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CRL4^{Cdt2}-Mediated Destruction of the Histone Methyltransferase Set8 Prevents Premature Chromatin Compaction in S Phase

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SUMMARY

The proper coordination between DNA replication and mitosis during cell-cycle progression is crucial for genomic stability. During G2 and mitosis, Set8 catalyzes monomethylation of histone H4 on lysine 20 (H4K20me1), which promotes chromatin compaction. Set8 levels decline in S phase, but why and how this occurs is unclear. Here, we show that Set8 is targeted for proteolysis in S phase and in response to DNA damage by the E3 ubiquitin ligase, CRL4^{Cdt2}. Set8 ubiquitylation occurs on chromatin and is coupled to DNA replication via a specific degron in Set8 that binds PCNA. Inactivation of CRL4^{Cdt2} leads to Set8 stabilization and aberrant H4K20me1 accumulation in replicating cells. Transient S phase expression of a Set8 mutant lacking the degron promotes premature H4K20me1 accumulation and chromatin compaction, and triggers a checkpoint-mediated G2 arrest. Thus, CRL4^{Cdt2}-dependent destruction of Set8 in S phase preserves genome stability by preventing aberrant chromatin compaction during DNA synthesis.

INTRODUCTION

DNA replication and other cell-cycle events, such as replication origin licensing in G1 and chromatin condensation in mitosis, are carefully coordinated to maintain genomic stability. The process of DNA replication is coupled with several other events, including chromatin assembly, sister-chromatid cohesion, ubiquitylation of specific cell-cycle regulators, activation of the DNA replication checkpoint, and DNA repair. Recent studies showed that the CRL4^{Cdt2} E3 ubiquitin ligase, which functions in a replication-coupled manner through binding to PCNA, plays a critical role in coordinating origin licensing in G1 and DNA replication in S phase (Jin et al., 2006; Kim et al., 2008; Lovejoy et al., 2006; Sansam et al., 2006; Zhong et al., 2003). To understand whether CRL4^{Cdt2} has additional roles in coordinating DNA replication with other cell-cycle events, we sought to identify additional CRL4^{Cdt2} substrates.

The CRL4^{Cdt2} E3 ligase complex is comprised of the scaffold protein Cul4, the adaptor protein Ddb1, and the putative substrate receptor protein Cdt2 (Angers et al., 2006; Higa et al., 2006; Jin et al., 2006; Sansam et al., 2006). The best-characterized substrate of CRL4^{Cdt2} is the "licensing" factor Cdt1, which is required to recruit the MCM2-7 complex to replication origins in G1. During DNA replication, Cdt1 binds to PCNA through a PCNA-interacting protein motif (PIP box), and is degraded on chromatin in a PCNA- and CRL4^{Cdt2}-dependent manner (Arias and Walter, 2005, 2006; Jin et al., 2006; Nishitani et al., 2006; Sansam et al., 2006; Senga et al., 2006). This replication-coupled mechanism for Cdt1 degradation ensures that fired replication origins cannot be relicensed in the same S phase. The CRL4^{Cdt2}-mediated degradation of Cdt1 occurs not only in S phase, but also after DNA damage (Higa et al., 2003, 2006; Hu et al., 2004; Hu and Xiong, 2006; Jin et al., 2006; Sansam et al., 2006; Senga et al., 2006). When it is bound to PCNA on chromatin, the PIP box of Cdt1 is presented as a degron and recognized by CRL4^{Cdt2}. Our analysis of the PIP degron of Cdt1 has identified three sequence elements critical for binding to PCNA and Cdt2, which are conserved among known CRL4^{Cdt2} substrates (Havens and Walter, 2009).

In a genome-wide search for PIP degron-containing proteins, we identified Set8 (KMT5A/PR-Set7/SETD8) as a potential substrate of CRL4^{Cdt2}. Set8 is the methyltransferase that monomethylates histone H4 on lysine 20 (H4K20me1) (Fang et al., 2002; Nishioka et al., 2002). Loss of Set8 in human, mouse, or *Drosophila* cells results in massive DNA damage during S phase and improper chromosome condensation in mitosis (Houston et al., 2008; Huen et al., 2008; Jorgensen et al., 2007; Karachentsev et al., 2005; Oda et al., 2009; Paulsen et al., 2009; Sakaguchi and Steward, 2007; Tardat et al., 2007). During the cell cycle, Set8 is most abundant during G2 and mitosis, and low during S phase (Huen et al., 2008; Oda et al., 2009; Yin et al., 2008). Concomitant with the elevation of its abundance in the G2 and M phases, Set8 promotes a transient accumulation of

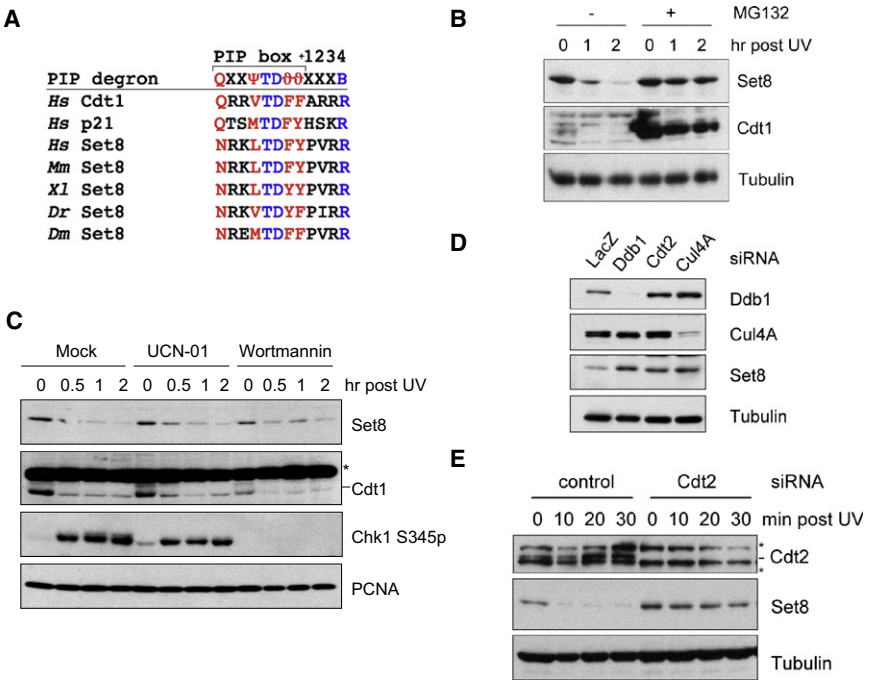

Figure 1. Set8 Is Degraded by CRL4^{Cdt2} in Response to DNA Damage

(A) Sequence alignment of the PIP degrons of human Cdt1, p21, and Set8 from different species. Ψ is any moderately hydrophobic amino acid L, V, I, or M. ϑ is an aromatic residue, Y or F. B is a positively charged residue, R or K. Red residues are conserved in the PIP boxes, and the blue residues are only conserved in the PIP degrons.

(B) UV-induced Set8 degradation is 26S proteasome dependent. U2OS cells were treated with 50 J/m^2 UV, and the levels of endogenous Set8, Cdt1, and tubulin were monitored at the indicated times by western blotting. Where indicated, 10 μM MG132 was added to cells 5 hr prior to irradiation.

(C) UV-induced Set8 degradation is independent of ATM, ATR, and Chk1. U2OS cells were treated with 1 μM UCN-01 (Chk1 inhibitor) or 100 μM wortmannin (PI3KK inhibitor) for 1 hr prior to UV irradiation. Inhibition of Chk1 phosphorylation serves as a positive control for wortmannin activity. Note that UCN-01 does not inhibit the phosphorylation of Chk1 by ATR, but prevents the mobility change of Chk1 indicative of Chk1 autophosphorylation.

(D) CRL4^{Cdt2} downregulates Set8 protein levels. U2OS cells were transfected with control siRNA or siRNAs targeting Ddb1, Cdt2, and Cul4A. The levels of the indicated proteins were monitored by western blotting.

(E) UV-induced Set8 degradation is Cdt2 dependent. U2OS cells transfected with Cdt2 siRNA or control siRNA were treated with UV followed by 100 μg/ml cycloheximide. The levels of the indicated proteins were analyzed in a time course. In all panels, asterisk labels nonspecific bands recognized by the indicated antibodies.

H4K20me1 (Houston et al., 2008; Huen et al., 2008; Oda et al., 2009; Rice et al., 2002). H4K20me1, which promotes chromatin compaction, may contribute to proper mitosis and impact the subsequent S phase (Houston et al., 2008; Oda et al., 2009; Sakaguchi and Steward, 2007; Trojer et al., 2007). While Set8 has a clear role in methylating H4K20 during mitosis, why and how it is downregulated during S phase is not clear. Interestingly, in the presence of proteasome inhibitors, Set8 is readily detected in S phase cells, and it colocalizes with the DNA replication protein PCNA (Huen et al., 2008; Jorgensen et al., 2007; Tardat et al., 2007). Furthermore, Set8 contains two PIP boxes that contribute to its binding to PCNA (Huen et al., 2008; Jorgensen et al., 2007).

In this study, we show that Set8 is degraded by a CRL4^{Cdt2}-mediated mechanism during S phase and in response to DNA damage. The degradation of Set8 relies on its PIP degron and its interactions with both PCNA and Cdt2. This mechanism of Set8 degradation is observed not only in human cells but also in *Xenopus* egg extracts. In the cell-free *Xenopus* system, Set8 is ubiquitylated on chromatin and destroyed in a PCNA-, Cdt2-, and PIP degron-dependent manner. Ablation of CRL4^{Cdt2} in human cells leads to stabilization of endogenous Set8 and aberrant accumulation of H4K20me1 in S phase cells. When a stabilized PIP box mutant of Set8 is expressed in S phase cells, it induces premature H4K20me1 accumulation and chromatin compaction, and triggers a checkpoint-mediated G2 arrest. We propose that CRL4^{Cdt2}-mediated degradation of Set8 prevents H4K20me1 accumulation during S phase, thereby preventing premature chromatin compaction that interferes with genome duplication. The replication-coupled downregulation of Set8 is a critical mechanism that defines the functional window of Set8 during the cell cycle, contributing to the orderly execution of DNA replication and mitosis.

RESULTS

Set8 Is Downregulated by CRL4^{Cdt2} in Cycling Cells and in Response to DNA Damage

Our recent studies revealed three sequence elements that are conserved in known CRL4^{Cdt2} substrates and together comprise a "PIP degron" (Figure 1A): one is a canonical PIP box that is essential for PCNA binding, another is a TD motif at positions 5 and 6 of the PIP box that confers high-affinity binding to PCNA, and the third is a basic residue located four amino acids downstream of the PIP box that is important for Cdt2 recruitment to the substrate-PCNA complex on chromatin (Havens and Walter, 2009). To identify additional substrates of the CRL4^{Cdt2} E3 ligase, we searched the Swiss-Prot database using ExPASy ScanProsite with the PIP degron consensus motif (Q/N-x-x-L/V/I/M-T-D-F/Y-F/Y-x-x-x-K/R). Like Cdt1 and p21, two known CRL4^{Cdt2} substrates (Abbas et al., 2008; Kim et al., 2008; Nishitani et al., 2008), Set8 was identified in this screen. Notably, the PIP degron sequence in Set8 was perfectly conserved across a wide range of metazoan organisms (Figure 1A). This finding prompted us to investigate whether the stabilities of Set8 and Cdt1 were similarly regulated in cells. Both Set8 and Cdt1 are downregulated during S phase, and Cdt1 is degraded in response to DNA damage (Higa et al.,

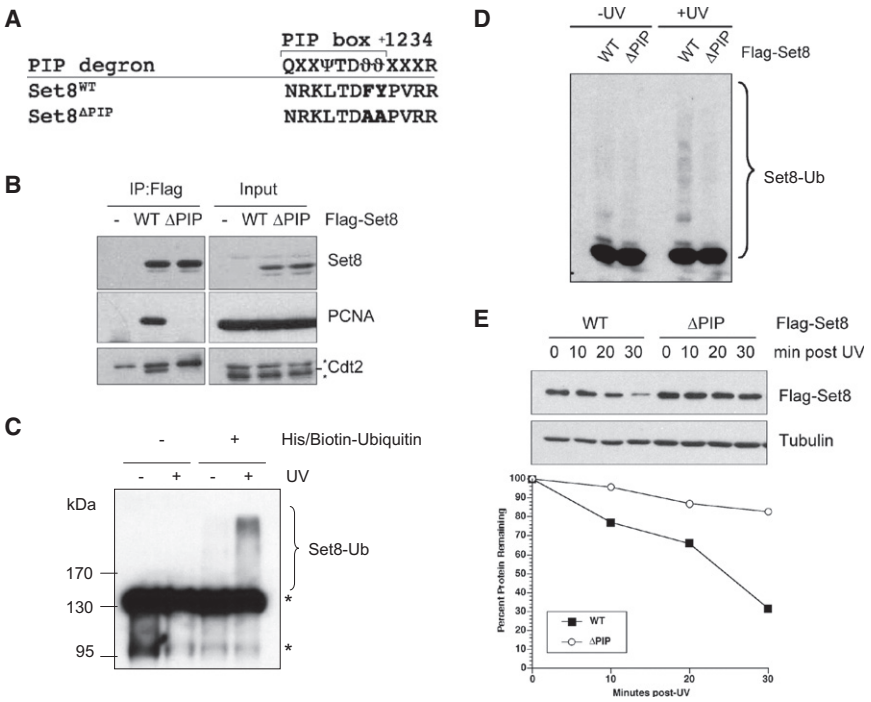

Figure 2. UV-Induced Set8 Degradation Is Dependent on Its PIP Degron

(A) The Set8$^{\Delta PIP}$ mutant lacks the two conserved aromatic residues in the putative PIP degron.

(B) The Set8$^{\Delta PIP}$ mutant is defective in binding to PCNA and Cdt2. Flag-tagged Set8^{WT} and Set8$^{\Delta PIP}$ were transiently expressed in U2OS cells and immunoprecipitated with anti-Flag antibody. The Set8, PCNA, and Cdt2 proteins in the immunoprecipitates and input extracts (2%) were analyzed by western blotting.

(C) Set8 ubiquitylation is induced by UV. HeLa cells stably expressing His/biotin-tagged ubiquitin and control HeLa cells were treated with UV or left untreated. Ubiquitylated proteins were captured with streptavidin beads under denaturing condition. Ubiquitylated Set8 was detected with Set8 antibody. Asterisk indicates nonspecific proteins bound to streptavidin beads and cross-reacted with Set8 antibody.

(D) UV-induced Set8 ubiquitylation requires the PIP degron. Cells expressing Flag-tagged Set8^{WT} or Set8$^{\Delta PIP}$ were synchronized in G1, pretreated with MG132 for 3 hr, and irradiated with UV. The Set8 proteins were analyzed with anti-Flag antibody 2 hr post-UV treatment.

(E) The Set8$^{\Delta PIP}$ mutant is more stable than Set8^{WT} after UV damage. U2OS cells with induced Flag-tagged Set8^{WT} or Set8$^{\Delta PIP}$ were treated with UV, and cultured in cycloheximide-containing media for the indicated times. The levels of Flag-Set8 and tubulin were analyzed by western blotting.

2003; Hu et al., 2004). A previous study showed that Set8 levels were reduced after DNA damage due to transcription repression (Shi et al., 2007). We found that in U2OS cells treated with 50 J/m^2 UV, the level of endogenous Set8 rapidly declined within 1 hr (Figure 1B, lanes 1–3). Furthermore, the UV-induced reduction of Set8 was prevented by MG132, suggesting that Set8 is degraded by the proteasome in response to DNA damage (Figure 1B). The DNA damage-induced degradation of Cdt1 is independent of the checkpoint kinases ATM and ATR (Higa et al., 2003). Analogously, wortmannin, an inhibitor of ATM and ATR, and UCN-01, an inhibitor of the ATR effector kinase Chk1, had no effect on the UV-induced degradation of Set8 (Figure 1C). These results show that Set8, like Cdt1, is degraded in a DNA damage-induced manner independently of ATM and ATR.

To address whether Set8 is a substrate of the CRL4^{Cdt2}, we used siRNA to knock down the components of this E3 ubiquitin ligase. Compared to cells treated with control siRNA, cells treated with siRNAs targeting Cdt2, Ddb1, or Cul4A exhibited elevated levels of Set8 (Figures 1D and see Figure S1A available online). In contrast, knockdown of Ddb2, a substrate receptor of the distinct CRL4^{Ddb2} E3 ligase, did not affect Set8 levels (Figure S1B). Cdt2 siRNA did not affect the level of Set8 mRNA (Figure S1C), excluding the possibility that the increase in Set8 protein is due to transcriptional changes. In cells treated with Cdt2 or Ddb1 siRNA, the UV-induced degradation of Set8 was significantly reduced as compared to that in control cells (Figures 1E and Figure S1D). Taken together, these results suggest that the CRL4^{Cdt2} E3 ligase downregulates the overall level of Set8 in an asynchronous cell population and mediates its degradation in response to DNA damage.

UV-Induced Set8 Degradation Is PIP Degron Dependent

To assess whether the putative PIP degron of Set8 is required for its degradation, we disrupted the degron using point mutations. The two conserved aromatic residues in the PIP degron of Cdt1 are essential for its binding to PCNA (Arias and Walter, 2006; Havens and Walter, 2009). To determine whether the corresponding residues in the PIP degron of Set8 are functionally important, we generated a Set8 point mutant lacking these residues (F184A, Y185A; referred to as Set8$^{\Delta PIP}$) (Figure 2A).

We first tested whether wild-type and mutant Set8 proteins are able to associate with PCNA and Cdt2. Immunoprecipitation of Flag-tagged Set8^{WT} captured both PCNA and Cdt2, showing that Set8 can interact with these proteins (Figure 2B). Consistent with a previous report (Jorgensen et al., 2007), Set8$^{\Delta PIP}$ failed to associate with PCNA (Figure 2B), confirming that this PIP box of Set8 is critical for PCNA binding. Moreover, Cdt2 did not coprecipitate with Set8$^{\Delta PIP}$, suggesting that the PIP box is also needed for Cdt2 binding. These results suggest that the PIP degron of Set8, like that of Cdt1, is required for its interactions with both PCNA and Cdt2. It should be noted that Cdt1 interacts with Cdt2 only in the presence of DNA-bound PCNA (Havens and Walter, 2009). Thus, the interaction between Flag-Set8 and Cdt2 reported here might also be mediated by PCNA and DNA.

We next asked if the PIP degron of Set8 is required for its degradation in response to DNA damage. In cells

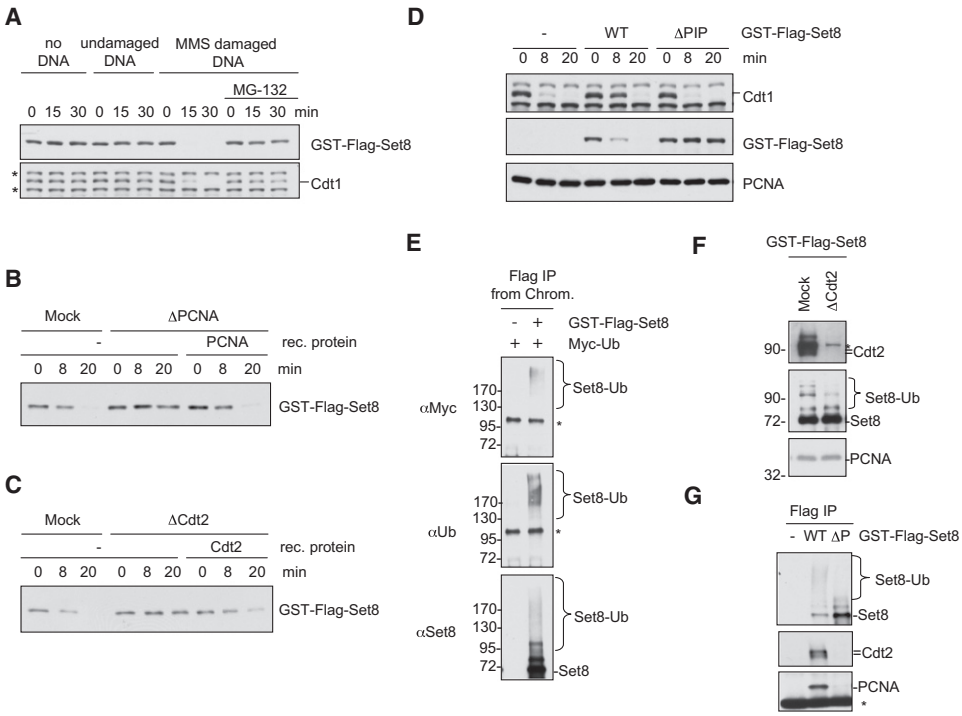

Figure 3. CRL4^{Cdt2}-Dependent and DNA Damage-Induced Set8 Degradation in *Xenopus* Egg Extracts

(A) DNA damage and proteasome-dependent destruction of Set8 in *Xenopus* egg extract. HSS was supplemented with human 50 nM GST-FLAG-Set8, as well as buffer (no DNA), undamaged plasmid, MMS-damaged plasmid, and MG132, as indicated, and at different times, samples were blotted for GST (top panel) or Cdt1 (bottom panel).

(B) Recombinant GST-FLAG-tagged human Set8 (50 nM) was added to mock-depleted or PCNA-depleted HSS that was optionally supplemented with 5 μM recombinant human PCNA. At the different times, samples were blotted for GST.

(C) Recombinant GST-Flag-tagged human Set8 (50 nM) was added to mock-depleted or Cdt2-depleted HSS that was optionally supplemented with 25 nM recombinant human Cdt2, as indicated. At different times, samples were blotted for GST.

(D) HSS was supplemented with MMS-treated plasmid, as well as buffer, 50 nM GST-Flag-tagged Set8^{WT}, or Set8$^{\Delta PIP}$. At different times, samples were blotted for the indicated proteins.

(E) HSS was supplemented with 1 mg/ml Myc-Ubiquitin and MG132, as well as buffer or 50 nM GST-Flag-tagged Set8^{WT}, as indicated. Immobilized, 1 kb MMS DNA was added and after 10 min, chromatin was recovered from the extract. Chromatin-bound proteins were denatured to release them from chromatin then diluted and immunoprecipitated with Flag antibody. The isolated material was blotted for Set8 (bottom panel), Ubiquitin (middle panel), or Myc (top panel).

(F) Immobilized, 1 kb MMS DNA was isolated from mock-depleted or Cdt2-depleted HSS, each of which was supplemented with 2 mg/ml methyl ubiquitin and 50 nM GST-Flag-tagged Set8 and incubated for 10 min. Samples were blotted for Cdt2, Set8, and PCNA.

(G) Methyl ubiquitin, GST-Flag-tagged Set8^{WT} or Set8$^{\Delta PIP}$, and 5 ng/μl MMS plasmid DNA were added to HSS. Set8^{WT}, Set8$^{\Delta PIP}$, and their associating proteins were immunoprecipitated from total extract with Flag antibody, and the indicated proteins were analyzed by western blotting. Asterisk indicates a protein nonspecifically recognized by PCNA antibody.

expressing His/biotin-tagged ubiquitin, endogenous Set8 was ubiquitylated and captured by streptavidin beads in a UV-induced manner (Figure 2C). Furthermore, Flag-tagged Set8^{WT}, but not Set8$^{\Delta PIP}$, underwent enhanced ubiquitylation after UV treatment (Figure 2D). When transiently expressed in cells, Set8^{WT} was less stable than Set8$^{\Delta PIP}$ after UV irradiation (Figure S2). To more precisely measure the effects of PIP box mutations on Set8 stability, we generated inducible cell lines that express Flag-tagged Set8^{WT} or Set8$^{\Delta PIP}$. We induced the Set8 proteins to similar levels, treated cells with UV and cycloheximide, and monitored the stabilities of the Set8 proteins in a time course. Set8$^{\Delta PIP}$ was more stable than Set8^{WT} after UV irradiation (Figure 2E). These data suggest that UV-induced Set8 ubiquitylation and degradation require the PIP degron of Set8.

CRL4^{Cdt2} Mediates Set8 Degradation in *Xenopus* Egg Extracts

To examine Set8 destruction in a biochemically tractable system, we turned to *Xenopus* egg extracts, which recapitulate the DNA replication and damage-dependent degradation of Cdt1 by CRL4^{Cdt2} (Jin et al., 2006). As shown in Figure 3A, Cdt1 was rapidly destroyed in a high-speed supernatant (HSS) of *Xenopus* egg cytoplasm supplemented with plasmid DNA that had been damaged with methyl methanesulfonate (MMS) (Figure 3A, bottom panel) (Jin et al., 2006). Under the same experimental conditions, GST-Flag-tagged human Set8 was also rapidly degraded (Figure 3A, top panel). As seen for Cdt1, Set8 degradation was induced by MMS-treated DNA, but not by undamaged DNA or in the absence of DNA (Figure 3A), showing that this is a DNA damage-dependent process.

Depletion of PCNA or Cdt2 efficiently prevented DNA damage-induced destruction of Set8 (Figures 3B and 3C and Figure S3A). In each case, Set8 degradation was rescued by reconstitution of the extracts with the corresponding recombinant protein (Figures 3B and 3C). Furthermore, Set8$^{\Delta PIP}$ was completely stable in egg extracts (Figure 3D, middle panel). Therefore, human Set8 is degraded in *Xenopus* egg extracts in a manner that depends on its PIP degron, PCNA, Cdt2, and DNA damage.

When Set8^{WT} was added to HSS, it bound to chromatin and recruited CRL4^{Cdt2} (Figure S3B). Moreover, Set8 was ubiquitylated on the chromatin (Figures 3E), but this was reduced when Cdt2 was depleted from HSS (Figure 3F). In contrast to Set8^{WT}, Set8$^{\Delta PIP}$ did not bind chromatin efficiently (Figure S3B), failed to recruit Cdt2 or Ddb1 to chromatin above background levels (Figures S3C), and was not efficiently ubiquitylated (Figure 3G and Figure S3C). Together, these results indicate that, like Cdt1, Set8 docks onto chromatin-bound PCNA, recruits CRL4^{Cdt2}, and then undergoes ubiquitylation. Interestingly, addition of Set8^{WT} but not Set8$^{\Delta PIP}$ to HSS resulted in decreased ubiquitylation of Cdt1 (Figure S3C), suggesting a competition between the two CRL4^{Cdt2} substrates.

Set8 Is Degraded during S Phase in a CRL4^{Cdt2}-Dependent Manner

Having established that Set8 is a substrate of CRL4^{Cdt2} following DNA damage, we next investigated whether Set8 is targeted for destruction by CRL4^{Cdt2} during DNA replication, as suggested by the elevated Set8 levels observed after silencing of CRL4^{Cdt2} components in unperturbed populations of asynchronous cells (Figure 1D). We first synchronized cells in S phase with hydroxyurea (HU), and then briefly induced expression of Flag-Set8^{WT}. Once Flag-Set8^{WT} became readily detectable, we stopped Set8 induction and released cells from HU. As cells resumed DNA replication after the release from HU, the levels of Flag-Set8^{WT} rapidly declined (Figure 4A; FACS profiles shown in Figure S4A). This decline of Set8^{WT} was inhibited by MG132 (Figure 4A), suggesting that Set8 is actively degraded by the proteasome in replicating cells. Consistently, endogenous Set8 was rapidly degraded in S phase cells synchronously released from a thymidine block (Figure 4B). Furthermore, Set8^{WT} was also rapidly degraded during chromosomal DNA replication in *Xenopus* egg extracts (Figure 4C). Geminin, which blocks replication initiation via inhibition of Cdt1 function, prevented Set8^{WT} destruction in this setting, demonstrating that Set8 is degraded in a replication-dependent manner in *Xenopus* egg extracts.

To test if the degradation of Set8 during DNA replication was mediated by CRL4^{Cdt2}, we monitored the effect of Cdt2 knockdown on the stability of endogenous Set8. Knockdown of Cdt2 but not Ddb2 significantly stabilized Set8 in replicating cells (Figure 4B). To determine whether the PIP degron of Set8 is needed for its degradation in S phase, we compared the stabilities of Set8^{WT} and Set8$^{\Delta PIP}$ during DNA replication. In *Xenopus* egg extracts, the degradation of Set8 during DNA replication was dramatically inhibited by mutations in the PIP degron (Figure 4C). In human cells synchronously released from HU, Set8$^{\Delta PIP}$ was more stable than Set8^{WT} (Figure S4B). Nonetheless, we noted that in replicating human cells, Set8$^{\Delta PIP}$ was still degraded at a slow rate even when Cdt2 was knocked down (Figures S4B and S4C), suggesting that a PIP degron- and Cdt2-independent mechanism also contributes to Set8

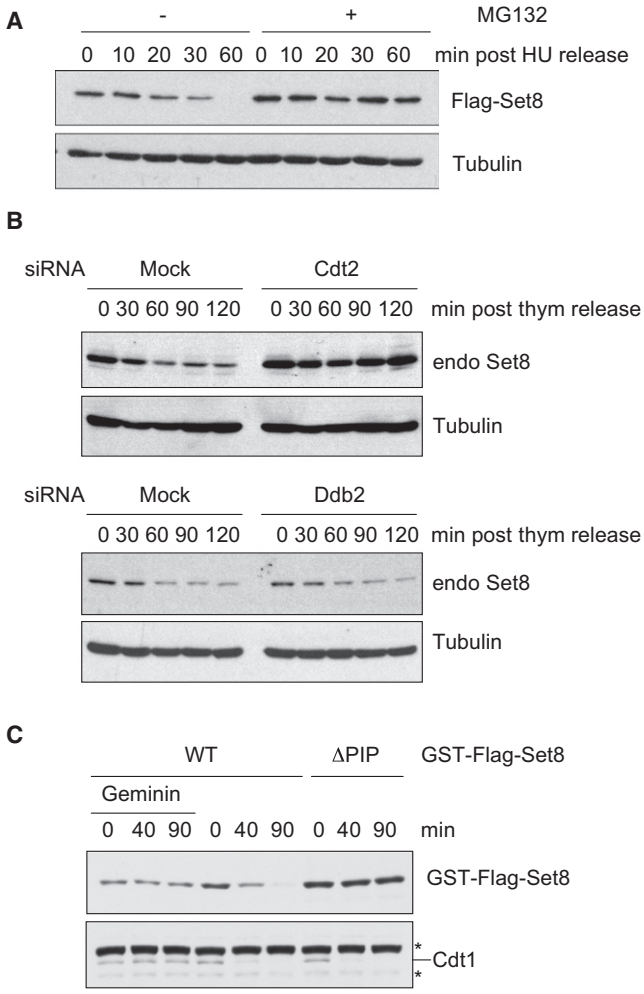

Figure 4. PIP Degron-Mediated Set8 Degradation during DNA Replication

(A) Set8 is actively degraded by the proteasome in replicating cells. Cells harboring inducible Flag-Set8 were synchronized in S phase with 1 mM HU for 24 hr. During the last 4 hr of HU treatment, Flag-Set8 was induced, and 10 μM MG132 was added when indicated. Subsequently, cells were released into HU-free and cycloheximide-containing media with or without MG132. Set8 levels were analyzed in a time course by western blotting.

(B) Endogenous Set8 is stabilized by knockdown of Cdt2, but not Ddb2. Cells transfected with siRNAs targeting Cdt2 or Ddb2 and cells mock transfected were synchronized with thymidine and released into cycloheximide-containing media. The levels of endogenous Set8 were analyzed at the indicated time points by western blotting.

(C) Set8 is degraded in *Xenopus* egg extracts in a replication- and PIP degron-dependent manner. Sperm chromatin was incubated with HSS for 30 min to promote replication licensing. Subsequently, a highly concentrated nucleoplasmic extract (Walter et al., 1998) containing GST-Flag-tagged Set8^{WT} or Set8$^{\Delta PIP}$ was added, which stimulated efficient replication initiation (data not shown). At different times after NPE addition, the reactions were stopped and samples were blotted for GST and Cdt1. In lanes 1–3, HSS was incubated with 200 nM Geminin before addition of sperm chromatin.

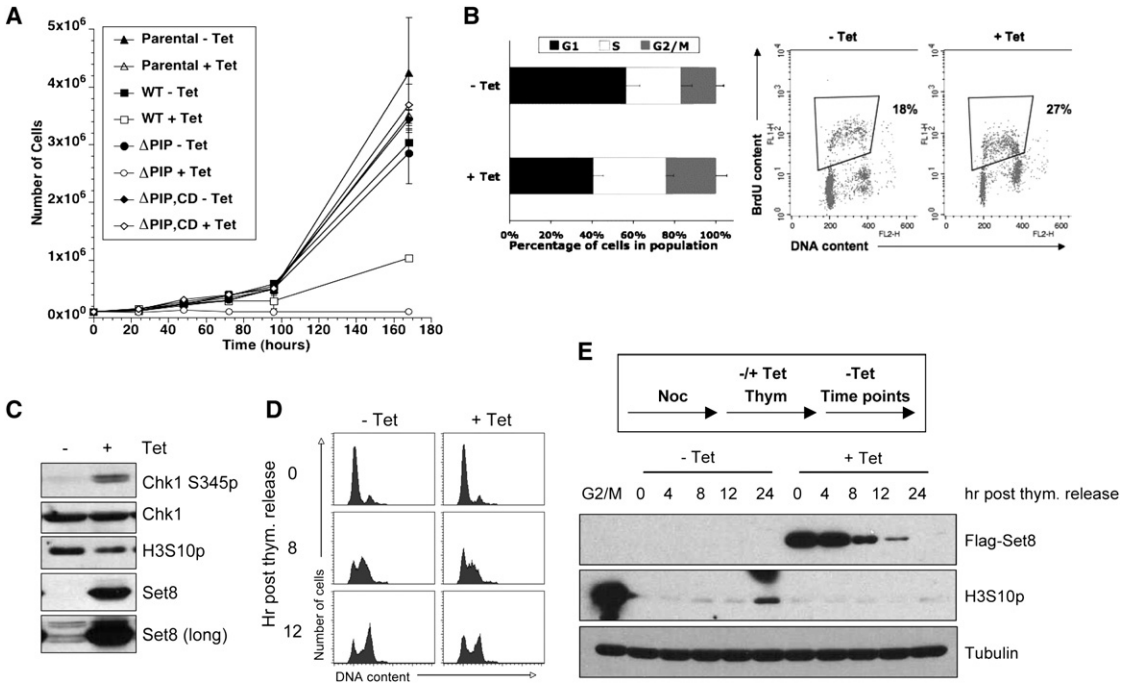

Figure 5. Forced Expression of Set8 Interferes with the Cell Cycle

(A) Forced Set8 expression slows cell proliferation. U2OS cells harboring inducible Flag-tagged Set8^{WT}, Set8$^{\Delta PIP}$, Set8$^{\Delta PIP,CD}$, or the parental cell line were cultured in the absence or presence of 0.1 μg/ml tetracycline (Tet). The total cell numbers at the indicated time points were plotted. Error bars indicate the standard deviation of three independent experiments.

(B) Constitutive Set8 expression leads to reduced DNA synthesis and accumulation of cells in G2/M. Cells were cultured for 48 hr with or without Set8 induction. Cell-cycle profiles (left panel) and BrdU incorporation (right panel) were analyzed by FACS. Error bars indicate the standard deviation of three independent experiments.

(C) Constitutive expression of Set8 elicits the ATR checkpoint. Cells were cultured for 48 hr with or without Set8 induction. The levels of Set8, phospho-Chk1 (Ser345), Chk1, and phospho-H3 (Ser10) were analyzed by western blotting.

(D and E) Overexpression of Set8 prior to S phase inhibits mitotic entry. Cells were synchronized in mitosis with 100 ng/ml nocodazole and then released into media containing 2 mM thymidine. Where indicated, Set8 was induced after the release from nocodazole. Cells synchronized at G1/S were then released from thymidine in the absence of Tet. Cell-cycle profiles are shown in (D), and protein levels of Flag-Set8, phospho-H3, and tubulin were analyzed by western blotting in (E).

destruction (see the Discussion). Taken together, these results suggest that CRL4^{Cdt2} plays an important role in repressing Set8 levels during S phase.

Forced Expression of Set8 Induces Replication Stress

To understand why Set8 is downregulated during S phase, we sought to override this mechanism. When Set8^{WT} was constitutively expressed at high levels, cell proliferation gradually slowed down (Figure 5A). FACS analysis of cells expressing Set8^{WT} revealed an increase in the population of S and G2/M phase cells (Figure 5B, left panel). BrdU labeling confirmed that the population of replicating cells was increased upon Set8^{WT} induction (Figure 5B, right panel). Moreover, for cells that were in S phase based on DNA content, the incorporation of BrdU was clearly less efficient when Set8^{WT} was induced relative to control cells (Figure 5B, right panel). These results suggest that constitutive Set8 expression interferes with DNA synthesis, leading to accumulation of cells in S phase and G2/M.

The reduction in DNA synthesis and accumulation of cells in S and G2/M prompted us to investigate whether the ATR-mediated replication checkpoint was activated by forced Set8^{WT} expression. Indeed, when Set8^{WT} was induced for 48 hr in asynchronous cells, the level of phospho-Chk1 (Ser345), a marker of ATR activation, was significantly increased (Figure 5C). Furthermore, consistent with compromised DNA replication and/or checkpoint-mediated cell-cycle arrest prior to mitosis, the levels of phospho-H3 were reduced (Figure 5C). These results provide further evidence that aberrant expression of Set8 interferes with proper DNA replication and activates the ATR checkpoint.

Since Set8 is known to function in mitosis, overexpression of Set8 may affect M phase and indirectly impact the subsequent S phase. To rule out this possibility, we synchronously released cells from a nocodazole block in mitosis, transiently induced Set8 expression, and arrested cells at the G1/S transition with thymidine. When cells were released into S phase with high levels of Set8, they progressed through S phase more slowly, as shown by FACS analysis (Figures 5D). Furthermore, cells with induced Set8 did not accumulate phospho-H3 (Ser10), indicating a failure to enter mitosis (Figure 5E). These results suggest that forced expression of Set8 prior to S phase interferes with DNA replication and prevents timely entry into mitosis.

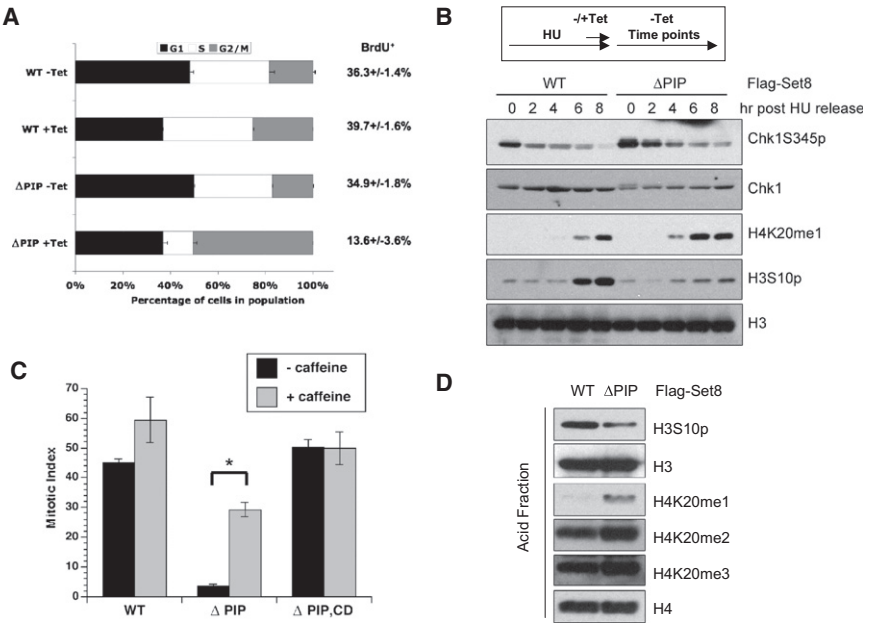

Figure 6. Expression of the Set8$^{\Delta PIP}$ Mutant Induces Premature H4K20me1 Accumulation

(A) Constitutive expression of the Set8$^{\Delta PIP}$ mutant leads to a dramatic loss of S phase cells and accumulation of G2/M cells. Shown are cell-cycle profiles and BrdU incorporation of cells expressing Set8^{WT} or Set8$^{\Delta PIP}$ for 48 hr. Error bars indicate the standard deviation of three independent experiments.

(B–D) Expression of the ΔPIP mutant prevents mitotic entry after release from HU arrest. In (B), cells harboring inducible Set8^{WT} or Set8$^{\Delta PIP}$ were synchronized in S phase with HU for 24 hr. During the last 4 hr in HU, expression of Set8^{WT} or Set8$^{\Delta PIP}$ was induced. Subsequently, cells were released from HU in the absence of Tet, and the levels of the indicated proteins in whole-cell extracts were analyzed by western blotting. In (C), Set8$^{\Delta PIP,CD}$ cells were included. Experiment was performed as in (B), except that cells were released into media containing nocodazole to trap mitotic cells. Where indicated, caffeine was added at 5 mM to bypass ATM/ATR-mediated checkpoint response. Twenty-four hours after HU release, phospho-H3-positive mitotic cells were scored by FACS. Error bars indicate standard deviation, and asterisk indicates $p < 0.005$ by Student's t test. In (D), experiment was performed as in (B), except that 4 hr after HU release, chromatin-bound histones were extracted with acid and analyzed by western blotting.

The Set8$^{\Delta PIP}$ Mutant Is a Potent Inhibitor of the Cell Cycle

Although induction of wild-type Set8 interferes with the cell cycle, this approach may not fully capture the effects of Set8 stabilization because even the induced Set8^{WT} is rapidly degraded (Figure 4). To more specifically address the role of CRL4^{Cdt2}-mediated Set8 degradation, we induced expression of Set8^{WT} and Set8$^{\Delta PIP}$ and compared their effects on the cell cycle. Compared to Set8^{WT}, Set8$^{\Delta PIP}$ induced a much more prominent increase in G2/M cells (Figure 6A). Furthermore, expression of Set8$^{\Delta PIP}$ dramatically reduced the BrdU-positive cells, suggesting that cells were trapped outside of S phase (Figure 6A and Figure S5A). Consistent with its effects on the cell cycle, Set8$^{\Delta PIP}$ reduced cell proliferation even more dramatically than Set8^{WT} (Figure 5A). Thus, Set8 is a potent inhibitor of the cell cycle when the PIP degron is disrupted.

To determine whether the effects of Set8$^{\Delta PIP}$ on the cell cycle are dependent upon its catalytic activity, we generated a catalytically "dead" Set8$^{\Delta PIP,\ CD}$ double mutant (Nishioka et al., 2002). In marked contrast to Set8$^{\Delta PIP}$, Set8$^{\Delta PIP,\ CD}$ did not induce a G2/M arrest (Figure 6C), nor did it inhibit cell proliferation (Figure 5A). These results show that Set8$^{\Delta PIP}$ remains catalytically active in cells, and that its activity is necessary for the induction of cell-cycle arrest.

The Set8$^{\Delta PIP}$ Mutant Triggers a Checkpoint-Mediated G2 Arrest

The loss of cells in S phase and accumulation of cells in G2/M indicates that Set8$^{\Delta PIP}$ may interfere with DNA replication and/or the G2/M transition. To assess this possibility, we synchronized cells in S phase with double-thymidine block or HU, briefly induced Set8^{WT} or Set8$^{\Delta PIP}$, and then terminated the induction and released the cells. As cells resumed DNA replication, the levels of phospho-Chk1 gradually declined (Figure 6B and Figure S5B). In cells with Set8$^{\Delta PIP}$, Chk1 was phosphorylated to higher levels in thymidine and HU, and phospho-Chk1 persisted for longer after cells resumed replication (Figure 6B and Figure S5B). Despite the persistent Chk1 phosphorylation, cells with Set8$^{\Delta PIP}$ progressed through S phase without obvious delay (data not shown). However, both FACS and western blotting analyses showed that while cells with Set8^{WT} gradually entered mitosis and accumulated phospho-H3 over time, cells with Set8$^{\Delta PIP}$ failed to accumulate significant levels of this mitotic mark (Figure 6B and Figures S5B and S5C). Thus, when briefly expressed in S phase cells, Set8$^{\Delta PIP}$ does not arrest replication but leads to a robust G2 arrest.

The modest but persistent Chk1 phosphorylation induced by Set8$^{\Delta PIP}$ during S phase suggests that although DNA replication can proceed, the process is not normal. Although the effects of Set8$^{\Delta PIP}$ are not sufficient to halt replication, they may contribute to the subsequent G2 arrest. Indeed, in the presence of caffeine, an inhibitor of ATM and ATR, the G2 arrest induced by Set8$^{\Delta PIP}$ was significantly bypassed (Figure 6C). Importantly, the catalytically inactive Set8$^{\Delta PIP,CD}$ mutant did not induce a G2 arrest (Figure 6C), showing that the catalytic activity of Set8$^{\Delta PIP}$ is needed to trigger the checkpoint.

The Set8$^{\Delta PIP}$ Mutant Leads to Aberrant H4K20me1 Accumulation during DNA Replication

As cells resumed DNA replication after the release from HU or thymidine, H4K20me1 accumulated significantly faster in cells expressing Set8$^{\Delta PIP}$ than in cells expressing Set8^{WT} (Figures 6B and 6D and Figures S5B and S5C). In cells expressing Set8^{WT}, the levels of H4K20me1 rose shortly before mitosis,

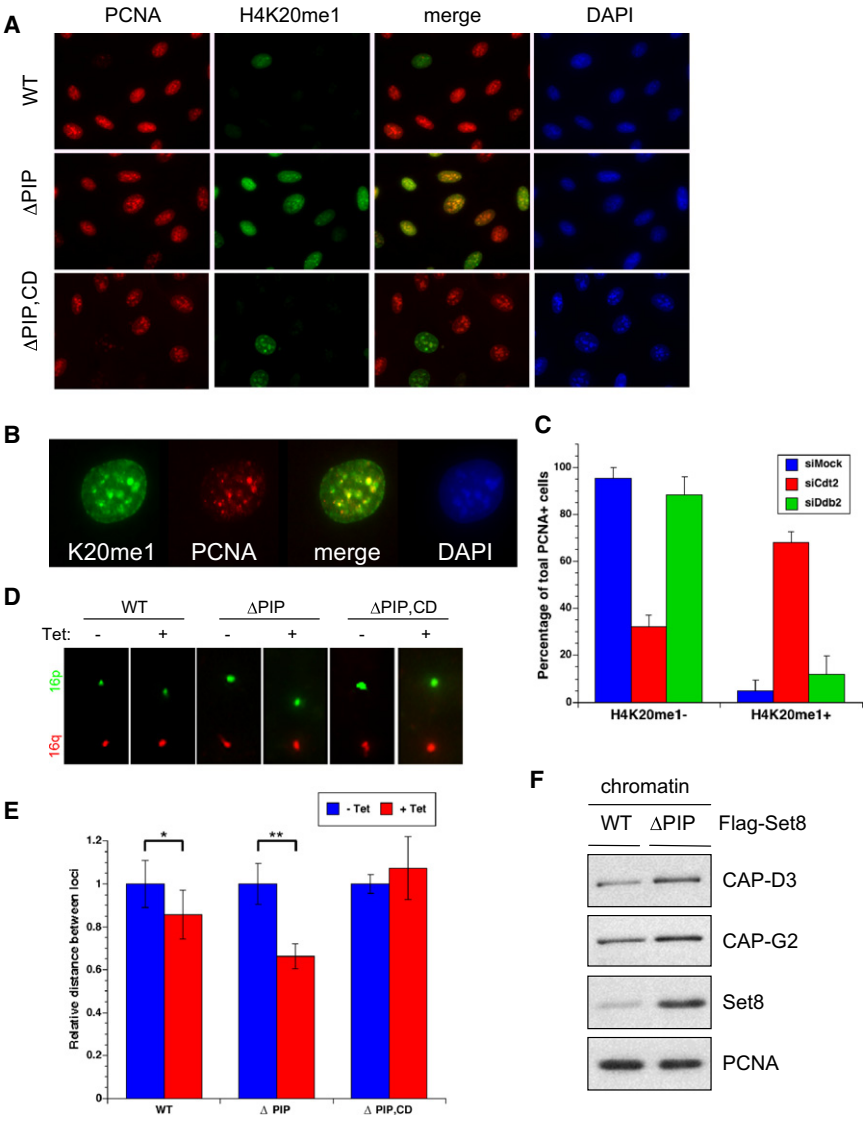

Figure 7. The Set8$^{\Delta PIP}$ Mutant Induces Aberrant Chromatin Compaction in Replicating Cells

(A) Transient expression of the ΔPIP mutant in S phase cells leads to aberrant costaining of PCNA and H4K20me1. Cells were arrested in S phase with HU for 24 hr, and Set8^{WT} or Set8$^{\Delta PIP}$ was transiently induced during the last 4 hr. Cells were subsequently released from HU and were stained with antibodies to PCNA and H4K20me1 4 hr after HU release.

(B) Colocalization of PCNA and H4K20me1 in the replicating cells that express Set8$^{\Delta PIP}$.

(C) Knockdown of Cdt2, but not Ddb2, leads to accumulation of H4K20me1 in PCNA-positive cells. Cells were transfected with siRNAs targeting Cdt2 and Ddb2, or mock treated. The levels of H4K20me1 and PCNA were analyzed by immunostaining. The averages of at least two independent experiments are plotted and error bars indicate standard deviation.

(D and E) The Set8$^{\Delta PIP}$ mutant reduces the distance between two loci on chromosome 16 in replicating cells. Cells were synchronized and induced to express Set8^{WT}, Set8$^{\Delta PIP}$, or Set8$^{\Delta PIP,CD}$ as in (A), and released for 6 hr. Dual colored FISH probes were used to visualize 16q22 and 16p13. Representative images of the two loci are shown in (D). The distances between the two loci in the indicated cell populations were measured using confocal microscopy (see the Experimental Procedures). Distances were normalized to the uninduced population of the same cell line, and the average of three independent experiments is plotted in (E). Error bars indicate standard deviation. *$p < 0.05$ and **$p < 0.001$ by Student's t test.

(F) The Set8$^{\Delta PIP}$ mutant promotes premature binding of condensin II to chromatin in S phase. Cells expressing Set8^{WT} or Set8$^{\Delta PIP}$ were synchronized and released as in (A), and were subjected to chromatin fractionation 6 hr after HU release. The levels of CAP-D3, CAP-G2, Set8, and PCNA in chromatin fractions were analyzed by western blotting.

and the levels of H4K20me2/me3 increased modestly following H4K20me1 accumulation (Figures S5B and S5C). In cells expressing Set8$^{\Delta PIP}$, the levels of H4K20me1 rose in S phase, whereas the levels of H4K20me2/me3 did not change significantly even after cells were arrested in G2 (Figure 6D and Figures S5B and S5C). These results suggest that H4K20me1 is the primary, if not the only, form of H4K20 methylation that is rapidly induced by Set8$^{\Delta PIP}$ during S phase.

We also examined the accumulation of H4K20me1 by immunostaining in cells expressing Set8^{WT} or Set8$^{\Delta PIP}$. In cells expressing Set8^{WT}, PCNA staining and H4K20me1 staining were mutually exclusive (Figure 7A and Figure S6A), consistent with the degradation of Set8 in replicating cells and its accumulation in G2/M. In marked contrast, a significant fraction of S phase cells expressing Set8$^{\Delta PIP}$ were positive for both PCNA and H4K20me1 (Figure 7A and Figure S6A). In addition, a fraction of the Set8$^{\Delta PIP}$ cells labeled with EdU were positive for H4K20me1, showing that this histone mark is aberrantly established in cells undergoing replication (Figure S6B). Furthermore, PCNA and H4K20me1 significantly colocalized with each other in cells containing Set8$^{\Delta PIP}$ (Figure 7B), indicating that H4K20me1 prematurely accumulated at or around replication forks. In contrast to Set8$^{\Delta PIP}$, Set8$^{\Delta PIP,CD}$ did not promote H4K20me1 accumulation in PCNA-positive cells (Figure 7A and Figure S6A), indicating that the ability of Set8$^{\Delta PIP}$ to induce H4K20me1 in replicating cells is dependent upon its catalytic activity.

To determine whether stabilization of endogenous Set8 in S phase promotes premature H4K20me1 accumulation, we monitored the effects of Cdt2 knockdown on the levels of H4K20me1 in PCNA-positive cells. Knockdown of Cdt2 led to a significant increase in the PCNA-positive cells with high levels of H4K20me1 (Figure 7C and Figure S6C), suggesting that stabilization of endogenous Set8 is sufficient to promote H4K20me1

accumulation in otherwise unperturbed, replicating cells. Similar premature H4K20me1 accumulation was induced by Ddb1 knockdown, but not by Ddb2 knockdown (Figure 7C and Figure S6C). In addition, a significant fraction of the Cdt2 knockdown cells with high H4K20me1 displayed phospho-RPA32 foci, indicating a link between aberrant H4K20me1 accumulation and activation of the ATM and/or ATR checkpoint kinases (Figures S6D and S6E).

Set8$^{\Delta PIP}$ Induces Premature Chromatin Compaction in Replicating Cells

H4K20me1 is a histone mark important for chromatin compaction, and its accumulation in mitosis is associated with chromatin condensation (Oda et al., 2009; Rice et al., 2002; Trojer et al., 2007). The premature accumulation of H4K20me1 in cells expressing Set8$^{\Delta PIP}$ suggested that chromatin may be aberrantly compacted during S phase. To assess this possibility, we first used dual color FISH and confocal microscopy to measure the distance between two loci on chromosome 16 (16q22 and 16p13). In synchronously replicating cells, the average distance between the two loci was significantly reduced by Set8$^{\Delta PIP}$ (Figures 7D and 7E). Compared to Set8$^{\Delta PIP}$, Set8^{WT} reduced the distance to a lesser extent, and Set8$^{\Delta PIP,CD}$ did not affect the distance at all (Figures 7D and 7E). These results suggest that the presence of Set8 activity during S phase promotes chromatin compaction.

In a second approach to monitor the effects of Set8 stabilization on chromatin compaction, we analyzed the association of linker histone H1 with chromatin by salt extraction. Coincident with the premature accumulation of H4K20me1 in Set8$^{\Delta PIP}$ cells 6 hr after the release from HU (Figure 6B), H1 was less extractable in Set8$^{\Delta PIP}$ cells than in Set8^{WT} cells (Figure S6F, left panel, lanes 4 and 5, 9 and 10), indicating that H1 is more tightly bound to chromatin in the replicating cells with Set8$^{\Delta PIP}$.

To further confirm the effects of Set8 stabilization on chromatin compaction, we analyzed chromatin using micrococcal nuclease (MNase). When Set8$^{\Delta PIP}$ was induced in S phase cells, it reduced the levels of mononucleosomes generated by partial MNase digestion (Figure S6G, left panel, compare mononucleosomes in lanes 2 and 3 versus lanes 6 and 7). The ratio of mono- to dinucleosomes was lower in Set8$^{\Delta PIP}$-expressing cells than in control cells after 15 min of MNase digestion (Figure S6G, left panel, lanes 2 and 6, and lower panel). Furthermore, after a longer MNase digestion, more nucleosomes remained in the di- and trinucleosome states in Set8$^{\Delta PIP}$-expressing cells (Figure S6G, left panel, lanes 4 and 8). These results suggest that Set8$^{\Delta PIP}$ renders chromatin more compacted than Set8^{WT}. In contrast to Set8$^{\Delta PIP}$, Set8$^{\Delta PIP,CD}$ had no effect on MNase cleavage (Figure S6G, right panel), suggesting that the ability of Set8$^{\Delta PIP}$ to promote chromatin compaction is dependent on its catalytic activity.

To understand how Set8 stabilization promotes aberrant chromatin compaction in S phase cells, we tested the possibility that condensin was prematurely recruited to chromatin during replication. The binding of condensin II to chromatin in prophase is the initial event that triggers normal mitotic chromatin condensation (Hirota et al., 2004). Expression of Set8$^{\Delta PIP}$ in S phase led to increased binding of CAP-D3 and CAP-G2, two specific components of condensin II, to chromatin in replicating cells (Figure 7F), consistent with a recent report that CAP-D3 binds H4K20me1 (Liu et al., 2010).

Together, the experiments above present four independent lines of evidence that Set8 stabilization during S phase leads to premature chromatin compaction.

DISCUSSION

Role of CRL4^{Cdt2} as a Coordinator of DNA Replication and Chromatin Compaction

The CRL4^{Cdt2} complex is a unique E3 ubiquitin ligase that specifically recognizes targets bound to PCNA on chromatin. This unique property of CRL4^{Cdt2} confers upon it the ability to ubiquitylate substrates and target them for degradation in a replication-coupled manner, suppressing cell-cycle events that are incompatible with ongoing DNA synthesis. During S phase, CRL4^{Cdt2} degrades the licensing factor Cdt1, and promotes the nuclear export of another licensing factor Cdc6 by degrading the CDK inhibitor p21 (Abbas et al., 2008; Arias and Walter, 2006; Jin et al., 2006; Kim et al., 2007, 2008; Nishitani et al., 2006, 2008; Sansam et al., 2006; Zhong et al., 2003). Thus, by restricting origin licensing to G1, CRL4^{Cdt2} plays a key role in coordinating G1 and S phases. Our results suggest that CRL4^{Cdt2} has another important role in coordinating S phase and mitosis. By suppressing Set8 function during S phase, CRL4^{Cdt2} ensures the orderly execution of DNA replication and H4K20me1-mediated chromatin compaction (Figure S6H). Failure to degrade Set8 in S phase leads to premature chromatin compaction in replicating cells, which appears to interfere with DNA synthesis and prevents proper entry into mitosis. Together with the previous studies on CRL4^{Cdt2}, our findings suggest that CRL4^{Cdt2} is a key factor that coordinates DNA replication with other cell-cycle events before and after S phase.

CRL4^{Cdt2}- and PCNA-Mediated Degradation of Set8

The mechanism by which Set8 is ubiquitylated is remarkably similar to that of Cdt1. In S phase and after DNA damage, destruction of both Set8 and Cdt1 is promoted by CRL4^{Cdt2}. Moreover, the PIP degron of Set8 closely resembles that of Cdt1. Finally, as seen for the interaction between Cdt1 and CRL4^{Cdt2}, Set8 and CRL4^{Cdt2} appear to assemble into a complex only in the context of chromatin-bound PCNA. In support of this model, we find that in egg extracts, the addition of Set8 leads to PIP box-dependent recruitment of CRL4^{Cdt2} onto chromatin. In addition, in mammalian cells, coimmunoprecipitation of Cdt2 and Set8 is dependent on the Set8 PIP box, and in egg extracts, the interaction between Set8 and Cdt2 requires the presence of DNA (data not shown). In summary, both Cdt1 and Set8 are degraded via a mechanism that involves docking of their PIP degrons onto chromatin-bound PCNA, followed by degron-dependent recruitment of CRL4^{Cdt2} and ubiquitylation.

Although Set8$^{\Delta PIP}$ is significantly stabilized after DNA damage, its degradation in S phase cells is delayed but not abolished. These features of Set8 degradation in human cells also resemble those of Cdt1 (Nishitani et al., 2006; Senga et al., 2006), and they suggest that during S phase, Set8 can be degraded by a PIP degron-independent mechanism. In addition to CRL4^{Cdt2}, the

CRL1^{Skp2} is implicated in the downregulation of Cdt1 (Kondo et al., 2004; Li et al., 2003), and it may play a secondary role in suppressing Cdt1 during S phase (Nishitani et al., 2006; Senga et al., 2006; Takeda et al., 2005). Interestingly, knockdown of Skp2 leads to cell-cycle arrest at the G1/S transition and elevated levels of Set8 (Yin et al., 2008). In our immunostaining analysis of unperturbed cycling cells, we noticed that a fraction of the PCNA-negative cells contained low levels of H4K20me1 (data not shown), suggesting that a PCNA-independent mechanism may suppress Set8 function outside of S phase. Since both Set8 and H4K20me1 accumulate during G2 and M phases, it is possible that Set8 is suppressed in G1. Consistent with this possibility, the levels of Set8 start to decline in G1 following nocodazole release (Huen et al., 2008; Yin et al., 2008). Our results suggest that while the CRL4^{Cdt2}-mediated degradation of Set8 is a critical mechanism that defines the functional window of Set8 during the cell cycle, additional mechanisms may exist.

The Need for Set8 Degradation during S Phase

Set8 promotes the accumulation of H4K20me1 during G2 and mitosis and is important for proper chromatin condensation (Houston et al., 2008; Oda et al., 2009; Sakaguchi and Steward, 2007). As cells exit from mitosis, the level of H4K20me1 rapidly declines and reaches its lowest point in early S phase (Huen et al., 2008; Oda et al., 2009). We found several lines of evidence that Set8 activity is incompatible with DNA replication. First, constitutive expression of Set8^{WT} reduces DNA synthesis and triggers the ATR checkpoint response. Second, prolonged expression of the stabilized Set8$^{\Delta PIP}$ mutant leads to a dramatic loss of replicating cells. Third, even when transiently expressed in S phase cells, the Set8$^{\Delta PIP}$ mutant induces premature H4K20me1 accumulation near DNA replication forks and triggers a checkpoint-mediated G2 arrest. Importantly, the ability of Set8$^{\Delta PIP}$ to induce aberrant H4K20me1 accumulation and to activate the checkpoint is dependent upon its catalytic activity. Thus, if Set8 is not degraded by CRL4^{Cdt2} during S phase, it interferes with DNA replication by monomethylating H4K20 in a PIP box-independent manner. Set8 has affinity for the N terminal tail of H4 with acetylated K5, K8, and K12 (Yin et al., 2008). Because newly synthesized H4 is hyperacetylated at K5 and K12, Set8, if not degraded during ongoing DNA synthesis, could be recruited to the newly assembled chromatin at replication forks. Consistent with the idea that aberrant H4K20me1 accumulation compromises DNA replication, loss of Suv4-20h1/2, the methyltransferases that convert H4K20me1 to H4K20me2/3, leads to genomic instability and defects in S phase (Schotta et al., 2008). Similarly, ablation of PHF8, an H4K20me1 demethylase, leads to a reduction of cells in S phase and accumulation of cells at G2/M (Liu et al., 2010).

Why is aberrant H4K20me1 accumulation in S phase a problem for the cell cycle? H4K20me1 is specifically recognized by the chromatin compaction factor L3MBTL1 and condensin II component CAP-D3 (Liu et al., 2010; Trojer et al., 2007). The aberrant H4K20me1 accumulation around replication forks may lead to local chromatin compaction that interferes with fork progression and/or other replication-coupled cellular events. Consistent with this possibility, we found multiple lines of evidence of premature chromatin compaction in S phase cells expressing the stabilized Set8$^{\Delta PIP}$ mutant. Coincident with premature chromatin compaction, Set8$^{\Delta PIP}$ elicited Chk1 phosphorylation during S phase and triggered a checkpoint-mediated G2 arrest. Furthermore, phospho-RPA foci were detected in Cdt2 knockdown cells with high H4K20me1, indicating that RPA-coated single-stranded DNA, a key structure for ATR activation, was induced by aberrant chromatin compaction (Zou and Elledge, 2003). These findings suggest that aberrant H4K20me1 accumulation not only compromises genome duplication but also impedes mitotic entry. In addition to its immediate impact on S phase and mitotic entry, aberrant H4K20me1 accumulation may have additional effects on the cell cycle through transcription repression (Congdon et al., 2010; Liu et al., 2010; Trojer et al., 2007). It remains possible that Set8 has substrates other than H4K20, whose aberrant methylation during S phase contributes to the faulty mitotic entry.

To ensure orderly cell-cycle progression, mitotic events must not occur prematurely during the cell cycle. Thus, the key mitotic regulator Cdk1-Cyclin B is fully activated only after S phase is completed (O'Farrell, 2001). In addition, the ATR-mediated checkpoint pathway monitors DNA replication and prevents premature chromatin condensation (Brown and Baltimore, 2000; Nghiem et al., 2001). This study shows that the process of DNA replication itself, via the PCNA and CRL4^{Cdt2}-mediated degradation of Set8, plays an active role in delaying chromatin compaction until S phase is completed. We expect that in the future, other substrates of CRL4^{Cdt2} will emerge whose presence in S phase is incompatible with the proper execution of DNA replication.

EXPERIMENTAL PROCEDURES

Cell Culture, Cell Synchronization, and Drug Treatments

U2OS, HeLa, and 293T cells were cultured in Dulbecco's modified Eagle's medium (DMEM) with 10% fetal bovine serum. U2OS-TR, RCZ11, RCZ23, and RCZ29 cells were cultured in DMEM with 10% serum and 50 μg/mL hygromycin (Invitrogen). Protein expression from stable cell lines was induced with 0.1 μg/mL tetracycline (Sigma). Zeocin (Invitrogen) was used for clonal selection at 100 μg/mL. To synchronize cells in S phase, 1 mM HU (Sigma) was added for 20–24 hr. For G1/S synchronization, cells were arrested in mitosis with 100 ng/mL nocodazole (Sigma), washed thoroughly with PBS, and released into media containing 2 mM thymidine (Sigma). Alternatively, cells were synchronized by standard double thymidine block. To measure protein stability, cycloheximide and MG132 (Sigma) were used at 100 μg/mL and 10 μM, respectively. Kinase inhibitors UCN-01 and wortmannin (Sigma) were used at 1 and 100 μM, respectively. For all experiments involving ultraviolet radiation exposure, 50 J/m^2 UV was used.

Antibodies and Immunological Techniques

Antibodies used for western blotting were from Millipore (Set8), Upstate (phospho-H3), Abcam (H4K20me1, H3, CAP-D3, CAP-G2), Active Motif (H1, H4, H4K20me2, H4K20me3), Cell Signaling Technologies (Cul4A, Tubulin, phospho-Chk1), Santa Cruz (Chk1), Bethyl (Cdt1, DDB1, Cdt2), Novus (Cdt2), Sigma (Flag), Chemicon (PCNA). Horseradish peroxidase-conjugated secondary antibodies were from Jackson ImmunoResearch. For immunofluorescence studies, antibodies used were from Abcam (PCNA), Bethyl (phospho-RPA32), and Active Motif (H4K20me1). Flag immunoprecipitations were performed with Flag M2-conjugated agarose beads (Sigma) in NETN buffer

containing 20 mM Tris-HCl (pH 8.0), 120 mM NaCl, 1 mM EDTA, 0.5% NP-40, and protease inhibitor cocktail (Sigma).

Dual Color FISH

Cells were harvested and incubated in hypotonic buffer (0.59% KC1) for 30 min at 37°C, fixed in ice-cold 3:1 MeOH:acetic acid, and spread on glass slides. Slides were prepared for FISH using fluorescently labeled probes specific for the arms of chromosome 16 (16q22, red; 16p13, green) according to the manufacturer's instructions (Cytocell; Cat# LPH 022). Coverslips were mounted and DNA was detected with 0.2 μg/ml DAPI/antifade solution (Cytocell). Fluorescent images were captured with a Hamamatsu Orca AG cooled CCD camera mounted on a Nikon TI/Yokagawa CSU-10 spinning disk confocal microscope with a 100×, 1.4 NA objective. A series of 0.25 μm optical sections were collected in the z axis for each channel (DAPI, fluorescein, and Texas red). Inter- and intrachromosome distances under each condition were measured with Slidebook analysis software. Approximately 25–40 intrachromosome distances were measured for each condition for each of three biological replicates.

Additional information can be found in the Supplemental Experimental Procedures.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures, Supplemental References, and six figures and can be found with this article online at doi:10.1016/j.molcel.2010.09.015.

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