

# SET8 is degraded via PCNA-coupled CRL4(CDT2) ubiquitylation in S phase and after UV irradiation

Stine Jørgensen,<sup>1</sup> Morten Eskildsen,<sup>1</sup> Kasper Fugger,<sup>1</sup> Lisbeth Hansen,<sup>1</sup> Marie Sofie Yoo Larsen,<sup>1</sup> Arne Nedergaard Kousholt,<sup>1</sup> Randi G. Syljuåsen,<sup>5</sup> Morten Beck Trelle,<sup>3,4</sup> Ole Nørregaard Jensen,<sup>3,4</sup> Kristian Helin,<sup>1,2</sup> and Claus Storgaard Sørensen<sup>1</sup>

<sup>1</sup>Biotech Research and Innovation Centre and <sup>2</sup>Centre for Epigenetics, University of Copenhagen, 2200 Copenhagen, Denmark

<sup>3</sup>Centre for Epigenetics and <sup>4</sup>Department of Biochemistry and Molecular Biology, University of Southern Denmark, 5230 Odense, Denmark

<sup>5</sup>Department of Radiation Biology, Institute for Cancer Research, The Norwegian Radium Hospital, 0310 Oslo, Norway

The eukaryotic cell cycle is regulated by multiple ubiquitin-mediated events, such as the timely destruction of cyclins and replication licensing factors. The histone H4 methyltransferase SET8 (Pr-Set7) is required for chromosome compaction in mitosis and for maintenance of genome integrity. In this study, we show that SET8 is targeted for degradation during S phase by the CRL4(CDT2) ubiquitin ligase in a proliferating cell nuclear antigen (PCNA)-dependent manner. SET8 degradation requires a conserved degron responsible for its interaction with PCNA and recruitment to chromatin

where ubiquitylation occurs. Efficient degradation of SET8 at the onset of S phase is required for the regulation of chromatin compaction status and cell cycle progression. Moreover, the turnover of SET8 is accelerated after ultraviolet irradiation dependent on the CRL4(CDT2) ubiquitin ligase and PCNA. Removal of SET8 supports the modulation of chromatin structure after DNA damage. These results demonstrate a novel regulatory mechanism, linking for the first time the ubiquitin-proteasome system with rapid degradation of a histone methyltransferase to control cell proliferation.

## Introduction

Eukaryotic cell cycle progression requires faithful DNA duplication and division of the chromosomes between daughter cells. These processes are tightly controlled and require massive restructuring of chromatin, which primarily consists of DNA repetitively wrapped around histone cores (Margueron and Reinberg, 2010). Posttranslational modifications of histones by phosphorylation, acetylation, methylation, and ubiquitylation are involved in the regulation of processes such as DNA replication, mitosis, DNA damage signaling, and repair (Kouzarides, 2007). Certain modifications can change the compaction of chromatin and thereby the accessibility of the surrounding DNA. Histone modifications can also serve as docking sites for effector proteins. Several proteins have been implicated in recognition of specific histone modifications that can facilitate recruitment of effector protein complexes to chromatin, where they elicit their cellular functions (Kouzarides, 2007). A well-characterized example of this is the dynamic

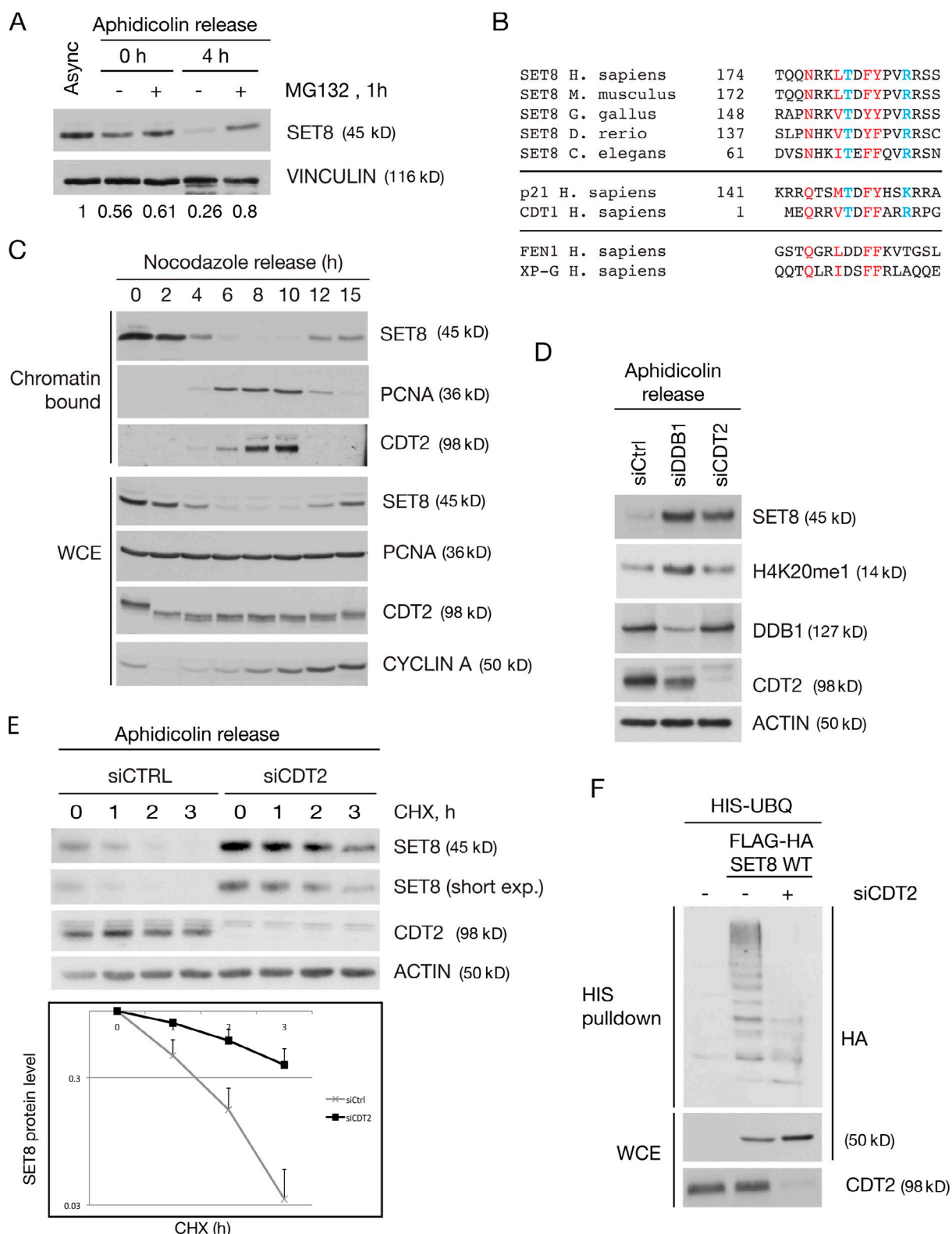
phosphorylation of Ser139 in histone H2AX ( $\gamma$ H2AX; Rogakou et al., 1998). This phosphorylation occurs near sites of DNA double-strand break lesions and is catalyzed by the ATM, DNA-PK, and ATR kinases.  $\gamma$ H2AX recruits the key DDR protein MDC1, which subsequently facilitates the localization of a number of effector proteins to the sites of DNA damage (Jackson and Bartek, 2009).

Another highly dynamic chromatin modification is the methylated form of histone H4 lysine 20 (H4K20; Margueron and Reinberg, 2010). This residue can be mono-, di-, and trimethylated. Monomethylation (H4K20me1) is catalyzed by the histone methyltransferase SET8 (Fang et al., 2002; Nishioka et al., 2002). SET8 plays a critical role in cell cycle progression, as its depletion in mammalian cells leads to aberrant progression through mitosis, DNA condensation failure, and slow progression through S phase accompanied by massive DNA damage (Jørgensen et al., 2007; Tardat et al., 2007; Houston et al., 2008;

Correspondence to Claus Storgaard Sørensen: [css@bric.ku.dk](mailto:css@bric.ku.dk)

Abbreviations used in this paper: DCAF, DDB1/CUL4-associated factors; IR, ionizing radiation; PCNA, proliferating cell nuclear antigen; WT, wild type.

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**Figure 1. CRL-CDT2 targets SET8 for degradation in S phase.** (A) SET8 protein level is low in S phase. U2-OS cells were treated with aphidicolin for 24 h and released for 4 h. The indicated cells were treated with MG132 1 h before harvest. Cells were processed for immunoblotting with the indicated antibodies. Vinculin was used as a loading control. Async, asynchronous control cells. Protein levels of SET8 are quantified and depicted below the figure. (B) SET8 contains a conserved PIP box degron. Cluster alignment of SET8 from different species show a highly conserved stretch of amino acids. When this stretch was aligned with CDT1 and p21, it revealed a conserved PIP box degron. (C) SET8 is mutually exclusive with PCNA and CDT2 on chromatin. U2-OS cells were synchronized with nocodazole, released into fresh medium, and collected at the indicated time points. Chromatin-bound fractions and whole cell extracts (WCE) were analyzed by immunoblotting with the indicated antibodies. (D) DDB1 and CDT2 depletion stabilizes SET8. U2-OS cells were treated with the indicated siRNAs 12 h before aphidicolin treatment. 24 h after aphidicolin treatment, cells were washed and released into fresh medium for 3 h and processed for immunoblotting with the indicated antibodies. Actin was used as a loading control. (E) CDT2 is important for the turnover of SET8

Huen et al., 2008; Oda et al., 2009). Similar observations were made in a genetic mouse model (Oda et al., 2009), in which SET8 deletion leads to early embryonic lethality (Huen et al., 2008; Oda et al., 2009). The phenotypes after loss of SET8 could be rescued by wild type (WT) but not a catalytically inactive version, suggesting that the methylation of H4K20 or additional targets is critical for cell cycle progression (Jørgensen et al., 2007; Tardat et al., 2007; Houston et al., 2008; Huen et al., 2008; Oda et al., 2009). The abundance of H4K20me1 is highly cell cycle regulated, being low in S phase and high in mitosis, a behavior that follows SET8 expression (Rice et al., 2002). This mark has been associated with chromatin condensation processes, which may be mediated in part by its direct recruitment of the MBT domain protein L3MBTL1 that can mediate compaction (Trojer et al., 2007). Monomethylated H4K20me1 can subsequently be di- (H4K20me2) and trimethylated (H4K20me3), which are triggered by the histone methyltransferases SUV4-20h1/2 (Schotta et al., 2008; Margueron and Reinberg, 2010). H4K20me3 has been reported to be directly involved in chromatin compaction, as the mark can induce a condensed chromatin state in vitro (Lu et al., 2008).

A key regulatory mechanism of cell cycle progression is ubiquitin-mediated proteolysis (O'Connell and Harper, 2007). This process involves the transfer of ubiquitin to lysine residues on the target protein by an E3 ubiquitin ligase that also mediates the substrate specificity of the ligase. Upon polyubiquitylation, the targeted proteins are degraded by the 26S proteasome (O'Connell and Harper, 2007). The cullin4 E3 ubiquitin ligases belong to a major subfamily of RING-H2 ubiquitin ligases that share a common architecture. The cullin subunit, CUL4A or CUL4B, associates with a common adapter protein, DDB1 (damage-specific DNA-binding protein 1), which mediates the interaction with the substrate receptor proteins known as DDB1/CUL4-associated factors (DCAFs; O'Connell and Harper, 2007; Jackson and Xiong, 2009). More than 50 DCAFs have been identified, allowing CUL4 E3s to regulate a large number of proteins and biological processes, such as cell cycle progression and the DNA damage responses to UV radiation (Jackson and Xiong, 2009). Mutations in two DCAFs, DDB2 and CSA, are associated with the human diseases Xeroderma pigmentosum and Cockayne Syndrome. Both of these disorders display defects in UV repair by the nucleotide excision repair mechanism, which is characterized by severe sensitivity to UV sunlight (Fousteri and Mullenders, 2008; Stoyanova et al., 2009). After UV irradiation and during S phase of the cell cycle, the DCAF CDT2 is also an important CUL4 E3 ligase. The CDT2 ubiquitin ligase is termed CRL4(CDT2), and it promotes the ubiquitylation and degradation of the replication factor CDT1 in a process that requires the interaction with proliferating cell nuclear antigen (PCNA; Zhong et al., 2003; Jin et al., 2006; Senga et al., 2006; Havens and Walter, 2009). PCNA recruits

the target protein to chromatin via a PCNA-interacting motif (PIP box) in which the protein becomes ubiquitylated in situ by chromatin-bound CRL4(CDT2) (Havens and Walter, 2009). In mammalian cells, the only other known target of CRL4(CDT2) is the Cdk inhibitor p21, whose destruction facilitates repair of UV lesions and limits overreplication of the genome (rereplication; Abbas et al., 2008; Kim et al., 2008; Nishitani et al., 2008). In addition, transcription factor E2F1 and DNA polymerase  $\epsilon$  have been shown to be targeted by CRL4(CDT2) in *Drosophila melanogaster* and *Caenorhabditis elegans*, respectively (Kim and Michael, 2008; Shibutani et al., 2008). CDT2 plays important roles in the regulation of cell cycle progression (Jin et al., 2006; Liu et al., 2007), and it is essential for mouse embryonic development (Liu et al., 2007).

To obtain further insight into how the chromatin status is coordinated with cell cycle progression, we investigated the biological regulation of SET8 in mammalian cells. In this study, we show that SET8 ubiquitylation and degradation in S phase and UV-irradiated cells are dependent on CRL4(CDT2). SET8 interacts with PCNA via a conserved PIP box, and this interaction is required for CRL4(CDT2)-dependent ubiquitylation. The ability to degrade SET8 under these conditions is critical to allow progression into mitosis. Furthermore, elevated SET8 induces unscheduled chromatin compaction, which is dependent on its methyltransferase activity. Our data reveal SET8 as the first methyltransferase to be tightly regulated by the ubiquitin proteasome system and highlights the key importance of this system in securing orderly cell cycle progression.

## Results

### CRL4(CDT2) targets SET8 for degradation in S phase

To understand the mechanisms underlying the S phase-specific regulation of SET8, we treated U2-OS cells with the DNA polymerase inhibitor aphidicolin, which induces an early S phase arrest. 24 h later, cells were released and allowed to progress into mid-S phase, where SET8 protein level was very low (Fig. 1 A and Fig. S1 A). Treatment of cells with the proteasomal inhibitor MG132 resulted in stabilization of SET8 after aphidicolin treatment and in cells released into S phase from the aphidicolin block (Fig. 1 A). This result indicates a role for proteasomal degradation in the regulation of SET8 protein levels. To understand how SET8 turnover is regulated, we scanned the amino acid sequence of SET8 searching for motifs that could support regulated proteolysis. The analysis revealed a conserved PIP box degron (PIP box is a motif that mediates PCNA interaction), which is also found in the CDT2 targets CDT1 and p21 (Havens and Walter, 2009). This finding could indicate an involvement of the CRL4(CDT2) E3 ubiquitin ligase in the degradation of SET8 (Fig. 1 B). To analyze this

in S phase. U2-OS cells were treated with siRNA 12 h before aphidicolin treatment. 24 h after aphidicolin treatment, cells were washed and released into fresh medium for 3 h. Cells were then treated with cycloheximide (CHX) as indicated and processed for immunoblotting with the indicated antibodies. The blots were quantified using an image reader. Representative data from three similar experiments are shown. Data are presented as mean  $\pm$  SD. (F) SET8 is ubiquitylated in vivo mediated by CDT2. HEK293 cells were depleted for CDT2 using siRNA 12 h before transfection with Flag-HA-SET8 and HIS-ubiquitin (HIS-UBQ) as indicated. Cells were processed for immunoprecipitation using cobalt beads and immunoblotted with the indicated antibodies.

aspect, we performed cell cycle synchronization assays to study the behavior of SET8, CDT2, and PCNA. We synchronized U2-OS cells in mitosis with the spindle inhibitor nocodazole, and cells were then released and collected through G1 and S phase. When we analyzed protein abundance on chromatin, it became obvious that SET8 was very low in S phase, which inversely correlated with the chromatin localization of PCNA and CDT2 (Fig. 1 C).

These data further indicated a role for CDT2 and PCNA in the control of SET8 accumulation on chromatin. We therefore depleted DDB1 and CDT2, two members of the CRL4(CDT2) E3 ubiquitin ligase complex (Jackson and Xiong, 2009). The siRNA treatments led to a marked increase in SET8 protein levels (Fig. 1 D). This result was confirmed for CDT2 siRNA in human diploid fibroblasts (Fig. S1 B).

To investigate whether CDT2 is involved in the turnover of SET8, U2-OS cells were depleted for CDT2 using siRNA, immediately followed by synchronization with aphidicolin and release into mid-S phase. As a measure of protein turnover, cells were treated with cycloheximide to inhibit protein synthesis and processed for immunoblotting. Although SET8 was unstable in control siRNA-treated cells, CDT2 depletion markedly increased the half-life of SET8 (Fig. 1 E). This was confirmed using a second siRNA targeting CDT2 (Fig. S1 C). Similar stabilization of SET8 was observed upon depletion of DDB1 and PCNA (Fig. S1, D and E). More efficient PCNA depletion could possibly further stabilize SET8; however, we deliberately chose partial PCNA depletion to avoid the severe cellular consequences of PCNA depletion (Moldovan et al., 2007). We obtained similar results on SET8 stability with two independent siRNAs targeting PCNA. CDT2 depletion has previously been shown to arrest cells in G2 phase, but our synchronization setup allowed cells to progress proficiently through S phase within the experimental timeframe (Fig. S1 F). In addition, we also observed largely similar cell cycle profiles in the presence and absence of cycloheximide treatment (Fig. S1 G). To investigate whether SET8 was targeted for polyubiquitylation in a CDT2-dependent manner, we expressed SET8 together with histidine-tagged ubiquitin (HIS-Ubi). Ubiquitylated proteins were purified via the HIS tag, and we observed marked polyubiquitylation of SET8. Importantly, this was very much reduced after CDT2 depletion using siRNA (Fig. 1 F). All together, we found that SET8 is a target for proteasomal degradation in S phase and that the degradation is mediated via the CRL4(CDT2) ubiquitin ligase complex.

#### **The interaction between PCNA and SET8 is important for CDT2-mediated degradation of SET8**

PCNA plays a major role in CRL4(CDT2)-mediated ubiquitylation and degradation, which is dependent on the PIP box degron in target proteins (Havens and Walter, 2009). We previously identified a functional PIP box in SET8 (Jørgensen et al., 2007); however, another more N-terminal PIP box has also been identified (Huen et al., 2008). Both PIP boxes are N-terminal to the SET domain, which contains the enzymatic activity as depicted in Fig. 2 A. We mutated the two published PIP box motifs and

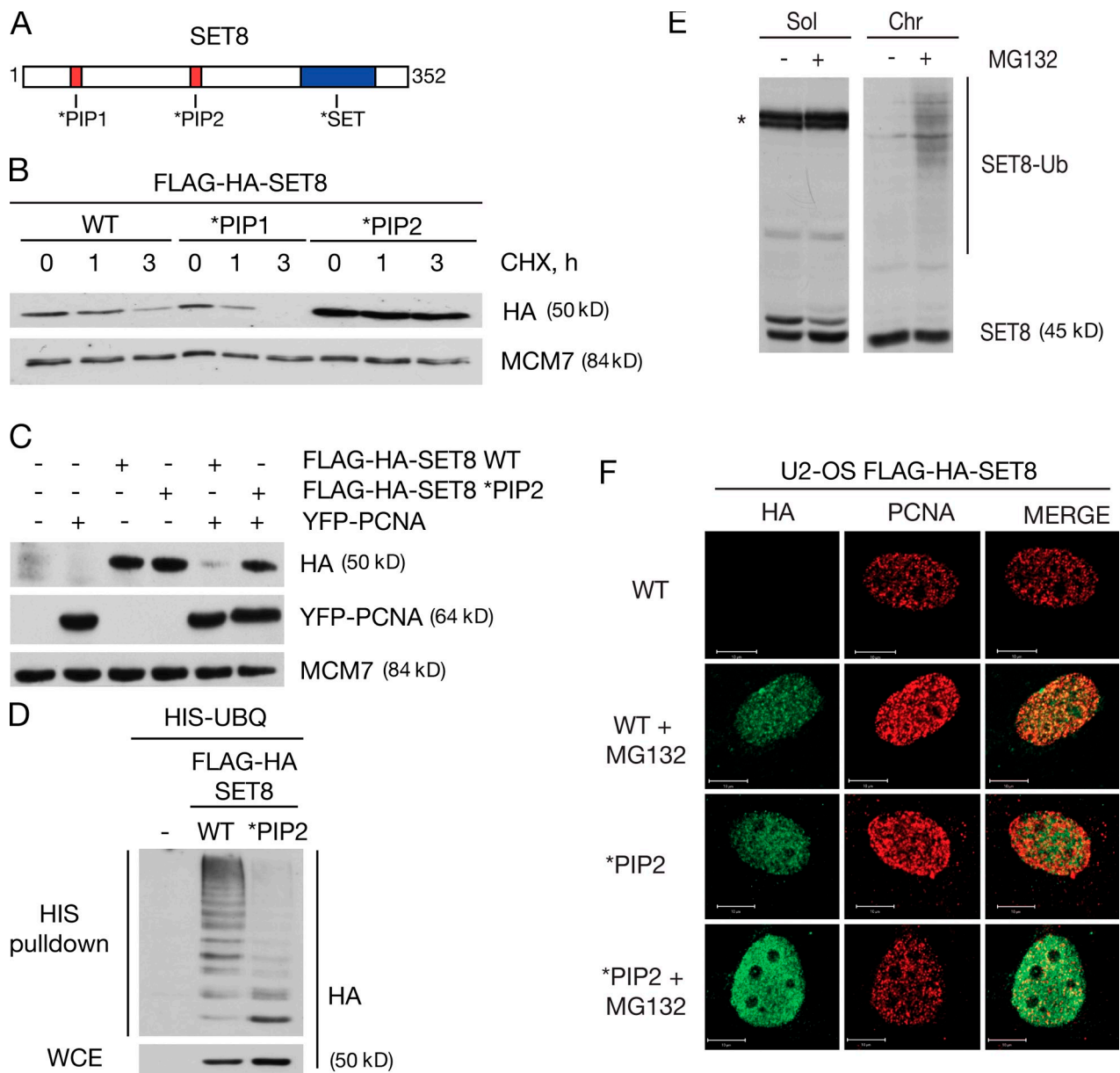
performed a cycloheximide chase experiment to investigate whether the PIP boxes were involved in the stabilization of SET8. Mutation of the second PIP box (\*PIP2) resulted in increased stability of SET8 compared with WT, whereas mutation of the first, more N-terminal PIP box (\*PIP1) did not alter the stability of SET8 (Fig. 2 B). Consistent with this result, we observed that the interaction between SET8 and PCNA is highly dependent on the PIP2 but not the PIP1 motif (Fig. S1 H). Furthermore, a deletion version of SET8 (aa 1–180) containing PIP1 but not PIP2 was also stable (unpublished data). We next investigated whether PCNA was involved in the turnover of SET8. Expression of exogenous PCNA was found to decrease the level of WT SET8 but not the SET8-\*PIP2 mutated version (Fig. 2 C). To determine whether the increased stability of SET8-\*PIP2 was caused by lack of polyubiquitylation, we performed a SET8 *in vivo* ubiquitylation assay. We found that polyubiquitylation of SET8-\*PIP2 was very much reduced compared with WT SET8 (Fig. 2 D).

To further understand the interplay between SET8 and PCNA, we investigated the localization of ubiquitylated SET8. U2-OS cells were treated with MG132 and chromatin fractionated, and we observed that endogenous SET8 is primarily polyubiquitylated on chromatin, as indicated by the appearance of high molecular mass SET8 species (Fig. 2 E). Finally, we investigated whether SET8 and PCNA were localizing to the same areas in the cell using immunofluorescence. We stabilized exogenous SET8 with MG132, and, consistent with the findings in cell lysates, we could clearly detect colocalization of PCNA and SET8 WT but not PCNA and SET8-\*PIP2 in U2-OS cells. SET8-\*PIP2 also localized to chromatin (Fig. S1 I); however, this version was not colocalizing with PCNA as clearly as wt-SET8 (Fig. 2 F). In conclusion, PCNA is important for the protein turnover of SET8 on chromatin, and the PIP2 motif is a functional PIP degron required for polyubiquitylation and degradation of SET8.

#### **SET8 turnover is accelerated after UV damage**

CRL4(CDT2) plays dual roles in protein degradation being active in S phase and after DNA damage (O'Connell and Harper, 2007). We therefore wished to investigate whether SET8 accumulation was affected by DNA damage. We treated cells with increasing dosages of UV and found that SET8 protein level was reduced in response to the irradiation (Fig. 3 A). H4K20me1 levels were also reduced by UV damage. To corroborate these findings, we performed a kinetics study using a fixed dosage of UV and observed that SET8 protein levels declined over time (Fig. 3 B). This was not because of UV-induced cell cycle alterations (Fig. S2, A and B), as only the 24-h time point was markedly affected. Consistently, H4K20me1 levels were also reduced after UV treatment, but with a slower kinetic than SET8, suggesting that this might be caused by loss of the methyltransferase. Similar data were obtained after ionizing radiation (IR), although SET8 degradation appeared less efficient than after UV treatment (Fig. S2, C and D). We also observed UV-mediated SET8 degradation in diploid human fibroblasts (Fig. S2 E).

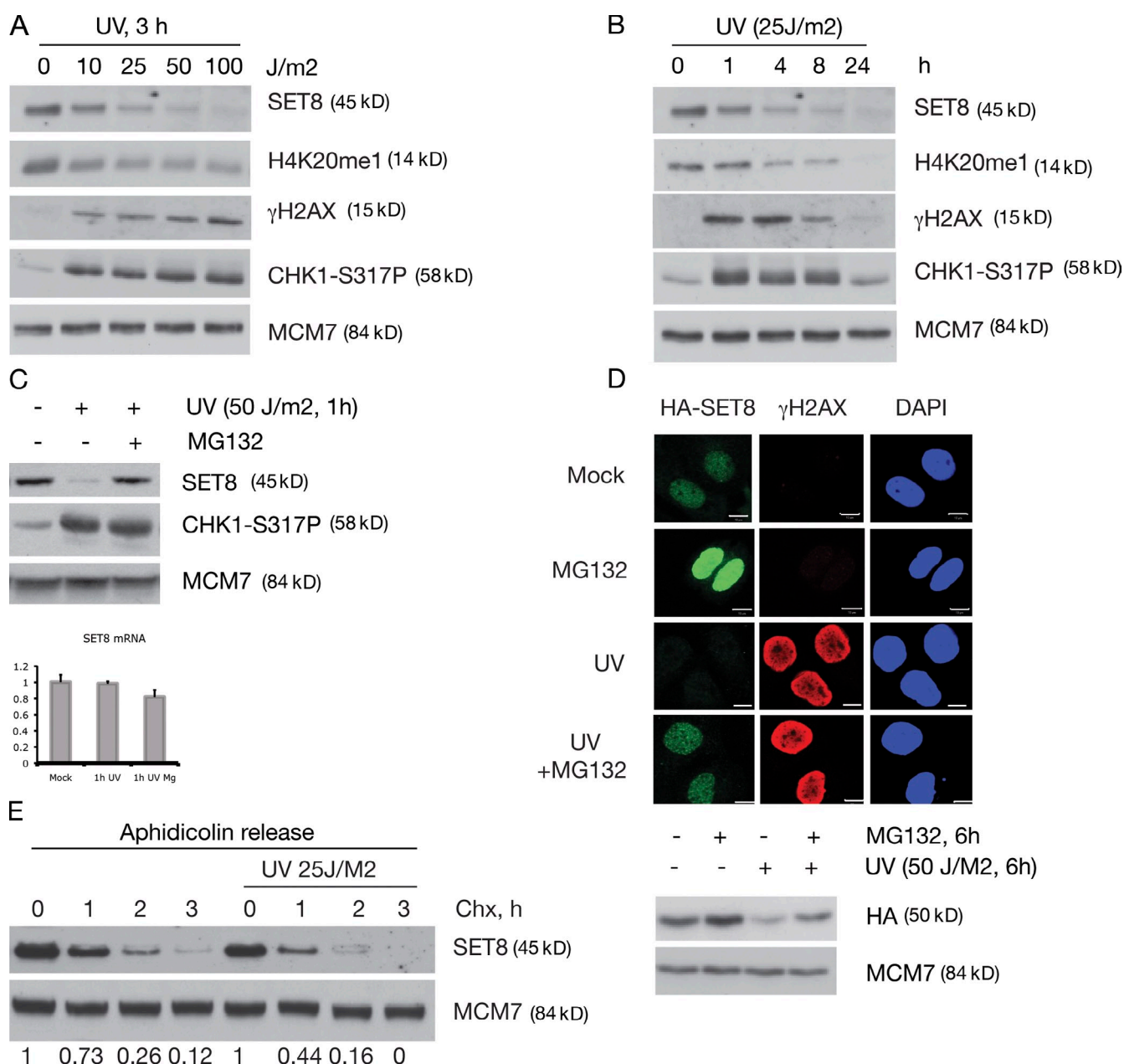




**Figure 2. The interaction between PCNA and SET8 is important for CDT2-mediated degradation of SET8.** (A) Schematic presentation of SET8. Two PIP boxes (PCNA-interacting domains) have been identified N terminally to the SET domain. (B) Mutation of PIP2 results in stabilization of SET8. HEK293 cells were transfected with plasmids expressing Flag-HA-SET8, Flag-HA-SET8-\*PIP1, and Flag-HA-SET8-\*PIP2. Cells were treated with cycloheximide and processed for immunoblotting with the indicated antibodies. MCM7 was used as a loading control. (C) Increased levels of PCNA result in degradation of WT SET8 but not SET8-\*PIP2. HEK293 cells were transfected as illustrated and processed for immunoblotting with the indicated antibodies. (D) In vivo ubiquitylation of SET8-\*PIP2 is reduced compared with SET8 WT. HEK293 cells were transfected with Flag-HA-SET8, Flag-HA-SET8-\*PIP2, and HIS-ubiquitin as indicated. Cells were processed for immunoprecipitation using cobalt beads and immunoblotted with the indicated antibodies. (E) SET8 is ubiquitylated on chromatin. U2-OS cells were either treated with MG132 for 3 h or left untreated. Soluble and chromatin-bound fractions were analyzed by immunoblotting with SET8 antibody. \*, nonspecific band. (F) SET8 WT and PCNA colocalize on chromatin after MG132 treatment. U2-OS cells expressing Flag-HA-SET8 or Flag-HA-SET8-\*PIP2 were seeded on coverslips and treated with MG132 for 3 h before preextraction and fixation. Cells were stained with antibodies against HA and PCNA. Bars, 10  $\mu$ m.

Next, we investigated whether the damage-mediated removal of SET8 was dependent on proteasomal degradation. We treated cells with a combination of UV and MG132, and although SET8 was unstable after UV treatment, the addition of MG132 rescued SET8 protein levels after UV (Fig. 3 C). Similar data were observed using immunofluorescence after UV treatment (Fig. 3 D) and IR (Fig. S2 F). It remained a possibility that the observed reduction in SET8 levels was a consequence of DNA

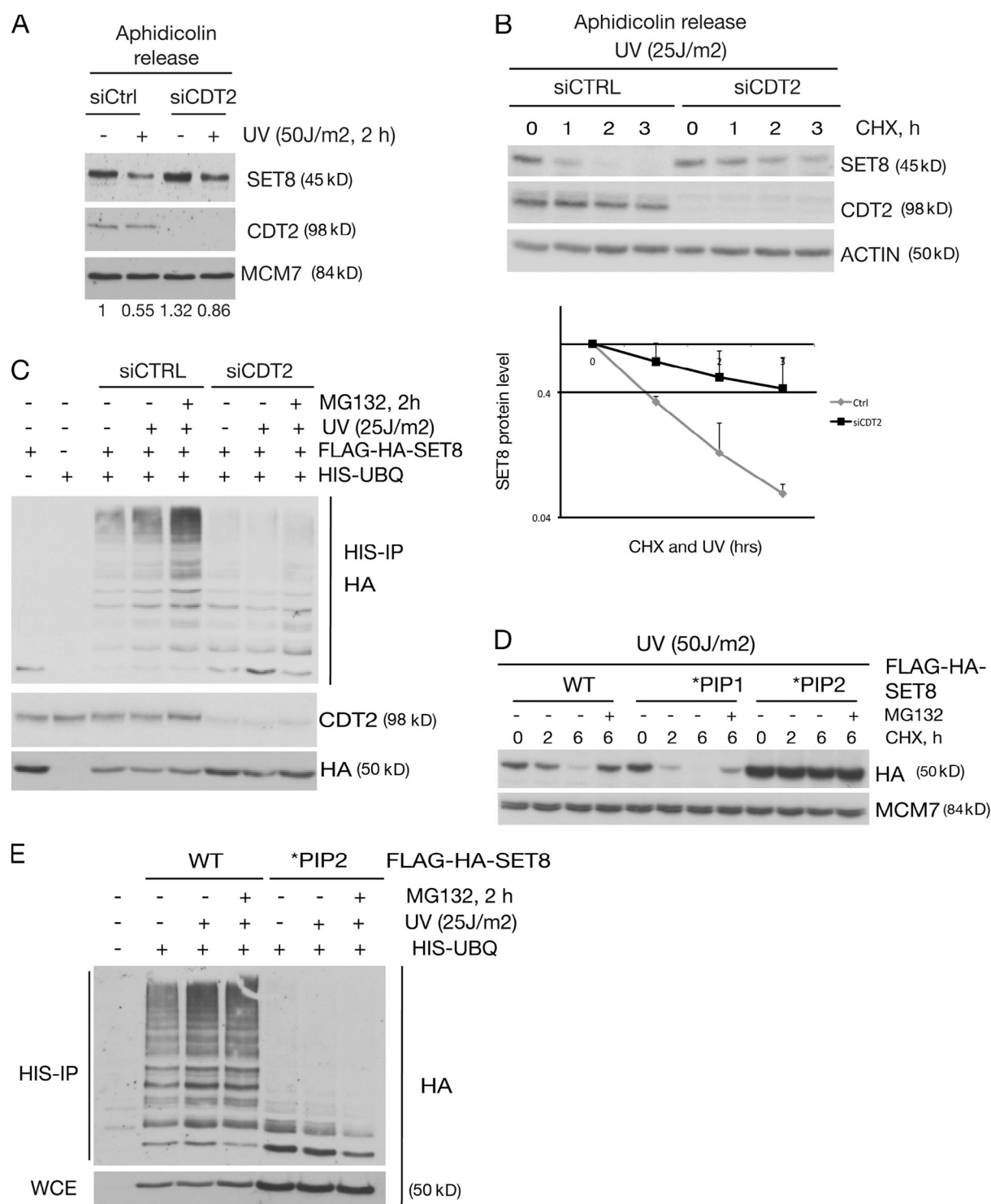
damage-induced cell cycle arrest blocking entry into mitosis, where the SET8 protein level is high. To investigate whether the turnover of SET8 was accelerated after UV treatment in S phase, we performed cycloheximide chase experiments. We released aphidicolin-synchronized cells into S phase and treated them with cycloheximide or a combination of cycloheximide and UV. We found that addition of UV irradiation resulted in an accelerated degradation of SET8 in S phase (Fig. 3 E).



**Figure 3. SET8 turnover is accelerated after UV damage, and SET8 is degraded after UV.** (A) U2-OS cells were treated with increasing amounts of UV damage and processed for immunoblotting 3 h after treatment as indicated. (B) U2-OS cells were treated with a fixed dose of UV, harvested at the indicated time points, and processed for immunoblotting. (C and D) Proteasomal inhibition blocks SET8 degradation. (C) U2-OS cells were treated with UV alone or a combination of UV and MG132 for 1 h. Cells were processed for immunoblotting and quantitative PCR as indicated. Data are represented as mean + SD. (D) U2-OS cells were infected with an HA-SET8-expressing virus and seeded onto coverslips. Cells were treated with MG132, UV, or a combination 6 h before fixation and harvest. Coverslips were processed for immunofluorescence with the indicated antibodies. The remaining cells were processed for immunoblotting with the indicated antibodies. Bars, 10  $\mu$ m. (E) UV treatment accelerates degradation of SET8. U2-OS cells were synchronized using aphidicolin and release into S phase, where they were treated with cycloheximide or a combination of cycloheximide and UV as indicated. Cells were processed for immunoblotting with the indicated antibodies.

To investigate whether the UV-induced degradation was dependent on CDT2, we knocked the protein down in U2-OS cells using siRNA and treated cells with UV. This revealed that CDT2 depletion increased SET8 level after UV (Fig. 4 A). Next, we wanted to analyze SET8 protein level in CDT2-depleted cells that had been released into S phase from aphidicolin synchronization in more details. After treatment with a combination of cycloheximide and UV, we found that depletion of CDT2 led to an increased half-life of SET8 (Fig. 4 B). We confirmed

these results with a second siRNA targeting CDT2 (Fig. S2 G), and SET8 was also stabilized when we depleted DDB1 and PCNA (Fig. S2, H and I). Furthermore, we wished to investigate whether UV treatment would affect the ubiquitylation of SET8. In vivo ubiquitylation assays showed that UV irradiation resulted in increased polyubiquitylation of SET8 that could be further increased by a combination of UV and MG132 (Fig. 4 C). In marked contrast, depletion of CDT2 reduced polyubiquitylation of SET8, and proteasomal inhibition with MG132 could



**Figure 4. CRL4(CDT2) targets SET8 for degradation after UV.** (A) CDT2 depletion stabilizes SET8 after UV. U2-OS cells were depleted for CDT2 and synchronized with aphidicolin. Cells were released into mid-S phase and treated with UV, harvested after 2 h, and processed for immunoblotting with the indicated antibodies. (B) Knockdown of CDT2 decreases SET8 turnover after UV. U2-OS cells were treated with siRNA 12 h before aphidicolin treatment. 24 h after aphidicolin treatment, cells were washed and released into fresh medium for 3 h. Cells were then treated with cycloheximide and UV as indicated and processed for immunoblotting with the indicated antibodies. The blots were quantified using an image reader. This is representative data from three similar experiments. Data are presented as mean + SD. (C) UV increases in vivo ubiquitylation of SET8. HEK293 cells were depleted for CDT2 using siRNA 8 h before transfection with Flag-HA-SET8 and HIS-ubiquitin as indicated. Cells were treated with UV or a combination of UV and MG132 2 h before harvest followed by immunoprecipitation using cobalt beads and immunoblotted with the indicated antibodies. (D) Mutation of PIP2 but not PIP1 increases stability of SET8 after UV. HEK293 cells were transfected with either Flag-HA-SET8, \*PIP1, or \*PIP2. They were treated with a combination of cycloheximide and UV or cycloheximide, UV, and MG132. Cells were processed for immunoblotting with the indicated antibodies. (E) WT SET8 but not SET8-\*PIP2 is ubiquitylated in vivo after UV treatment. Cells were transfected with Flag-HA-SET8, Flag-HA-SET8-\*PIP2, and HIS-ubiquitin as indicated. Cells were processed for immunoprecipitation using cobalt beads and immunoblotted with the indicated antibodies.

not rescue this (Fig. 4 C). To investigate whether PIP boxes affected the UV-mediated degradation of SET8, we performed cycloheximide chase experiments including the two PIP box mutants and WT SET8. Although both WT and SET8-*\*PIP1* were unstable after the combined cycloheximide and UV treatment, the SET8-*\*PIP2* version was stable (Fig. 4 D). Finally, we performed an *in vivo* ubiquitylation assay, which revealed that SET8-*\*PIP2* cannot be efficiently polyubiquitylated in response to UV irradiation. WT SET8 was efficiently polyubiquitylated in response to UV (Fig. 4 E). In conclusion, SET8 is degraded by the proteasome in response to UV irradiation, a degradation mediated via CDT2 and PCNA-dependent ubiquitylation.

### Degradation of SET8 allows cell cycle progression beyond G1 phase

To investigate the biological relevance of the oscillating level of SET8 protein through the cell cycle, we performed flow cytometry analysis of cells expressing WT and mutant forms of SET8. We used a mitotic trap with the spindle inhibitor nocodazole to evaluate whether cells were able to proceed into mitosis (Fig. 5 A). WT SET8 expressed from a weak promoter is subjected to proteolysis, and as a consequence, the protein level was oscillating and present at a lower level than the nondegradable SET8-*\*PIP2* (Fig. 5 B). We observed that cells expressing WT and catalytically inactive SET8 behaved almost as control cells regarding their progression through the cell cycle into mitosis (Fig. 5 A). In contrast, expression of nondegradable SET8-*\*PIP2* markedly affected cell cycle progression, as very few cells entered mitosis in the experimental setup (Fig. 5 A). Importantly, the nondegradable and catalytically inactive SET8-*\*PIP2/\*SET* mutant did not disturb cell cycle progression in the course of the experiment. This indicates that SET8 levels are reduced by proteolysis during S phase to avoid unscheduled methyltransferase activity in S and G2 phase. As positive control, we expressed elevated levels of WT SET8 saturating the degradation capacity, and as expected, this also blocked cell cycle progression into mitosis (Fig. 5 A). Importantly, flow cytometry analysis of cells expressing SET8-*\*PIP2* and SET8-*\*PIP2/\*SET* did not reveal alterations in progression through S phase when compared with control cells (Fig. S3 A). We did not observe checkpoint activation upon expression of nondegradable SET8, as assayed by immunoblotting (Fig. S3 B) or flow cytometry (Fig. S3 C). We also investigated the consequences of stable SET8 expression by analyzing the fitness of these cells in clonogenic survival assays. In agreement with the cell cycle progression defect, SET8-*\*PIP2*-expressing cells formed few colonies in clonogenic survival assays, whereas cells expressing the catalytically inactive SET8-*\*PIP2/\*SET* version displayed better fitness in this assay (Fig. S3 D). We were not able to properly address the survival of cells expressing SET8-*\*PIP2* after UV treatment because these cells performed poorly in clonogenic survival assays even in the absence of UV treatment (Fig. S3 D).

Data from several model systems including SET8 knockout mice have shown that deletion of SET8 results in reduced chromatin compaction (Houston et al., 2008; Oda et al., 2009). We reasoned that the degradation of SET8 could serve to modulate chromatin structure, and we subsequently

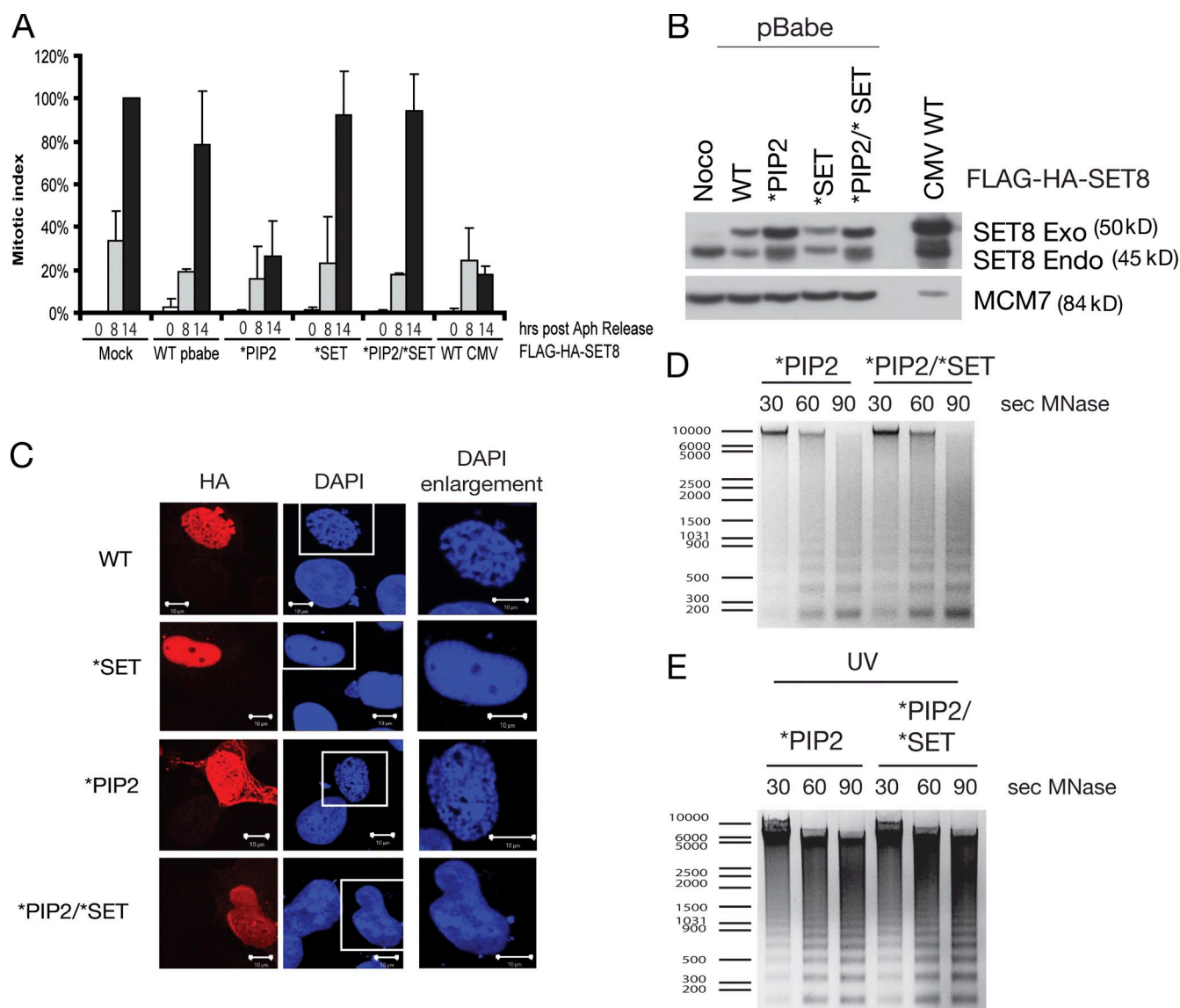
investigated how expression of various mutant forms of SET8 affected chromatin compaction. Cells expressing SET8-*\*PIP2* and elevated levels of WT SET8 could induce marked chromatin compaction in cells released from aphidicolin arrest. This phenotype was dependent on the methyltransferase activity, as catalytically inactive SET8 versions had little effect on chromatin compaction (Fig. 5 C and Fig. S3 E). In agreement with this, we observed that chromatin was in a more-compacted state, as indicated by reduced MNase cleavage in SET8-*\*PIP2*-expressing cells compared with cells expressing SET8-*\*PIP2/\*SET* (Fig. 5 D and Fig. S3 F). Similar data were obtained when comparing MNase digest from cells expressing wt-SET8 and SET8-*\*SET* (Fig. S3 G). Finally, we wished to investigate the chromatin status of UV-treated cells expressing SET8-*\*PIP2* compared with SET8-*\*PIP2/\*SET* using a MNase digest. In line with the aforementioned data, we observed that expression of SET8-*\*PIP2* resulted in a more compact chromatin structure compared with SET8-*\*PIP2/\*SET* also after UV treatment (Fig. 5 E and Fig. S3 H). In conclusion, we propose that SET8 is a target of ubiquitylation and proteasomal degradation to allow a flexible state of chromatin that facilitates chromatin modulation during the cell cycle.

## Discussion

In this study, we discovered how cells control the ubiquitylation and degradation of the H4K20 methyltransferase SET8, a key regulator of cell cycle progression and genome integrity (Oda et al., 2009; Yang and Mizzen, 2009). It was established that SET8 levels oscillate during the cell cycle, being low in S phase and high in mitosis (Rice et al., 2002). We now show that the CRL4(CDT2) E3 ubiquitin ligase promotes ubiquitylation and subsequent degradation of SET8 dependent on a conserved PIP box degron. In cells depleted of DDB1, CDT2, or PCNA, the degradation and ubiquitylation of SET8 were impaired. This proteolysis of SET8 is important because its impairment affects cell cycle progression at the G2/M transition. Remarkably, CDT2 depletion also induces a G2 arrest, and this further suggests a close linkage between the CRL4(CDT2) ubiquitin ligase and SET8 turnover. We therefore propose that PCNA-CRL4(CDT2) is required to degrade SET8 when PCNA loads on chromatin at the G1/S transition (Fig. 1 C). Endogenous levels of SET8 clearly do not exceed the capacity of PCNA-CRL4(CDT2), and we noted that cells have high capacity to degrade SET8.

Most cells expressing the SET8-*\*PIP2* version were able to enter S phase even when the protein was expressed at a high level from a cytomegalovirus promoter. We did not observe aberrant S phase progression (Fig. S3 A), and these cells accumulated in G2 phase. PCNA-CRL4(CDT2) is also required in S phase to promote the ubiquitylation and degradation of the Cdk inhibitor p21 (Abbas et al., 2008; Kim et al., 2008; Nishitani et al., 2008) and the replication licensing factor CDT1 (Zhong et al., 2003; Jin et al., 2006; Senga et al., 2006). Elevated levels of CDT1 did not block replication; rather, its PCNA-CRL4(CDT2)-mediated proteolysis served to prevent overreplication of the





**Figure 5. Degradation of SET8 allows cell cycle progression beyond G1 phase.** (A) Nondegradable SET8 reduces entry into mitosis. U2-OS cells were cotransfected with H2B-GFP and the indicated Flag-HA-SET8 constructs in a 1:3 ratio and simultaneously treated with aphidicolin for 24 h. Cells were released into fresh medium containing nocodazole, harvested, and fixed at the indicated time points. Cells were stained with H3S10P antibody, and cells positive for both GFP and H3S10P were identified by flow cytometry. Data are presented as mean  $\pm$  SD. (B) Expression levels of SET8 constructs. U2-OS cells were treated with nocodazole for 16 h (Noco) or transfected with the indicated pBabe or pCMV constructs 36 h before harvest. Cells were processed for immunoblotting with the indicated antibodies. Only 0.1 of the pCMV SET8 WT lysate was loaded compared with pBabe SET8 lysates. (C) SET8 WT and SET8-\*PIP2 expression induces compaction of chromatin. pCMV Flag-HA-SET8 constructs were expressed in U2-OS cells seeded on coverslips and simultaneously treated with aphidicolin for 24 h. Cells were released into fresh medium and fixed after 4 h. Chromatin structure in SET8-expressing cells was analyzed by immunofluorescence stainings for DAPI in HA-positive cells. Bars, 10  $\mu$ m. (D) Elevated levels of SET8-\*PIP2 but not SET8-\*PIP2/\*SET results in a more compact chromatin structure. HEK293 cells were transfected with either Flag-HA-SET8-\*PIP2 or Flag-HA-SET8-\*PIP2/\*SET constructs. Cells were harvested for MNase digest followed by separation of the DNA on an agarose gel. (E) Elevated levels of SET8-\*PIP2 but not SET8-\*PIP2/\*SET results in a more compact chromatin structure in response to UV. HEK293 cells were transfected with either Flag-HA-SET8-\*PIP2 or Flag-HA-SET8-\*PIP2/\*SET constructs. Cells were treated with UV (100 J/m<sup>2</sup>) 3 h before harvest. Cells were harvested for MNase digest followed by separation of the DNA on an agarose gel.

genome (Jin et al., 2006). However, we have not observed re-replication in experiments with elevated or nondegradable SET8 (unpublished data). Given that SET8-\*PIP2-expressing cells can replicate efficiently, we expect that these G2-arrested cells can eventually initiate rereplication reaching  $>4N$  DNA content without intervening mitosis. However, this will be a delayed secondary event relative to the rapidly occurring issues described in this study.

It is remarkable that PCNA plays a unique role in CRL4(CDT2)-mediated ubiquitylation processes. Recently, CDT1 ubiquitylation via CRL4(CDT2) has been clarified, and it was apparent that the docking of CDT1 onto chromatin-bound PCNA via the PIP degron generates a signal for recruiting the CRL4(CDT2) ligase and, ultimately, CDT1 ubiquitylation and destruction (Havens and Walter, 2009). A similar mechanism is likely to operate for SET8, which we identified in this study,

and p21. All three proteins are involved in cell cycle control and DNA replication, and their elevated levels will disturb these processes.

In contrast to SET8-\*PIP2, cells expressing catalytically inactive SET8-\*PIP2 were able to enter mitosis. Thus, the presence of SET8 methyltransferase activity during S and G2 phase leads to G2 arrest. Importantly, the expression of wt-SET8 and SET8-\*PIP2 induced marked chromatin compaction, which is dependent on methyltransferase activity. These data support recent studies identifying an important role of SET8 in chromosome condensation (Houston et al., 2008; Oda et al., 2009). Oda et al. (2009) showed that conditional knockout of SET8 in mouse ES cells results in chromosomal decondensation of mitotic and in interphase nuclei. In line with this, SET8 and H4K20me1 have been previously reported to induce chromatin compaction via H4K20me1-dependent recruitment of L3MBTL1 (Trojer et al., 2007). We addressed the potential role of L3MBTL1 by depleting it in cells ectopically expressing SET8; however, L3MBTL1 depletion only had a minor effect on chromatin compaction (unpublished data). These findings are in line with a recent study showing absence of chromatin compaction phenotypes in L3MBTL1-deficient cells (Qin et al., 2010). We favor the notion that L3MBTL1 and additional proteins respond to elevated SET8 and H4K20 methylation states to alter chromatin structure. Very recently, monomethylated H4K20 was shown to specifically interact with condensin-II subunits, and this may also drive the unscheduled compaction of chromatin (Liu et al., 2010). Finally, structural experiments of the nucleosome revealed that trimethylated H4K20 could increase the compaction of nucleosomal arrays, further suggesting that the H4K20 residue is involved in chromatin compaction (Lu et al., 2008). Because of the inability to deplete and reconstitute with mutant versions of histone H4, we cannot address the role of H4K20 methylation directly in our experimental setup. However, our results are in agreement with the emerging data from Liu et al. (2010) suggesting that SET8-mediated monomethylation of histone H4 mediates chromatin compaction that subsequently negatively affects cell cycle progression.

In this study, we also discovered that SET8 is actively degraded after UV radiation by polyubiquitylation. The E3 ligase that promotes the ubiquitylation in response to UV irradiation is also PCNA-CRL4(CDT2). Silencing components of the CRL4(CDT2) E3 ubiquitin ligase by siRNA not only elevated the basal levels of SET8, it also reduced SET8 degradation after UV (Fig. 4). To our knowledge, this is the first study in mammalian cells that identifies a regulated role of histone methyltransferase proteolysis in the DNA damage response. It is noteworthy that we did not observe a role for the checkpoint kinases ATR, ATM, or CHK1 in the regulation of SET8 turnover (negative data not depicted). This is similar to previous data on CDT1 and p21 destruction after DNA damage, and it may be the structural modulation of PCNA in areas with DNA lesions that accelerates the proteolysis of CDT2 targets.

SET8 has been previously linked with the DNA damage response, as we and others have discovered that the depletion of SET8 leads to loss of genomic integrity even in the absence of exogenous DNA damage (Jørgensen et al., 2007;

Tardat et al., 2007; Houston et al., 2008; Huen et al., 2008; Oda et al., 2009). Experiments performed with conditional SET8 knockout ES cells revealed that the cell cycle can progress through the first S and M phase without SET8, but catastrophic events occur in the following cell cycle where spontaneously arising DNA damage leads to checkpoint activation (Oda et al., 2009). A very tightly regulated expression of SET8 is therefore critical to allow cells to proliferate productively and at the same time protect themselves against exogenously arising DNA damage. Furthermore, we propose that the DNA damage-mediated reduction of SET8 is involved in relaxation of the chromatin structure, which is supported by our MNase digest data. This is reminiscent of the role of the transcriptional repressor KAP-1, whose depletion leads to chromatin relaxation and more efficient DNA repair after IR (Ziv et al., 2006; Goodarzi et al., 2008). We do not exclude that nonhistone targets of SET8 can modulate the phenotypic outcome. As an example, SET8 has been shown to methylate a nonhistone protein, p53. The methylation was found to inhibit the transactivating activity of p53 on the target genes p21 and PUMA and thereby also part of the damage response (Shi et al., 2007). We have however not been able to detect an effect of ectopic SET8 expression on p53 target genes in our cellular system (unpublished data).

All together, we propose that SET8 protein levels are kept low from the G1/S transition and during S phase by the CRL4(CDT2) ubiquitin ligase complex to support a chromatin structure that favors cell cycle progression. In addition, we suggest that the CRL4(CDT2)-mediated degradation of SET8 after UV treatment supports a chromatin structure that facilitates repair. Three recent studies also identified a role for PCNA-CRL4(CDT2)-mediated regulation of SET8 in cell cycle progression (Abbas et al., 2010; Centore et al., 2010; Tardat et al., 2010). Centore et al. (2010) also observed marked chromatin compaction in SET8-expressing cells. Intriguingly, Abbas et al. (2010) observed some H2AX phosphorylation after SET8-\*PIP2 expression, whereas Centore et al. (2010) observed increased CHK1 phosphorylation (Abbas et al., 2010; Centore et al., 2010). We have not observed this in our experiments (Fig. S3, B and C), which may relate to different levels of SET8-\*PIP2 expression between the three studies. Tardat et al. (2010) also observed H2AX phosphorylation; however, this was linked with DNA replication issues at later stages after SET8-\*PIP2 expression (Tardat et al., 2010). The issue of genome integrity upon expression of stable SET8 is an important topic for future studies.

## Materials and methods

### Cell culture and chemicals

Human U2-OS osteosarcoma, HEK293, and Tig3 TERT cell lines were grown in DME with 10% fetal bovine serum. Nocodazole, cycloheximide, MG132, and aphidicolin were used at 100 ng/μl, 20 μg/ml, 5 μM, and 4 μM, respectively (Sigma-Aldrich). IR was performed using an x-ray apparatus (Faxitron X-Ray LLC) calibrated to give 1 Gy  $\gamma$ -irradiation for 2 min. UV was performed using a UV Stratalinker 1800 (AH Diagnostics).

### Chromatin fractionation

To obtain soluble and chromatin-enriched cellular fractions, cell fractionation was performed. In brief, cells were lysed for 30 min in a small volume of CSK buffer [0.5% Triton X-100, 10 mM Pipes, pH 6.8, 300 mM sucrose, 100 mM NaCl, and 1.5 mM MgCl<sub>2</sub>]. The lysed cells were pelleted by

centrifugation at 2,000 g, and supernatant was collected (soluble fraction). The pellet was washed once with CSK buffer, resuspended in 0.2 M HCl, and incubated at 4°C for 2 h. The supernatant represented the chromatin-enriched fraction. HCl-containing samples were neutralized with Tris buffer before SDS-PAGE. Whole cell extracts were prepared by lysing cells in RIPA buffer (50 mM Hepes, pH 7.5, 150 mM NaCl, 1 mM EDTA, 2.5 mM EGTA, 10% glycerol, 1% IgePal630, 1% deoxycholic acid [sodium salt], 0.1% SDS, 1 mM PMSF, 5 µg/ml leupeptin, 1% vol/vol aprotinin, 50 mM NaF, and 1 mM DTT) followed by sonication using a digital sonifier (102C CE Converter; Branson).

### Immunoblotting and antibodies

Cells were lysed on ice in RIPA buffer followed by sonication (see previous paragraph). Protein were separated on SDS-PAGE gels and transferred to a nitrocellulose membrane. The membranes were incubated in primary antibody diluted in 5% milk as indicated in Figs. 1–5 followed by incubation with secondary antibody (peroxidase-labeled anti-mouse or -rabbit IgG [1:10,000; Vector laboratories]). Films were developed using an x-ray machine (Valso; Ferrania). Immunoblots were quantified using an image reader (LAS-3000; Fujifilm) and analyzed using Image Gauge software (Fujifilm) when indicated. Antibodies to MCM7 (DCS-141) and CHK1 (DCS310) have been described previously (Sørensen et al., 2005). Commercially available antibodies used in this study were cyclin A (sc-751) and rabbit (Y11) anti-HA tag (Santa Cruz Biotechnology, Inc.). Phospho-CHK1 antibody (CHK1-pSer317; #23445) was purchased from Cell Signaling Technology. Phosphorylated H2AX (05-636), phosphorylated H3S10 (06-570), and SET8 (06-1304) were purchased from Millipore. Monomethylated H4 lysine20 (ab9051) was purchased from Abcam. Vinculin (V9131) was purchased from Sigma-Aldrich. PCNA (PC10) was purchased from Abnova, and CDT2 (NB-10-40840) was obtained from Novus Biologicals. The DDB1 antibody (34-2300) was purchased from Invitrogen, and actin (MAB1501) was purchased from Millipore. Fluorescence-conjugated anti-mouse and anti-rabbit IgG were purchased from Invitrogen (Alexa Fluor 488 and 594; Table I).

### In vivo ubiquitylation

Cells were lysed in ubiquitin lysis buffer (50 mM NaH<sub>2</sub>PO<sub>4</sub>, 300 mM NaCl, 5 mM imidazole, and 0.05% Tween 20 + inhibitors) and sonicated. Lysate was incubated with cobalt beads (His tag purification resins; Takara Bio Inc.) for 1 h at 4°C followed by 10 washes with ubiquitin wash buffer (50 mM NaH<sub>2</sub>PO<sub>4</sub>, 300 mM NaCl, 10 mM imidazole, and 0.05% Tween 20 + inhibitors). Bound proteins were eluted in 500 mM imidazole, mixed with Laemmli sample buffer, and immunoblotted with the indicated antibody.

### Microscopy and immunofluorescence

Cells were grown on coverslips and treated as indicated in Figs. 1–5. The coverslips were washed briefly in PBS and fixed for 10 min with 4% paraformaldehyde. Cells were permeabilized in PBS supplemented with 0.3% Triton X-100 for 10 min and incubated with primary antibodies in PBS supplemented with 3% BSA for 1 h at room temperature. Cells were washed in PBS supplemented with 0.05% Tween 20 three times for 10 min and incubated with Alexa Fluor 488- or 594-conjugated secondary antibodies (1:1,000; Invitrogen) for 1 h at room temperature. Cells were washed in PBS supplemented with 0.05% Tween 20 three times for 10 min and mounted using mounting medium containing DAPI (Vectashield; Vector Laboratories) followed by confocal microscopy analysis. Pictures were acquired at room temperature on an inverted microscope (Axiovert 200M LSM 510; Carl Zeiss, Inc.) using a 40× NA 1.2 c-Apochromat objective in H<sub>2</sub>O or a 63× NA 1.2 c-Apochromat objective in H<sub>2</sub>O. Pictures were analyzed using LSM 510 META software and LSM image examiner software (Carl Zeiss, Inc.). All pictures were exported in preparation for printing using Photoshop (Adobe).

### Quantitation of mRNA levels by quantitative PCR

RNA was purified from cells using the RNeasy kit (QIAGEN). cDNA was generated by RT-PCR using reverse transcription reagents (TaqMan; Applied Biosystems). Relative mRNA levels were determined using the SyBR green I detection chemistry system (Applied Biosystems) and a sequence detection system (Prism 7300; Applied Biosystems). Ubiquitin expression was used as a control for normalization.

### FACS

Cells were cotransfected with H2B-GFP or GFP-actin and Flag-HA-SET8 constructs in a 1:3 ratio and treated as described in Figs. 1–5. In brief,

Table I. siRNA oligonucleotides used in this study

siRNA	Sequence
CDT2 #1	5'-GAAUUUACUGCUUAUCGA-3'
CDT2 #2	5'-AUACAAGAGUGACUCUAUA-3'
DDB1 #1	5'-ACAGAGUGGCGAGAGCAUU-3'
DDB1 #2	5'-CCUGUUGAUUGCCAAAAC-3'
PCNA #1	5'-GGAGAAAGUUUCAGACUAU-3'
PCNA #2	5'-GCCGAGAUUCAGCCAUUAU-3'

cells were fixed in 70% ethanol and stained with rabbit H3S10P and mouse γH2AX for 1 h. This was followed by 1-h incubation with conjugated anti-mouse IgG and anti-rabbit IgG (Alexa Fluor 488 and 647 at 1:1,000). DNA was counterstained by 0.1 mg/ml propidium iodide supplemented with RNase for 30 min at 37°C. Analysis was performed on a FACSCalibur using CellQuest software (BD). Profiles are based on GFP-positive cells. Quantification and analysis of cell cycle profiles were obtained using FlowJo (version 7.2.2; Tree Star, Inc.).

### Micrococcal nuclease digestion

Cells were transfected as indicated and harvested in cold PBS. Cells were then collected by centrifugation (300 g for 5 min at 4°C), and the pellet was resuspended in cytosolic lysis buffer (10 mM Tris-HCl, pH 7.5, 10 mM NaCl, 5 mM MgCl<sub>2</sub>, 0.5% NP-40, and 0.25 mM PMSF) and left on ice for 8 min. Nuclei were pelleted by centrifugation (500 g for 5 min at 4°C). The pellet was washed once in nuclei buffer (60 mM KCl, 15 mM NaCl, 0.34 M sucrose, 0.25 mM PMSF, and 1 mM DTT) and resuspended in nuclei buffer to a final absorbance at 0.1. 2 mM CaCl<sub>2</sub> was added, and the samples were prewarmed to 25°C. Micrococcal nuclease (1:1,000 U/µl; Sigma-Aldrich) was added to the sample and incubated at 30°C for the indicated time period. The digest was stopped by addition of STOP buffer (0.7% SDS and 30 mM EDTA, pH 8). Genomic DNA was purified using a DNeasy Blood and Tissue kit (QIAGEN) and run on a 1% agarose gel. Pictures were acquired using a Gel Doc XR (Bio-Rad Laboratories).

### Online supplemental material

Fig. S1 shows characterization of SET8 protein levels and cell cycle progression through S phase upon depletion of CDT2, DDB1, and PCNA. An interaction study between mutant forms of SET8 and endogenous PCNA and a cellular localization study of SET8-WT versus SET8\*PIP2 are also shown. Fig. S2 shows that DNA damage-mediated degradation of SET8 can be rescued by addition of MG132 or depletion of CDT2, DDB1, or PCNA. Fig. S3 shows that expression of SET8\*PIP2 does not affect progression through S phase or lead to DNA damage up to 48 h after transfection. It also shows that SET8\*PIP2 expression results in poor survival and compaction of chromatin. Online supplemental material is available at <http://www.jcb.org/cgi/content/full/jcb.201009076/DC1>.

This work was supported by the Novo Nordic Foundation, the Danish Cancer Society, the Lundbeck Foundation, the Danish Medical Research Council, the Danish National Research Foundation, the Norwegian Cancer Society, Harald Andersens Foundation, The Norwegian Research Council and South-Eastern Norway Regional Health Authority.

Submitted: 14 September 2010

Accepted: 5 December 2010

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