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A Family of Diverse Cul4-Ddb1-Interacting Proteins Includes Cdt2, which Is Required for S Phase Destruction of the Replication Factor Cdt1

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Summary

Cul4 E3 ubiquitin ligases contain the cullin 4 scaffold and the triple β propeller Ddb1 adaptor protein, but few substrate receptors have been identified. Here, we identify 18 *D*db1- and *C*ul4-*a*ssociated *f*actors (DCAFs), including 14 containing WD40 repeats. DCAFs interact with multiple surfaces on Ddb1, and the interaction of WD40-containing DCAFs with Ddb1 requires a conserved "WDXR" motif. DCAF2/Cdt2, which is related to *S. pombe* Cdt2, functions in *Xenopus* egg extracts and human cells to destroy the replication licensing protein Cdt1 in S phase and after DNA damage. Depletion of human Cdt2 causes rereplication and checkpoint activation. In *Xenopus*, Cdt2 is recruited to replication forks via Cdt1 and PCNA, where Cdt1 ubiquitylation occurs. These studies uncover diverse substrate receptors for Cul4 and identify Cdt2 as a conserved component of the Cul4-Ddb1 E3 that is essential to destroy Cdt1 and ensure proper cell cycle regulation of DNA replication.

Introduction

Ubiquitin-mediated proteolysis is a central mechanism by which regulatory proteins and their consequent signaling events are controlled. Attachment of ubiquitin to a target protein proceeds through a cascade involving an E1 activating enzyme, an E2 conjugating enzyme, and an E3 ubiquitin ligase (Pickart, 2004). E3s provide specificity to ubiquitylation reactions by bridging substrates and E2s. Cullin-RING-based E3s (CRLs) constitute a major subclass of RING finger E3s (Petroski and Deshaies, 2005). Seven cullins have been identified in human cells, each of which functions as a "scaffold" around which the active ubiquitin ligase assembles. All cullins interact with an E2 binding RING finger protein (Rbx1 or Rbx2) through their C-terminal domain and with substrate receptors via the N terminus (Zheng et al., 2002; Petroski and Deshaies, 2005).

The best-understood CRL is the SCF complex, which employs Skp1 to link Cul1 with members of the F-box family of substrate receptors (Bai et al., 1996). F-box proteins interact with Skp1 through an N-terminal F-box motif and with substrates through C-terminal WD40 or leucine-rich repeats (reviewed in Petroski and Deshaies [2005]). Similarly, Cul2 and Cul5 complexes employ the Skp1-like protein Elongin C to interact with SOCS-box proteins while Cul3 employs BTB proteins as receptors. Together, these CRLs may assemble more than 200 distinct E3s.

In contrast with the cullins described above, receptors for Cul4A and Cul4B are poorly understood. Cul4A and Cul4B interact through their N terminus with Ddb1 (damage-specific DNA binding protein 1). Ddb1 appears to function as a Skp1-like adaptor, as it can interact with at least three potential substrate receptors in human cells (Ddb2, CSA, Det1-Cop1), and with Cdt2 in *S. pombe*. In human cells, Cul4-Ddb1^{Ddb2} promotes the ubiquitylation of histone H3, histone H4 (Wang et al., 2006), and the xeroderma pigmentosum (XP) group C protein, XP-C (Sugasawa et al., 2005) to regulate their activity. Ddb2 is mutated in humans of the XP-complementation group E subtype (Kapetanaki et al., 2006). In contrast, Cul4-Ddb1^{CSA} and Cul4-Ddb1$^{Det1\text{-}Cop1}$ promote proteolysis of CSB and c-*jun*, respectively (Wertz et al., 2004; Groisman et al., 2006). Both CSA and CSB are mutated in patients with Cockayne syndrome. Finally, *S. pombe* Cul4-Ddb1^{Cdt2} is required for the degradation of Spd1, an inhibitor of ribonucleotide reductase that is destroyed at the onset of S phase and after DNA damage (Liu et al., 2005). Interestingly, Ddb2, Cop1, CSA, and *S. pombe* Cdt2 contain WD40 repeats, which are frequently used in CRLs such as the SCF to bind substrates (Petroski and Deshaies, 2005).

Another substrate of Cul4-Ddb1 is the DNA replication factor Cdt1, although no substrate receptor for Cdt1 has been identified. In G1, Cdt1 cooperates with the origin recognition complex (ORC) and Cdc6 to recruit MCM2-7 into a prereplication complex or pre-RC (the "licensing" reaction) (Blow and Dutta, 2005). In S phase, the MCM2-7 helicase is activated, and it travels away from the origin, leading to pre-RC disassembly. In vertebrates, origin refiring in S, G2, and M phase is prevented because de novo MCM2-7 loading is prohibited, primarily through the inhibition of Cdt1 activity via Geminin and ubiquitin-mediated proteolysis. Available evidence suggests that Cdt1 ubiquitylation and degradation involve Ddb1 and Cul4 in humans, *Xenopus*, and *C. elegans* (Zhong et al., 2003; Higa et al., 2003; Hu et al., 2004; Nishitani et al., 2006; Arias and Walter, 2006). In S phase, Cul4-Ddb1-dependent Cdt1 ubiquitylation occurs exclusively on chromatin in a manner that is dependent on DNA replication (Arias and Walter, 2005, 2006; Nishitani et al., 2001, 2006). Cdt1 contains a highly conserved PCNA interaction motif (called a PIP box), which is required for its destruction in S phase (Arias and Walter, 2006; Senga et al., 2006; Nishitani et al., 2006). Ddb1 is recruited to chromatin during replication initiation, suggesting that Cdt1 and its E3 ubiquitin ligase both bind to the replication fork via PCNA, leading to ubiquitin transfer (Arias and Walter, 2006). Interestingly, human Cdt1 is rapidly destroyed in response to DNA damage by a proteolysis pathway that requires Cdt1's PIP box, PCNA, Cul4, and Ddb1 (Higa et al., 2003; Hu et al., 2004; Hu and Xiong, 2006; Nishitani et al., 2006; Senga et al., 2006).

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Structural analysis of Ddb1 in complex with the simian paramyxovirus SV5 protein—which co-opts Ddb1-Cul4 to promote STAT degradation—shows that Ddb1 contains three seven-bladed β propellers (designated βPA, βPB, and βPC), potentially providing a large surface area for recruitment of additional specificity factors (Li et al., 2006). SV5 contacts βPC while Cul4 binds βPB. Using a proteomic approach, we identified 18 previously unknown human *D*db1- and *C*ul4-*a*ssociated *f*actors (DCAFs), the majority of which contain WD40 repeats. WD40-containing DCAFs employ "WDXR" motifs to bind Ddb1, and many DCAFs, like SV5, make contacts with βPC. Furthermore, we found that DCAF2, the apparent vertebrate ortholog of *S. pombe* Cdt2, is required for destruction of Cdt1 during S phase and after DNA damage in human cells and in *Xenopus* egg extracts, through a mechanism that involves Cdt1 and PCNA-dependent recruitment of Cul4-Ddb1^{Cdt2} to chromatin. These experiments provide insight into the composition of the Cul4-Ddb1 ligase family and shed light on the mechanism by which cells restrict DNA replication to a single round per cell cycle.

Results

Identification of DCAFs, Candidate Substrate Receptors for the Cul4-Ddb1 Ubiquitin Ligase

To search for Ddb1-associated proteins, 293T cells expressing endogenous levels of Flag-HA-Ddb1 (Figure 1A) were subjected to tandem affinity purification (TAP). SDS-PAGE analysis revealed a number of Ddb1-associated proteins absent from control samples (Figure 1B), the identities of which were determined by mass spectrometry. Several previously identified Cul4-Ddb1-interacting proteins, including Cul4A, Cul4B, all eight subunits of the COP9 signalosome, CSA, Det1, Ddb2, and Cop1 were identified, indicating the presence of physiological Ddb1 partners (Figure 1C). In addition, 18 previously unidentified human Ddb1-interacting proteins were found, which we generally refer to as DCAFs (Figure 1C). Interestingly, like CSA, Ddb2, and Cop1, 14 of the DCAFs contained WD40 repeats (Figure 1C). One of these, DCAF2, is the closest human relative of the *S. pombe* protein Cdt2, which interacts with Ddb1 (Liu et al., 2005). DCAF2 is 26% identical and 44% similar to *Sp*Cdt2, and we will refer to the human protein as *Hs*Cdt2. Metazoan homologs of Cdt2 each contain seven WD40 repeats (data not shown), suggesting they assemble into a classical seven-bladed propeller. DCAF1 was previously identified as VprBP, a protein that interacts with the Vpr protein from human immunodeficiency virus (Zhang et al., 2001), but its connection to Ddb1 remained unknown. Five additional interaction domains were identified in DCAFs (Figure 1C): a calmodulin binding (IQ) domain in DCAF6, tetratricopeptide repeat (TPR) domains in DCAF9, a SOF motif in DCAF13, bromodomains in DCAF14, and a Lissencephaly type-1-like homology motif (LisH) in DCAF1. Like Det1, four Ddb1-associated proteins (DCAF15–17 and Dda1) lack known protein interaction domains (Figure 1C). Dda1, although a Ddb1 interaction protein, was not designated as a DCAF because it interacts with multiple Ddb1-DCAF complexes (Ning Zheng, personal communication) and therefore is unlikely to serve as a substrate receptor.

Validation of Ddb1-Interacting Proteins

To validate our TAP results, vectors expressing 15 DCAF proteins (DCAFs 1, 3–12, 14–17, and *Hs*Cdt2/DCAF2), as well as Dda1, Ddb2, CSA, and Det1, were cotransfected into 293T cells in combination with vectors expressing Flag-Cul4A, Flag-Cul4B, and Flag-HA-Ddb1 (or Flag-Ddb1) and tested for interaction (Figure 1E and see Figure S1B in the Supplemental Data available with this article online). All DCAF proteins tested were found to interact with Ddb1 with an efficiency comparable to that seen with Ddb2, CSA, and Det1, and they also interacted with Cul4A (and, in many cases, Cul4B) (Figure 1E and Figure S1B). In addition, we found that endogenous Ddb1 interacts with endogenous *Hs*Cdt2 and with DCAF1 in coimmunoprecipitation experiments (Figure 1D). These data greatly expand the number of known Ddb1- and Cul4-interacting proteins, as summarized in Figure S1A.

Distinct Modes of Interaction between Ddb1 and Its Interacting Proteins

While Ddb1 shares the adaptor function of Skp1, it is structurally more complex and contains three seven-bladed β propellers (βPA, βPB, and βPC) (Li et al., 2006). While Ddb1 is known to bind the viral SV5 protein through the "top" face of βPC and Cul4 through the "bottom" face of βPB, as defined by Li et al., (2006) (Figure 2A), it is not clear whether other Ddb1-associated proteins use the same or distinct surfaces to bind Ddb1.

To examine this question, we generated four Ddb1 mutants in βPA and βPC, which encompass conserved single residues or clusters of conserved residues on the top face of βPC and the bottom face of βPA (Figure 2A). Vectors encoding Ddb1 mutants were coexpressed with a variety of DCAF proteins as well as Dda1, CSA, Ddb2, and Det1, and interactions were tested by coprecipitation (Figure 2B and Figure S1C). The results, summarized in Figure 2C, revealed that different surfaces in Ddb1 are required for interaction with different classes of DCAF proteins. Thus, *Hs*Cdt2; Det1; DCAFs 1, 3, 5, 8, and 11; and CSA each required at least one of the three sets of residues mutated on the surface of βPC, although these proteins display clear differences in their requirements for these βPC residues (Figure 2C). For example, while Det1, DCAF1, and DCAF8 required only the M910/L912/Y913 cluster (Figure S1C, lanes 6–10, 16–20, and 33–37), *Hs*Cdt2 required this cluster as well as the E840/E842 cluster (Figure 2B, lanes 1–5). For CSA, mutation of W953 resulted in a substantial reduction in binding but the M910/L912/Y913 and E840/E842 clusters were not required (Figure S1C, lanes 48–52). DCAF3 and DCAF5 required all three sets of residues on βPC (Figure S1C, lanes 1–5 and 38–42). In contrast, Ddb2, DCAF12, and Dda1 did not require any of the residues examined in βPC (Figure 2B, lanes 5–10; Figure S1C, lanes 11–15 and 43–47). Strikingly, Dda1 was found to require a conserved cluster of residues (Y316/D318/N319) on the bottom face of βPA, revealing that Ddb1 also uses βPA to interact with proteins (Figures 2A–2C). GST alone did not associate with Ddb1 (Figure S1C, lanes 21–26), indicating the specificity of this approach. In summary, the majority of Ddb1-associated proteins interact with Ddb1 through distinct