonstrate that activation of CDK1 inhibits degradation of CRL4<sup>CDT2</sup> substrates. We show here that CDK1 activity (directly and/or indirectly) inhibits CRL4<sup>CDT2</sup> activity itself by preventing its accumulation on chromatin, an event necessary for CRL4<sup>CDT2</sup> activity. Activation of CDK1 as S phase completes is necessary for the normal reaccumulation of substrates, such as CDT1 and SET8, and we show that, like CDT1, failure to reaccumulate SET8 *de novo* prior to mitosis leads to mitotic progression defects (21). The temporal control of CRL4<sup>CDT2</sup> activity ensures the accumulation of CRL4<sup>CDT2</sup> substrates during mitosis, thereby preventing chromosome instability. We propose that purposeful protection from replication-coupled destruction anticipates the end of S phase and primes efficient progression through mitosis.

## EXPERIMENTAL PROCEDURES

**Cell Culture and Manipulations**—HCT116, 293T, and HeLa cells were cultured in Dulbecco's modified Eagle's medium (DMEM) (Difco) supplemented with 10% fetal calf serum (Sigma). HCT116 and HeLa cells were synchronized in early S phase by double thymidine block (2 mM thymidine for 17 h, release into fresh medium for 9 h, 2 mM thymidine for 15 h) or in prometaphase by treatment with 2 mM thymidine for 18 h followed by release into 100 nM nocodazole for 10 h as described previously (28, 29). Kinase inhibitors were used at the following concentrations: c-Jun N-terminal kinase (JNK) inhibitor VIII at 10  $\mu$ M (EMD Millipore), p38 inhibitor SB203580 at 30  $\mu$ M (LC Laboratories), and the CDK1 inhibitor RO-3306 (Sigma) at 10  $\mu$ M or as indicated. DNA damage was induced by a single treatment of 20 J/m<sup>2</sup> UV. Small interfering RNA (siRNA) transfections were performed with a 400 nM concentration of each siRNA duplex using Dharmafect 1 reagent (Dharmacon) according to the manufacturer's guidelines. This high concentration of siRNA was required for efficient knock-down, given the brief siRNA treatment time. Synthetic duplexed RNA oligonucleotides were synthesized by Invitrogen: luciferase (5'-CUUACGCGAGUACUUCGA-3'), SET8-ORF (5'-GATGCAACTAGAGAGACA-3') (10), and SET8-UTR (5'-AAGCAUACAAGCCGAACGUU-3') (30).

**Antibodies**—Antibodies were purchased from the following sources: MAPKAP kinase 2, phospho-MAPKAP kinase 2, phospho-c-JUN (Ser-63), and SET8 from Cell Signaling Technologies; hemagglutinin (HA) from Roche Applied Science;  $\alpha$ -tubulin (DM1A) from Sigma; PCNA (PC-10), p21, cyclin A, cyclin B1, and Orc2 from Santa Cruz Biotechnology, Inc.; H4K20me1

from EMD Millipore (catalog no. 04-735); H4K20me2 from Active Motif (catalog no. 39174). CDT2 and p12 antibodies were a gift from A. Dutta. Cul4B and Cul4A antibodies were a gift from Y. Xiong. Antibodies to human CDT1 have been described previously (31). AlexaFluor 488 and Rhodamine Red-X-labeled donkey secondary antibodies for immunofluorescence microscopy were obtained from Jackson Immuno-Research Laboratories.

**Plasmids and Protein Lysate Preparation**—HA-tagged mutant CDT1 and p21 were generated via PCR and cloned into pLX302 (Addgene plasmid 25896) (32) or pBABE (Addgene plasmid 51070) (33) expression vectors, respectively, as were the tagged wild type constructs. Plasmids expressing glutathione S-transferase (GST)-p21<sub>WT</sub> and GST-p21<sub>PIP</sub> fusions were generated by recombinational cloning between pENTR-p21 derivatives and pDEST15 for N-terminal GST fusion (Gateway LR clonase, Invitrogen) and expressed in BL21 cells. The PIP box was inactivated by site-directed mutagenesis to generate the p21<sub>PIP</sub> mutant (MTDFY to AAAA). Whole-cell lysates were prepared either by direct lysis of equal cell numbers in 2 $\times$  Laemmli sample buffer with 10%  $\beta$ -mercaptoethanol or in CSK buffer (supplemented with 0.1% Triton X-100 and protease and phosphatase inhibitors) (34). Chromatin-enriched fractions were isolated as described previously (35) by the addition of micrococcal nuclease and CaCl<sub>2</sub> to Triton-insoluble pellets to release DNA-bound material into the soluble pool and clarified by centrifugation. Alternatively, sonication was used to solubilize the chromatin-bound proteins.

**Protein-Protein Interaction Assays**—Recombinant GST fusion proteins GST-p21<sub>WT</sub> or GST-p21<sub>PIP</sub> (mutated PIP box) were immobilized on glutathione-Sepharose 4B beads (GE Healthcare). Whole cell extracts (from HCT116 cells) were prepared in CSK buffer (supplemented with 0.1% Triton X-100 and protease and phosphatase inhibitors), and 1 mg of cell lysate was incubated with the bound GST-p21 derivatives for 3 h at 4 °C. The bound complexes were washed three times in complete CSK buffer, resuspended in 50  $\mu$ l of 2 $\times$  SDS sample buffer, and boiled for 5 min to elute bound proteins.

**Immunofluorescence Microscopy**—Cells were rinsed briefly in 1 $\times$  phosphate-buffered saline (PBS, pH 7.4) prior to fixation. Cells were typically prefixed with 4% paraformaldehyde for 60 s, permeabilized with 0.5% Triton X-100 for 5 min, and then fixed for 15 min using 4% paraformaldehyde. For all immunostaining procedures involving PCNA, the cells were first prefixed in 2% paraformaldehyde for 60 s and then permeabilized with 0.5% Triton X-100 for 5 min and fixed with 2% paraformaldehyde for 15 min followed by  $-20$  °C methanol for 4 min. Blocking steps, antibody incubations, and washes were all performed in 1 $\times$  PBS buffer plus 0.1% BSA. All antibody incubations were for 1 h at 37 °C, and washes were for 10 min at room temperature. DAPI staining (0.1  $\mu$ g/ml) was performed for 10 min, and cells were mounted using either Prolong Antifade (Invitrogen/Molecular Probes) or VECTASHIELD mounting medium (Vector Laboratories, Inc.).

For indirect immunofluorescence microscopy of interphase nuclei, images were acquired using Metamorph Software and a 60 $\times$ /1.4 numerical aperture (PlanApo) differential interference contrast oil immersion objective (Nikon) mounted on a Nikon

TE300 inverted microscope equipped with a Yokogawa CSU10 spinning disk and a Hamamatsu Orca ER cooled CCD camera. For fluorescence intensity measurements, the average values for integrated nuclear PCNA fluorescence from control and experimental cell samples were subjected to background subtraction to obtain the specific nuclear fluorescence levels. For indirect immunofluorescence microscopy of mitotic cells, images were acquired using Metamorph Software and a 100×/1.4 numerical aperture (PlanApo) differential interference contrast oil immersion objective mounted on a Leica DMIRB inverted microscope equipped with a Photometric HQ2 cooled CCD camera. *Scale bars* represent 5 μm in all figures.

**Flow Cytometry**—Prior to flow cytometry analysis, cells were trypsinized, fixed in 70% ethanol, and treated with propidium iodide/RNase solution according to standard methods. Flow cytometry analysis was performed using a Cyan FACScan (DakoCytomation) and Summit version 4.3 software (DakoCytomation) as described previously (36).

**RESULTS**

**CRL4<sup>CDT2</sup> Substrate Accumulation Occurs in Late S Phase Prior to PCNA Unloading**—Previous work described replication-coupled destruction of CRL4<sup>CDT2</sup> substrates and further documented their robust accumulation at later cell cycle stages (5, 11, 19, 37) illustrated in Fig. 1A. Given that PCNA loading at replication and repair sites (PCNA<sup>DNA</sup>) is a prerequisite for CRL4<sup>CDT2</sup> targeting, the normal substrate reaccumulation as S phase ends could simply be a consequence of PCNA unloading as replication completes. Nevertheless, replication can extend close to the time of mitosis, and this late replication has been implicated in the genome instability associated with common fragile sites (38). Because PCNA<sup>DNA</sup> triggers CRL4<sup>CDT2</sup>-mediated degradation, persistence of PCNA<sup>DNA</sup> so late in S phase was not fully consistent with observations by us and others that CRL4<sup>CDT2</sup> substrates reaccumulate well in advance of mitosis (5, 11, 19, 39). We therefore considered the possibility that substrates may be actively stabilized during the transition from S phase to mitosis. If so, then CRL4<sup>CDT2</sup> substrates may in fact accumulate prior to PCNA<sup>DNA</sup> unloading.

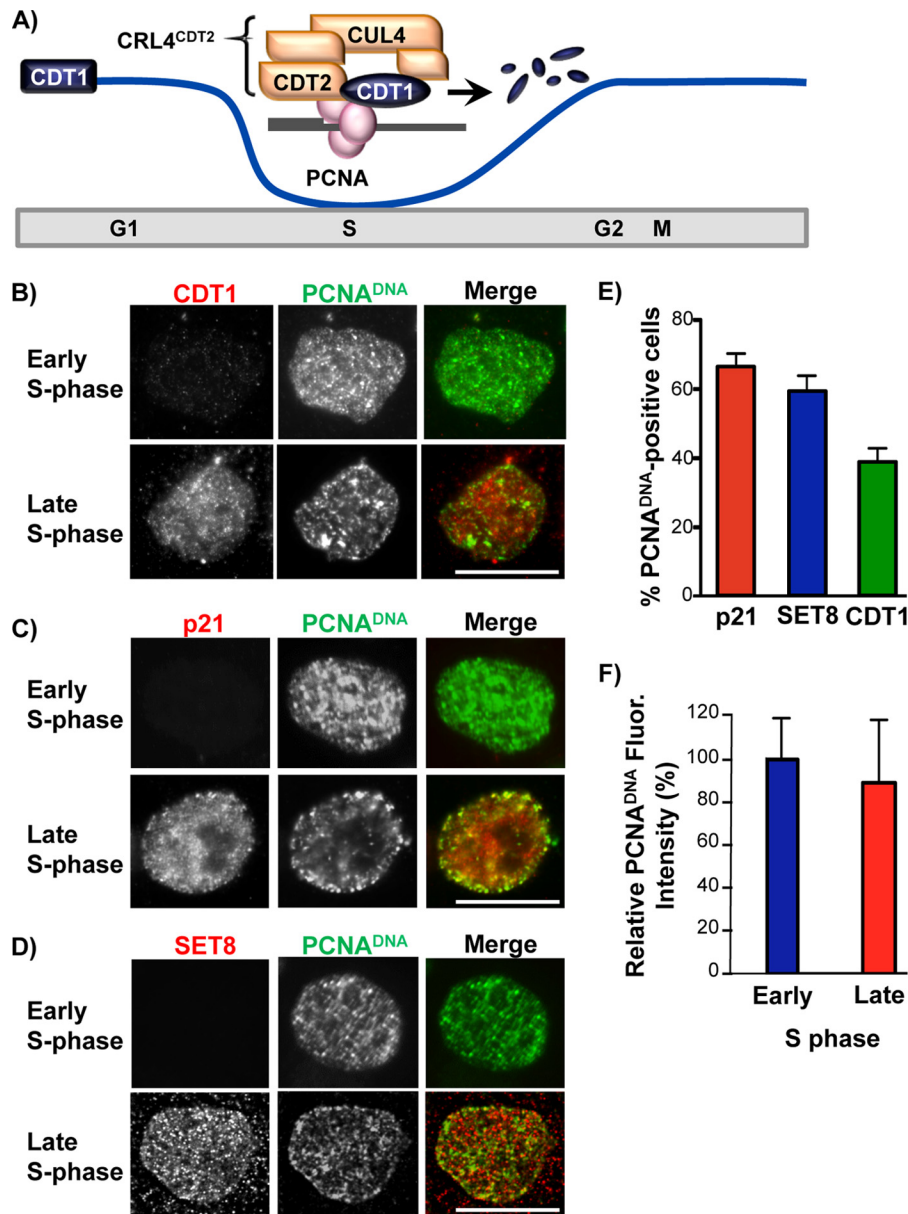
To test this idea, we interrogated the timing of CRL4<sup>CDT2</sup> substrate accumulation in individual cells relative to the dynamics of PCNA<sup>DNA</sup> unloading as cells completed S phase. We synchronized cells in early S phase, released them to proceed to late S phase, and then extracted soluble proteins prior to fixation and immunostaining for endogenous PCNA<sup>DNA</sup> and the CRL4<sup>CDT2</sup> substrates CDT1, p21, and SET8. As expected, cells in early S phase had abundant PCNA<sup>DNA</sup> in a characteristic early S phase pattern (40, 41) but had very little CDT1 (Fig. 1B, *top row*) and similarly low levels of p21 and SET8 (Fig. 1, C and D, *top rows*). In late S phase (7 h postrelease), PCNA was still DNA-loaded in some cells, and CDT1, p21, and SET8 were readily detectable in those same cells (Fig. 1, B–D, *bottom rows*). Late S phase cells are characterized by foci of chromatin-bound PCNA at the nuclear periphery with more diffuse nuclear staining elsewhere, as seen in Fig. 1, B–D (40, 41). (Multiple mechanisms recruit these proteins to chromatin, resulting in only partial co-localization with PCNA foci.) We counted the number of cells that retained PCNA<sup>DNA</sup> foci 7 h into S phase (14%, *n* =

150) and then scored those that also contained nuclear p21, SET8, or CDT1 using antibodies to the endogenous proteins. We found that 40–65% of PCNA<sup>DNA</sup>-positive cells already had abundant CRL4<sup>CDT2</sup> substrates (Fig. 1E; we cannot distinguish differences among the individual substrate kinetics from differences in antibody avidity). Importantly, individual late S phase cells that retained PCNA<sup>DNA</sup> had nearly equivalent amounts of PCNA<sup>DNA</sup> relative to their early S phase counterparts (Fig. 1F). Thus, the accumulation of substrates was not accounted for by the amount of PCNA<sup>DNA</sup> declining in individual late S phase cells. The fact that cells with similar amounts of PCNA<sup>DNA</sup> showed dramatically different abilities to support replication-coupled destruction indicates a qualitative difference between early and late S phase cells with regard to CRL4<sup>CDT2</sup> activity. Moreover, the presence of CDT1, SET8, and p21 in late S phase nuclei with PCNA<sup>DNA</sup> suggested an active mechanism to inhibit CRL4<sup>CDT2</sup>-mediated degradation.

**CRL4<sup>CDT2</sup> Substrates Cannot Be Targeted during Mitosis**—It is worth noting that at 7 h postrelease (Fig. 1), not every cell contained both PCNA<sup>DNA</sup> and CRL4<sup>CDT2</sup> substrates, consistent with the observation that even in synchronized cells, progression through S phase is not perfectly uniform. Our time courses indicate that the onset of CRL4<sup>CDT2</sup> substrate accumulation occurs during the final ~10–15% of S phase based on an average 7–8-h S phase and mitotic entry at 8–9 h (*e.g.* see Fig. 4A). Because we could not arrest cells in very late S phase for biochemical analysis, we took advantage of the next available robust cell cycle block in prometaphase, which is 1–2 h later, to generate homogeneous cell populations. To determine if CRL4<sup>CDT2</sup> substrates are actively protected from degradation late in the cell cycle, we deliberately stimulated synchronous replication-coupled destruction by UV-irradiating prometaphase cells. UV irradiation causes robust and simultaneous PCNA loading onto DNA during nucleotide excision repair, and thus UV treatment causes highly synchronous replication-coupled destruction of CRL4<sup>CDT2</sup> substrates by the same mechanism that operates during S phase (reviewed in Ref. 42). (Note that CRL4<sup>CDT2</sup>-mediated degradation is stimulated by DNA synthesis per se and is not a component of the DNA damage checkpoint response (25)). We monitored the levels of CDT1 as well as three other CRL4<sup>CDT2</sup> substrates by immunoblotting lysates from the two conditions. As expected in asynchronous cells, CDT1, SET8, p21, and p12 were degraded following UV irradiation (Fig. 2, A and B, *lanes 1 and 2*). In stark contrast, however, none of the four substrates were degraded in prometaphase cells (Fig. 2, A and B, *lanes 3 and 4*).

CRL4<sup>CDT2</sup> substrates are resistant to degradation in some quiescent cells because the substrate adapter subunit, CDT2, is itself degraded (43, 44). CDT2 was equally present in both asynchronous and prometaphase cells, so the substrate protection we observed could not be attributed to loss of CDT2 under these conditions (Fig. 2B, *lanes 3 and 4*). Importantly, releasing mitotic cells into the subsequent G<sub>1</sub> fully restored sensitivity of SET8 and CDT1 to CRL4<sup>CDT2</sup>-mediated degradation (Fig. 2C). SET8 is also an APC/C target (23), and we observed the expected low SET8 levels in untreated G<sub>1</sub> cells relative to mitotic cells. Taken together, we conclude that the substrates of





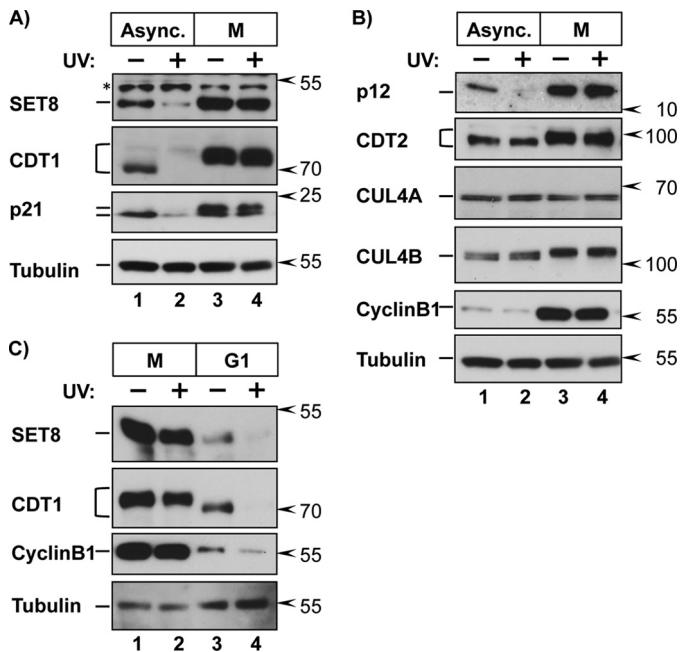
**FIGURE 1. Targets of replication-coupled destruction reaccumulate prior to the end of S phase.** *A*, in early S phase, PCNA is loaded onto DNA and is bound by CRL4<sup>CDT2</sup> substrates (e.g. CDT1). The interaction of substrates with PCNA<sup>DNA</sup> recruits the CRL4<sup>CDT2</sup> E3 ubiquitin ligase via the CDT2 substrate receptor subunit for replication-coupled destruction. *B*, HCT116 cells were synchronized in early S phase by double thymidine block. Soluble proteins were extracted, and bound proteins were fixed in early S phase (during the arrest) or in late S phase 7 h after release into fresh medium. Endogenous PCNA<sup>DNA</sup> and CDT1 were detected by immunostaining. *C* and *D*, as in *B* except that p21 or SET8 was detected by immunostaining. Scale bars, 5 μm in *B–D*. *E*, percentage of late S phase cells containing PCNA<sup>DNA</sup> that also contained SET8 ( $n = 60$ ), p21 ( $n = 100$ ), or CDT1 ( $n = 122$ ) was quantified. *F*, total PCNA nuclear fluorescence was quantified for the early and late S phase samples in *B* ( $n = 15$ ). A total of 60 cells were counted over three biological replicates ( $\geq 15$  cells were counted per replicate). Averages and S.D. values (error bars) are indicated in both *E* and *F*.

CRL4<sup>CDT2</sup> are protected from degradation in a cell cycle-dependent manner.

**Mitotic Kinase Activity Is Required for Protection from CRL4<sup>CDT2</sup>-mediated Degradation**—Our observation that CRL4<sup>CDT2</sup> substrates are stable from late S phase through mitosis but susceptible to replication-coupled destruction during G<sub>1</sub> suggested that a mitotic activity, such as one or more mitotic protein kinases, confers protection from CRL4<sup>CDT2</sup>-mediated degradation. To test that possibility, we employed pharmacological inhibition of candidate kinases: cyclin-dependent kinases (CDKs) and the stress-activated mitogen-activated protein kinases (MAPK) p38 and JNK, which we previously showed

could stabilize CRL4<sup>CDT2</sup> substrates (39). We briefly treated prometaphase cells with kinase inhibitors prior to UV irradiation and then tested for reversal of mitotic stability by immunoblotting (Fig. 3, *A* and *B*). In control cells as before, CRL4<sup>CDT2</sup> substrates were stable in mitotic cells, but strikingly, treatment with the CDK1-specific inhibitor RO-3306 effectively reversed the protection (Fig. 3, *A* (compare lanes 4 and 6) and *B* (compare lanes 4 and 5)). In contrast, treatment with the p38 or JNK inhibitors had little to no effect on substrate protection (Fig. 3*B*, lanes 6 and 7). Note that both p38 and JNK are not only active in mitosis but also induced by UV (45, 46); the JNK and p38 inhibitors were effective for inhibiting their respective kinases at

## Inhibition of CRL4<sup>CDT2</sup>



**FIGURE 2. CRL4<sup>CDT2</sup> substrates are protected in mitosis.** A and B, HCT116 cells were grown asynchronously (Async.) or synchronized in prometaphase by release from a thymidine block into 100 nM nocodazole for 10 h (M). Cells were irradiated with 20 J/m<sup>2</sup> UV to induce PCNA<sup>DNA</sup> loading (DNA repair synthesis) and harvested 2 h later or left untreated as indicated. Endogenous proteins were detected in whole cell lysates by immunoblotting (asterisk in A denotes a nonspecific background band). C, HCT116 cells were synchronized in prometaphase as in A and released into fresh medium for 2.5 h to proceed to G<sub>1</sub>. Cells were UV-irradiated and harvested 90 min later, followed by immunoblotting for the indicated proteins.

these concentrations, as measured by phosphorylation of representative substrates (Fig. 3B, bottom panels). CDK1 inhibition also caused cyclin B1 degradation (Fig. 3B, lanes 5 and 8), which is consistent with prior studies showing that inhibiting CDK1 in mitotic cells activates APC<sup>Cdc20</sup> (47, 48). (SET8 is also an APC<sup>Cdc20</sup> target, but SET8 phosphorylation during mitosis blocks APC<sup>Cdc20</sup> binding, so SET8 was not degraded in cells treated with the CDK1 inhibitor alone (23).) Importantly, only CDK1 inhibition resensitized CRL4<sup>CDT2</sup> substrates in mitosis (Fig. 3B, compare lane 4 with lanes 5–7). Even simultaneous inhibition of p38 and JNK could not substitute for CDK1 inhibition (data not shown). Furthermore, increasing concentrations of the CDK1 inhibitor resulted in progressive loss of CRL4<sup>CDT2</sup> substrate protection (Fig. 3C).

We confirmed that reacquisition of UV-induced degradation in CDK1-inhibited cells was still CUL4-dependent by co-treatment with the neddylation inhibitor, MLN4924 (Fig. 3D, compare lanes 6 and 8). Together, these data indicate that CDK1 activity is required for the protection of CRL4<sup>CDT2</sup> substrates from replication-coupled destruction.

**CDK1 Activity Is Required for Substrate CRL4<sup>CDT2</sup> Reaccumulation in Late S Phase**—To understand the relationship of CDK1 activity in late S phase cells to the kinetics of CRL4<sup>CDT2</sup> substrate reaccumulation, we treated synchronized cells in mid-S phase (5 h post-thymidine release) with the CDK1 inhibitor. We then monitored both cell cycle progression and the anticipated reaccumulation of CDT1 and SET8 as S phase completed (Fig. 4, A and B). Treatment with the CDK1 inhibitor, RO-3306, in asynchronous cells results in a G<sub>2</sub> arrest (49), and

as expected, it blocked cells released from early S synchronization, but CDK1 inhibition did not prevent S phase completion (Fig. 4A). However, CDK1 inhibition strongly and reproducibly dampened the normal late S and G<sub>2</sub> increase in both SET8 and CDT1 protein levels (Fig. 4B, compare lanes 5–8 with lanes 10–13). The levels of cyclin A and cyclin B1 at the 7 and 8 h time points corresponding to late S and G<sub>2</sub> phases were unperturbed by the inhibitor, but at the normal time of mitosis (at 9 h), CDK1 inhibition caused premature cyclin B1 loss, consistent with results in nocodazole-arrested cells (Figs. 3B (lane 5) and 4B (lanes 9–11)). Importantly, these effects on cyclin B1 levels were evident 2 h after the delay in CDT1 and SET8 reaccumulation.

We once again examined individual late S phase cells (7 h postrelease) for the presence of both PCNA<sup>DNA</sup> and SET8 or CDT1. In a substantial proportion of control PCNA<sup>DNA</sup>-positive cells, SET8 and CDT1 had already reaccumulated, similar to Fig. 1E (Fig. 4C). When CDK1 was inhibited starting at 5 h post-S phase release, however, the percentage of double positive cells decreased nearly 2-fold (Fig. 4C). Because the effects of CDK1 inhibition on CRL4<sup>CDT2</sup> substrate accumulation occurred well in advance of mitosis, CDK1 activity inhibits CRL4<sup>CDT2</sup> not only in prometaphase-arrested cells but also during the transition from S phase into G<sub>2</sub> and mitosis.

**CRL4<sup>CDT2</sup> Activity Is Itself Inhibited**—Our prior work provided evidence for stabilization of CDT1 by direct phosphorylation as cells transit from S phase to M phase (39). Furthermore, our study and that of Kim *et al.* (50) suggested that a similar direct p21 phosphorylation mechanism could control p21 stability. To determine whether protection from CRL4<sup>CDT2</sup>-mediated degradation during late cell cycle stages is solely the consequence of direct substrate phosphorylation, we expressed epitope-tagged WT p21 (HA-p21-WT) or p21 harboring alanine substitutions at the two phosphorylation sites that were reported to stabilize p21, Thr-57 and Ser-130 (HA-p21-AA) (50). In synchronized cells, both HA-p21-WT and HA-p21-AA (as well as endogenous p21) migrated slower by SDS-PAGE compared with asynchronous cells, and this mobility shift was reversed by phosphatase treatment (Fig. 5A, lanes 5 and 10). The HA-p21-AA mutant migrated faster than WT HA-p21 in lysates of mitotic cells but was just as stable as both ectopic and endogenous WT p21 (Fig. 5A, compare lanes 4 and 8). Residual sites of p21 phosphorylation affect p21 gel mobility but have not been shown to affect p21 stability (51) (reviewed in Ref. 52). Nevertheless, mitotic phosphorylation of p21 at Thr-57 and Ser-130 is not the principal reason p21 is stable in mitotic cells.

We similarly expressed epitope-tagged versions of WT CDT1 and CDT1 harboring five alanine substitutions (CDT1-5A) at the mitotic phosphorylation sites we had previously shown to stabilize CDT1. Specifically, we had shown that CDT1-5A is not protected from CRL4<sup>CDT2</sup> by stress MAPK activation (39). These cells were then subjected to UV irradiation during asynchronous culture or after synchronization in prometaphase. Both endogenous CDT1 and epitope-tagged WT CDT1 were protected in synchronized cells (Fig. 5B, compare lanes 6 and 8), and as expected, both WT proteins migrated slower by SDS-PAGE compared with asynchronous cells. CDT1-5A migrated slightly faster than WT CDT1 from

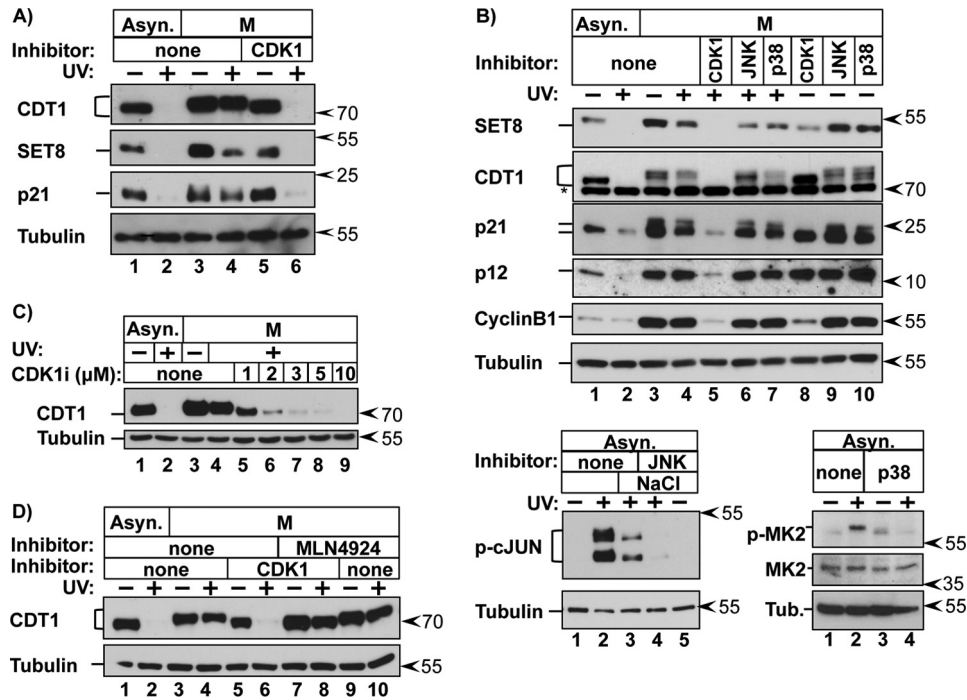


FIGURE 3. **CDK1 activity is required for CRL4<sup>CDT2</sup> substrate protection.** *A*, asynchronous or prometaphase HCT116 cells were treated with the CDK1 inhibitor RO-3306 as indicated 30 min prior to PCNA<sup>DNA</sup> induction by UV and harvested 2 h later. *B*, *top panel*, asynchronous or prometaphase cells were treated with the indicated kinase inhibitors or DMSO (*none*) for 30 min prior to PCNA<sup>DNA</sup> induction by UV irradiation. Endogenous proteins were detected by immunoblotting of whole cell lysates. *Bottom panels*, asynchronous cells were treated with the inhibitor JNK VIII or SB203580 (p38 inhibitor) 30 min prior to treatment with 250 mM NaCl and/or UV irradiation, as indicated. (Hyperphosphorylated c-JUN migrates significantly slower than the partially phosphorylated form). *C*, asynchronous or prometaphase HCT116 cells were treated with the indicated concentrations of RO-3306 for 30 min prior to UV irradiation and harvested 1 h later. *D*, asynchronous or prometaphase cells were untreated (–) or treated with UV (+) to induce PCNA<sup>DNA</sup> and harvested 1 h after treatment. The CDK1 inhibitor RO-3306 and/or the CUL4 neddylation inhibitor MLN4924 (5 μM) were added 30 min prior to UV irradiation as indicated.

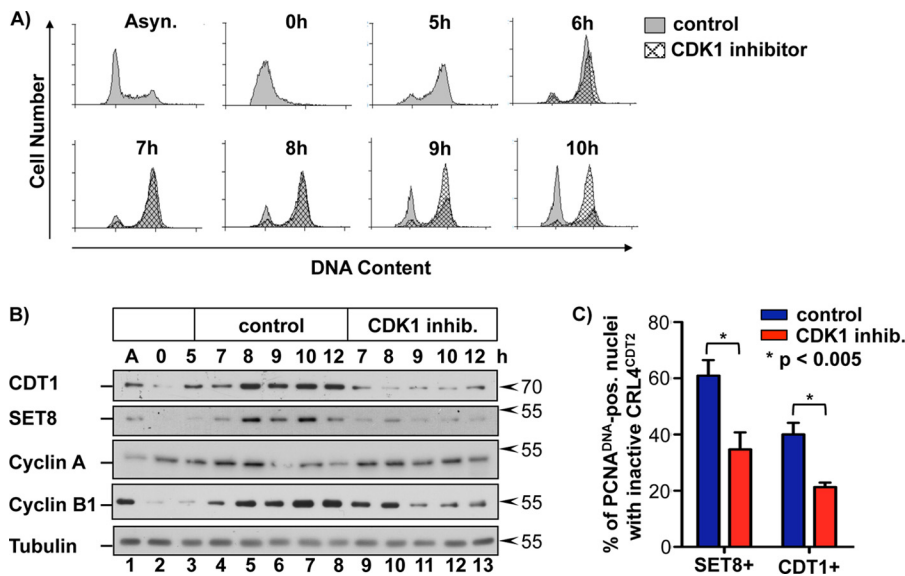


FIGURE 4. **CDK1 activity is required for substrate reaccumulation in late S phase.** *A*, HCT116 cells were synchronized in early S phase via double thymidine block and 5 h after release were treated with RO-3306 or DMSO (control). Cells were harvested at the indicated time points and analyzed for DNA content by flow cytometry. *B*, HCT116 cells were treated as in *A*, harvested at the indicated time points, and analyzed via immunoblotting. *C*, HCT116 cells were treated as in *A*. Soluble proteins were extracted, and bound proteins were fixed in early S phase (during the arrest) or in late S phase 7 h after release into fresh medium. Endogenous PCNA<sup>DNA</sup>, SET8, and CDT1 were detected by immunostaining as in Fig. 1. Late S phase cells containing PCNA<sup>DNA</sup> were scored for the presence of SET8 or CDT1 as an indicator of inactive CRL4<sup>CDT2</sup> ( $n \geq 75$  for each sample). Error bars, S.D.

lysates of mitotic cells, consistent with our prior analysis of this mutant. The clear residual mitotic shift of this mutant is consistent with additional phosphorylation sites in CDT1 that may or may not affect its stability (53–55). Like the p21-AA mutant, CDT1–5A was just as stable as both ectopic and endogenous

CDT1 in prometaphase cells, indicating that phosphorylation at these sites previously shown to stabilize CDT1 is not solely responsible for CDT1 mitotic stability (Fig. 5*B*, lanes 9–12). Together, these observations indicate that protection from CRL4<sup>CDT2</sup> probably involves more than phosphorylation of the



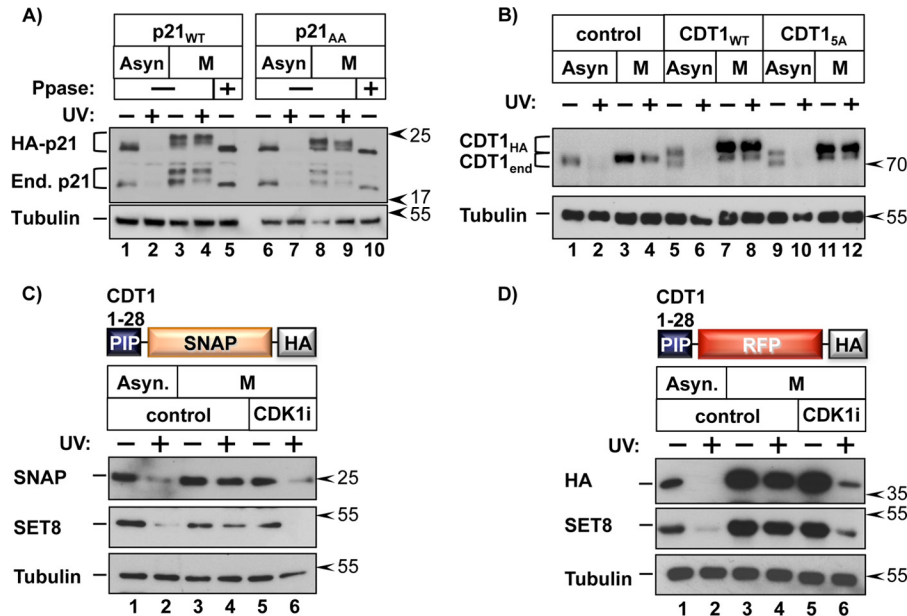


FIGURE 5. **CDK1-dependent inhibition of CRL4<sup>CDT2</sup> activity.** A, HCT116 cells stably expressing HA-tagged WT or p21-AA (T57A and S130A mutations) were grown asynchronously or arrested in prometaphase. Cells were harvested 2 h after PCNA<sup>DNA</sup> induction by UV irradiation; cell lysates were treated with  $\lambda$ -phosphatase (*Ppase*) prior to SDS-PAGE, as indicated. B, HeLa cells stably expressing HA-tagged WT or CDT1-5A (39) were grown asynchronously or arrested in prometaphase. Cells were harvested 1 h after PCNA<sup>DNA</sup> induction by UV. C, HeLa cells stably expressing the illustrated PIP degnon-SNAP fusion were grown asynchronously or arrested in prometaphase and treated with the CDK1 inhibitor RO-3306 or control DMSO 30 min prior to PCNA<sup>DNA</sup> induction by UV irradiation; cells were harvested 1 h after UV. D, as in C except with HeLa cells stably expressing a PIP degnon-RFP (mCherry) fusion.

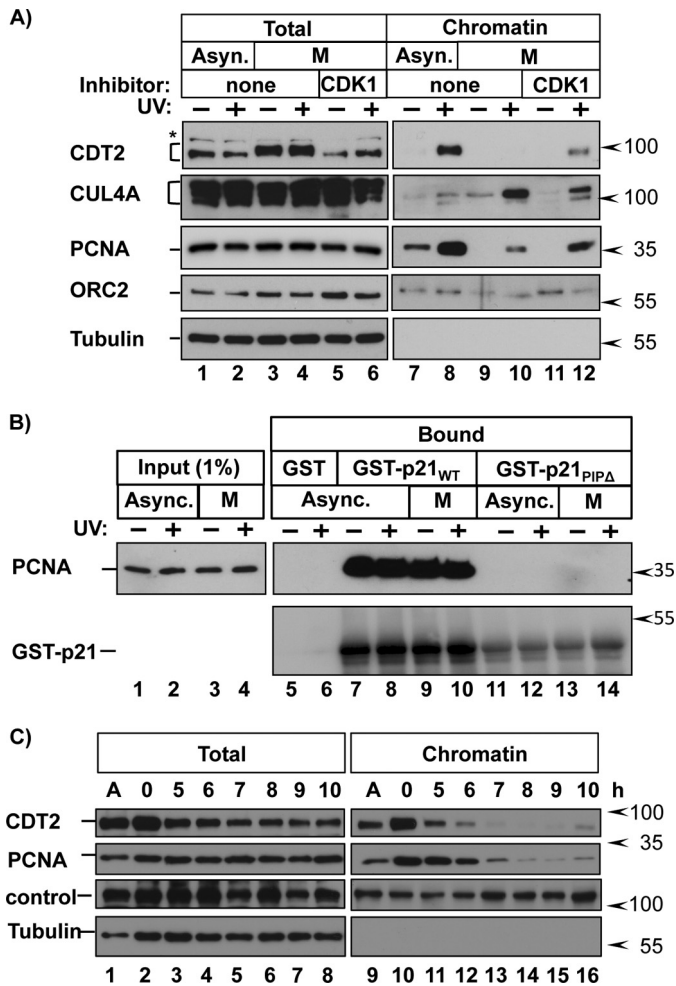
substrates themselves. Moreover, the fact that all four of the tested CRL4<sup>CDT2</sup> substrates were resistant to degradation suggests that CRL4<sup>CDT2</sup> is globally suppressed during progression from late S phase into mitosis.

To probe the activity of CRL4<sup>CDT2</sup> itself in mitosis, we designed two reporter constructs for stable expression in which the N-terminal 28 amino acids of CDT1 were fused to either red fluorescent protein (RFP) or the SNAP tag (56). The N-terminal 28 amino acids of CDT1 contain the PIP degnon and was previously shown to confer CUL4-dependent degradation to a heterologous protein (57). Importantly, neither reporter construct contains canonical CDK or MAPK phosphorylation sites. In asynchronously growing cells, the SNAP reporter fusion was degraded following UV irradiation (Fig. 5C, lanes 1 and 2); however, in prometaphase cells, the reporter was stable (Fig. 5C, lanes 3 and 4). Furthermore, CDK1 inhibition resensitized the SNAP reporter to degradation (Fig. 5C, lanes 5 and 6), indicating that regulation of this reporter is similar to endogenous CRL4<sup>CDT2</sup> substrates. We observed nearly identical CDK1-dependent stabilization of the RFP reporter (Fig. 5D). These findings indicate that the ability of CRL4<sup>CDT2</sup> to target substrates is generally inhibited via a CDK1-dependent event independently of any additional phosphorylation-mediated mechanisms of direct substrate stabilization.

**CDK1 Activity Prevents Chromatin Association of CDT2—**During productive CRL4<sup>CDT2</sup> targeting, PCNA is first loaded onto DNA for replication (S phase) or repair (e.g. post-UV). Proteins containing PIP degnons bind to PCNA<sup>DNA</sup>, and the resulting complex is then recognized by the substrate receptor, CDT2 (58) (illustrated in Fig. 1A). We postulated that one or more of these steps is inhibited by CDK1. We first tested whether PCNA can be successfully loaded onto mitotic chro-

matin by UV-irradiating nocodazole-synchronized cells and then analyzing chromatin fractions. We detected PCNA in chromatin fractions from untreated asynchronous cells (reflecting the S phase fraction; Fig. 6A, lane 7), and PCNA<sup>DNA</sup> was further induced by UV irradiation, which stimulates PCNA loading in all cell cycle phases (Fig. 6A, lane 8). As reported previously, CUL4A is also recruited to chromatin following UV irradiation (59–61), and as expected, a resident chromatin protein, ORC2, is unaffected (Fig. 6A). In otherwise untreated mitotic cells, PCNA was not DNA-associated (as expected) but was loaded following UV irradiation (Fig. 6A, lane 10). Thus, mitotic chromatin is not *intrinsically* resistant to PCNA loading. In addition, PCNA from extracts of mitotic and asynchronous cells showed similar binding to recombinant p21 (but not a p21 PIP box mutant) *in vitro* (Fig. 6B). In stark contrast to PCNA, CDT2 could not be inducibly recruited to mitotic chromatin, although it was readily recruited to chromatin in asynchronously growing cells (Fig. 6A, compare lanes 8 and 10). Finally, CDK1 inhibition restored CDT2 chromatin recruitment in mitotic cells (Fig. 6A, compare lanes 10 and 12). Thus, the CDK1-dependent inhibition of CRL4<sup>CDT2</sup>-mediated degradation operates by interfering with chromatin recruitment of the CDT2 substrate receptor. Consistent with this finding, we noted that CDT2 is lost from chromatin fractions in late S phase earlier than PCNA itself (Fig. 6C, lanes 11–13) coincident with the normal reaccumulation time points for CDT1 and SET8 (e.g. Figs. 1 (B and D) and 4B).

**De Novo SET8 Reaccumulation Is Essential for Normal Mitotic Progression—**Having demonstrated active inhibition of CRL4<sup>CDT2</sup> targeting as cells progress from S phase to M phase, we next considered the possible biological significance of this mechanism. Replication-coupled destruction via CRL4<sup>CDT2</sup>



**FIGURE 6. CDK1 activity prevents CDT2 chromatin recruitment.** *A*, HCT116 cells were grown asynchronously or synchronized in prometaphase. Cells were treated with DMSO (*none*) or the CDK1 inhibitor RO-3306 for 30 min prior to PCNA<sup>DNA</sup> induction by UV irradiation and harvested 90 min later. Endogenous proteins in whole cell extracts and chromatin-bound fractions were analyzed by immunoblotting. *B*, GST, GST-p21<sub>WT</sub>, or GST-p21<sub>PIPΔ</sub> was produced in *E. coli* and bound to glutathione beads. The protein-coated beads were then incubated with whole cell lysates from asynchronous or mitotic cells as described previously (39). Endogenous PCNA and GST-tagged p21 were detected by immunoblotting. *C*, HCT116 cells were synchronized in early S phase via double thymidine block and released into fresh medium. Cells were harvested at the indicated time points. Endogenous proteins in whole cell extracts and chromatin-bound fractions were analyzed by immunoblotting.

during S phase ensures a single round of replication through the destruction of both CDT1 and SET8; their abnormal persistence in S phase leads to repeated rounds of origin relicensing and rereplication (1, 3). On the basis of these facts alone, it should be detrimental to actively accumulate proteins like CDT1 and SET8 prior to the subsequent G<sub>1</sub>. However, CDT1 also has an essential function in mitosis that we recently documented (21), thus providing incentive for high levels of CDT1 before mitosis. In addition, sufficient levels of p21 are required in mitosis for normal mitotic progression (22). Given that all CRL4<sup>CDT2</sup> substrates follow a similar pattern of mitotic stabilization, we postulated that CDT1 is not the only substrate needed in abundance and *de novo* at the end of S phase, and we turned our attention to SET8. Depleting SET8 from asynchronous cells leads to multiple defects, including not only ineffi-

cient origin licensing in G<sub>1</sub> but also impaired chromatin condensation in mitosis. Thus far, these phenotypes have all been attributed to the loss of SET8-deposited histone H4K20me1 (3, 23, 62). However, it is not yet known whether the mitotic defects from SET8 depletion are due to the absence of SET8 specifically during G<sub>2</sub> and mitosis or if they arise *secondarily* from the absence of SET8 in a prior cell cycle phase, such as G<sub>1</sub>. It is possible that SET8 activity deposits the essential methylations for chromosome condensation during G<sub>1</sub>, making SET8 dispensable during G<sub>2</sub>.

To determine whether SET8 is required specifically *during* transit from late S phase into mitosis, we employed a synchronization-knockdown procedure we developed during the investigation of the mitotic function of CDT1. We synchronized cells in early S phase after the G<sub>1</sub> function of SET8 had been fully completed and released them in the presence of either control or SET8 siRNA to proceed otherwise unperturbed through S phase (Fig. 7A). SET8 siRNA effectively prevented accumulation of both SET8 and monomethylation of histone H4K20 after S phase (Fig. 7B, compare lanes 2–7 with lanes 8–13). Of note, SET8 depletion also caused a reduction in H4K20me2, indicating that establishment of histone H4K20me1 during late S phase is required to maintain H4K20me2 levels during G<sub>2</sub> and mitosis. H4K20me1 has recently been shown to be required for proper kinetochore assembly (63). Our data clearly support this finding and further indicate that this deposition occurs during G<sub>2</sub>/M as SET8 reaccumulates (Fig. 7B).

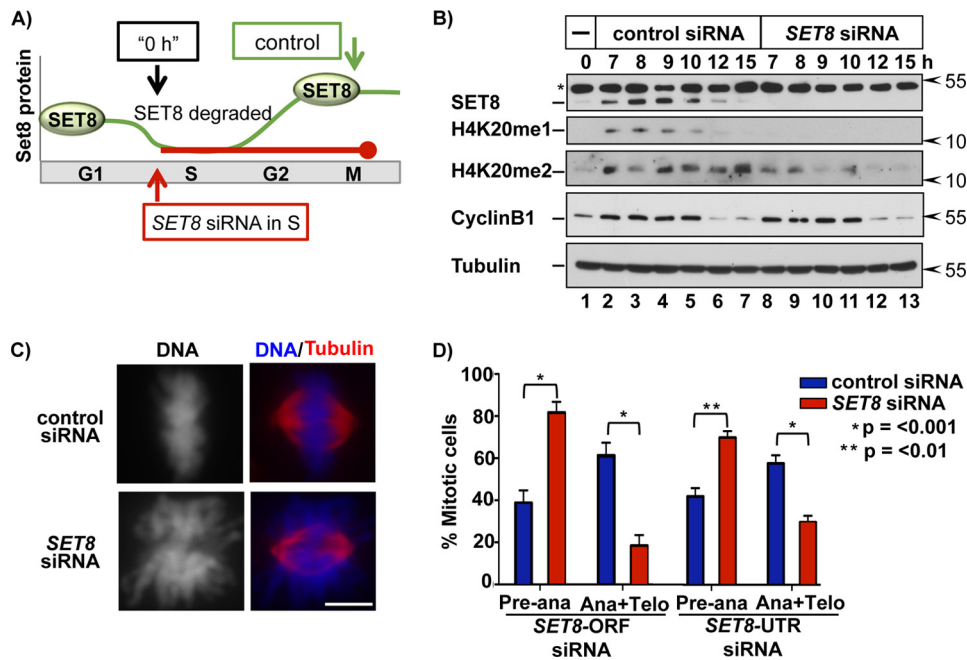
We examined mitotic chromosome morphology in the synchronized SET8-depleted cells by staining for DNA with DAPI and for the mitotic spindle with anti-tubulin. Control cells analyzed 10 h after release from the early S phase block contained metaphase chromosomes that were fully condensed and properly aligned at the metaphase plate (Fig. 7C, *top panel*). In contrast, chromosomes in cells that had normal SET8 in G<sub>1</sub> but contained little to no SET8 only in G<sub>2</sub> were less dense and formed a “cloud” of DNA that failed to align properly in metaphase (Fig. 7C, *bottom panels*). We replicated both the failure to reaccumulate normal H4K20me1 and the mitotic chromosome condensation defects using a different SET8 siRNA in synchronized cells (Fig. 7D). Furthermore, we quantified mitotic progression 10 h after thymidine release; cells with normal SET8 in G<sub>1</sub> but blocked from reaccumulating SET8 in late S/G<sub>2</sub> were significantly enriched in preanaphase states with a corresponding decrease in cells that had completed anaphase at 10 h (Fig. 7D). These data highlight the importance of SET8 reaccumulation and, by extension, general CRL4<sup>CDT2</sup> inhibition prior to mitosis for proper chromosome condensation and mitotic progression. Thus, CDK1 activity in late S phase and G<sub>2</sub> phase is required for the normally robust rebound in the levels of CRL4<sup>CDT2</sup> substrates, and the reaccumulation (of CDT1 and SET8) is important for proper mitotic progression.

## DISCUSSION

At least three substrates (CDT1, p21, and SET8) have known roles in mitosis (21–23, 64), and it is possible that other CRL4<sup>CDT2</sup> substrates also have mitotic roles, making general substrate stabilization important for mitosis. Independent of these mitotic roles, the known substrates of replication-cou-



## Inhibition of CRL4<sup>CDT2</sup>



**FIGURE 7. De novo SET8 reaccumulation after S phase is necessary for normal mitotic progression.** *A*, illustration of the experimental approach to block SET8 reaccumulation only after S phase. *B*, HCT116 cells were synchronized in early S phase via double thymidine block. Cells were released into fresh medium containing control (luciferase) or SET8-ORF siRNA and harvested at the indicated time points for immunoblotting of the indicated endogenous proteins (\*, a nonspecific background band). *C*, cells were synchronized as in *A* and then released into siRNA-containing medium for 10 h prior to fixation and immunofluorescence staining for tubulin and DAPI staining for DNA. Scale bar, 5  $\mu$ m. *D*, the number of mitotic cells from *C* in preanaphase versus anaphase/telophase was quantified in three biological replicates ( $n = 300$  for each replicate) using two different siRNAs as indicated. Error bars, S.E.

pled destruction via CRL4<sup>CDT2</sup> are proteins with clear roles in G<sub>1</sub> or S phase. For example, the early step of origin licensing, minichromosome maintenance complex recruitment to chromatin, begins in telophase (65, 66). Thus, with respect to origin licensing, once the nuclear envelope reforms in telophase, the daughter nuclei are G<sub>1</sub>-like nuclei in a shared cytoplasm. Because telophase occurs just 2–3 h after the end of S phase in cultured somatic cells, active inhibition of replication-coupled destruction may also facilitate G<sub>1</sub> events, a notion that has been suggested regarding CDT1 accumulation (67–69). Based on our findings, this need for early accumulation may apply to the entire cohort of CRL4<sup>CDT2</sup> substrates.

PCNA<sup>DNA</sup> is an efficient and potent trigger for replication-coupled destruction. The bulk of CDT1 can be degraded within minutes of UV irradiation (39) and occurs nearly simultaneously with S phase onset (1, 12, 70). The potency of PCNA<sup>DNA</sup> then presents a challenge near the end of S phase because CRL4<sup>CDT2</sup> substrates are needed in abundance for mitosis and the subsequent G<sub>1</sub>. (Although CDT1 can also be targeted by CRL1<sup>SKP2</sup> (54, 57, 70), we have noted in multiple cell lines that CRL4<sup>CDT2</sup> is the major regulator of CDT1 degradation in S phase (39).) Replication of at least 1% of the genome occurs as late as 90 min prior to the onset of mitosis (71), and this late replication requires the continued presence of PCNA<sup>DNA</sup>. Instead of a long G<sub>2</sub> phase following complete PCNA<sup>DNA</sup> unloading to allow buildup of essential mitotic proteins, including those that are actively degraded at the onset of S phase, G<sub>2</sub> can be short because that buildup begins during late S phase. We detected significant amounts of CRL4<sup>CDT2</sup> substrates in the same cells that still contained nearly as much PCNA<sup>DNA</sup> as they did at the start of S phase. Our findings indicate that CDK1

itself or a CDK1-dependent activity overrides the ability of PCNA<sup>DNA</sup> to stimulate CRL4<sup>CDT2</sup> recruitment for replication-coupled destruction. The first active CDK1 complex that appears contains cyclin A, and this form of CDK1 may be responsible for initiating CRL4<sup>CDT2</sup> inhibition; later inhibition would be accomplished via cyclin B-CDK1. In keeping with this idea, pharmacological CDK1 inhibition blunted CRL4<sup>CDT2</sup> substrate reaccumulation at time points that coincide with the expected time of cyclin A-CDK1 activity (Fig. 4B). Interestingly, depleting cyclin A in early S phase-arrested embryonic stem cells prevented accumulation of CDT1 8 h after release (72).

It is clear that the ultimate target(s) of CDK1 (either direct or indirect) is neither PCNA<sup>DNA</sup> nor PCNA<sup>DNA</sup>-substrate interactions but rather the subsequent chromatin recruitment of CDT2 (illustrated in Fig. 8). This inhibition is distinct from the recently reported SCF-dependent CDT2 degradation and underscores the importance of regulating the CDT2 substrate receptor for cell cycle control (43, 44). In fact, phosphorylation of CDT2 during S phase promotes interaction with 14-3-3 proteins that protect CDT2 from this SCF-mediated degradation (73). We and others have noted extensive mitotic phosphorylation of CDT2 in mitotic cells, and human CDT2 bears 19 conserved Ser/Thr-Pro sites, but it is currently unknown if phosphorylation at these sites affects CDT2 substrate interactions. We have found, however, that CDT2 binding to CRL4 is unaffected in mitosis (data not shown). Given the number of potential mitotic proline-directed kinases, the number of potential CDT2 phosphorylation sites, and observations that other members of the CRL4<sup>CDT2</sup> complex (CUL4A/B and Roc1) are phosphorylated in mitosis (74, 75), further studies are required to define the mechanism of CRL4<sup>CDT2</sup> inhibition.

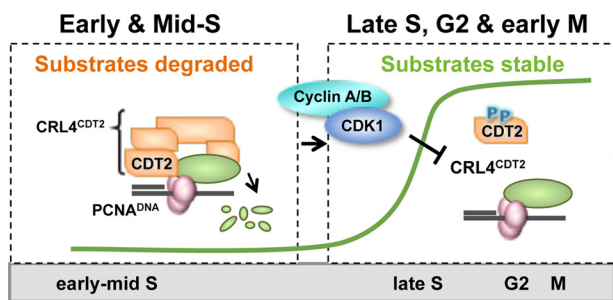


FIGURE 8. **Model.** CRL4<sup>CDT2</sup> substrates are efficiently degraded in early S phase as a result of PCNA loading onto DNA (PCNA<sup>DNA</sup>). Beginning in late S phase, active CDK1 blocks substrate-mediated chromatin recruitment of the CDT2 receptor, allowing reaccumulation of the cohort of CRL4<sup>CDT2</sup> substrates that were subject to replication-coupled destruction. Many of these substrates are required for normal mitotic progression.

Because the details of how CDT2 contacts the substrate-PCNA<sup>DNA</sup> remain unknown, we cannot yet explain how a CDK1-dependent event blocks this interaction. Importantly, however, CDT2 chromatin recruitment could conceivably be achieved by any PIP degraon-PCNA complex in sufficient abundance; we infer that not only is the interaction between CDT2 and the substrates tested here inhibited, but the interaction between CDT2 and any as yet undiscovered substrates is also inhibited. For example, two recent reports identified thymine DNA glycosylase as a target of CRL4<sup>CDT2</sup> in *Xenopus* and human cells (6, 7). The behavior of our two reporter substrates and accumulated findings herein suggest that thymine DNA glycosylase is also protected via CDK1.

Substrates of replication-coupled destruction accumulate beginning in late S phase and are abundant in mitosis. In contrast, experimental manipulations that stabilize the substrates CDT1 or SET8 throughout S phase cause severe genome damage from origin relicensing and refiring (1, 10–12, 14, 37, 76). If the continued presence of either CDT1 or SET8 during S phase is toxic, how then do G2 cells tolerate the high levels of both CDT1 and SET8 prior to mitosis? In other words, why do the high levels of CDT1 and SET8 not trigger rereplication in late S/G<sub>2</sub>/M? Replication-coupled destruction is only one of many mechanisms that prevent rereplication, but interestingly, many of the other mechanisms are not fully in place until late in S phase. For example, the CDT1 inhibitor geminin is induced in S phase, but its levels are substantially lower in early S phase than in late S phase (77, 78). Other mechanisms, such as the degradation of the largest subunit of the origin recognition complex, Orc1, and the nuclear export of the CDC6 licensing protein are also most evident in middle to late S phase (reviewed in Ref. 24). Further, cyclin A-CDK2 (middle to late S phase) is a better inhibitor of origin licensing than cyclin E-CDK2 (early to middle S phase) (79). In addition, we recently provided evidence that the mitotic phosphorylation of CDT1 not only stabilizes CDT1 but also inhibits its licensing activity (39). Of note, these additional mechanisms impacting CRL4<sup>CDT2</sup> substrate targeting probably fine tune the kinetics of individual substrate degradation and reaccumulation. Therefore, in early S phase, while both geminin and cyclin A are low, we postulate that replication-coupled destruction of CDT1 and SET8 is most critical, but by late S phase, it is “safe” for CDT1 and SET8 to accumu-

late to high levels because the many other mechanisms that block origin relicensing are by then fully established.

The anticipatory accumulation of proteins and activities prior to the cell cycle phase in which they are needed is well established. For example, a series of mid-G<sub>1</sub> events, including CDC6 accumulation, results in replication origin licensing during late G<sub>1</sub> that is in turn essential for S phase execution (80). Likewise, cyclin B1 accumulation in G<sub>2</sub> occurs in advance of M phase. Moreover, the concept of a checkpoint that prevents a cell cycle transition until the preceding one is complete is also well known. We have explored here a variation on these themes in which an essential regulatory mechanism (replication-coupled destruction) is actively inhibited immediately before and during the next cell cycle phase (prior to and during M phase). In contrast to a checkpoint that blocks the transition to the next phase, this mechanism blocks regulation of the current phase so that the next phase can be launched efficiently. Reaccumulation of CRL4<sup>CDT2</sup> targets to their maximal levels in anticipation of their requirement primes cells for subsequent cell cycle events. As such, our discovery of CDK1-mediated inhibition of CRL4<sup>CDT2</sup> adds a new dimension to a general cell cycle theme: proactive preparation for cell cycle phase launch.

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