Direct binding of Cdt2 to PCNA is important for targeting the CRL4<sup>Cdt2</sup> E3 ligase activity to Cdt1

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The CRL4<sup>Cdt2</sup> ubiquitin ligase complex is an essential regulator of cell-cycle progression and genome stability, ubiquitinating substrates such as p21, Set8, and Cdt1, via a display of substrate degrons on proliferating cell nuclear antigens (PCNAs). Here, we examine the hierarchy of the ligase and substrate recruitment kinetics onto PCNA at sites of DNA replication. We demonstrate that the C-terminal end of Cdt2 bears a PCNA interaction protein motif (PIP box, Cdt2<sup>PIP</sup>), which is necessary and sufficient for the binding of Cdt2 to PCNA. Cdt2<sup>PIP</sup> binds PCNA directly with high affinity, two orders of magnitude tighter than the PIP box of Cdt1. X-ray crystallographic structures of PCNA bound to Cdt2<sup>PIP</sup> and Cdt1<sup>PIP</sup> show that the peptides occupy all three binding sites of the trimeric PCNA ring. Mutating Cdt2<sup>PIP</sup> weakens the interaction with PCNA, rendering CRL4<sup>Cdt2</sup> less effective in Cdt1 ubiquitination and leading to defects in Cdt1 degradation. The molecular mechanism we present suggests a new paradigm for bringing substrates to the CRL4-type ligase, where the substrate receptor and substrates bind to a common multivalent docking platform to enable subsequent ubiquitination.

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Introduction

The integrity of genomic information is maintained in the cell cycle by faithful replication during the S phase and segregation of duplicated chromosomes during mitosis, which is critical for proper cell reproduction, cell function, and cell survival. In addition, cells are continuously challenged by genotoxic agents and environmental stress, and have complex mechanisms to activate DNA damage checkpoints, prevent cell-cycle progression, and repair the damaged DNA (Hoeijmakers, 2001; Branzei & Foiani, 2010). Many of the cell cycle transition events, as well as responses to DNA damage, are driven by E3 Cullin-RING ubiquitin Ligases (CRLs) that catalyse the ubiquitination and destruction of specific protein targets. Such cell cycle–regulated E3 ligases include CRL1<sup>F-box</sup> and CRL4<sup>DCAF</sup>, which target many substrates crucial for cell cycle regulation and DNA damage responses (Cardozo & Pagano, 2004; Petroski & Deshaies, 2005; Jackson & Xiong, 2009). These CRLs comprise a scaffolding protein (cullin 1 or cullin 4 [Cul4]), an adapter protein (Skp1 and DDB1, respectively), and a RING domain protein that interacts with the E2 (such as Rbx1 or Rbx2). Finally, CRL1 and CRL4 ligases contain either an F-box or DCAF substrate recognition factor (SRF, or substrate receptor), respectively, responsible for interacting with the substrate and targeting it for ubiquitination. F-box proteins in CRL1, such as Fbw7 or β-TRCP, recognize specific degrons in substrates that often contain phosphorylated residues, whereas CRL4 include DCAFs such as DDB2, which directly recognizes UV-damaged DNA (Scrima et al, 2008).

The CRL4<sup>Cdt2</sup> ligase uses Cdt2 as the SRF, and functions both during the S phase and after DNA damage (Abbas & Dutta, 2011; Havens & Walter, 2011; Sakaguchi et al, 2012; Statopoulos et al, 2012). Cdt2, targets substrates such as p21 and Set8, and the DNA replication licensing factor Cdt1 for ubiquitin-mediated proteolysis, both in S phase and following DNA damage (Abbas et al, 2008; Kim et al, 2008; Nishitani et al, 2008; Centore et al, 2010; Oda et al, 2010; Tardat et al, 2010; Jorgensen et al, 2011). In addition, an increasing number of Cdt2 target proteins have been identified, including thymidine DNA glycosylase, Cdc6, the DNA polymerase δ subunit p12 (Terai et al, 2013; Clijsters & Wolthuis, 2014; Shibata et al, 2014; Slenn et al, 2014), and xeroderma pigmentosum group G (XPG), a
structure-specific repair endonuclease of the nucleotide excision repair pathway (Han et al., 2015).

Cdt1 and Cdt2 were originally identified as Cdc10-dependent transcript 1 and 2 in fission yeast, but have no sequence similarity (Hofmann & Beach, 1994). Cdt1 has a critical role in establishing the DNA replication licensing complex in the G1 phase: it associates with chromatin through the origin recognition complex and operates together with Cdc6 to load the MCM2-7 complex onto chromatin, thereby licensing DNA for replication (Bell & Dutta, 2002; Diffley, 2004; Nishitani & Lygerou, 2004; Blow & Dutta, 2005; Tsakraklides & Bell, 2010; Symeonidou et al., 2012).

Preventing re-licensing of replicated regions is essential (Blow & Dutta, 2005; Arias & Walter, 2007). One of the mechanisms to achieve this is by CRL1<sup>Skp2</sup> and CRL4<sup>Cdt2</sup> redundantly mediating Cdt1 destruction in higher organisms. CRL1<sup>Skp2</sup> (also known as SCF<sup>Skp2</sup>) recognizes a phospho-degron motif on Cdt1 that is created at the initiation of S phase by CDKs (Li et al., 2003; Sugimoto et al., 2004; Nishitani et al., 2006). In contrast, CRL4<sup>Cdt2</sup> recognizes Cdt1 when bound to the proliferating cell nuclear antigen (PCNA) trimmer, through a binding motif (PIP box) in its N-terminal end (Arias & Walter, 2006; He et al., 2006; Higa et al., 2006; Jin et al., 2006; Nishitani et al., 2006; Ralph et al., 2006; Sansam et al., 2006; Senga et al., 2006; Kim & Kipreos, 2007). Both initiation of DNA replication and DNA damage trigger PCNA loading onto chromatin and Cdt association with PCNA through its PIP box (Arias & Walter, 2006; Havens & Walter, 2009; Raman et al., 2011; Shiomi et al., 2012). DNA damage–induced degradation of Cdt1 and other substrates appears to facilitate repair (Mansilla et al., 2013; Tsanov et al., 2014; Tanaka et al., 2017).

Cdt2 recruitment onto chromatin is not fully characterized: recruitment through the Cdt1 PIP box bound to PCNA and a specific basic residue downstream of the Cdt1 PIP box (Havens & Walter, 2009; Michishita et al., 2011; Havens et al., 2012) or independently of Cdt1 (Roukos et al., 2011) has been reported. Following CRL4<sup>Cdt2</sup>-mediated ubiquitination, Cdt1 is degraded, thus blocking further licensing. The N-terminal region of Cdt2 contains a WD40 repeat domain, predicted to form a substrate-recognizing propeller structure similar to the one shown for DDB2 (Scrima et al., 2008; Havens & Walter, 2011). In analogy with DDB2 (Fischer et al., 2011), the N-terminal domain of Cdt2 should bind to the DDB1 WD40 repeat domains, β-propeller A and β-propeller C, on one side. The other side would be expected to recognize the substrate, in analogy to DDB2. Higher eukaryotic Cdt2 proteins have an extended C-terminal region, not present in fission yeast. In Xenopus, the C-terminal domain of Cdt2 binds to PCNA and is important for the turnover of the Xic1 cyclin kinase inhibitor (Kim et al., 2010). Recently, we reported that Cdt2 mutated at multiple CDK consensus phosphorylation sites co-localized with PCNA throughout the S phase even when most of the substrates were degraded, also suggesting that Cdt2 interacts with PCNA independent of its substrates (Nukina et al., 2018).

To understand how Cdt2 recognizes PCNA and localizes CRL4<sup>Cdt2</sup> activity during the S phase and following UV irradiation, we investigated the role of Cdt2 domains in localization and ubiquitination. Unexpectedly, we found a PIP box in the C-terminal end of Cdt2, and showed that it directly mediates interaction of Cdt2 with PCNA. Both Cdt1 and Cdt2 PIP box peptides bind the PCNA binding pocket in a similar manner, but Cdt2 has a significantly higher affinity for PCNA. We suggest that Cdt2 and Cdt1 could simultaneously recognize different subunits of the PCNA trimer, and we put forward a new paradigm for localizing E3 ligase activity onto the PCNA docking platform, allowing simultaneous docking of substrates in the same platform, enabling ubiquitination by proximity.

Results

Cdt2 is stably bound to UV-damaged sites independently of Cdt1

CRL4<sup>Cdt2</sup> recognizes Cdt1 bound on PCNA and recruitment of CRL4<sup>Cdt2</sup> to damaged chromatin was reported to require Cdt1 in Xenopus extracts (Havens & Walter, 2009). In contrast, experiments in live human cells suggested that Cdt2 was recruited to DNA damage sites independently of Cdt1 (Roukos et al., 2011). To verify whether Cdt2 binding to damaged sites requires Cdt1, we performed a time-course analysis of Cdt2 recruitment to sites of localized UV-C irradiation in control and Cdt1 depleted HeLa cells. Cells were exposed to UV-C through micro pore filters, and locally induced damage was detected by the staining of cyclobutane pyrimidine dimers (CPDs). In control cells, Cdt1 was rapidly recruited to damaged sites (Fig 1A, 10 min) and proteolyzed by 30 min (Fig 1A and B), consistent with earlier studies (Ishii et al., 2010; Roukos et al., 2011). Cdt2 was recruited at sites of damage by 10 min whereas cells showing Cdt2 recruitment increased at subsequent time points (30 and 45 min post-irradiation) even-though Cdt1 was degraded. Depletion of Cdt1 by siRNA had no effect on either the kinetics or the extent of Cdt2 recruitment to sites of UV-C damage (Fig 1A and B). These results show that Cdt2 is recruited to UV-C–irradiated sites independently of Cdt1 and remains there long after Cdt1 proteolysis.

To assess the binding properties of Cdt2 at sites of UV-C damage in the presence and absence of Cdt1, fluorescence recovery after photobleaching (FRAP) was used. MCF7 cells transiently expressing Cdt1, Cdt2, or PCNA tagged with GFP were first analysed in the presence and absence of local UV-C irradiation (Figs 1C and S1A–C). All factors exhibit fast recovery in the absence of damage (Fig S1A and C), consistent with transient interactions (Xouri et al., 2007b). Following recruitment to the sites of damage, PCNA shows a significant immobile fraction, as expected for stable binding, whereas Cdt1 shows dynamic interactions at UV-C–damaged sites (Roukos et al., 2011; Rapsomaniki et al., 2015). Cdt2 has a slower fluorescence recovery rate at the site of damage than Cdt1, with a half-recovery time of 0.83 s and an immobile fraction of 16%, underlining long-term association at UV-C–damaged sites. To assess whether Cdt2 binding to sites of damage is affected by the presence of Cdt1, Cdt2 kinetics were assessed by FRAP in MCF7 cells synchronized in G1, depleted of Cdt1 and locally UV-C irradiated. As shown in Fig 1D and in Fig S1D and E, the binding kinetics of Cdt2 at the sites of damage remain the same despite the absence of Cdt1.

We conclude that Cdt2 binds to sites of damage stably independently of Cdt1.

The C-terminal part of Cdt2 is required for recruitment to DNA damage sites

The above results suggested that Cdt2 contains a domain that mediates its association with UV-damaged sites independently
of Cdt1. Human Cdt2 is a 730 amino acid polypeptide, with an N-terminal WD40 domain predicted to form a substrate receptor and a long C-terminal domain (Fig 2A). Constructs expressing Cdt21–417, which contains the N-terminal WD40 domain, the C-terminal Cdt2390–730, and the full-length Cdt21–730 as a control fused with GFP were transiently expressed in MCF7 cells, followed by localized UV-C irradiation (Fig 2B and C). Cdt21–730 was robustly detected at DNA-damage sites, as previously reported. The C-terminal Cdt2390–730 construct was also efficiently recruited to DNA-damage sites. On the contrary, there was no evidence for recruitment of Cdt21–417 to sites of damage. This suggests that the C-terminal region of Cdt2, but not the predicted N-terminal substrate receptor domain, is important for its recruitment to the sites of damage.

The C-terminal part of Cdt2 is required for interaction with PCNA and has a PIP box in its C-terminal end

Consistent with the observation that the C-terminal region of Cdt2 is important for recruitment to the CPD-stained sites where PCNA also localizes, we identified PCNA as a Cdt2 C-terminus interacting protein in a yeast two-hybrid screening (Fig S2). To confirm the interaction with PCNA, we transiently transfected 3FLAG-tagged Cdt21–730, Cdt21–417, and Cdt2390–730 into HEK293T cells. Before cell lysis preparation, cells were cross-linked. Immunoprecipitation (IP) experiments using α-FLAG antibody showed that the C-terminal half, but not the N-terminal half, interacts with PCNA (Fig 3A). The Cdt21–417 domain, although it lost most of its affinity for PCNA, is still able to bind DDB1 as expected. In contrast, the Cdt2390–730 domain,
although it still binds to PCNA, has lost its ability to bind DDB1, and therefore to form a functional CRL4 complex.

To narrow the region of Cdt2 required for interaction with PCNA, we made a series of additional constructs fused to a triple FLAG tag. We confirmed that when the C-terminal 30 amino acids were deleted in the Cdt21 construct, this was sufficient to lead to a loss of interaction with PCNA (Fig 3B). Consistently, the C-terminal 130 amino acids alone, Cdt2600–730, were sufficient to mediate the interaction with PCNA.

We postulated that the C-terminal part of Cdt2 directly interacts with PCNA via a PCNA interaction protein (PIP) box; a common motif found in PCNA-dependent DNA replication and repair factors. We used PROSITE to search the UniProtKB protein sequence database for human sequences that contained consensus PIP boxes ([KMQ]-x-x-[ILMV]-x-x-[FY]-[FY]). Our search revealed a PIP box sequence (SSMRKICTYFHRKS) with a carboxytetramethylrhodamine fluorescent label at the N-terminus (Cdt2PIP) and characterized its binding to recombinant PCNA, by fluorescence polarization (FP). Cdt2PIP binds PCNA with high affinity (57 ± 3 nM, Fig 4A). The corresponding Cdt2PIP-3A peptide showed practically no detectable binding to PCNA, and to quantify that interaction, we synthesized the binding p21 PIP box peptide (50 – 85 nM) (Bruning & Shamoo, 2004; De Biasio et al, 2012), as it was confirmed in our assays (RRQTSMDFYHSKR, 36 ± 2 nM, Fig 4A).

A C-terminal PIP box in Cdt2 directly interacts with PCNA with high affinity

To confirm that this PIP box motif in Cdt2 interacts directly with PCNA, and to quantify that interaction, we synthesized the peptide corresponding to the human Cdt2 PIP box (704–717) (SSMRKICTYFHRKS) with a carboxytetramethylrhodamine fluorescent label at the N-terminus (Cdt2PIP) and characterized its binding to recombinant PCNA, by fluorescence polarization (FP). Cdt2PIP binds PCNA with high affinity (57 ± 3 nM, Fig 4A). The corresponding Cdt2PIP-3A peptide showed practically no detectable binding to PCNA in the same assay (Fig 4A), suggesting that the Cdt2 PIP box binds PCNA in a manner very similar to other known PCNA complexes with PIP box peptides.

As Cdt2 is recruited to PCNA on chromatin during the S phase and after DNA damage through the Cdt1 N-terminal PIP box (residues 3–10) (Havens & Walter, 2009; Ishii et al, 2010; Roukos et al, 2011), we then synthesized a Cdt2PIP-TAMRA (MEQRRVTDFFARRR) peptide, to compare its affinity with PCNA. Remarkably, the affinity of the Cdt2PIP peptide to PCNA (7,200 ± 200 nM, Fig 4A) is two orders of magnitude weaker than that for the Cdt2PIP-3A, and similar to what is reported for other peptides derived, for example, from the pol-δ p66 and FEN1 (10–11) (Havens & Walter, 2009; Ishii et al, 2010; Roukos et al, 2011), we then synthesized a Cdt1PIP peptide (57 ± 3 nM, Fig 4A). The corresponding Cdt1PIP-3A peptide showed practically no detectable binding to PCNA in the same assay (Fig 4A), suggesting that the Cdt2 PIP box binds PCNA in a manner very similar to other known PCNA complexes with PIP box peptides.

Next, we examined the interaction of CRL4<sup>Cdt2</sup> ligase with its target protein Cdt1, using HEK293 cells alone and cells transfected with Cdt2<sup>WT</sup> or Cdt2<sup>PIP-3A</sup>, with or without UV irradiation. Using an anti-FLAG antibody resin to precipitate Cdt2<sup>WT</sup>, both PCNA and Cdt1 were precipitated, and their amounts were notably increased after UV irradiation (Fig 3D). In contrast, neither Cdt1 nor PCNA co-precipitated well with Cdt2<sup>PIP-3A</sup>, before or after UV irradiation. These results suggested that the PIP box of Cdt2 was required for forming a stable complex with its substrates on PCNA.
To confirm the binding mode of the Cdt2PIP and Cdt1PIP peptides to PCNA, we determined the crystal structure of both complexes by X-ray crystallography, to a 3.5- and 3.4-Å resolution, respectively. PCNA adopts its well-characterized trimer conformation, with one peptide bound per monomer. The binding mode of both the peptides was very clear (Figs 4B, C, S3A and B and Table 1, see the Materials and Methods section for details). Both peptides bind in a similar manner to other PCNA complex structures. Some notable differences in the binding mode are the conserved Phe713/Phe11 side-chain ring that rotates 180 degrees between the two structures and the Tyr712/Phe10 that repositions so as to extend more towards a hydrophilic environment in Cdt2. The Ile709 and Val7 side chains occupy a similar space in the hydrophobic recognition pocket. Albeit the peptide main chain is in a very similar conformation between the structures, some of the other side chains adopt rather different conformations. The average buried area upon the binding of the Cdt2 peptide is $692 \pm 6 \text{Å}^2$ and average calculated energy of binding is $-13 \pm 0.2 \text{ kcal mol}^{-1}$; these confirm the tighter binding of the Cdt2 peptide to PCNA.

**Cdt2 directly binds to PCNA on DNA through its PIP box independently of Cdt1 in vitro**

The above results suggested that Cdt2 was recruited to the PCNA sites primarily through its PIP box rather than the N-terminal substrate receptor domain, which contrasts to the model that Cdt1 and CRL4Cdt2 are sequentially recruited to PCNA on Chromatin. To investigate the mechanism of Cdt1 and CRL4Cdt2 recruitment to PCNA, we set up an in vitro analysis with purified and defined human proteins. FLAG-tagged Cdt1 (Cdt1-3FLAG) and CRL4Cdt2 (Cdt2-3FLAG) were expressed in insect cells and purified on anti-FLAG resin. PCNA and its loader replication factor C (RFC) were purified as described in the experimental procedures (Fig S4). First, we examined the interaction of Cdt1 or CRL4Cdt2 with PCNA in the absence of DNA. Figure 3. The C-terminal domain of Cdt2 has a conserved PIP box and interacts with PCNA.

(A) Full-length Cdt2 (Cdt21–730) and Cdt2390–730, but not Cdt21–417, interact with PCNA. HEK293 cells were transfected with plasmids expressing the indicated Cdt2 fusions to a C-terminal FLAG tag, cross-linked, and precipitated with anti-FLAG antibodies (arrows). Precipitates were examined for PCNA, Cul4A, and DDB1. * indicates nonspecific bands derived from IgGs. The bands in the blank lanes for Cul4A blotting correspond to the cross-reacting bands to pre-stained marker. (B) Cdt12600–730, but not Cdt21–700, interacts with PCNA. HEK293 cells were transfected with the indicated plasmids and treated as in A. * indicates nonspecific bands derived from IgGs. (C) A schematic drawing of the Cdt2 domain structure highlighting the C-terminal PIP box and a sequence alignment of PIP boxes in various organisms. The alanine-changed PIP box mutant of Cdt2 was shown (PIP-3A) together with Cdt1 PIP box. (D) HEK293 cells were transfected with Cdt2360–730 or PIP box mutant (Cdt21–730-3A) C terminally fused to a FLAG tag, treated as in B, and Cdt2 was precipitated with anti-FLAG resin. The interaction with PCNA and DDB1 was examined by immunoblotting. * indicates nonspecific bands. (E) HEK293 cells were transfected with Myc-tagged PCNA and Cdt2360–730-FLAG or Cdt2360–730-3FLAG; PCNA was immunoprecipitated by anti-Myc antibodies and the interaction with Cdt2 was examined by immunoblotting.
The purified Cdt1 or Cdt2 were fixed on anti-FLAG beads, incubated with free PCNA, and the amount of bound PCNA was analysed. In contrast to the above-mentioned results that both Cdt1 and Cdt2 PIP box peptides bound to PCNA (Fig 4), PCNA was detected on Cdt1-beads, but much less on CRL4Cdt2-beads (Fig 5B). Because Cdt1 and Cdt2 bind to PCNA only when PCNA is loaded on DNA in Xenopus egg extracts (Havens & Walter, 2009), we prepared PCNA loaded on nicked circular DNA (ncDNA) with RFC (PCNAon DNA) (Fig S5A and B). In our in vitro conditions, three molecules of PCNA trimer were loaded on one plasmid DNA. Then, we analysed the binding of Cdt1 and CRL4Cdt2 to PCNAon DNA as described in Fig S5A. After incubation, Cdt1 was efficiently recovered on the PCNAon DNA beads, but not on the control beads (Fig 5C). Although we could not show CRL4Cdt2 binding to free PCNA, we show that CRL4Cdt2 binds to PCNAon DNA in the absence of Cdt1. The interaction was not mediated by RFC proteins bound on ncDNA (Fig 5D, lane 6). The molar ratio of Cdt2 bound to trimeric PCNAon DNA was the same or somewhat higher than that of Cdt1 (408 fmol of Cdt2 bound to 158 fmol trimeric PCNAon DNA, that is, 2.57 Cdt2 molecules on one PCNA trimer, while 301 fmol of Cdt1 to 148 fmol of trimeric PCNAon DNA 2.04 Cdt1 molecules on one PCNA trimer). These results indicate that CRL4Cdt2 directly and efficiently binds to DNA-loaded PCNA without substrate, consistent with data in cells showing that Cdt2 was recruited to the sites of damage in the absence of Cdt1 (Fig 1).

To confirm that the interaction of CRL4Cdt2 with PCNAon DNA was mediated by the PIP box of Cdt2, the CRL4 complex having Cdt2PIP-3A (CRL4Cdt2PIP-3A) was purified like CRL4Cdt2 (Fig 5A) and used in a binding assay. As above, CRL4Cdt2 interacted with PCNAon DNA. In contrast, the binding activity of CRL4Cdt2PIP-3A to PCNAon DNA was severely reduced as compared with CRL4Cdt2 (Fig 5D). These results demonstrate that the PIP box of Cdt2 was directly responsible for the interaction of CRL4Cdt2 with PCNAon DNA.

**The Cdt2 PIP box promotes Cdt2 recruitment to UV-irradiated sites and PCNA foci**

To investigate the role of the Cdt2 PIP box in cells, we isolated HEK293 cells stably expressing FLAG-tagged Cdt2PIP-3A and showed...
that Cdt2PIP-3A was not recruited to UV-irradiated sites (Fig 6A). XPA, a component of nucleotide excision repair, staining was used to confirm that similar extents of DNA damage were induced both in Cdt2WT- and Cdt2PIP-3A-expressing cells. Next, we verified that the recruitment of Cdt2 onto PCNA during the S phase is also dependent on its PIP box. To detect the chromatin-associated fraction of PCNA and Cdt2, asynchronous cells were pre-extracted before fixation and co-stained with PCNA and FLAG antibodies. A similar percentage of PCNA-positive S-phase cells was detected both in Cdt2WT- and Cdt2PIP-3A-expressing cells (Fig S6). Cdt2PIP-3A was co-localized with PCNA foci, corresponding to sites of DNA replication. More than 80% of PCNA-positive cells were co-stained with Cdt2WT, with the early S-phase cells displaying stronger Cdt2WT staining than the late S-phase cells as reported (Nukina et al, 2018) (Figs 6B and C, and S6A). In contrast, almost no signal was detected for Cdt2PIP-3A irrespective of the presence of PCNA. Consistent with the immunofluorescence analysis, following cell fractionation, Cdt2WT was recovered in a chromatin-containing fraction, whereas Cdt2PIP-3A was undetectable (Fig 6D).

These results collectively suggest that although Cdt2PIP-3A is capable of forming a complex with the rest of the components of the CRL4 ligase, the Cdt2 PIP box is required for efficient recruitment of CRL4Cdt2 to the PCNA-loaded sites.

Cdt1 ubiquitination is abortive in Cdt2PIP-3A-expressing cells

Because the recruitment of Cdt2PIP-3A to PCNA sites was compromised, it would imply that ubiquitination of Cdt1 was also abrogated. To consider this possibility, asynchronously growing control HEK293 cells and cells expressing Cdt2WT or Cdt2PIP-3A were treated with proteosome inhibitor MG132 and the levels of poly-ubiquitinated Cdt1 were examined. Although poly-ubiquitinated

Figure 5. Cdt1 and CRL4Cdt2 independently associate with PCNA loaded on DNA through PIP box in vitro. (A) Purified CRL4Cdt2(WT), CRL4Cdt2(PIP-3A), and Cdt1 proteins. (B) Cdt1, but not CRL4Cdt2, associates with free PCNA. The purified Cdt1 and CRL4Cdt2 proteins were fixed on anti-FLAG–resins and incubated with PCNA for 2h at 4°C and the bound PCNA was analysed. The relative amounts of bound PCNA were shown, normalized with FLAG signal, and the level on Cdt1-3FLAG beads set to 1.0. Error bars represent SD from three independent experiments. (C) The ncDNA beads (DNA+) or control beads (DNA−) were loaded with PCNA by RFC or not. Then, beads were incubated with the purified Cdt1 or CRL4Cdt2, and the bound proteins were recovered for Western blotting. The amounts of PCNA (as a trimer) loaded on plasmid DNA and the amounts of bound Cdt1-3FLAG and Cdt2-3FLAG were measured and shown (f mol). The number of bound Cdt1 and Cdt2 on PCNA trimer was shown (Cdt1/PCNA and Cdt2/PCNA). Error bars represent SD from independent experiments (n = 3 or n > 3). (D) CRL4Cdt2(WT) and CRL4Cdt2(PIP-3A) were incubated with control beads, ncDNA beads, or ncDNA beads which had been pre-incubated with RFC alone or together with PCNA for 1h at 4°C. Beads were recovered and bound proteins were analysed.
Cdt1 was clearly detected in Cdt2WT-expressing cells, its levels were reduced in Cdt2<sub>PIP-3A</sub>-expressing cells (Fig 6E), indicating that the ability of Cdt2<sub>PIP-3A</sub> to ubiquitinate Cdt1 was compromised. Fractionation of cell extracts revealed that most of the poly-ubiquitinated Cdt1 and p21 were recovered in the chromatin-containing precipitate fraction prepared from Cdt2WT-expressing cells, in agreement with CRL4Cdt2 operating on chromatin (Fig 6E). Cdt2PIP-3A-expressing cells however had reduced levels of both poly-ubiquitinated Cdt1 and p21 on chromatin. Interestingly, Cdt2 itself is poly-ubiquitinated on chromatin, and this is severely reduced in Cdt2<sub>PIP-3A</sub>-expressing cells (Fig 6E). Increased levels of poly-ubiquitination of Cdt1 and p21 were also observed in another cell line, U2OS cells expressing Cdt2<sub>WT</sub>, but not in cells expressing Cdt2<sub>PIP-3A</sub> (Fig S7B).

Next, we examined the accumulation of poly-ubiquitinated Cdt1 after UV irradiation. For this experiment, the endogenous Cdt2 was depleted with an siRNA targeted to the 3' UTR to assay for the activity of introduced Cdt2. Cells were treated with MG132, irradiated with UV and the levels of poly-ubiquitinated Cdt1 were monitored by Western blotting. In HEK293 cells treated with control siRNA, poly-ubiquitinated Cdt1 was detected 15 min after irradiation (Fig S6B). Depletion of Cdt2 blocked the poly-ubiquitination of Cdt1. The defect was rescued by Cdt2WT expression. In contrast, Cdt2<sub>PIP-3A</sub>-expressing cells were defective in poly-ubiquitination of Cdt1 (Fig S6B), suggesting that Cdt2 PIP box is required for efficient poly-ubiquitination of substrates.

The Cdt2 PIP box is required for efficient Cdt1 degradation

Because the poly-ubiquitination activity of Cdt2<sub>PIP-3A</sub> was reduced, we monitored the degradation of Cdt1 after UV irradiation. Asynchronously growing control U2OS cells and cells expressing Cdt2WT or Cdt2<sub>PIP-3A</sub> (Fig S7) were depleted of endogenous Cdt2 using an siRNA targeted to the 3' UTR, and irradiated with UV. When the cells were exposed to UV (20 J/m<sup>2</sup>) radiation, degradation of Cdt1 was almost prevented in U2OS cells depleted of endogenous Cdt2 (Fig 7A and B). Ectopic expression of Cdt2WT restored Cdt1 degradation to similar kinetics to that of control siRNA-transfected U2OS cells. In contrast, Cdt2<sub>PIP-3A</sub> was much less effective in rescuing Cdt2 depletion. Similarly, at a lower dose of UV irradiation (5 J/m<sup>2</sup>), Cdt1...
remained stable 1 h post UV irradiation in Cdt2PIP-3A cells, when endogenous Cdt2 was depleted, both on immunofluorescent analyses and Western blotting (Figs 7C and D, and S7C).

Next, we examined Cdt1 degradation after the onset of the S phase in the Cdt2PIP-3A-expressing cells. Cyclin A is present in the S and G2 phase, when Cdt1 should be degraded. Thus, Cdt1 should be present in cells that are negative for cyclin A, and absent in cells that are positive for cyclin A (Nishitani et al., 2006; Xouri et al., 2007a, 2007b). Cdt1 degradation after the onset of the S phase is carried out by two ubiquitin ligases, CRL1Skp2 and CRL4Cdt2. We depleted the substrate recognition subunits, Cdt2 and/or Skp2, by siRNA, and the percentage of Cdt1 positive cells among cyclin A-positive cells was assessed by immunofluorescence. In control siRNA-transfected U2OS cells, ~2% of cyclin A-positive cells stained positive for Cdt1. In contrast, in Cdt2-depleted and Skp2-depleted, and Cdt2/Skp2 co-depleted cells, 50%, 30%, and more than 80% of cyclin A-positive cells stained positive for Cdt1, respectively (Fig 8A~C), consistent with earlier work (Nishitani et al., 2006). Then, we depleted Cdt2 and Skp2 and examined Cdt1 degradation in Cdt2WT or Cdt2 PIP-3A-expressing cells. Ectopic expression of Cdt2WT fully complemented the depletion of Cdt2, and also the co-depletion of Skp2. This was probably because the levels of ectopically expressed Cdt2WT were higher than the endogenous Cdt2 levels. On the other hand, Cdt1 degradation was not fully rescued by the expression of Cdt2PIP-3A protein both in Cdt2-depleted and Cdt2 and Skp2 co-depleted cells (Fig 8A and B). Upon depletion of endogenous Cdt2, many cells exhibited significantly enlarged nuclei, consistent with re-replication. This phenotype was rescued by ectopic expression of Cdt2WT, but not of Cdt2PIP-3A (Figs 8D and S8), suggesting that the PIP box of Cdt2 is essential to prevent re-replication.

Taken together, our in vitro and in vivo analyses show that the Cdt2 PIP box brings CRL4 ubiquitin ligase to PCNA sites and is required for efficient substrate degradation both during the S phase and following UV irradiation, to maintain genome stability.

Discussion

Cdt2 substrates are degraded rapidly as cells enter the S phase or when cells are exposed to DNA-damaging agents. Cdt2 of higher
Eukaryotic cells have an extended C-terminal region. We demonstrate that Cdt2 has a PIP box in its C-terminal end that recognizes chromatin-bound PCNA with high affinity. This interaction is important for CRL4Cdt2 function in recognizing its targets before their ubiquitin-dependent degradation: this previously unnoticed Cdt2 PIP box is required for recruitment of CRL4Cdt2 to chromatin-bound PCNA, and indispensable for efficient Cdt1 ubiquitination, thus for prompt degradation of Cdt1 both in the S phase and after UV irradiation. The Cdt2 PIP box peptide directly interacts with PCNA with high affinity (about 50 nM) in vitro, similar to the unusually high-affinity p21 PIP box peptide, and significantly tighter than the Cdt1 PIP box peptide. Molecular modelling experiments suggest that both the Cdt1 and Cdt2 PIP boxes bind PCNA in the classical way and their binding to PCNA occurs independently of each other (Fig 4). Furthermore, the purified CRL4Cdt2 ligase interacted directly with the PCNA loaded on DNA via Cdt2 PIP box (Fig 5). Consistently, the C-terminal domain of Cdt2 has been shown to bind to PCNA in Xenopus egg extracts (Kim et al, 2010). Our analysis now shows that the PIP box is conserved in Xenopus, and thus likely to be responsible for this interaction.

PCNA is a trimer, hence it offers three PIP box binding sites that are proposed to be asymmetric when PCNA is loaded onto DNA (Ivanov et al, 2006). Consequently, both Cdt1 and Cdt2 can be simultaneously recruited onto PCNA, effectively utilizing PCNA as a common docking platform for bringing the CRL4Cdt2 ligase and the Cdt substrate in proximity, allowing more efficient transfer of a ubiquitin moiety from the E2 to Cdt1 (Fig 9). This Cdt2 PIP box assisted ubiquitination is likely to be used by other Cdt2 substrates, as p21 poly-ubiquitination was also defective in Cdt2PIP-3A-expressing cells (Figs 6E and S7B).

**Figure 8.** The Cdt2 PIP box plays an important role for Cdt1 degradation during cell cycle. (A, B) U2OS cells and U2OS cells stably expressing Cdt2WT-3FLAG or Cdt2PIP-3A-3FLAG were transfected with siCdt2 and/or siSkp2 or control siLuc for 72 h. Cells were fixed and stained with Cdt1 and cyclin A antibodies. The frequency of Cdt1-positive (+) cells among cyclin A-positive (+) cells was calculated ((n = 3). (C) Cells transfected with siRNAs as above (C+S; siRNAs for Cdt2 and Skp2) were examined for indicated protein levels. (D) Cells treated with siCdt2 or control siLuc were measured for their nuclear size (n = 200 for each), and are shown in a scatter plot. Frequency of cells with nuclear size larger than 450 μm² is shown (%).
same protein platform brings the E3 ubiquitin ligase and its substrate in proximity, is a new paradigm in ubiquitin conjugation. This model fits recent results which show that Cdt2 itself can be auto-ubiquitinated by CRL4<sup>Cdt2</sup> (Abbas et al., 2013): CRL4<sup>Cdt2</sup> and Cdt2 (or a second CRL4<sup>Cdt2</sup>) may both bind PCNA through the Cdt2 PIP box and catalyse this auto-ubiquitination event. Consistently, the poly-ubiquitination of Cdt2 which was observed on chromatin in Cdt2<sup>wt</sup>-expressing HEK293 cells was severely reduced in Cdt2<sup>PIP-3A</sup>-expressing cells (Fig 6E).

The mono-ubiquitination (mUb) of PCNA is carried out by Rad6-Rad18, but CRL4<sup>Cdt2</sup> also contributes to it. In non-damaged cells, mUb of PCNA is present at a basal level and its levels increase in response to DNA damage (Terai et al., 2013). The Cdt2 PIP box may also be important for mUb of PCNA, as mUb-PCNA levels appeared decreased in Cdt2<sup>PIP-3A</sup> in comparison with Cdt2<sup>wt</sup>-expressing cells (Fig S7B). Therefore, Cdt2 PIP box may be required for various events driven by CRL4<sup>Cdt2</sup> in the cell; degradation of PIP-degron proteins, auto-ubiquitination of Cdt2, and PCNA mUb.

We show that the Cdt2 PIP box is crucial for recruitment of the CRL4<sup>Cdt2</sup> ligase onto PCNA in human cells. However, further interactions of Cdt2 domains, mediated by its N-terminal WD40 repeats, with the PCNA-bound Cdt1 PIP box (the PIP degron) also enhance the affinity of the CRL4<sup>Cdt2</sup> (Havens & Walter, 2009).

Here, we propose a synergistic mechanism where the Cdt2 PIP box and the Cdt1 PIP-degron operate in concert to promote CRL4<sup>Cdt2</sup>-dependent target ubiquitination (Figs 9 and S9). It is also noteworthy that the binding of Cdt1 onto PCNA is transient in cells, whereas Cdt2 interactions are more stable (Roukos et al., 2011 and Fig 1B); a synergistic mechanism would imply that interactions of pre-bound Cdt2 with transiently formed Cdt1 PIP-degron, are important for localizing CRL4<sup>Cdt2</sup> and Cdt1 onto PCNA. The property that affinity of Cdt2 PIP box to PCNA is higher than that of Cdt1 PIP box (Fig 6A) is compatible with such a model. In addition, weaker affinity of Cdt1 PIP box can help to release Cdt1 after poly-ubiquitination, whereas CRL4<sup>Cdt2</sup> could remain on PCNA to trap the next substrate, leading to an efficient degradation cycle of substrates (Fig 5F). In the absence of Cdt2 PIP box, the process needs to be performed sequentially, transient Cdt1 binding to PCNA and recognition by Cdt2 via its N-terminal WD40 repeats, which would be, however, less effective for Cdt2 degradation.

Our biochemical analysis demonstrated that CRL4<sup>Cdt2</sup> interacts more strongly with PCNA<sup>wt</sup> DNA than with PCNA that is not associated with DNA (free PCNA) (Fig S5B and C). Because Cdt2 PIP peptide can bind to free PCNA (Fig 4), there is a mechanism that enhances the affinity of CRL4<sup>Cdt2</sup> to the PCNA<sup>wt</sup> DNA. This is compatible with models that suggest that a conformational change upon DNA binding can modulate affinity. It is tempting to speculate that the high-affinity CRL4<sup>Cdt2</sup> complex docking into DNA-bound PCNA triggers changes that allow the transient loading of low-affinity substrates such as Cdt1, enabling rapid ubiquitination molecules in the vicinity. On the other hand, we reproducibly observed that Cdt2 was recovered, although at lower levels, on DNA beads in the absence of PCNA (Fig 5C), suggesting that CRL4<sup>Cdt2</sup> has a DNA-binding activity. Furthermore, the RFC1 complex may have a role connecting PCNA and Cdt2, as Cdt2 PIP-3A was detected to some more levels in the presence of the PCNA and RFC1 complex (Fig 5D). As reported, PCNA loaders appear to have an additional role in the CRL4<sup>Cdt2</sup>-mediated ubiquitination after loading PCNA (Shiomi et al, 2012). The extended C-terminal region of Cdt2 might have an additional domain involved in such a regulation, or an unknown factor might be involved. The exact molecular mechanisms that interplay to regulate these interactions need to be investigated further.

Although the Cdt2 PIP box mediates direct interaction with PCNA<sup>wt</sup> DNA, the affinity of Cdt2 to PCNA decreases as cells progress into and complete the S phase. The chromatin levels of Cdt2 are reduced during the late S phase (Rizzardi et al, 2015), and the co-localization of Cdt2<sup>wt</sup> with PCNA was mostly observed in the early S phase cell nuclei (Fig 6B). It was suggested that the CDK-dependent phosphorylation of Cdt2 down-regulates Cdt2 interaction with PCNA, as Cdt1 inhibition recovered the chromatin interaction of Cdt2 at the late S phase, and Cdt2 mutated at CDK phosphorylation sites displayed strong interaction with PCNA throughout the S phase (Rizzardi et al, 2015; Nukina et al, 2018). We propose that the C-terminal region of Cdt2 contributes to regulating the activity of CRL4<sup>Cdt2</sup> in the cell cycle by controlling its affinity to PCNA; Cdt2 PIP box-mediated binding to PCNA is important for rapid substrate degradation at the onset of the S phase, whereas the phosphorylation of the C-terminal region of Cdt2 inhibits its PCNA interaction and thus reduces ubiquitination activity during the late S phase, leading to substrate re-accumulation. It remains, however, to be elucidated how phosphorylation of Cdt2 affects its interaction to PCNA. We noticed that our Cdt2 preparation from insect cells was phosphorylated (Fig S4D).
at levels found in human cells. The assay with de-phosphorylated Cdt2 and kinase-treated Cdt2 could help to understand how the phosphorylation on Cdt2 regulates its binding activity.

CRL-type ligases are thought to use the “SRF” or “substrate receptor” subunit to recognize a specific degron in the substrate itself. However, CRL4-type ligases can deviate from that mechanism. For example, CRL4DDB2 recognizes specific UV-damaged bases in DNA, creating a “ubiquitination zone” around the repair site, to ubiquitinate specific substrates (Fischer et al, 2011). Here, we show a novel specific mechanism, where the ligase and substrate recognize the same molecule of a cellular platform, to bring the enzyme and substrate into close proximity and thus to facilitate substrate trapping: our data constitute a new paradigm for the use of a docking platform in ubiquitin conjugation. Creating such a three-component system offers many possibilities for regulation and robustness of the ubiquitination response: the recognition of PCNA by both substrate and Cdt2 through specialized PIP boxes likely enables further interactions between substrate and Cdt2 (as, for example, the recognition of the PIP degron by its N-terminal WD40 repeat domain), creating a set of redundant molecular recognition events that regulate this system in the cellular context.

Recently published work is consistent with and supports our data (Leng et al, 2018).

**Experimental Procedures**

**Cell culture**

HeLa cells, HEK293 cells, 293T cells, and U2OS cells were cultured in Dulbecco’s modified Eagle’s medium with 10% fetal bovine serum and 5% CO₂. MCF7 cells were cultured in Dulbecco’s modified Eagle’s medium with 20% fetal bovine serum and 5% CO₂ at 37°C. HEK293 cells were cultured in dishes was performed at 5 to 100 J/m² using a UV lamp (SUV-16, inhibitor MG132 was used at 25 pCMV-HA-Cdt2 WT(1–417), and p3FLAG was constructed by cloning the PCR-amplified BamH1-DBB1-Ecor1 fragment and the EcoR1-DBB1-Xhol fragment into pBacPAK9 at BamH1-Xho1 sites. pcDNA-6myc-PCNA was constructed by ligating the PCR-amplified SacI-Cdt1-3FLAG-Xho1 fragment using pCMV-Cdt1-3FLAG as a template into pBacPAK9. The GFP-Cdt2wt(417), GFP-Cdt21–417, and GFP-Cdt2(390–730) expression plasmids were constructed by subcloning of the respective FLAG-tagged constructs into pEGFP-C3 (Clontech). For the GFP-Cdt2–expressing plasmid, pCMV-HA-Cdt2(390–730) was cut with NcoI and Kpn1, Kpn1 and Xmal. To produce blunt ends for the NcoI sites, Klenow was used. The fragment was then cloned into the HindIII and Xmal sites of pEGFP-C3. The ends produced by HindIII were made blunt by Klenow. For the GFP-Cdt2(1–417), the pCMV-HA-Cdt2(2–417)-3FLAG construct was cut by NcoI and Xmal. Klenow was used for the NcoI ends. The fragment was then cloned into the HindIII and Xmal sites of pEGFP-C3. The ends produced by HindIII were made blunt by Klenow. To achieve a better nuclear localization for the GFP-Cdt2wt and GFP-Cdt2(1–417) constructs, these were subcloned into p3FLAG-3NLS-myc by Xhol and BamH1. Then, they were subcloned again into pEFP-C3 by using the HindIII-BamH1 sites. For the GFP-Cdt2(390–730) expression plasmid, the p3FLAG-3NLS-myc Cdt2(390–730) construct was cut with NcoI and Xmal. Klenow was used for the NcoI ends. The fragment was then cloned into the HindIII and Xmal sites of pEFP-C3. The ends produced by HindIII were made blunt by Klenow. For Western blotting, whole-cell lysates were prepared by lysing cell pellets directly in SDS–PAGE buffer. For immunofluorescence, U2OS or HEK293 cells were fixed in 4% paraformaldehyde (PFA; WAKO) for 10 min, permeabilized in 0.25% (vol/vol) Triton X-100 in PBS, and stained with the indicated antibodies as described previously. For double-staining with PCNA and Cdt2wt or Cdt2(2–417), cells were permeabilized in 0.1% Triton X-100 for 1 min on ice, and then incubated in 0.5 N NaOH for 5 min for DNA denaturation before immunofluorescence. Alexa488-conjugated anti-mouse and Alexa592-conjugated anti-rabbit antibodies were used as secondary antibodies with Hoechst 33258 to visualize DNA. The following primary antibodies were used: Cdt1 (Nishitani et al, 2006), Cdt2 (Nishitani et al, 2008), cyclin A (mouse; Ab-6; Neomarkers; rabbit, H-432; Santa Cruz), Myc (mouse, 9E10; Santa Cruz), FLAG (F3165, F7425; Sigma-Aldrich), PCNA (PC10; Santa Cruz; rabbit, serum, a gift from Dr. Tsurimoto), KP-A (FL-273; Santa Cruz), RCC1 (Shiomi et al, 2012), DDB1 (Bethyl Laboratories), Rbx1 (ROC1, Ab-1;
Neomarkers, Cul4A (Bethyl Laboratories), RFC4 (H-183; Santa Cruz Biotechnology), DYKDDDK Tag (Cell Signaling), and CPDs (CosmoBio). Protein levels were analysed by ImageJ software.

**Micropore UV irradiation assay**

We used a method to induce DNA photoproducts within localized areas of the cell nucleus as described in Ishii et al (2010). To perform micropore UV irradiation, cells were cultured on cover slips, washed twice with PBS, and subsequently covered with an isopore polycarbonate membrane (Millipore) with a pore size of either 3 or 5 μm in diameter, and irradiated. UV irradiation was achieved using a UV lamp (SUV-16, As One, Japan) with a dose rate of 0.4 J/m² s, which was monitored with a UV radiometer (UVX Radiometer, UVP) at 254 nm, or with a UV box equipped with a UV-C lamp monitored with a VLX-3W radiometer (Vilber Lourmat) at 254 nm (CX-254 sensor). The filter was removed and cells were either fixed or cultured for the indicated time before fixation, and processed for immunofluorescence. For FRAP analysis, cells were cultured for 20 min to allow recruitment to sites of damage before photobleaching.

**IP from Cdt2-FLAG–expressing cells**

Control or Cdt2-FLAG expressing 293 cells that were UV irradiated (50 J/m²), or not UV irradiated, or in the middle S phase (5 h after release from aphidicolin arrest) were lysed using 0.1% Triton X-100-containing modified cytoskeleton (mCSK) buffer (10 mM Pipes, pH 7.9, 100 mM NaCl, 300 mM sucrose, 0.1% [vol/vol] Triton X-100, 1 mM phenylmethylsulfonyl fluoride, 10 mM β-glycerophosphate, 1 mM NaVO₄, 10 mM NaF), and then sonicated. After centrifugation (120,000 g, Beckman Type 45Ti rotor, for 20 min at 4°C), the supernatants were mixed with anti-FLAG antibody-conjugated magnetic beads (M8823; Sigma–Aldrich) for 1 h at 4°C to obtain immunoprecipitates. The precipitate was washed with ice-cold 0.1% Triton X-100 containing mCSK buffer and subsequently suspended in SDS sample buffer.

**RNAi knockdown experiments**

Double-stranded RNAs were transfected at 100 nM using Oligofectamine (Invitrogen) or HiPerFect (QIAGEN). Twenty-four hours after the first transfection, a second transfection was performed and cells were cultured for two more days. The following siRNAs were made by Dharmacon: Skp2; GCAUGUACAGUGGCGUUGU, Cdt2_3’UTR; GCGUGCCUUUGCUCCUAUA. The siRNA for siLuc, known as GL2, was used as a control siRNA. For Cdt1 silencing in Fig 1A and B, unsynchronized HeLa cells were transfected twice with 200 nM of Cdt1 siRNA or control luciferase siRNA using Lipofectamine 2000 with a time interval of 24 h and were analysed 48 h after the second transfection. In synchronized MCF7 cells (Fig 1D), a single transfection with 200 nM of Cdt1 siRNA or control luciferase siRNA using Lipofectamine 2000 5 h after release from thymidine and before adding nocodazole was carried out. The Cdt1 siRNA was made by MWG: AACGUGGAUGAAGUACCCGACTT.

**Chromatin fractionation**

Cell extracts were prepared using 0.1% Triton X-100–containing mCSK buffer (200 μg of total protein in 400–800 μl) After centrifugation (17,900 g for 10 min at 4°C), soluble and chromatin-containing pellet fractions were obtained. The pellets were washed with ice-cold 0.1% Triton X-100–containing mCSK buffer and subsequently suspended in SDS sample buffer.

**Protein purification**

**PCNA**

PCNA was purified essentially as a trimer as described (Fukuda et al, 1995). Cell lysate was prepared from pT7-PCNA–transformed Rosetta (DE3) and sequentially subjected to Resource Q, Superdex 200, and Mono Q (GE Healthcare Life Science) column chromatography.

**Cdt1-3FLAG**

The baculoviruses for Cdt1-3FLAG expression were infected into SF21 insect cells, and cultured at 27°C for 60 h. Cell lysate was prepared with 0.5 M NaCl–containing buffer B (50 mM Tris–HCl, pH 8.0, 0.15 M NaCl, 1 mM EDTA, 10% glycerol, 1× Protease inhibitor cocktail [Roche Applied Science], 1 mM PMSF, 2 μg/ml Leupeptin), and passed on Diethylaminoethylation (DEAE) column. The flowthrough fraction was mixed with anti-FLAG resin (Sigma–Aldrich) and Cdt1-3FLAG was eluted with 200 μg/ml 3× FLAG peptides (Sigma–Aldrich).

**RFC complex**

The baculoviruses for FLAG-Rfc1, Rfc2, Rfc3, and Rfc5 were co-infected to High Five cells, expressed, and purified as described (Shioni et al, 2000).

**CRL4<sup>Clf2</sup>**

The baculoviruses for Cdt2-3FLAG (WT or Pip-3A), DDB1, HA-Cul4, and His-myc-Rbx1 were co-infected into SF21 cells, and purified as described (Hayashi et al, 2014). Briefly, the cell lysate was prepared with 0.5 M NaCl and 0.5% Nonidet-P-40–containing buffer B, clarified by centrifugation, and passed on a DEAE column. The flowthrough fraction was mixed with anti-FLAG resin and CRL4<sup>Clf2-3FLAG</sup> was eluted with buffer B containing 0.1 M NaCl, 0.1% NP-40 and 200 μg/ml 3FLAG peptide (Sigma–Aldrich). If necessary, CRL4<sup>Clf2-3FLAG</sup> was loaded on a 15–35% glycerol gradient in buffer G (25 mM Hepes, pH 7.8, 0.1 M NaCl, 1 mM EDTA, 0.01% NP-40) and subjected to ultra-centrifugation (Beckman TLS-55) at 214,000 g for 18 h at 4°C. 100 μl fractions were collected.

**In vitro binding assay for Cdt1 and CRL4<sup>Clf2</sup> with PCNA**

Cdt1-3FLAG (WT) and CRL4<sup>Clf2-3FLAG</sup> (WT) beads were prepared as follows (Fig 5B): Cleared lysates were made from insect cells infected with baculoviruses for Cdt1-3FLAG (WT) or CRL4<sup>Clf2-3FLAG</sup> (WT), incubated with anti-FLAG magnetic beads pre-blocked with buffer B containing 50% FBS, 0.5 M NaCl, and 0.5% NP-40; and washed with 0.5 M NaCl and 0.5% NP-40–containing buffer B and then with 0.1 M NaCl and 0.1% NP-40–containing buffer B. The protein beads prepared as above were incubated with PCNA protein at 4°C for 2 h, washed, and subjected to Western blotting.
Preparation of ncDNA beads

Closed circular (cc) DNA beads were prepared as described (Higashi et al., 2012). Briefly, single-stranded DNA templates prepared using pBluescript II KS(–) were annealed with a biotinated oligonucleotide primer (5’-GGAGACGGACCTGAATGAAGC-3’). After second-strand synthesis and ligation, covalently cc double-stranded DNA was purified by CsCl/EtBr density gradient centrifugation, incubated with streptavidin, and bound to biotin-Sepharose beads to produce the cc DNA beads (around 100 ng per centrifugation, incubated with streptavidin, and bound to biotin-Sepharose beads to produce the ncDNA beads. A single nick was introduced by Nb.BbvCI (New England Biolabs) treatment to produce the ncDNA beads.

PCNA loading and binding assay with purified streptavidin alone (sa-beads).

As control beads, biotin-sepharose beads were incubated with PCNA and streptavidin to produce the ncDNA beads.

In vitro binding of PIP box peptides to PCNA

The expression plasmid for full-length human PCNA has been described (Hibbert & Sixma, 2012). Human PCNA was expressed in E.coli Bl21 (DE3) cells (Merck KGaA) and purified as described (Hibbert & Sixma, 2012). The peptides Cdt2PIP (704–717), Cdt1PIP (1–14), and Cdt1PIP-TAMRA (1–14) were synthesized in-house using Fmoc synthesis.

FP was monitored using a PheraStar plate reader (BMG Labtech) in non-binding 96-well plates (Corning), using an excitation wavelength of 540 nm and an emission wavelength of 590 nm. Experiments were performed in a buffer containing 10 mM Hepes, pH 7.5, 125 mM NaCl, and 0.5 mM Tris (2-carboxyethyl) phosphine hydrochloride. In the direct binding experiments, 1 nM of labelled peptide was mixed with PCNA at final concentrations ranging from 0 to 2 μM. Error bars represent standard deviations from triplicate experiments. The data were fitted according to standard equations (Littler et al., 2010).

X-ray crystallography structure determination

The purified PCNA was co-crystallized with Cdt2 and Cdt1 peptides, using the sitting drop vapour diffusion method in MRC 3-well crystallization plates (Swissci), with standard screening procedures (Newman et al., 2005). The protein solution at 17 mg·ml⁻¹ was pre-incubated with an equimolar amount of peptide, and 0.1 μl of this solution was mixed with 0.1 μl of reservoir solution and equilibrated against a 30 μl reservoir. Crystals for the Cdt2 complex were obtained in 10% (wt/vol) PEG 6000 and 100 mM MES/HCl, pH 6.0. Crystals for the Cdt1 complex were obtained in 15% (wt/vol) PEG 400, 100 mM calcium acetate, and 100 mM MES/HCl, pH 6.0. Crystals appeared at 4°C within 72 h. Crystals were briefly transferred to a cryo-protectant solution containing the reservoir solution and 25% (wt/vol) PEG200 and vitrified by dipping in liquid nitrogen.

Data collection and structure refinement

X-ray data were collected on beamline ID29 at the European Synchrotron Radiation Facility. The images were integrated with XDS (Kabsch, 2010) and merged and scaled with AIMLESS (Evans, 2011). The starting phases were obtained by molecular replacement using Phaser (McCoy et al., 2007; Winn et al., 2011) with an available PCNA structure (PDB, 1AXC) as the search model. The models were built using COOT (Emsley et al, 2010) and refined with REFMAC (Murshudov et al., 2011) in iterative cycles. Model re-building and refinement parameter adjustment were performed in PDB-REDO (Joosten et al., 2014); homology-based hydrogen bond restraints (van Beusekom et al., 2018) were used at some stages of the procedure. The quality of the models was evaluated by MOLPROBITY (Chen et al., 2010). Data collection and refinement statistics are presented in Table 1. PISA, “Protein Interfaces, Surfaces and Assemblies” service PISA at the European Bioinformatics Institute (http://www.ebi.ac.uk/pdbe/prot_int/pistart.html) (Krissinel & Henrick, 2007) was used to calculate binding energies for Cdt2 PIP and Cdt1 PIP.

FRAP experiments and data analysis

Cells (HeLa or MCF7) were plated on 35-mm glass-bottom dishes in phenol-red-free medium (Invitrogen). FRAP experiments were conducted as previously described (Xouri et al., 2007b; Giakoumakis et al., 2017). A Leica TCS SP5 microscope equipped with a 63x magnification 1.4 NA oil-immersion lens and FRAP booster were used. During FRAP, cells were maintained at 37°C and 5% CO2. A defined circular region of interest with 2 μm diameter (ROI1) was placed in the nucleus and at the recruitment sites. GFP was excited using a 488-nm argon laser line. 50 pre-bleach images were acquired with 3% of the 488-nm line at 60% argon laser line intensity, followed by a single bleach pulse on ROI1 using 476-nm and 488-nm laser lines combined at maximum power. In this manner, at least 60% of the fluorescence in ROI1 was bleached successfully. Next, 400 post-bleach images with a 0.052 s interval were recorded. Mean intensities of ROI1, the whole nucleus (ROI2) and an area outside of the nucleus (ROI3) were quantified and exported as .csv format files. Data analysis was performed using easyFRAP (Rapsomaniki et al., 2012; Giakoumakis et al., 2017; Koulouras et al., 2018).

Supplementary Information

Supplementary information is available at https://doi.org/10.26508/lsa.201800238.
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A Panagopoulos: data curation, formal analysis, and investigation.
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A Perrakis: supervision, funding acquisition, investigation, and writing.
Z Lygerou: supervision, funding acquisition, investigation, and writing.
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M Stadnik-Spiewak: data curation, formal analysis, and investigation.
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Conflict of Interest Statement

The authors declare that they have no conflict of interest.

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