THE FUNCTIONS OF *C. ELEGANS* CUL-4/DDB-1 COMPLEXES IN DNA REPLICATION AND CELL CYCLE REGULATION

by

YOUNGJO KIM

(Under the Direction of Edward T. Kipreos)

ABSTRACT

The replication of genomic DNA is strictly regulated to occur only once per cell cycle. This regulation centers on the temporal-restriction of replication licensing factor activity. In all eukaryotic species examined, the regulation of Cdt1 is critical to prevent re-replication. In humans, two distinct ubiquitin ligase (E3) complexes, CUL4/DDB1 and SCF(Skp2), have been shown to target the replication licensing factor Cdt1 for ubiquitin-mediated proteolysis. However, it is unclear to what extent these two distinct Cdt1 degradation pathways are conserved. The work described here shows that *C. elegans* CUL-4/DDB-1 functions as the major E3 ligase to target CDT-1 for degradation. A *ddb-1* null mutant exhibits extensive DNA re-replication in post-embryonic blast cells, similar to what is observed in *cul-4(RNAi)* larvae. In contrast, SKPT-1 has no appreciable role in CDT-1 degradation during S phase.

This dissertation also describes additional CUL-4/DDB-1 functions. The CUL-4/DDB-1 complex is required to target the degradation of the CIP/KIP family CKI-1 and cyclin E homolog CYE-1. It is shown that CKI-1 is required for DNA re-replication

associated with loss of CUL-4 or DDB-1. Evidence is provided that the 'WDXR' motif protein CDT-2 functions as the SRS (substrate recognition subunit) for a *C. elegans* CUL-4/DDB-1 complex that targets the degradation of CKI-1. CDT-2 is required to target both CDT-1 and CKI-1 for degradation. CDT-2 can directly bind to both CDT-1 and CKI-1, as expected for an SRS.

Germ cells in *ddb-1* mutants and *cul-4(RNAi)* larvae exhibit corrupted cell and nucleolar morphology. The *ddb-1* mutant germ cells do not exhibit re-replication phenotypes, suggesting that the CUL-4/DDB-1 ubiquitin ligase is required for germ cell integrity independently of its known function in regulating DNA replication. It is shown that a 'WDXR' motif protein VprBP phenocopies *ddb-1* in the germ cells, suggesting that it functions as an SRS to regulate germ cell integrity.

In total, this work demonstrates that the *C. elegans* CUL-4/DDB-1 E3 ligase is a central regulator that controls the extent of DNA replication, is required for germ cell viability, and regulates the critical cell cycle regulators CKI-1 and cyclin E.

INDEX WORDS: *Caenorhabditis elegans*, CUL-4, DDB-1, CDT-2, SCF, Skp2, CDT-1, CKI-1, CYE-1, VprBP, cullin, cell cycle, proteolysis, ubiquitin, DNA replication

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DEDICATION

This dissertation is dedicated to my wife and parents. Without their encouragement, support, and discipline, it would be impossible for me to make achievements. My deep gratitude goes to my wife and my mother specially. My wife was always durable. Without her patience, I would not be able to complete my doctoral study. My mother made me who I am. Without her deep love and sacrifice to me, I would not be able to complete my education.

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CHAPTER I

GENERAL INTRODUCTION

UBIQUITIN-MEDIATED PROTEOLYSIS

Selective proteolysis by the ubiquitin-proteasome system allows specific removal of key regulators to control diverse cellular pathways, including the cell cycle, signal transduction, transcription, protein quality control, viral modulation, circadian clock and development (Petroski and Deshaies, 2005). Alterations in ubiquitination reactions have been implicated in the pathogenesis of multiple human diseases (Ciechanover and Schwartz, 2004). The ubiquitin-mediated proteolysis pathway involves the covalent transfer of ubiquitin to one or more lysine residues of the target protein in a three-enzyme cascade (E1, E2 and E3) and the degradation of the ubiquitinated protein by the 26S proteasome (Hershko and Ciechanover, 1998).

Ubiquitin

Ubiquitin is a conserved protein of 76 amino acid residues (Hershko and Ciechanover, 1998). In most cases, ubiquitin is covalently linked to specific lysine residues of the target protein and the ubiquitin chain is polymerized through a K48-G76 isopeptide bonds between ubiquitin monomers (Hochstrasser, 1996). Poly-ubiquitination marks the target protein for degradation by the 26S proteasome. In other cases, poly-ubiquitin chains can be modified by alternate

covalent linkages between K63 and G76 residues of ubiquitin monomers, leading to subcellular compartment sorting of target proteins instead of degradation (Pickart, 2001). Mono-ubiquitination of target proteins such as Histone H2A, H3, H4 and the DNA damage response protein XPC potentially provides signals for chromatin remodeling and DNA damage response pathways, respectively (O'Connell and Harper, 2007; Pickart, 2001). Besides ubiquitin, there are multiple ubiquitin-like (UBL) proteins, which can be also covalently attached to substrates. However, these ubiquitin-like proteins use different enzyme systems, and they do not mark proteins for proteolysis (Wilkinson, 2000).

Ubiquitin-activating enzyme (E1)

The ubiquitin-mediated proteolysis pathway starts with the activation of ubiquitin (Figure 1.1). The C-terminal Gly residue of ubiquitin is activated by an ubiquitin-activating enzyme (E1) in an ATP-dependent manner. This biochemical reaction can be divided into two intermediate steps; an initial intermediate ubiquitin adenylate is formed with the release of PPi and then the activated ubiquitin is transferred from ubiquitin adenylate to E1 to form an intermolecular thioester bond between C-terminal Gly residue of ubiquitin and Cys residue of E1, releasing AMP (Hershko and Ciechanover, 1998). Then the E1-ubiquitin transfers the ubiquitin moiety to a ubiquitin-conjugating enzyme (E2). Usually there is a single E1 enzyme in an organism (McGrath et al., 1991).

Ubiquitin-conjugating enzymes (E2s)

The ubiquitin-conjugating enzyme receives ubiquitin from the E1 and forms a thioester linkage with ubiquitin through a Cys residue (Figure 1.1) (Hershko and Ciechanover, 1998). In contrast to the E1, multiple E2s have been identified. Thirteen genes encode E2-like proteins in the genome of budding yeast and at least 50 E2s have been described in humans (Glickman and Ciechanover, 2002; Hochstrasser, 1996; Pickart, 2001). In most cases, E2 enzymes transfer ubiquitin to substrates with the help of an E3 ubiquitin ligase (Hershko and Ciechanover, 1998). In a few cases, E2s have been shown to directly bind to substrates without the presence of an E3 in vitro (Goebl et al., 1988; Kalchman et al., 1996). However it is not clear whether E2s directly bind to their substrates in vivo (Ciechanover et al., 2000).

Ubiquitin-ligases (E3s)

Ubiquitin is attached to the substrate via an amide isopeptide bond between the C-terminal Glycine residue of ubiquitin and an ε-amino group of a Lysine residue of the target protein (Hershko and Ciechanover, 1998). In most cases, E3 enzymes are required for the last step of ubiquitination reaction cascade (Figure 1.1) (Hershko and Ciechanover, 1998). The substrate specificity of the ubiquitin-mediated proteolysis pathway is largely regulated at the level of the E3. Different targets are recognized by different E3s. In contrast to E1 and E2 enzymes, there are a huge number of E3 or E3 complexes that allow the specific recognition of a diverse range of substrates (Pickart, 2001). In humans, several percent of genome are taken up by E3s or E3 complex

components (Semple, 2003). E3 enzymes can be classified into two major groups based on their catalytic mechanism to ligate ubiquitin to their substrates: HECT-domain E3s that utilize a covalent mechanism and RING-domain E3s that do not (Pickart, 2004). Several other minor groups of E3s, which do not fall into the two major types, also have been identified (Gonen et al., 1996; Orian et al., 1995; Stancovski et al., 1995). However the lack of sequence similarity between these E3s makes difficult it to classify them into a single group (Hershko and Ciechanover, 1998).

HECT-domain E3s

The HECT-domain class encompasses E3s that harbor a 350 amino acid region called HECT (Homology to the E6-AP Carboxyl Terminus) domain (Glickman and Ciechanover, 2002; Hershko and Ciechanover, 1998; Pickart, 2001). The HECT-type of E3s form a thioester bond with ubiquitin at the HECT-domain and then transfer the ubiquitin to the substrates that are recruited to the E3 through N-terminal unique domain (Glickman and Ciechanover, 2002; Hershko and Ciechanover, 1998; Pickart, 2001). The prototype of E3s with HECT-domains is E6-AP (E6 associating protein). E6-AP targets p53 for degradation in the presence of the HPV oncoprotein E6 (Scheffner et al., 1993; Scheffner et al., 1995).

RING-domain E3s

The RING (Really Interesting New Gene)-domain family is potentially the

largest group of E3s and its members have a RING-finger motif (Pickart, 2001). The RING-finger motif, which is responsible for bringing the E2 to the E3, consists of a Cys-rich consensus sequence flanked by one or two His residue(s) and is capable of binding Zn^{2+} (Borden, 2000; Saurin et al., 1996). Some RING-domain E3s consist of just one multi-domain polypeptide, whereas a majority of RING-domain E3s function in multi-protein complexes in which the RING subunit acts to recruit E2 to the complex (Lorick et al., 1999; Seol et al., 1999). RING-domain E3s can be subcategorized into three classes based on the type of their RING domain: RING-HC (C3HC4), RING-H2 (C3H2C3), and RING-IBR-RING (Jackson et al., 2000; Tanaka et al., 2004).

26S Proteasome

Ubiquitin-marked proteins are recognized and degraded by the 26S proteasome (Figure 1.1). The 26S proteasome is a huge protein complex that is composed of at least 64 protein subunits, which are encoded by 32 independent genes (Baumeister et al., 1998). The 26S proteasome can be divided into two subcomplexes; the 670 kDa 20S core particle (CP), which contains proteolytic active sites, and the 900 kDa 19S regulatory particle (RP), which binds to either or both ends of the 20S CP (Baumeister et al., 1998). The 20S CP functions to proteolyze target proteins and the 19S RP has a regulatory function. Both the structure and subunits of 26S proteasome are evolutionarily conserved from Archaebacteria to eukaryotes, suggesting the importance of the ubiquitin-mediated proteolysis pathway (Glickman and Ciechanover, 2002; Hershko and

Ciechanover, 1998).

The eukaryotic 20S proteasome subcomplex is composed of two outer rings and two inner rings, forming a barrel-shaped complex, which is 15 nm in length and 11 nm in diameter (Baumeister et al., 1998). The interior of the cavity is responsible for its proteolytic activity. The 19S RP can be divided into two subcomplexes, known as 'base' and 'lid' (Glickman et al., 1998). The base is composed of six ATPases (Rpt1-Rpt6) and has two large regulatory components, Rpn1 and Rpn2 that function as presumptive receptor(s) for ubiquitin (Leggett et al., 2002). The entry of substrates into the cavity of 20S proteasome is controlled by the base 19S complex, which binds to the outer ring of the 20S proteasome and opens a narrow gate in an ATP-dependent manner (Smith et al., 2005). The lid contains multiple non-ATPase subunits (Rpn3, Rpn5-9, Rpn11-13, and Rpn15) and catalyzes the rapid disassembly of the ubiquitin (Ub) chain (Glickman and Ciechanover, 2002). In the course of proteolysis, ubiquitin is not degraded but, instead is regenerated from proteolytic intermediates by deubiquitinating enzymes, providing the fodder for new ubiquitination reactions (Pickart, 2004).

CULLIN/RING-H2 E3S

Cullin/RING-H2 E3s are potentially the largest superfamily of E3 complexes that target a wide range of proteins for degradation (Petroski and Deshaies, 2005). In a Cullin/RING-H2 E3, multiple subunits are assembled on a

cullin scaffold. Although there is a great diversity of Cullin/RING-H2 E3 ligases in terms of their composition and function, this group of E3 complexes can be classified by a set of general principles (Petroski and Deshaies, 2005). In humans, there are seven cullin paralogs, which each nucleate a multisubunit E3 ligase. In addition, the APC2 subunit of APC/C (anaphase promoting complex/cyclosome) also has a 'cullin-homology' domain (Yu et al., 1998a; Zachariae et al., 1998). The APC/C is active from the metaphase-to-anaphase transition to the beginning of S phase and ubiquitinates cell cycle regulators (Koepp et al., 1999). However the APC/C complex is clearly distinct from other cullin-based E3 complexes in its structure and regulation (Petroski and Deshaies, 2005).

The composition of Cullin/RING-H2 E3 ligases

Cullin homologs have been found across eukaryotic phyla and their primary sequences are conserved (Kim and Kipreos, 2007a; Kipreos et al., 1996). The cullin gene family has six known members in *C. elegans*, seven in *H. sapiens*, three in *S. cerevisiae*, and three in *S. pombe*. Humans have two members of the CUL-4 group, CUL4A and CUL4B, while *C. elegans* has two members of the CUL-1 group, CUL-1 and CUL-6. Budding yeast has A (Cdc53), B and C type cullins, while fission yeast has CUL-1, -3 and -4 homologs (Kipreos et al., 1996; Wood et al., 2002). The highly conserved primary sequences of the cullins suggest that they share a common biochemical mechanism (Kipreos et al.,

1996). Indeed all cullins have been reported to form ubiquitin ligase complexes in humans and nematodes (Petroski and Deshaies, 2005).

The best-studied Cullin/RING-H2 complex is the CUL1 ubiquitin ligase complex that is also called SCF complex in recognition of three of four common subunits: Skp1; Cul-1 (or Cdc53); F-box protein; and Rbx1 (Figure 1.2). F-box proteins encompass a large and diverse protein family, which is named for a small region of protein that is required for binding with the Skp1 adaptor. F-box proteins also have another protein-protein interaction module, such as leucine rich repeats (LRR) or WD40 repeats, that bind directly to specific substrates (Petroski and Deshaies, 2005; Skowyra et al., 1997). The adaptor protein, SKP1 links the cullin and F-box protein. It is highly conserved between organisms; every CUL1 complex has Skp1 as the adaptor protein. The N-terminal Skp1/POZ domain is responsible for binding to the cullin (Bai et al., 1996). The cullin subunit serves as a structural platform that binds both the SKP1/Fox box protein subcomplex and the RING H2 protein. RBX1, which is a RING-H2 protein, recruits E2-ubiquitin with the assistance of cullins (Schulman et al., 2000).

The protein complexes in which the other cullins function have also been determined. Mammalian CUL2 forms a cullin/RING-H2 complex similar to the SCF complex that contains elongin C (a Skp1-related protein), elongin B (an ubiquitin-related protein), RBX1, and VHL-box proteins (Petroski and Deshaies, 2005). VHL-box protein functions as the substrate-recognition subunit to recruit

substrate to the complex in humans and nematodes (Kamura et al., 2004; Vasudevan et al., 2007). CUL3 forms a cullin/RING H2 complex with the BTB-containing proteins and RBX1. The BTB-containing proteins serve as a substrate-specific adaptor; in contrast to CUL1 and 2 complexes, it serves both as the adapter and the substrate binding component. In *C. elegans*, the microtubule-severing protein MEI-1/katanin is targeted for degradation by the CUL-3/MEL-26 complex (Pintard et al., 2003b; Xu et al., 2003). It has been shown that viral proteins recruit a CUL5 complex to degrade host proteins. In these complexes, CUL5 binds to Elongin B, C, RBX1, and viral proteins. The viral proteins function as substrate-recognition subunits through interaction with Elongin B, C (Querido et al., 2001; Yu et al., 2003). Although CUL2 and CUL5 complexes share Elongin B, C as the adaptor, they appear to have distinct groups of SRSs. Recent report showed that in mammalian cells, SOCS-box proteins specifically bind to endogenous CUL5-Rbx2 complex whereas VHL-box proteins bind to endogeous CUL2-Rbx1 complex (Kamura et al., 2004).

Structure of Cullin/RING-H2 complexes

The structural studies of cullin complexes provide an understanding of the whole complex's protein topology and offer clues about how the complex ubiquitinates its substrate. Zheng et al. reported the 3.2-Å scale crystal structure of a cullin/RING H2 complex containing Cul1, Rbx1, Skp1 and Skp2 (Zheng et al., 2002). CUL1, which organizes the overall complex, consists of a long stalk-like N-terminal domain (NTD) and globular C-terminal domain (CTD). The CUL1

NTD stalk, which binds to SKP1/SKP2, consists of three repeats of similar α-helix-bundles. The CUL1 CTD globule is responsible for interaction with RBX1 and E2, and covalent modification by the ubiquitin-like protein Nedd8 (neddylation). The first repeat of the NTD is responsible for binding to SKP1. This region is conserved among CUL1 orthologs but not other paralogs. However, other cullins also show homology with their orthologs within the first repeat, indicating that it is the binding site for adaptor proteins specific for each class of cullin complexes (Zheng et al., 2002). Indeed Elongin C and BTB-domain proteins bind to the NTD of their corresponding cullins, CUL2 and CUL3, respectively. A structural model of the CUL2 complex has been proposed based on VHL/Elongin B, Elongin C crystallography and primary sequence similarity between CUL1 and CUL2 (Stebbins et al., 1999). In this model, CUL2 binds to VHL/Elongin B, C at the NTD, and to RBX1 and E2 at the CTD as in the CUL1 complex, suggesting that the CUL1 and CUL2 complexes are very similar in their overall organization. As all cullin paralogs have conserved primary sequences, it has been proposed that all cullins form ubiquitin ligase complexes similar to the CUL1 and CUL2 complexes. Recently a structural study of a CUL4A complex indicates that the CUL4 complex shares the same modular structure as CUL1 and CUL2 complexes (Angers et al., 2006; Li et al., 2006b).

Cellular functions of cullins in *C. elegans*

Cullin complexes regulate many cellular processes such as cell cycle progression, transcription, and signal transduction in yeast and metazoa,

depending on their target substrates. The phenotypic analysis of cullins has provided useful information to screen for complex components and substrates, and characterize their cellular functions. The phenotypes of each cullin are well characterized in *C. elegans*. The invariant pattern of cell divisions, cell positions, and cell fates of *C. elegans* has facilitated this analysis (Sulston and Horvitz, 1977).

In *C. elegans*, the loss of *cul-1* gene function results in abnormal DTC (distal tip cell) migration and a failure in cell cycle exit (Kipreos et al., 1996). In the *cul-1* null mutant, postembryonic blast cells do not exit the cell cycle normally, which produces *hyperplasia* (many abnormally-small cells). As expected, the RNAi of the F-box proteins (*lin-23* and *skp2*) and Skp1 homologs (*skr-1* and *skr-2*) show overlapping phenotypes with *cul-1*, suggesting they form ubiquitin ligase complex similar to those in yeast and other metazoa (Kipreos et al., 1996; Nayak et al., 2002).

CUL-2 regulates several key processes in cell division and embryonic development, including the G1-to-S phase transition, meiotic progression, anterior-posterior polarity and mitotic chromatin condensation (Feng et al., 1999; Vasudevan et al., 2007). The *cul-2* deletion mutant has four major phenotypes: 1) a G1 phase arrest of germ cells; 2) no detectable chromosome condensation at prometaphase of the first mitotic division in zygote; 3) delayed mitotic progression through prometaphase, resulting in mitoses six-fold longer on

average than in wild type; and 4) basic defects in cytoskeletal organization that are apparent from three sub-phenotypes - the migration of pronuclei is defective, mitotic spindles are mispositioned, and there are extensive cytoplasmic projections from cells (Feng et al., 1999). Feng et al. found that in *C. elegans*, elongins B and C function as complex components with CUL-2 as in humans (Feng et al., 1999); unpublished data).

CUL-3 is required to target MEI-1/Katanin for degradation at the meiosis-mitosis transition (Pintard et al., 2003a). Neddylation defects or RNAi of *cul-3* causes a failure in MEI-1 degradation, resulting in unorganized mitotic spindle positions at the first mitotic division in the zygote. The loss-of-function of the BTB containing protein, *mel-26* has the same phenotype, and its physical interaction with CUL-3 has been verified, suggesting that MEL-26 functions in a complex with CUL-3 (Pintard et al., 2003b; Xu et al., 2003).

CUL4/RING-H2 E3 COMPLEXES

CUL4 has high sequence similarity with other cullin paralogs that have been shown to form Cullin/RING E3 ligase complexes. Therefore, it has been proposed that CUL4 forms ubiquitin ligase complexes to target a distinct group of proteins for degradation. Since I started to investigate the function of CUL-4 in 2001, several research groups have demonstrated that CUL4 indeed forms ubiquitin ligase complexes with various protein components to target the degradation of substrates.

The function of CUL4

As with other cullin paralogs, CUL-4 also regulates an important cellular process in *C. elegans*. CUL-4 is required to maintain genome stability by restraining the DNA replication licensing factor CDT-1 (Zhong et al., 2003). Upon RNAi inactivation of *cul-4*, animals arrest at the L2 larval stage and blast cells undergo DNA re-replication. The *cul-4* RNAi blast cells do not divide, but instead enlarge dramatically with the DNA content of these cells increasing up to *100C* DNA content in a few days. The DNA licensing factor CDT-1 is normally degraded as cells enter S phase, which helps to prevent DNA re-replication (Nishitani et al., 2000). In *cul-4* RNAi cells, the Cdt1 level is not decreased even after S phase, indicating that CUL-4 promotes CDT-1 degradation (Zhong et al., 2003).

Groisman et al. reported that in humans, CUL-4A forms two complexes that contain DDB1 (damaged DNA-binding protein1) and the RING protein RBX1, and either DDB2 (damaged DNA-binding protein2) or CSA (Cockayne syndrome protein A) (Groisman et al., 2003). The CUL4A complexes regulate different steps of nucleotide excision repair pathways in response to DNA damage. The finding that DDB1 protein is detected in two different CUL4A complexes (DDB2- and CSA-containing) raised the hypothesis that DDB1 functions as the adaptor protein in CUL4 complexes to bind different SRSs. It has also been demonstrated that DDB1 protein can physically interact with CUL4A to target Cdt1 in response to UV irradiation (Hu et al., 2004). In that study, recombinant

DDB1 and Cdt1 bind directly each other *in vitro*. Therefore, it has been proposed that DDB1 can single-handedly bridge CUL4 and the substrate Cdt1, functioning as a substrate specific adaptor, similar to the BTB-domain proteins in CUL3 E3 ligase complexes. However, more recent studies report DDB1 binding substrates through SRSs.

CUL4-DDB1 has been demonstrated to target multiple substrates through various SRSs. CUL4-DDB1^{CSA} targets the degradation of the nucleotide excision repair protein CSB (Groisman et al., 2006). CUL4-DDB1$^{hDET1\text{-}hCOP1}$, which includes a dimeric SRS consisting of hDET1 and hCOP1, targets the degradation of the transcription factor c-jun (Wertz et al., 2004). CUL4-DDB1^{DDB2} mediates stable ubiquitin modifications of histones H2A, H3 and H4, and the nucleotide excision repair protein XPC (Kapetanaki et al., 2006; Sugasawa et al., 2005; Wang et al., 2006). Finally, CUL4-DDB1^{CDT2} has been implicated in the degradation of mammalian p53, fission yeast Spd1, and Cdt1 (Banks et al., 2006; Higa et al., 2006a; Jin et al., 2006; Liu et al., 2005; Ralph et al., 2006; Sansam et al., 2006). It is likely that there will be additional CUL4-DDB1 complexes containing different SRS proteins that function in a wide-range of molecular and physiological processes.

The structure of CUL4-DDB1 ubiquitin ligase complexes

The crystal structure of the CUL4A-DDB1 E3 complex has recently been determined (Angers et al., 2006; Li et al., 2006b). Like CUL1, CUL4A adopts an

arc shaped N-terminal domain and globular C-terminal domain. DDB1 is a large multi-domain protein that contains three β-propeller domains (BPA, BPB, and BPC). One β-propeller domain (BPB) binds to the rod-shaped N-terminus of CUL4, while the other two β-propeller domains (BPA and BPC) form a bi-lobed domain that has multiple potential interaction sites for protein binding and serves as the docking site for SRSs or substrates (Angers et al., 2006; Li et al., 2006b) (Figure 1.2). A number of proteins have been identified as DDB1 interactors that are presumed or known to function in substrate binding: hDET/hCOP1; CDT2/L2DTL/DTL; DDB2; CSA; and paramyxovirus V proteins (Groisman et al., 2003; Li et al., 2006b; Liu et al., 2005; Ulane et al., 2005; Wertz et al., 2004). Additionally, four research groups have used biochemical and proteomic approaches to identify a novel family of WD40-repeat proteins that bind to the CUL4-DDB1 complex (Angers et al., 2006; He et al., 2006; Higa et al., 2006b; Jin et al., 2006). A majority of these WD-repeat proteins possess a variant WD-repeat sequence that is referred to as a 'WDXR', or 'DXR', and which mediates binding to DDB1 (Angers et al., 2006; He et al., 2006; Higa et al., 2006b; Jin et al., 2006). This WD repeat protein family has been referred to by three names: DCAF (DDB1- and CUL4-associated factors); CDW (CUL4 and DDB1-associated WD40 repeat proteins); and DWD (DDB1-binding and WD40 repeat proteins) (Angers et al., 2006; He et al., 2006; Higa et al., 2006b; Jin et al., 2006). The DCAF family is predicted to contain ~90 members in mammals, of which at least 49 have been shown to physically interact with CUL4 or DDB1, mainly by co-expression/co-IP in mammalian cells (Angers et al., 2006; He et al., 2006; Higa

et al., 2006b; Jin et al., 2006). Five members of the DCAF family are known to function as SRSs, and the functions of their respective complexes are as described above.

CONTROL OF DNA REPLICATION

Cdt1 degradation and the control of DNA replication

To maintain genome integrity, DNA replication must be strictly regulated to occur only once per cell cycle. Replication is, therefore, tightly regulated to prevent the re-initiation of DNA replication within the same S phase. A failure to restrict DNA replication results in 're-replication', in which the genome is over-replicated within the same cell cycle via origin re-firing. In eukaryotes, the extent of DNA replication is controlled by temporally restricting the assembly of the pre-replication complex (pre-RC) through a process termed 'replication licensing' (reviewed in (Blow and Dutta, 2005; Machida et al., 2005)). Pre-RCs form on replication origins through the sequential binding of DNA replication proteins during late mitosis or G1 phase (Figure 1.3). The six-member origin recognition complex (ORC) binds replication origins on newly-synthesized chromatin. During late mitosis or G1 phase, the replication licensing factors Cdt1 and Cdc6 are recruited to the origin. Cdt1 and Cdc6 together load the presumptive replicative helicase, the Mcm2-7 complex, onto the origin to complete pre-RC formation. During S phase, pre-RCs are activated by phosphorylation via CDK and DDK (Dbf4-dependent kinase) activity. This phosphorylation allows the recruitment of essential replication factors, including Cdc45, Mcm10, RPA, proliferating cell

nuclear antigen (PCNA), and DNA polymerases α and δ.

Cdt1 and Cdc6 are essential loading factors for the Mcm2-7 complex, and they are negatively regulated during S phase to ensure that the Mcm2-7 complex cannot re-bind to origins that have already fired. In budding yeast, Cdt1 is exported from the nucleus during S phase (Tanaka and Diffley, 2002). In contrast, fission yeast and metazoan Cdt1 homologs are degraded during S phase (Blow and Dutta, 2005; Feng and Kipreos, 2003; Machida et al., 2005). The other replication licensing factor, Cdc6, is regulated by degradation during S phase in budding and fission yeast; while in metazoa, Cdc6 is exported from the nucleus (Blow and Dutta, 2005; Feng and Kipreos, 2003; Kim et al., 2007; Machida et al., 2005). In most eukaryotic species examined, redundant regulation prevents reassembly of pre-RCs in S phase. The exact regulation varies between eukaryotes, and can include controls of each of the pre-RC components: Cdt1, Cdc6, ORC subunits, and the Mcm2-7 complex (Blow and Dutta, 2005; Feng and Kipreos, 2003; Machida et al., 2005). In all eukaryotic species examined, Cdt1 is a major focus of replication licensing regulation.

In *Drosophila* and vertebrates, Cdt1 activity is redundantly regulated by its degradation and the binding of a Cdt1-inhibitor called Geminin (Machida et al., 2005). Loss of Geminin leads to re-replication in *Drosophila* and in certain human cell lines but not in others (Kulartz and Knippers, 2004; Melixetian et al., 2004; Mihaylov et al., 2002; Nishitani et al., 2004; Quinn et al., 2001; Zhu et al.,

2004). In human HeLa cells, Cdt1 is degraded prior to the expression of Geminin, suggesting that Geminin is a back-up system that functions after the majority of Cdt1 has been degraded (Nishitani et al., 2001). HeLa cells do not undergo re-replication when Cdt1 degradation is blocked or when Geminin is inactivated; however, when both pathways are deregulated simultaneously, re-replication is observed, indicating that the two pathways redundantly restrain Cdt1 activity (Nishitani et al., 2006).

In both *C. elegans* and *Xenopus* embryos, loss of Geminin is not associated with re-replication (Kim and Kipreos, 2007b; McGarry, 2002; McGarry and Kirschner, 1998). In contrast, Cdt1 degradation is more critical for regulating DNA replication in these species. A failure to degrade CDT-1 in *C. elegans* is associated with re-replication (Zhong et al., 2003). Likewise, the expression of a non-degradable Cdt1 (but not wild-type Cdt1) induces re-replication in *Xenopus* egg extract (Arias and Walter, 2006).

Cdt1 is degraded by the ubiquitin-proteasome system. In humans, two distinct E3 complexes, CUL4-DDB1^{CDT2} and SCF^{Skp2}, have been reported to target Cdt1 for ubiquitin-mediated degradation. The two E3 complexes utilize distinct mechanisms for targeting Cdt1 ubiquitination.

The CUL4-DDB1 complex targets Cdt1 for degradation

Studies in *C. elegans* first suggested the involvement of CUL4 in Cdt1

degradation. As described above, the inactivation of the *C. elegans cul-4* gene by RNAi causes proliferating cells to arrest in S phase and undergo massive levels of DNA re-replication (Zhong et al., 2003). In *C. elegans*, as in vertebrates and fission yeast, CDT-1 is degraded as cells enter S phase (Zhong et al., 2003). However, when *cul-4* is inactivated, CDT-1 is not degraded in S phase, but instead accumulates in the re-replicating cells (Zhong et al., 2003). Reduction of CDT-1 levels by half abolishes the re-replication in *cul-4* RNAi cells, indicating that CDT-1 accumulation is a critical factor in causing the re-replication. This work showed that CUL-4 negatively regulates CDT-1 levels, but did not address whether CDT-1 is a direct target of the CUL-4 complex.

In humans and *Drosophila*, Cdt1 is rapidly degraded in response to DNA damage induced by UV- or γ-irradiation, presumably to prevent DNA replication until the DNA damage can be repaired (Higa et al., 2003). CUL4 is also required for this Cdt1 degradation pathway (Higa et al., 2003; Hu et al., 2004). The CUL4-mediated degradation of Cdt1 upon DNA damage occurs independently of DNA replication or the classic DNA damage pathway that includes the ATM/ATR and CHK1/CHK2 kinases (Higa et al., 2003). The CUL4-dependent Cdt1 degradation in response to DNA damage can occur throughout the cell cycle (in G1, S, and G2 phases) (Higa et al., 2003; Hu et al., 2004; Nishitani et al., 2006; Ralph et al., 2006).

CDT2 and PCNA are required for Cdt1 degradation

The DCAF (DDB1-CUL4 Associated Factor) protein CDT2 physically interacts with CUL4 and DDB1 *in vivo* (Higa et al., 2006a; Jin et al., 2006; Sansam et al., 2006). In humans, *Xenopus*, zebrafish, and fission yeast, CDT2 is required for Cdt1 degradation during S phase and in response to DNA damage (Higa et al., 2006a; Jin et al., 2006; Ralph et al., 2006; Sansam et al., 2006). In *Xenopus* egg extract, CDT2 is required to load the CUL4-DDB1^{CDT2} complex onto chromatin in response to Cdt1 binding to PCNA, suggesting that the interaction of CDT2 with Cdt1 mediates the localization (Jin et al., 2006). These findings suggest that CDT2 is the SRS for the complex. However, this has not yet been formally established, as there are no reports that CDT2 can bind directly to Cdt1. There is also an intriguing finding that inactivating human CDT2 reduces the association of DDB1 with CUL4, suggesting that CDT2 may have an additional or alternative function to regulate CUL4-DDB1 complex formation (Higa et al., 2006a). There is also evidence that DDB1 may function directly as the SRS for Cdt1 binding. It has been reported that purified human Cdt1 can bind directly to purified DDB1 (Hu et al., 2004).

In *Xenopus* egg extract, the degradation of Cdt1 by CUL4-DDB1^{CDT2} in S phase requires the interaction between Cdt1 and PCNA (Arias and Walter, 2006; Jin et al., 2006). Cdt1 binds PCNA through a PCNA-interacting protein (PIP) box motif in the Cdt1 N-terminus (Arias and Walter, 2006). The degradation of Cdt1 in response to UV irradiation has similarly been shown to require the association

of Cdt1 with PCNA in humans, *Drosophila*, and fission yeast (Arias and Walter, 2006; He et al., 2006; Higa et al., 2006a; Nishitani et al., 2006; Senga et al., 2006). This suggests that the two distinct Cdt1 degradation events, occurring in response to DNA damage or S-phase entry, are triggered by the same molecular signal: Cdt1 binding to chromatin-associated PCNA. PCNA forms a trimeric ring structure that is loaded onto DNA during both DNA replication and DNA repair (Barsky and Venclovas, 2005; Maga and Hubscher, 2003). One can hypothesize that chromatin-loaded PCNA (potentially in conjunction with other factors) is sufficient to promote Cdt1 binding and its subsequent degradation (Figure 1.4).

SCF^{Skp2} functions redundantly with $CUL4\text{-}DDB1^{CDT2}$ to degrade Cdt1 in humans

In humans, the SCF^{Skp2} E3 complex also targets Cdt1 for degradation. Human Cdt1 is phosphorylated by cyclin-CDK complexes, and the phosphorylation is dependent on a cyclin-binding (Cy) motif within Cdt1 (Liu et al., 2004; Sugimoto et al., 2004). The phosphorylation on threonine 29 within the N-terminus of Cdt1 is required for its interaction with Skp2 (Takeda et al., 2005). Mutating the N-terminal CDK-phosphorylation sites of Cdt1 increases its half-life in asynchronous human cells (Liu et al., 2004). Similarly, siRNA depletion of Skp2 increases the level of Cdt1 in asynchronous human cells (Li et al., 2003; Nishitani et al., 2006). These results indicate that SCF^{Skp2} regulates Cdt1 levels in response to CDK-phosphorylation (Figure 1.4).

There have been differing reports on the effect of inactivating the SCF^{Skp2} pathway on Cdt1 levels in human S-phase cells. One study indicated that Skp2 was required to allow S-phase degradation of Cdt1 (Li et al., 2003). A second study indicated that mutation of the cyclin-binding motif of Cdt1 (which prevents Skp2 binding) does not block the majority of Cdt1 degradation in S phase, although higher residual levels of Cdt1 protein are observed in S-phase cells (Sugimoto et al., 2004). Finally, three other reports indicate that inactivation of the SCF^{Skp2}-mediated Cdt1 degradation pathway does not stabilize Cdt1 during S phase (Nishitani et al., 2006; Senga et al., 2006; Takeda et al., 2005). Recent work has clarified these apparent contradictions by showing that in human cells both SCF^{Skp2} and CUL4-$DDB1^{CDT2}$ pathways redundantly target Cdt1 for degradation during S phase (Nishitani et al., 2006; Senga et al., 2006).

Analysis of published results suggests that SCF^{Skp2} mediates Cdt1 degradation throughout the cell cycle. This conclusion is based on the observation that Skp2 siRNA depletion in asynchronous cells leads to a three-fold increase in Cdt1 levels, even though Skp2 siRNA treatment does not affect S- or G2-phase levels of Cdt1 (because Cdt1 is still targeted for degradation by CUL4-$DDB1^{CDT2}$) (Nishitani et al., 2006). This implies that Cdt1 levels must increase in non-S- or G2-phase cells upon Skp2 siRNA treatment (presumably G1 phase cells). As described above, Skp2 redundantly targets Cdt1 for degradation during S and G2 phases. Therefore, it can be concluded that Skp2 targets Cdt1 degradation throughout the cell cycle. In contrast, CUL4-DDB1-

mediated Cdt1 degradation is S-phase specific (Nishitani et al., 2006).

Figure 1.1. Ubiquitin mediated proteolysis.

Ubiquitin is activated by a ubiquitin activating enzyme (E1). The activated ubiquitin is transferred to a ubiquitin conjugating enzyme (E2), which transfers it to a target protein with the help of a ubiquitin ligase (E3). Once the substrate protein is ubiquitinated it is recognized and degraded by the 26S proteasome. (Diagram provided by E.T. Kipreos)

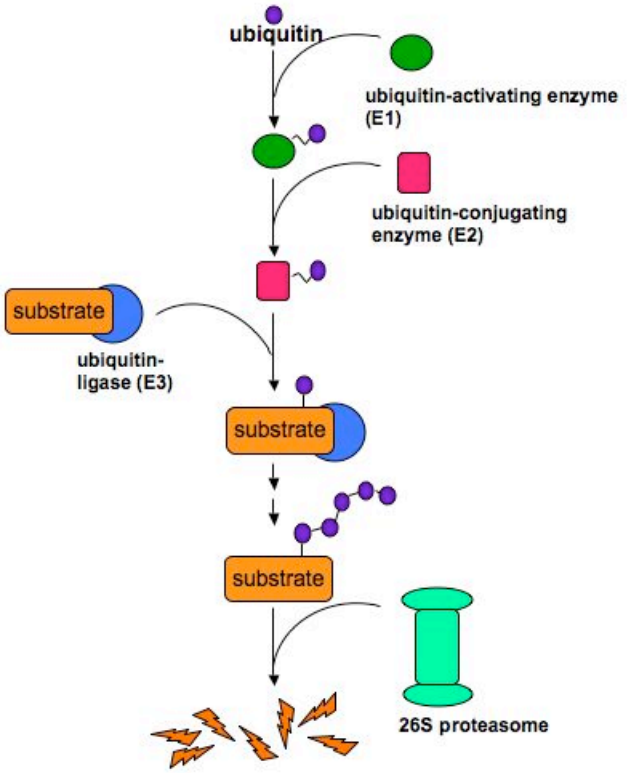
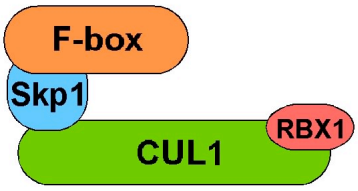
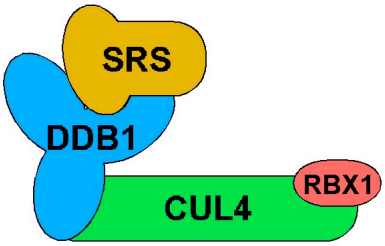

ubiquitin
ubiquitin-activating enzyme (E1)
ubiquitin-conjugating enzyme (E2)
substrate
ubiquitin-ligase (E3)
substrate
substrate
26S proteasome

Figure 1.2. Modular structure of CUL1- and CUL4-RING H2 E3 ligase. CUL1 complexes have CUL1 as a structural scaffold, SKP1 as the adaptor, F box proteins as SRS (substrate recognition subunit) and RBX1 as RING H2 protein. CUL4 complexes share a similar modular structure. In CUL4 complexes, DDB1 potentially functions as the adaptor and a group of WDXR-domain proteins has been proposed to function as SRSs.



SCF(CUL1)



CUL4-DDB1

Figure 1.3. A model of DNA replication licensing.

In metazoa, ORC is bound to DNA replication origins immediately after daughter chromosomes are synthesized. In G1 phase, the DNA licensing factors Cdt1 and Cdc6 are loaded onto origins. The presence of Cdt1 and Cdc6 enables the putative replication helicase Mcm2-7 complex to be loaded onto chromatin to allow DNA replication. In S phase, Cdt1 is degraded by the CUL4 E3 ligase and Cdc6 is exported from the nucleus. The removal of Cdt1 and Cdc6 ensures that the Mcm2-7 complex cannot be reloaded to the origins, and therefore prevents origins from re-firing.

(Diagram provided by E.T. Kipreos)

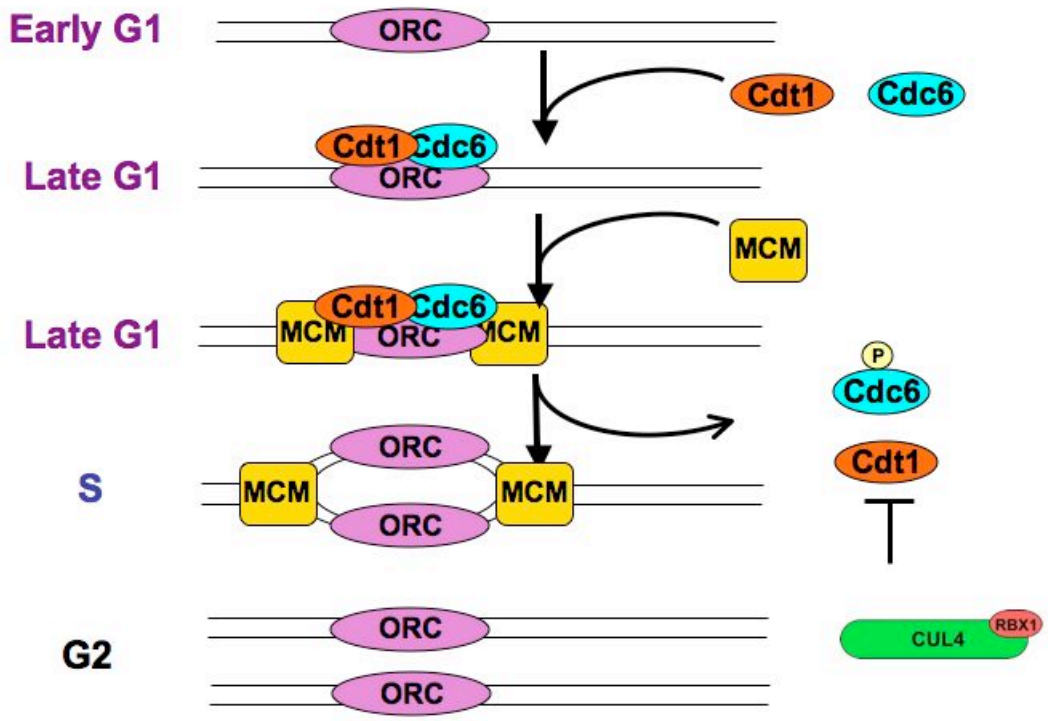
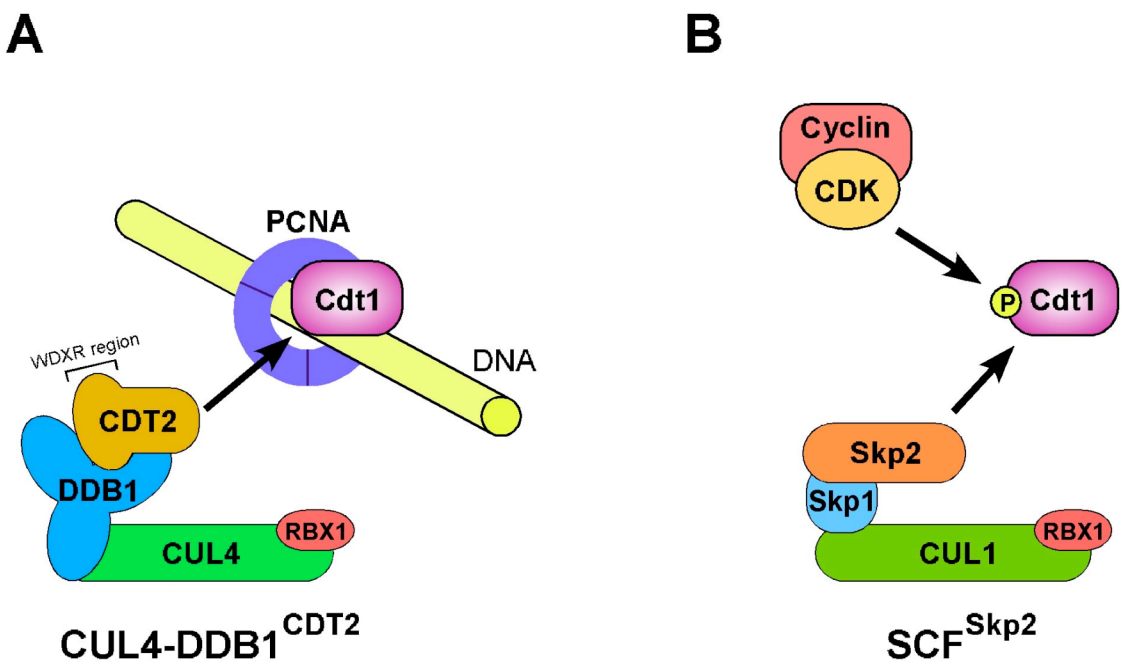

Early G1
ORC
Cdt1
Cdc6
Late G1
Cdt1
Cdc6
ORC
MCM
Late G1
Cdt1
Cdc6
MCM
ORC
MCM
P
Cdc6
Cdt1
S
ORC
MCM
MCM
ORC
G2
ORC
ORC
CUL4
RBX1

Figure 1.4. Two distinct molecular pathways for Cdt1 degradation.

(A and B) CUL4-DDB1^{CDT2} and SCF^{Skp2} CRL ubiquitin ligase complexes have similar modular structures: a cullin; a common RING H2-finger protein Rbx1; an adaptor protein, DDB1 or Skp1; and an SRS, CDT2 or Skp2. In the CUL4-DDB1^{CDT2} complex, CDT2 binds to DDB1 through a WD-repeat region with a specific signature, termed a 'WDXR' or 'DXR' domain (marked in figure). In the SCF^{Skp2} complex, Skp2 binds to Skp1 through an F-box motif (not marked). (A) CUL4-DDB1^{CDT2} targets Cdt1 for degradation after Cdt1 binds to PCNA on chromatin. CDT2 is proposed to directly bind Cdt1 after Cdt1 binds to PCNA, although the CDT2-Cdt1 interaction has not yet been formally demonstrated. (B) SCF^{Skp2} targets Cdt1 for degradation after CDK/Cyclin complexes phosphorylate Cdt1. See text for details.


A
PCNA
Cdt1
DNA
WDXR region
CDT2
DDB1
CUL4
RBX1
CUL4-DDB1CDT2
B
Cyclin
CDK
P
Cdt1
Skp2
Skp1
CUL1
RBX1
SCFSkp2

CHAPTER II

THE *C. ELEGANGS* REPLICATION LICENSING FACTOR CDT-1 IS TARGETED FOR DEGRADATON BY THE CUL-4/DDB-1 COMPLEX

BACKGROUND

Cullin-RING ubiquitin ligase complexes are potentially the largest superfamily of multisubunit E3s in eukaryotic cells, and they function in a wide-range of cellular processes (Petroski and Deshaies, 2005). The prototype of the cullin-RING E3s is the SCF complex, which includes the cullin CUL1, the RING H2-finger protein Roc1/Rbx1/Hrt1, the adaptor protein Skp1, and a variable F-box protein, which functions as a substrate recognition subunit (SRS). Other cullins form complexes that contain the same RING finger protein but have different adaptors and SRSs. CUL2 and CUL5 employ elongin C as the adaptor, and use BC-box/VHL-box proteins as SRSs. In contrast, CUL3 complexes do not employ a separate adaptor, instead a single BTB-domain protein directly binds to both CUL3 and substrates.

The structure of the CUL4 complex varies based on the role of the complex component DDB1. DDB1 (damaged-DNA binding protein 1) was initially identified as a component of the DDB complex, which functions in nucleotide excision repair, and is defective in individuals with xeroderma pigmentosum and Cockayne syndromes (Chu and Chang, 1988; Keeney et al., 1993). DDB1 can

function as an adaptor to link SRSs to the core CUL4 complex. In humans, DDB1 binds a heterodimeric SRS comprised of hDET1 and hCOP1 to target the degradation of the c-jun transcription factor (Wertz et al., 2004). Human DDB1 also binds the V proteins of paramoxyviruses, which act as SRSs in the degradation of STAT proteins (Higa et al., 2006c; Ulane and Horvath, 2002). Other proteins that have been implicated as SRSs for CUL4 complexes include: mammalian DDB2, which targets the ubiquitination of histones H3, histone H4, and the xerodosum pigmentosum protein XP-C; *S. pombe* Cdt2, which targets the degradation of the ribonucleotide reductase regulator Spd1; and mammalian CSA, which targets the degradation of the Cockayne syndrome protein CSB (Groisman et al., 2006; Groisman et al., 2003; Liu et al., 2005; Sugasawa et al., 2005; Wang et al., 2006). However, there is also evidence that DDB1 can act as an SRS and bind substrates directly. Human DDB1 binds to Cdt1 to mediate Cdt1 degradation in response to a DNA damage checkpoint (Hu et al., 2004). In *C. elegans*, CUL-4 negatively regulates the Cdt1 ortholog, CDT-1, to prevent re-replication during S phase (Zhong et al., 2003).

Maintaining genome integrity depends on the accurate replication of the genome during each cell division cycle. DNA replication is strictly regulated to ensure that origins of replication can initiate DNA synthesis only once per cell cycle. In eukaryotes, this regulation is focused on the assembly of pre-replicative complexes (pre-RCs) prior to S phase. Pre-RCs form on origins through the sequential binding of key DNA replication proteins: the six-member origin

recognition complex (ORC), the replication licensing factors Cdt1 and Cdc6, and the presumptive replicative helicase, the Mcm2-7 complex (Blow and Dutta, 2005; Machida et al., 2005). Cdt1 and Cdc6 are essential loading factors for the MCM2-7 complex, and they are regulated to ensure that the MCM2-7 complex cannot re-bind origins that have already fired during S phase. In yeast and metazoa, Cdt1 and Cdc6 are removed from the nucleus during S phase by degradation or nuclear export, thereby ensuring that they are unable to reload the MCM2-7 complex (Blow and Dutta, 2005; Feng and Kipreos, 2003; Machida et al., 2005).

In fission yeast and metazoa, Cdt1 is regulated by proteolysis at the onset of S phase and in response to DNA damage (Arias and Walter, 2005; Higa et al., 2003; Hu et al., 2004; Kondo et al., 2004; Nishitani et al., 2000; Nishitani et al., 2001; Thomer et al., 2004; Zhong et al., 2003). Our laboratory has shown that in *C. elegans*, loss of CUL-4 leads to a failure to degrade CDT-1, which is associated with extensive DNA re-replication (Zhong et al., 2003). In mammals and *Drosophila*, expression of Cdt1 to high levels induces limited DNA re-replication (Thomer et al., 2004; Vaziri et al., 2003).

It has been reported that in humans, the degradation of Cdt1 in response to DNA damage or S phase entry is mediated by the SCF^{Skp2} E3 complex (Kondo et al., 2004; Li et al., 2003). The binding of SCF^{Skp2} to Cdt1 is dependent on prior phosphorylation of Cdt1 by CDK/cyclin kinases (Liu et al., 2004; Sugimoto et al.,

2004). The CUL4 E3 complex has also been reported to be required for the DNA damage-induced degradation of Cdt1 in both human and *Drosophila* cells (Higa et al., 2003; Hu et al., 2004). In *Xenopus* egg extracts, a CUL4/DDB1 complex is the predominant E3 for Cdt1 degradation during S phase, although a role for Skp2 has not been explicitly ruled out (Arias and Walter, 2005). More recent reports indicate that both CUL4/DDB1 and SCF^{Skp2} are required for the complete degradation of human Cdt1 in S phase, suggesting that in humans, both E3s target Cdt1 for degradation (Nishitani et al., 2006; Senga et al., 2006). It is unclear to what extent the two pathways of Cdt1 degradation are evolutionarily conserved.

In this work, we characterize the loss-of-function phenotypes of the *C. elegans* orthologs of DDB1 and Skp2, and determine the extent to which they contribute to the degradation of CDT-1 in S phase. We find that *ddb-1* mutants are inviable due to defects in postembryonic cell divisions. The CUL-4/DDB-1 complex is required for CDT-1 degradation during S phase and for restraining DNA re-replication. DDB-1 physically associates with CDT-1, indicating that CDT-1 is a direct substrate of the CUL-4/DDB-1 complex. In contrast, the Skp2 ortholog, SKPT-1, is not required for viability and only exhibits an impenetrant defect in gonad migration. SKPT-1 provides no measurable contribution to the degradation of CDT-1 either in a wild-type or a *ddb-1* mutant background.

RESULTS

C. elegans has a single DDB1 ortholog, *ddb-1*, which is located on chromosome IV (Figure 2.1A). The DDB-1 protein has significant sequence identity across its entire length with orthologous DDB1 proteins, displaying 37% amino acid identity with *H. sapiens* DDB1, 37% for *X. laevis*, 36% for *D. melanogaster*, and 22% for *S. pombe* DDB1. Recent studies suggest that DDB1 interacts with CUL4 to form a ubiquitin ligase complex in fission yeast and vertebrates (Arias and Walter, 2006; Groisman et al., 2003; Hu et al., 2004; Liu et al., 2003; Ulane et al., 2005; Wertz et al., 2004). Consistent with this, we observed that DDB-1 interacts specifically with CUL-4, but not with other cullins, in the yeast two-hybrid assay (Figure 2.1D).

DDB-1 developmental expression pattern

To determine the DDB1 developmental expression pattern, DDB-1 was ectopically expressed as a GFP translational fusion using its own promoter. In larvae, DDB-1::GFP expression was observed in blast cells that proliferate during the larval stages, including seam cells in the lateral hypodermis, P cells in the ventral hypodermis, and intestinal cells (Figure 2.2A–D, H). The P-cell lineage has the longest quiescent period between rounds of cell division, and within the P-lineage, DDB-1::GFP expression correlates with the proliferative state. P blast cells did not express DDB1::GFP in newly-hatched L1 larvae; however, expression was observed later in the L1 stage as the cells began to proliferate (Figure 2.2B). During the L2 stage, the P-cell descendents are either quiescent

or postmitotic, and they lack DDB-1::GFP expression (data not shown). DDB-1::GFP expression resumes during the L3 stage in the P-cell descendents that create the vulva (the vulval precursor cells, VPCs), as they move from quiescence to proliferation (Euling and Ambros, 1996); and expression continues through the L4 stage (Figure 2.2C, D). In adults, all P cell descendents are postmitotic, and DDB-1::GFP is not expressed (data not shown).

DDB-1::GFP expression was also observed in a subset of cells that do not proliferate during the larval stages, including the lateral hyp7 hypodermal cells, rectal gland and epithelial cells, and a subset of neuronal cells in the head and tail regions (Figure 2.2F, G). Additionally, adult hermaphrodites exhibit high-level expression in the spermatheca (Figure 2.2E). DDB-1::GFP expression from the transgenic array was not observed in germ cells, but this may be due to transgene silencing in the germ line (Kelly and Fire, 1998). In embryos, we did not observe significant zygotic DDB-1::GFP expression. Overall, our expression studies suggest that DDB-1 expression is linked to the proliferative state of post-embryonic blast cells. DDB-1 is also expressed in a subset of non-proliferative cells, suggesting it may have functions that are unrelated to cell cycle progression.

ddb-1 loss-of-function phenotypes

We have characterized a *ddb-1* mutant deletion allele, *ddb-1(tm1769)*, that was isolated by the National Bioresource Project, Japan. The *tm1769* allele is a

deletion of 540 bp that removes the promoter region and exons 1 and 2 of the *ddb-1* gene; it is therefore expected to be a null allele (Figure 2.1A). *ddb-1(tm1769)* is recessive and homozygotes are inviable, and thus the allele is maintained as a heterozygous strain. Homozygous *ddb-1(tm1769)* mutant progeny from heterozygous parents exhibit normal embryogenesis. Approximately 15% (7/47) of *ddb-1(tm1769)* homozygotes arrest at the L2 or L3 stages, while the remaining 85% become sterile adults with a protruding vulva phenotype (Figure 2.4D). The timing of larval development is significantly slower for homozygous *ddb-1(tm1769)* mutants than for wild-type larvae (81.3 ± 5.6 hrs vs. 64.8 ± 3.9 hrs from laid eggs to adult at 20°C, n = 10 each; $p < 1 \times 10^{-6}$).

We also analyzed *ddb-1* RNAi phenotypes. Wild-type L4 stage larvae were fed bacteria expressing *ddb-1* dsRNA, and progeny that were laid 20 hours after the start of the RNAi treatment were analyzed. A small percentage of *ddb-1(RNAi)* progeny (5%, 7/149) arrested as late, pretzel-stage embryos. The remaining *ddb-1(RNAi)* embryos hatched normally but then arrested at the L2 or L3 larval stages. The RNAi phenotype contrasts with the phenotype of *ddb-1(tm1769)* homozygotes, most of which develop to the adult stage. The weaker *ddb-1* mutant phenotype is likely due to the effect of maternal product, in which *ddb-1* mRNA or protein is provided by the parent is sufficient to allow early development, while later developmental defects occur as maternal product decreases. By exposing wild-type L1 stage larvae to *ddb-1* feeding RNAi, we can mimic the loss of DDB-1 later in development. These *ddb-1(RNAi)* larvae

developed to the adult stage but were sterile with protruding vulva, similar to homozygous *ddb-1* mutants (data not shown).

Loss of DDB-1 induces DNA re-replication

Loss of *cul-4* causes proliferating blast cells to undergo DNA re-replication, resulting in dramatically-enlarged nuclei and increased DNA content (Zhong et al., 2003). We observed that arrested *ddb-1(tm1769)* homozygotes have similarly enlarged seam cells (Figure 2.3A). Other blast cell lineages also exhibit increased size; however, non-proliferating cells are not affected (data not shown). The enlarged *ddb-1* mutant cells show markedly increased DNA levels (25.9 ± 15.4 vs. 2.0 ± 0.2 C in wild type; n = 20 each, 1C = 1n DNA content, $p < 1x10^{-7}$, Figure 2.3B), suggesting that DDB-1 prevents DNA re-replication, similar to CUL-4. Enlarged seam cells are also observed in the 85% of *ddb-1* homozygotes that develop to the adult stage.

In our previous study, loss of *cul-4* function was derived solely from RNAi depletion, as a mutant allele was not available (Zhong et al., 2003). We have now obtained a *cul-4* mutant allele, *gk434,* which is a deletion of the promoter region and exons 1 and 2, and is therefore expected to be a null (Figure 2.1B). The *cul-4(gk434)* allele is recessive and homozygotes are inviable. *cul-4(gk434)* homozygotes uniformly arrest at the L2 stage with enlarged blast cells that have increased DNA content, similar to *cul-4(RNAi)* and *ddb-1(RNAi)* larvae (Figure 3.1B, C). The physical interaction between DDB-1 and CUL-4, and their largely

indistinguishable mutant phenotypes, are consistent with a CUL-4/DDB-1 complex mediating the known CUL-4 functions.

Vulval is defective in *ddb-1* deficient animals

To investigate whether *ddb-1* mutants that develop to the adult stage have defects associated with re-replication, we focused on the late-dividing vulval lineage. The vulva is composed of 22 cells that are generated from three progenitor VPC cells during the L3 stage (Sulston and Horvitz, 1977). *ddb-1(tm1769)* homozygotes have a significantly reduced number of vulval cells (11.0 ± 1.2; n = 23 vs. 22 in wild type (Sulston and Horvitz, 1977), and these cells are enlarged (Figure 2.4A, B). *ddb-1* mutants exhibit abnormal vulval morphology during larval development and protruding vulvae in adults (Figure 2.4A-D), similar to defects associated with inadequate vulval cell numbers (Eisenmann and Kim, 2000; Seydoux et al., 1993). DNA levels in *ddb-1* mutant vulval cells are significantly increased (7.2 ± 1.4 vs. 2.1 ± 0.4 C in wild type; n = 14 each, $p < 1x10^{-9}$), suggesting that the enlargement of vulval cells results from DNA re-replication (Figure 2.4E-H). Somatic gonadal cells also divide during the L3 stage, and we observed that these cells are also larger and contain increased DNA levels (Figure 2.4A, B, E-H).

DDB-1 is required for CDT-1 degradation during S phase

In fission yeast and metazoa, the essential DNA replication licensing factor CDT-1 is degraded upon entry into S phase to ensure that DNA re-

replication does not occur (Blow and Dutta, 2005; Machida et al., 2005). In *C. elegans*, CUL-4 is required for CDT-1 degradation during S phase (Zhong et al., 2003). We asked whether DDB-1 is similarly required for CDT-1 degradation during S phase. The V1 - V6 seam cells enter S phase at approximately 120 min post-hatch at 20°C (Zhong et al., 2003). Immunofluorescence staining with anti-CDT-1 antibody shows that CDT-1 is present in late G1 phase nuclei (90 - 110 min post-hatch) in both wild-type and *ddb-1(RNAi)* seam cells (Figure 2.5A, B). In wild-type larvae, the level of CDT-1 protein abruptly decreases as seam cells enter S phase, and CDT-1 is not detected at 180 - 200 min post-hatch (Figure 2.5A) (Zhong et al., 2003). In *ddb-1* RNAi larvae, however, the CDT-1 protein levels do not drop after cells enter S phase, but instead remain elevated even at 180 - 200 min post-hatch, indicating that DDB-1 is required for CDT-1 degradation during S phase (Figure 2.5B).

We observed a similar requirement for DDB-1 in the degradation of a CDT-1::GFP fusion protein during S phase (Figure 2.6B, C). CDT-1::GFP was expressed in a strain that contains AJM-1::GFP to visualize seam cell boundaries, and an S phase-marker transgene, in which RFP was expressed from the ribonucleotide reductase promoter (*Prnr-1*::RFP). The CDT-1::GFP signal is much higher relative to the background compared to anti-CDT-1 immunofluorescence; and it is clearly apparent that CDT-1::GFP is degraded during S phase rather than undergoing redistribution to the cytoplasm (Figure 2.6B). The observed requirement of DDB-1 for CDT-1 degradation during S

phase is similar to that observed for CUL-4 (Zhong et al., 2003), strongly suggesting that DDB-1 functions with CUL-4 to negatively regulate DNA replication *in vivo*.

In humans and *Xenopus*, PCNA (Proliferating Cell Nuclear Antigen) is required for CUL4/DDB1-mediated CDT-1 degradation at the onset of S phase or in response to DNA damage (Arias and Walter, 2006; Hu and Xiong, 2006; Nishitani et al., 2006; Senga et al., 2006). PCNA interacts with Cdt1 through a PCNA-Interacting Protein motif (PIP box) in Cdt1. Cdt1 mutant proteins that lack the PIP box are unable to be degraded by the CUL4/DDB1 complex (Arias and Walter, 2006; Nishitani et al., 2006; Senga et al., 2006). To assess the requirement for the PIP box in *C. elegans* CDT-1, a mutant CDT-1 in which six conserved amino acids of the PIP box were mutated to alanine, CDT-$1^{PIP(6A)}$, was ectopically expressed as a GFP fusion protein. In S phase seam cells, CDT-$1^{PIP(6A)}$::GFP expression in nuclei remains high, whereas the wild-type CDT-1::GFP is not detected in S phase (Figure 2.6B, D), indicating that *C. elegans* CDT-1 degradation requires an intact PIP box. However the re-replication was not detected in CDT-$1^{PIP(6A)}$::GFP expressed cells, suggesting that the failure to degrade CDT-1 is not enough to cause re-replication.

DDB-1 physically interacts with CDT-1

DDB1 has been observed to function in the CUL4 E3 complex either as the SRS, which directly binds to substrates, or as an adaptor that links the SRS

to the complex (Hu et al., 2004; Ulane and Horvath, 2002; Wertz et al., 2004). To determine whether DDB-1 can interact with CDT-1, we expressed the proteins in human 293T cells. We observed that DDB-1 and CDT-1 reciprocally co-immunoprecipitated each other (Figure 2.7A). We also tested for interaction between DDB-1 and CDT-1 using an *in vitro* system. ^{35}S-labelled FLAG-tagged DDB-1, which was *in vitro* translated using a wheat germ extract and then affinity purified, specifically bound to purified, recombinant GST-tagged CDT-1 *in vitro* (Figure 2.8A). The interaction between DDB-1 and CDT-1 in both systems supports the proposal that DDB-1 can physically interact with CDT-1.

It has been suggested that human DDB1 preferentially binds to phosphorylated Cdt1 (Hu et al., 2004). It also has been proposed that cyclinE/CDK2 phosphorylation of the *Drosophila* Cdt1 ortholog promotes its degradation at the G1/S phase transition (Thomer et al., 2004). We therefore investigated whether phosphorylation of *C. elegans* CDT-1 on CDK sites affects its interaction with DDB-1. The phosphorylation of GST-CDT-1 by CDK did not increase the interaction with ^{35}S-labeled FLAG-DDB-1 *in vitro* (Figure 2.8A), suggesting that CDK phosphorylation of CDT-1 is not essential for CUL-4/DDB-1 mediated degradation.

C. elegans Skp2 is not required for CDT-1 degradation during S phase

It has been proposed that the human SCF^{Skp2} ubiquitin ligase targets Cdt1 for degradation during S phase (Li et al., 2003; Liu et al., 2004; Nishitani et al.,

2006; Sugimoto et al., 2004). *C. elegans* has only one *Skp2* ortholog, *skpt-1* (Skp-two related protein) (Figure 2.1C). The SKPT-1 protein has 28% sequence identity with human Skp2, and reciprocal BLAST analysis provides matches with e-values of 10^{-24}. To investigate the function of SKPT-1 in *C. elegans*, we analyzed *skpt-1(ok851)* mutants. The *skpt-1(ok851)* allele deletes exons 2 and 3 and parts of exons 1 and 4, with the resulting exon 1–exon 4 fusion creating a premature truncation, strongly suggesting that it is a null allele. *skpt-1(ok851)* homozygotes are viable and produce similar numbers of eggs compared to wild type (277 ± 26 vs. 288 ± 36 eggs, n = 6 and 10, respectively). *skpt-1(ok851)* homozygotes appear overtly wild type, with the exception of a low-penetrance gonad mismigration phenotype, in which 7% (8/113) of *skpt-1(ok851)* mutants exhibit an improper trajectory of one gonad arm (Figure 9A). *skpt-1* RNAi produces a similar level of gonad migration defects (7%; 13/180) (H. Jin and E.T.K., unpublished observation). A strain heterozygous for the *skpt-1(ok851)* allele and a deficiency (*sDf121*) that eliminates the *skpt-1* locus (so that the heterozygotes have only one copy of the *skpt-1(ok851)* allele) also has a 7% (9/132) level of gonad mismigration. The observation that one copy of the *skpt-1(ok851)* allele gives the same phenotype as two copies provides genetic evidence that *skpt-1(ok851)* is a null allele.

To determine if SKPT-1 is required for CDT-1 degradation during S phase, we analyzed the degradation of CDT-1 in seam cells as they progress from G1 to S phase. The time course of CDT-1 degradation in *skpt-1(ok851)* homozygotes

was similar to that of wild-type larvae (Figures 2.5A, C and 2.9B). This suggests that SKPT-1 is not required for either the proper timing or the extent of CDT-1 degradation during S phase. To address whether SKPT-1 has a minor, redundant role in degrading CDT-1 and preventing DNA re-replication, we asked whether the co-elimination of SKPT-1 and DDB-1 would increase the extent of the replication compared to loss of DDB-1 alone. A comparison of *ddb-1(RNAi)* and *ddb-1(RNAi), skpt-1*(*ok851*) larvae did not reveal significant differences in either: the size of re-replicating seam cells (107.2 ± 48.6 μm^2 and 112.6 ± 48.0 μm^2 , respectively, n = 34 each); their DNA levels (25.9 ± 15.4 and 21.7 ± 20.0 C, respectively; n = 20 each); or their CDT-1 levels (data not shown). Therefore, SKPT-1 appears to have no detectable role in regulating CDT-1 or DNA replication even in a sensitized *ddb-1* mutant background.

To test whether *C. elegans* SKPT-1 can bind CDT-1, we analyzed interaction in 293T cells. Reciprocal immunoprecipitations of CDT-1-GFP and SKPT-1-MYC pulled down only very low levels of the other protein. The extent of interaction between CDT-1 and SKPT-1 was not higher than that between CDT-1 and a non-specific F-box protein, LIN-23 (Figure 2.7B). In contrast, the interaction of CDT-1 and DDB-1 was considerably stronger than the interaction between DDB-1 and LIN-23 (Figure 2.7A). In an *in vitro* binding assay, the interaction between GST-CDT-1 and *in vitro* translated DDB-1 was 7.3-fold higher than between GST-CDT-1 and *in vitro* translated SKPT-1 (Figure 2.8A, B). Skp2 recognizes only CDK-phosphorylated substrates (Petroski and Deshaies,

2005). Significantly, phosphorylation of CDT-1 by CDK did not increase interaction with SKPT-1 (Figure 2.8A), further suggesting that it is not a real substrate of SKPT-1. Taken together with the failure of SKPT-1 to regulate CDT-1 levels *in vivo*, these results suggest that SKPT-1 does not contribute significantly to the degradation of CDT-1.

When did genes for the two Cdt1-degradation pathways arise during evolution?

To determine when the genes for the CUL4-DDB1^{CDT2} and SCF^{Skp2} complexes arose during evolution, we analyzed divergent species using reciprocal BLAST searches (Altschul et al., 1997). We limited our analysis to those organisms in which the whole genome had been sequenced, so that a failure to detect a gene would be meaningful. Cullin genes were not found in bacteria or archaea, but at least two cullins were found in all of the eukaryotic genomes that we examined (Table 2.1). The observation of cullins in protists suggests that the cullin gene family arose early in the eukaryotic lineage (Table 2.1, Figure 2.10). All eukaryotic species examined contain cullins that were most similar to metazoan CUL1 and CUL4 in reciprocal BLAST analysis, with the exception of budding yeast (which lacks a CUL4-like gene) (Table 2.1). This suggests that an ancestral duplication that gave rise to CUL1-like and CUL4-like genes occurred early in eukaryotic evolution. This result matches a phylogenetic analysis of cullins, in which the first branch point of the cullin phylogeny creates two clades, with the first clade giving rise to CUL1, CUL2 and CUL5, and the

second clade giving rise to CUL3 and CUL4 (Nayak et al., 2002). The adaptor proteins Skp1 and DDB1 are present whenever CUL1-like and CUL4-like genes are observed, suggesting that the association between the cullins and their adaptor proteins is ancient (Table 2.1, Figure 2.10). The substrate-specific components CDT2 and Skp2 appear to have arisen at different points in eukaryotic evolution. CDT2 is observed in all animals analyzed, and a majority of fungi and plants, but is not observed in protists (Table 2.1, Figure 2.10). This suggests that CDT2 arose in the main ancestral eukaryote lineage after the protist lineages diverged, but prior to the genesis of plants. In contrast, Skp2 apparently arose later in evolution. Skp2 is present in animals, but is not detected in fungi or plants, suggesting that it arose after the branching of fungi from the main eukaryotic lineage but prior to the genesis of metazoa (Table 2.1, Figure 2.10). This analysis implies that CDT2, and by extension the CUL4-DDB1^{CDT2} complex, is more ancient than Skp2 and the SCF^{Skp2} complex.

DISCUSSION

In this study, we report the expression pattern and mutant phenotype of the *C. elegans* ortholog of DDB1. DDB-1 is expressed in proliferating postembryonic blast cells, and expression correlates with cell cycle progression in the P-blast cell lineage. The P blast cells and their descendents express DDB1 while proliferating in the L1 and L3 larval stages, but expression disappears in cells that permanently exit the cell cycle and also during the extended period of quiescence in the L2 stage. Additionally, DDB-1 is observed

in a subset of non-proliferative cells, suggesting that it has functions unrelated to cell cycle progression.

DDB-1 is required for viability and shares mutant phenotypes with *cul-4*. *ddb-1* RNAi produces an L2-stage larval arrest similar to that observed in *cul-4* mutants. The arrested *ddb-1(RNAi)* larvae exhibit signs of re-replication with large, polyploid blast cells, similar to what we have previously described for *cul-4(RNAi)* larvae (Zhong et al., 2003) and now observe in *cul-4* mutants. We showed that DDB-1 interacts specifically with CUL-4 but not with other *C. elegans* cullins. This physical interaction, coupled with their similar mutant phenotypes, strongly suggests that CUL-4 regulates DNA replication solely through a CUL-4/DDB-1 complex.

Zhong et al. had previously observed re-replication in a wide range of somatic blast cells in arrested *cul-4(RNAi)* larvae (Zhong et al., 2003). Our current data suggests that CUL-4/DDB-1 is also required to restrain DNA replication in later somatic cell divisions. In *ddb-1* mutants, early larval cell divisions occur normally (apparently due to rescue by maternal product), but mutant phenotypes are observed in later larval stages. *ddb-1* mutants exhibit cell division failures associated with increased DNA levels in the late-dividing vulva and the somatic gonad lineages.

CDT-1 is a direct target of CUL-4/DDB-1

Zhong et al. had previously shown that CUL-4 is required for the degradation of CDT-1 in S phase (Zhong et al., 2003). We also reported that the deletion of one copy of genomic *cdt-1* can significantly suppress the re-replication phenotype of *cul-4(RNAi)* larvae, suggesting that CDT-1 perdurance is a decisive factor in causing the re-replication. In this study, we found that CDT-1 is not degraded during S phase in *ddb-1* mutants, strongly supporting the proposal that CUL-4 mediates CDT-1 degradation through a conserved CUL-4/DDB-1 complex.

We observed that *C. elegans* DDB-1 and CDT-1 can physically associate with each other when co-expressed in 293T cells, and that *in vitro* translated DDB-1, made with a wheat germ extract, binds to bacterially-produced recombinant GST-CDT-1. We found that CDK-mediated phosphorylation of CDT-1 does not improve the efficiency of *in vitro* binding to DDB-1, suggesting that CDT-1 degradation does not require prior phosphorylation. We cannot conclude that CDT-1 binds directly to DDB-1, as it is conceivable that a human or wheat protein bridges the two proteins in the 293T and wheat extract systems, respectively. We have been unable to isolate soluble recombinant DDB-1 protein, which has precluded a direct test of this question. However, purified human Cdt1 has been shown to bind directly to purified human DDB1 (Hu et al., 2004), indicating that in humans, the two proteins can interact directly. Our data in two different expression systems is consistent with this result.

SCF^{Skp2} functions redundantly with $CUL4\text{-}DDB1^{CDT2}$ to degrade Cdt1 in humans

In humans, the SCF^{Skp2} E3 complex also targets Cdt1 for degradation. Human Cdt1 is phosphorylated by cyclin-CDK complexes, and the phosphorylation is dependent on a cyclin-binding (Cy) motif within Cdt1 (Liu et al., 2004; Sugimoto et al., 2004). The phosphorylation on threonine 29 within the N-terminus of Cdt1 is required for its interaction with Skp2 (Takeda et al., 2005). Mutating the N-terminal CDK-phosphorylation sites of Cdt1 increases its half-life in asynchronous human cells (Liu et al., 2004). Similarly, siRNA depletion of Skp2 increases the level of Cdt1 in asynchronous human cells (Li et al., 2003; Nishitani et al., 2006). These results indicate that SCF^{Skp2} regulates Cdt1 levels in response to CDK-phosphorylation (Figure 2.10B).

There have been differing reports on the effect of inactivating the SCF^{Skp2} pathway on Cdt1 levels in human S-phase cells. One study indicated that Skp2 was required to allow S-phase degradation of Cdt1 (Li et al., 2003). A second study indicated that mutation of the cyclin-binding motif of Cdt1 (which prevents Skp2 binding) does not block the majority of Cdt1 degradation in S phase, although higher residual levels of Cdt1 protein are observed in S-phase cells (Sugimoto et al., 2004). Finally, three other reports indicate that inactivation of the SCF^{Skp2}-mediated Cdt1 degradation pathway does not stabilize Cdt1 during S phase (Nishitani et al., 2006; Senga et al., 2006; Takeda et al., 2005). Recent work has clarified these apparent contradictions by showing that in human cells

both SCF^{Skp2} and CUL4-$DDB1^{CDT2}$ pathways redundantly target Cdt1 for degradation during S phase (Nishitani et al., 2006; Senga et al., 2006).

The *C. elegans* Skp2 homolog is not required for CDT-1 degradation

In humans, SCF^{Skp2} functions redundantly with CUL4-$DDB1^{CDT2}$ to degrade Cdt1 as described in Chapter I. However it was not clear whether SCF^{Skp2}-mediated Cdt1 degradation pathway is conserved in other systems. We have characterized a *skpt-1* deletion allele, which is a null by genetic criteria. *skpt-1* mutants appear wild type with the exception of an impenetrant gonad mismigration phenotype. Gonad migration is an active process in which the distal tip cell (DTC) migrates in response to extracellular guidance cues and leads the developing gonad into its final shape (Lehmann, 2001). It is presently unclear whether SKPT-1 functions cell autonomously in the DTC or non-cell autonomously in the surrounding tissues. However, since 93% of *skpt-1* homozygous mutants have a normal gonad migration pattern, SKPT-1 does not appear to have a major, non-redundant role in this process.

We have not observed any role for SKPT-1 in the S phase degradation of CDT-1. In homozygous *skpt-1* mutant larvae, CDT-1 is degraded as cells enter S phase with the same kinetics as in wild type, indicating that SKPT-1 is not required for CDT-1 degradation. The simultaneous inactivation of DDB-1 and SKPT-1 did not increase the size of re-replicating cells, their DNA content, or CDT-1 levels. This suggests that SKPT-1 has no discernable redundant function

with DDB-1 to degrade CDT-1. Co-immunoprecipitation assays in 293T cells and *in vitro* binding studies suggest that SKPT-1 does not interact specifically with CDT-1, as the weak interaction observed is not higher than non-specific interaction between LIN-23 and CDT-1.

Cdt1 degradation in other metazoa and yeast

In this study, the roles of Skp2 and CUL4 in degrading Cdt1 have been explicitly compared in *C. elegans*. Inactivation of *C. elegans cul-4* or *ddb-1* fully stabilizes CDT-1 during S phase. In contrast, the *C. elegans* Skp2 homolog, *skpt-1,* does not contribute to CDT-1 degradation or re-replication even in a sensitized *ddb-1* mutant background. *skpt-1* null mutant homozygotes are completely viable and appear overtly wild-type with the exception of a low-penetrance gonad migration defect, indicating that the gene is not required for any essential functions.

In *Xenopus* egg extract, CDK-phosphorylation of Cdt1 is not required for Cdt1 degradation (Arias and Walter, 2005). This implies that SCF^{Skp2} is not required for Cdt1 degradation because CDK-phosphorylation of Cdt1 is a prerequisite for recognition by human Skp2 (Liu et al., 2004; Sugimoto et al., 2004), and most SCF^{Skp2} substrates must be phosphorylated to be recognized (Petroski and Deshaies, 2005). In contrast, CUL4-DDB1 is essential for Cdt1 degradation in *Xenopus* egg extract, with DDB1 depletion blocking Cdt1 degradation during S phase (Arias and Walter, 2006). These results suggest that

CUL4-DDB1^{CDT2} is the predominant E3 for Cdt1 degradation in *Xenopus*, and that SCF^{Skp2} either has no role or has only a minor, subservient role in Cdt1 degradation.

In *Drosophila*, mutation of all of the N-terminal CDK-phosphorylation sites of Cdt1 is not able to block S-phase degradation, although it does provide a limited increase in overall stability (Thomer et al., 2004). This indicates that a phosphorylation-dependent pathway (and by implication SCF^{Skp2}) either is not involved or is redundant for Cdt1 degradation during S phase in *Drosophila*. There are currently no reports on the function of the fly Skp2 homolog.

Fission yeast does not have a recognizable Skp2 homolog, but does express the CUL4-DDB1^{CDT2} complex. Fission yeast CUL4-DDB1^{CDT2} is essential for the degradation of Cdt1 during S phase and in response to DNA damage, indicating that it is the dominant pathway for regulating Cdt1 levels (Ralph et al., 2006). Taken together, these studies suggest that SCF^{Skp2}-mediated degradation of Cdt1 is not conserved in non-mammalian species (summarized in Table 2.2).

Is the SCF^{Skp2}-dependent Cdt1 degradation pathway conserved in mice?

The studies described above suggest that SCF^{Skp2}-mediated degradation of Cdt1 is not conserved in yeast, invertebrates, or even the vertebrate *Xenopus laevis*. It is therefore valid to ask whether SCF^{Skp2}-mediated Cdt1 degradation is

conserved among mammals; and in fact, there is evidence that casts doubt on the conservation of the pathway in mice. Inactivation of Skp2 by siRNA treatment in human cells leads to an approximately three-fold increase in the steady state level of Cdt1 (Li et al., 2003; Nishitani et al., 2006). However, $Skp2^{-/-}$ knockout mice or $Skp2^{-/-}$ MEFs (mouse embryonic fibroblasts) do not have elevated levels of Cdt1 (Nakayama et al., 2004; Nishitani et al., 2006). In contrast, $DDB1^{-/-}$ knockout mice have elevated Cdt1 levels in proliferating tissues (Cang et al., 2006). Further, Cdt1 protein level is stabilized after UV-irradiation in $DDB1^{-/-}$ MEFs (Cang et al., 2006). These results indicate that in mice, the CUL4-DDB1 complex is required non-redundantly for proper Cdt1 degradation during normal cell cycle progression and in response to DNA damage; in contrast, loss of Skp2 does not perturb these processes.

It is interesting that $Skp2^{-/-}$ knock-out mice are completely viable and fertile (Nakayama et al., 2000). This is particularly striking in light of the long list of human Skp2 substrates, including important cell cycle and transcriptional regulators: Cdt1, Orc1, $p27^{Kip1}$, $p21^{Cip1}$, cyclin E, cyclin D, cyclin A, c-Myc, b-Myb, p130/pRb2, E2F-1, $p57^{Kip2}$, MKP-1, RAG-2, FOXO1, and Cdk9 (Barboric et al., 2005; Bendjennat et al., 2003; Bhattacharya et al., 2003; Bornstein et al., 2003; Carrano et al., 1999; Charrasse et al., 2000; Huang et al., 2005; Jiang et al., 2005; Kamura et al., 2003; Kiernan et al., 2001; Kondo et al., 2004; Li et al., 2003; Lin and Yang, 2006; Liu et al., 2004; Marti et al., 1999; Mendez et al., 2002; Nakayama et al., 2000; Sugimoto et al., 2004; Sutterluty et al., 1999;

Tedesco et al., 2002; Tsvetkov et al., 1999; von der Lehr et al., 2003; Yu et al., 1998b). Although $Skp2^{-/-}$ mice are viable, they exhibit a minor defect of polyploidy and extra centrosomes in the cells of a few tissues (Nakayama et al., 2000). Both of these defects arise as secondary consequences of a failure of these cells to enter mitosis, with the affected cells subsequently re-entering the next cell cycle and duplicating their DNA and centrosomes (Nakayama et al., 2004). Significantly, the mitotic defect is suppressed by co-inactivation of $p27^{Kip1}$, suggesting that the inability to degrade $p27^{Kip1}$ causes the defect (Nakayama et al., 2004). The lack of phenotypes associated with a failure to degrade other potential substrates suggests either that they are not substrates in mice, that their degradation is not important for development, or that they are under redundant control with other degradation pathways. Taken together, the available evidence suggests that CUL4-$DDB1^{CDT2}$ is the predominant ubiquitin ligase to mediate Cdt1 degradation in mice, and that SCF^{Skp2} either does not target Cdt1 for degradation or does so only as a minor pathway that cannot compensate for loss of CUL4-$DDB1^{CDT2}$.

CUL4/DDB1CDT2 is an ancient pathway to control DNA replication

The finding that the CUL4-$DDB1^{CDT2}$ complex targets Cdt1 for degradation in fission yeast and *C. elegans*, while SCF^{Skp2} does not, suggests that the CUL4-$DDB1^{CDT2}$ pathway is the ancient, conserved pathway for controlling the extent of DNA replication via Cdt1 degradation. A prediction of this hypothesis is that yeast or metazoan species that have lost genes for the CUL4-$DDB1^{CDT2}$ complex

would have to employ a different strategy to restrict Cdt1 activity during S phase. In this regard, it should be noted that budding yeast (unlike other fungi) does not contain CUL4, DDB1, or CDT2 (Table 2.1). Strikingly, budding yeast employ a strategy for regulating Cdt1 that has not been observed in any other species: Cdt1 is exported from the nucleus with the Mcm2-7 complex rather than being degraded (Tanaka and Diffley, 2002). The fungal ancestor of budding yeast must have originally had the genes for the CUL4-DDB1^{CDT2} complex and then lost them, because the genes are found in plants and other fungi (Table 2.1 and Figure 2.10). It is possible that the loss of these genes put pressure on budding yeast to develop a novel strategy to regulate Cdt1 during S phase. Alternatively, the nuclear-export strategy may have developed and co-existed with the CUL4-DDB1^{CDT2} pathway, but the redundancy between the two pathways subsequently allowed the loss of the CUL4-DDB1^{CDT2} genes.

Conclusion

In humans, both CUL4-DDB1^{CDT2} and SCF^{Skp2} redundantly target Cdt1 for degradation. SCF^{Skp2}-mediated degradation of Cdt1 is not restricted to S phase in humans, but instead occurs throughout the cell cycle. In contrast, CUL4-DDB1^{CDT2}-mediated degradation of Cdt1 is S-phase specific. The current evidence suggests that in fission yeast, *C. elegans*, *Xenopus*, and potentially even in mice, SCF^{Skp2} does not contribute significantly to Cdt1 regulation, while the CUL4-DDB1^{CDT2} complex is a major regulator of Cdt1 degradation in these species. The extent to which SCF^{Skp2}-mediated Cdt1 degradation is conserved

in mammals other than humans is not yet clear. Genome comparisons suggest that the CUL4-DDB1^{CDT2} complex arose earlier in evolution than SCF^{Skp2} based on the finding that a CDT2 ortholog is present in plants and fungi, while a Skp2 homolog is absent in these organisms. We propose that CUL4-DDB1^{CDT2} is the ancient and paramount ubiquitin ligase for the degradation of Cdt1 in response to S-phase entry and DNA damage. Further experiments will be required to address the interesting question of when during early eukaryotic evolution the CUL4-DDB1 complex first began to regulate DNA replication.

MATERIALS AND METHODS

Strains and RNAi

The following *C. elegans* strains were used: N2, wild type; ET263, *ddb-1(tm1769)/dpy-20(e2017)*; VC1033, *cul-4(gk434)/mIn1* [*mIs14 dpy-10(e128)*]; RB956, *skpt-1(ok851)*; ET278, *unc-119(e2498), ekEx12* [pPD95.75/*ddb-1 (ddb-1::GFP) + unc-119(+)*]; ET285, *skpt-1(ok851) unc-36(e251)*/*sDf121 unc-32(e189)*; PS3729, *syIs78*[AJM-1::GFP + *unc-119*(+)], *unc-119(ed4).* Plasmid pPD95.75/*ddb-1* was created by cloning genomic *ddb-1*, including 254 base pairs upstream of the translation start, into plasmid pPD95.75, which allows a C-terminal GFP fusion (primers used, gccgctgcagTTCTTCTTCGCTCATTTTAAAAAC and cgtcccggggGTGCATTCTCGCCAAATCC). *ddb-1* RNAi was performed by providing bacteria expressing *ddb-1* dsRNA to L4-stage larvae as a food source, as described (Kamath et al., 2001). The *ddb-1* feeding protocol employed *E. coli*

strain HT115 containing plasmid pDEST129.36/*ddb-1*, which is a modification of pPD129.36 that contains a full-length *ddb-1* cDNA cloned into a Gateway recombination cloning site situated between double T7 primers.

Two-hybrid assay

Two-hybrid analysis was performed with the full-length *ddb-1* gene in the pACTII (activation domain) vector and full-length cullin genes in the pAS2 (DNA binding domain) vector (Clontech). Transformation of the *S. cerevisiae* strain pJ69-4A (James et al., 1996) was performed as described (Janssen, 1995). Interaction in the two-hybrid system was tested by growth on both histidine- and adenine-deficient selective media.

Immunofluorescence

Affinity purified polyclonal anti-CDT-1 was as described (Zhong et al., 2003). Anti-AJM-1 (MH27), which highlights gap junctions (Koppen et al., 2001), was obtained from the Developmental Studies Hybridoma Bank. Anti-rabbit Alexa Fluor 488 (Molecular Probes) and anti-mouse rhodamine (Cappel) were used as secondary antibodies. DNA was stained with 1 µg/ml Hoechst 33258 dye. Immunofluorescence was performed using the “freeze-crack” method, as described (Miller and Shakes, 1995). For analysis of CDT-1 expression in L1 larvae at set times post-hatch, timed cohorts of newly-hatched L1 larvae were collected at 15 min intervals and allowed to develop for the required length of

time on plates containing OP50 bacteria as a food source, as previously described (Zhong et al., 2003).

Microscopy

Animals were observed by differential interference contrast (DIC) and immunofluorescence microscopy using a Zeiss Axioskop microscope. Images were taken with a Hamamatsu ORCA-ER digital camera with Openlab 4.0.2 software (Improvision). Images were processed with Adobe Photoshop 7.0. Matched images were taken with the same exposure time and processed identically. Matched images of anti-CDT-1, anti-AJM-1, and DAPI staining (Figures 2.3B and 2.5) were deconvolved to equivalent extents to minimize background fluorescence using the multineighbor deconvolution program of Openlab.

Quantitation of DNA content

DNA content was measured using described methods (Flemming et al., 2000; Hedgecock and White, 1985). In brief, single digital images were taken of nuclei stained with 1 µg/ml of Hoechst 33258. Every image included either 2n post-mitotic cells or 1n sperm to be used as an internal reference. The signal for a given nucleus in the images was determined as the average signal of the nucleus minus the average background signal multiplied by the area of the nucleus. The signals were normalized to known 2n or 1n reference nuclei within the same image, with the data converted to C units (units of DNA content),

wherein 1n = 1C (Swift, 1950).

Ectopic expression of CDT-1::GFP and *Prnr-1*::RFP

To generate extragenic transgenic lines, we microinjected into the strain PS3729 (which expresses AJM-1::GFP) N2 genomic DNA (200mg/ml), the marker plasmid pRF4 containing *rol-6(su1006)* (5mg/ml) (Mello and Fire, 1995), an S phase marker plasmid pRED95.67/*Prnr-1* (5mg/ml), and either pPD95.75/*Pwrt-2-cdt-1WT* (5mg/ml) or pPD95.75/*Pwrt-2-cdt-1PIP(6A)* (5mg/ml). To create plasmid pPD95.75/*Pwrt-2-cdt-1WT*, we fused the *wrt-2* promoter and *cdt-1* genomic coding sequence using an overlap extension mutagenesis method (Mikaelian and Sergeant, 1992). The fusion of *wrt-2* promoter and *cdt-1* was introduced into plasmid pPD95.75, which provides a C-terminal GFP fusion. For pPD95.75/*Pwrt-2-cdt-1PIP(6A),* site-directed mutation of six conserved amino acids in the PIP box was introduced using an overlap extension PCR method with the following primer pairs:
CCGCATGCTTACGACGAATAATTTTATTGAATTTTG /
GcagCAGCAGTCgcCGGAGTCTGGGACC; and
gcGACTGCTGctgCtGcCgcTgctAAGGTAAATTGGAGTTTGAAG /
CTTCCACGAGATTCGGTC. Plasmid pRED95.67 was created by replacing the GFP cDNA sequence of pPD95.67 with RFP cDNA from plasmid pPD158.114. The *rnr-1* promoter and SV40 NLS sequences, which came from pVT501, were introduced into SphI/AgeI sites of pRED95.67 to create plasmid pRED95.67/*Prnr-1*.

Transfection, immunoprecipitation, and immunoblot analysis

Human 293T cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum (Gibco-BRL). For expression in 293T cells, full-length cDNA sequences of *ddb-1* and *cdt-1* were cloned into pCMV-Tag2 (Stratagene) and pEGFP-N1 (Clontech), respectively, and full-length cDNA sequences of *skpt-1* and *lin-23* were cloned into pcDNA3.1(+)/myc-His (Invitrogen). 293T cells were transfected using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. After 60 hours incubation at 37^{o}C, cells were lysed with NP-40 buffer containing 10 mM Sodium phosphate (pH 7.2), 150 mM NaCl, 1% NP-40, 2 mM EDTA, complete protease inhibitors cocktail (Roche), Ser/Thr and Tyr phosphatase inhibitors (Upstate), and 50 μM N-acetyl-L-leucinyl-L-leucinal-L-norleucinal (LLnL; Sigma). To minimize proteasome-mediated degradation, transfected cells were treated with 50 μM LLnL 10 hours before harvest. The primary antibodies used in immunoprecipitation and western detection were anti-MYC (9E10; Covance), anti-FLAG (M2; Sigma), rabbit polyclonal anti-MYC tag (Bethyl laboratory), and anti-CDT-1 (Zhong et al., 2003). The secondary antibodies used were anti-rabbit-HRP (Pierce) and anti-mouse-HRP (GE Healthcare and Pierce). Western blots were visualized using the Advanced ECL chemiluminescence system (GE healthcare).

***In vitro* binding**

Plasmid pGEX-2T/*cdt-1* contains the full-length *cdt-1* gene in the pGEX-2T vector (Smith and Johnson, 1988). pGEX-2T/*cdt-1* was expressed in the *E. coli*

strain *BL21cp*. The glutathione *S*-transferase (GST)-tagged CDT-1 protein was solubilized and purified using a modification of a described method (Frangioni and Neel, 1993). Briefly, bacteria were induced with 1 mM IPTG for 4 hours at 37^{o}C, pelleted, and resuspended in STE (10 mM Tris pH 8.0, 150 mM NaCl, and 1 mM EDTA) containing 100µg/ml of lysozyme. N-laurylsarcosine (sarcosyl) was added to a final concentration 0.5% to solubilize the recombinant CDT-1 proteins. After a 1 hr incubation, Triton X-100 was added to 2%, and GST-CDT-1 protein was purified with Glutathione Sepharose 4 Fast Flow beads (GE Healthcare), according to the manufacturer's instructions. To phosphorylate GST-CDT-1 on CDK sites, the purified protein was incubated with recombinant human cdk1/cyclinB (Upstate) in phosphorylation buffer (40 mM Tris pH 7.4, 5 mM MgCl2, 1 mM DTT, 500 µM ATP, and 1 µM okadaic acid) for 90 min at 30^{o}C. ^{35}S-labeled FLAG-DDB-1 and SKPT-1-MYC proteins encoded by the pCMV-Tag2/*ddb-1* and pcDNA3.1(+)/myc-His/*skpt-1* constructs, respectively, were translated using the TNT T7/T3 Coupled Wheat Germ Extract System (Promega). ^{35}S-labeled FLAG-DDB-1 protein was further purified through affinity binding to anti-FLAG M2 agarose (Sigma) and elution with FLAG peptide (Sigma). Purified GST, GST-CDT-1 or phosphorylated GST-CDT-1 proteins were incubated with ^{35}S-labeled FLAG-DDB-1 or SKPT-1-MYC for 30 min on ice. Glutathione Sepharose 4 Fast Flow beads were added and incubated for an additional 30 min with rotation. After washing, proteins associated with the beads were analyzed with SDS-PAGE followed by autoradiography. 10% of the ^{35}S-labeled

FLAG-DDB-1 or SKPT-1-MYC proteins that were used for each binding reaction were loaded in input lanes.

ACKNOWLEDGEMENTS

We thank S. Mitani and the National Bioresource Project for the Experimental Animal Nematode *C. elegans* for providing the *ddb-1*(*tm1769*); the *C. elegans* Gene Knockout Consortium for providing the *cul-4*(*gk434*) allele; A. Fire for providing plasmid vectors; the *Caenorhabditis* Genetics Center for providing strains. This work was supported by grant R01 GM055297 from the National Institute of General Medical Sciences (NIH). Part of the research presented in this chapter has been published (Kim and Kipreos, 2007a; Kim and Kipreos, 2007b). The published figures and texts are used in this chapter with permissions of Molecular and Cellular Biology, and Cell Division.

Figure 2.1. Deletion alleles of the *ddb-1, cul-4* and *skpt-1* genes and two-hybrid interaction between DDB-1 and CUL-4.

A-C, Line drawings of the *ddb-1*, *cul-4*, and *skpt-1* genomic regions on chromosome IV, II, and III, respectively. Exons are represented as boxes and shaded areas represent coding regions. Arrows indicate the start point and direction of translation. Broken boxes with diagonal shading indicate the nearest exon of the closest upstream gene. Regions deleted in mutant alleles are represented below the gene as the missing regions bounded by dashed lines. **A,** The *ddb-1(tm1769)* allele is a 540 bp deletion that removes 177 bp of the promoter region and the complete exons 1 and 2. **B,** The *cul-4(gk434)* allele is a 580 bp deletion that removes 305 base pairs of the promoter region and the complete exons 1 and 2. **C,** The *skpt-1(ok851)* allele is a 698 bp deletion that removes the last 41 bp of exon 1, the complete exons 2 and 3, and the first 117 bp of exon 4. The scale bar is 1 kilobase. **D,** DDB-1 specifically binds to CUL-4. The interactions between DDB-1 and the six *C. elegans* cullins (CUL-1 through CUL-6) were examined in the two-hybrid system using the yeast strain pJ69-4A, which enables selection for growth on histidine- or adenine-deficient media. DDB-1 was fused to the GAL4 activation domain and the cullins were fused to the GAL4 DNA binding domain.

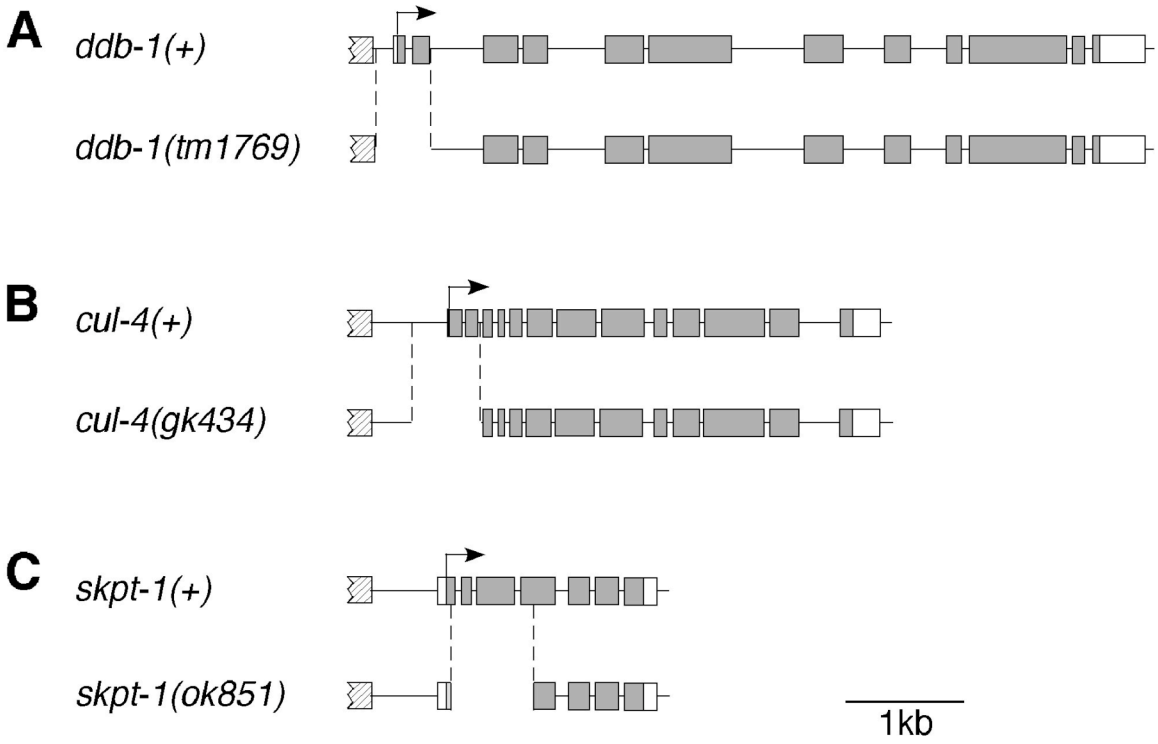

A
ddb-1(+)
ddb-1(tm1769)
B
cul-4(+)
cul-4(gk434)
C
skpt-1(+)
skpt-1(ok851)
1kb
D
DDB-1+
CUL-6
CUL-1
CUL-5
CUL-2
CUL-4
CUL-3
L- W-
L- W- H-
L- W- A-

Figure 2. 2. Developmental expression of *ddb-1.*

For all panels (**A-H**), DIC image is on top and GFP epifluorescence image of DDB-1::GFP transgene is below. **A,** The lateral hypodermis of an L2-stage larva showing seam cells and hyp7 syncytial hypodermal cells. **B,** L1-stage larva showing ventral P blast cells expressing DDB-1::GFP. **C,** L3-stage larva showing P-lineage VPC descendents expressing DDB-1::GFP. **D,** L4-stage larva showing expression in vulval cells after the completion of VPC divisions. **E-H,** Adults demonstrating expression of DDB-1::GFP in: spermathecal cells (**E**); rectal gland and rectal epithelial cells (**F**); neuronal cells in the head region (**G**); and intestinal cells (**H**). Se, seam cells; H, hyp7 syncytial cells; P, P blast cells; VPC, vulval precursor cells; Sp, spermathecal cells; RG, rectal gland cells; RE, rectal epithelial cell; and I, intestinal cells. Scale bar is 10 μm.

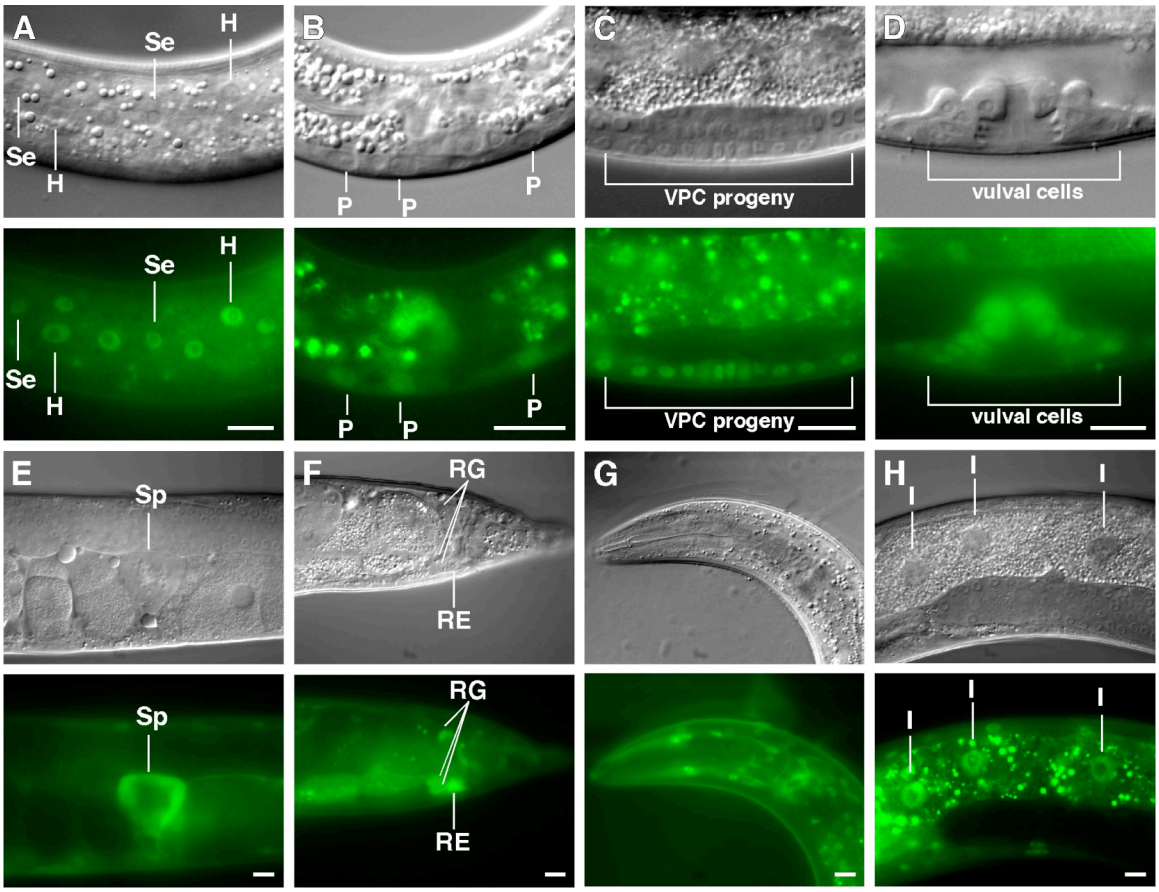

A
Se
H
Se
H
B
P
P
P
C
VPC progeny
D
vulval cells
Se
H
Se
H
P
P
P
VPC progeny
vulval cells
E
Sp
F
RG
RE
G
H
I
I
I
Sp
RG
RE
I
I
I

Figure 2.3. Loss of DDB-1 is associated with enlarged blast cells with excessive DNA content.

A, DIC image of lateral hypodermis of wild-type L2-stage larvae, and *ddb-1(tm1769)* arrested L2-stage larvae (3 days post-hatch). Se, seam cell nuclei; H, hyp7 cell nuclei. **B,** Seam cell nuclei of wild-type L2-stage larvae, and *ddb-1(tm1769)* arrested L2-stage larvae (3 days post-hatch), stained with DAPI (blue) and anti-CDT-1 (green). Anti-AJM-1 staining (red), which highlights gap junctions (Koppen et al., 2001), was overlaid to mark seam cell boundaries.

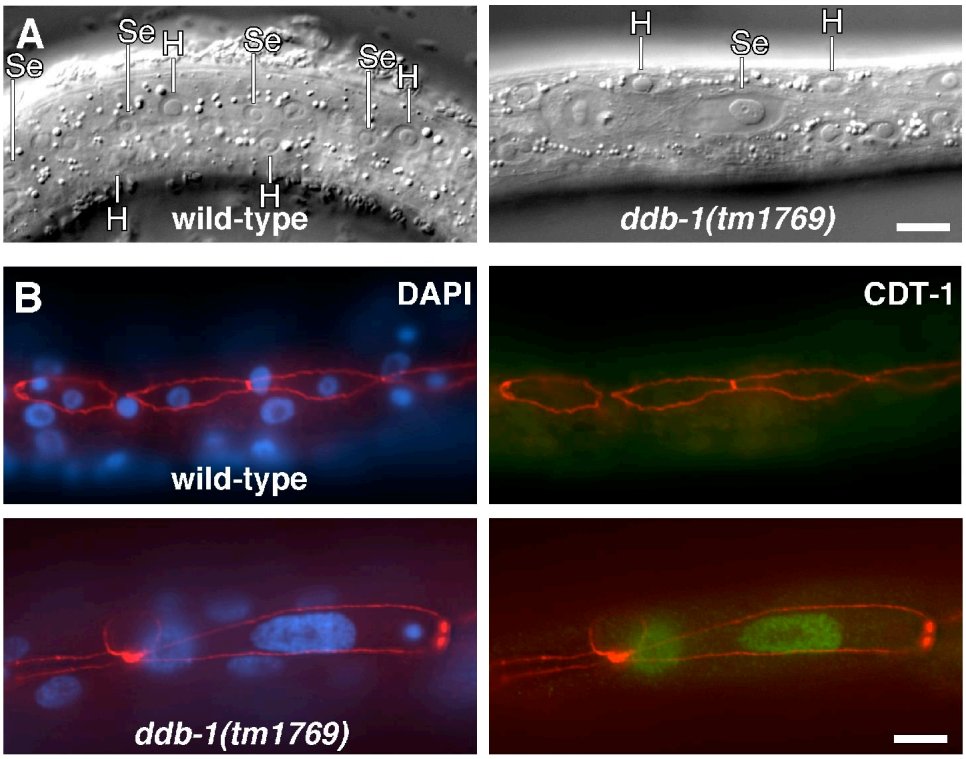

A
Se
Se
H
Se
Se
H
H
H
wild-type
H
Se
H
ddb-1(tm1769)
B
DAPI
wild-type
CDT-1
ddb-1(tm1769)

Figure 2.4. Loss of zygotically-expressed *ddb-1* causes defects in late developmental stages.

A-D, DIC images of vulval region in wild-type (**A**) and *ddb-1(tm1769)* (**B**) mid-to-late L4-stage larvae, and wild type (**C**) and *ddb-1(tm1769)* (**D**) adults. White arrowheads in B indicate enlarged vulval cells in *ddb-1(tm1769)* vulva. **E-H,** DAPI staining of wild-type (**E**) and *ddb-1(tm1769)* mutant (**F**) young adults, mid-to-posterior-region shown. The vulval regions encompassed in the white boxes in **G** and **H** are magnified in **E** and **F**, respectively. Scale bars, 10 μm.

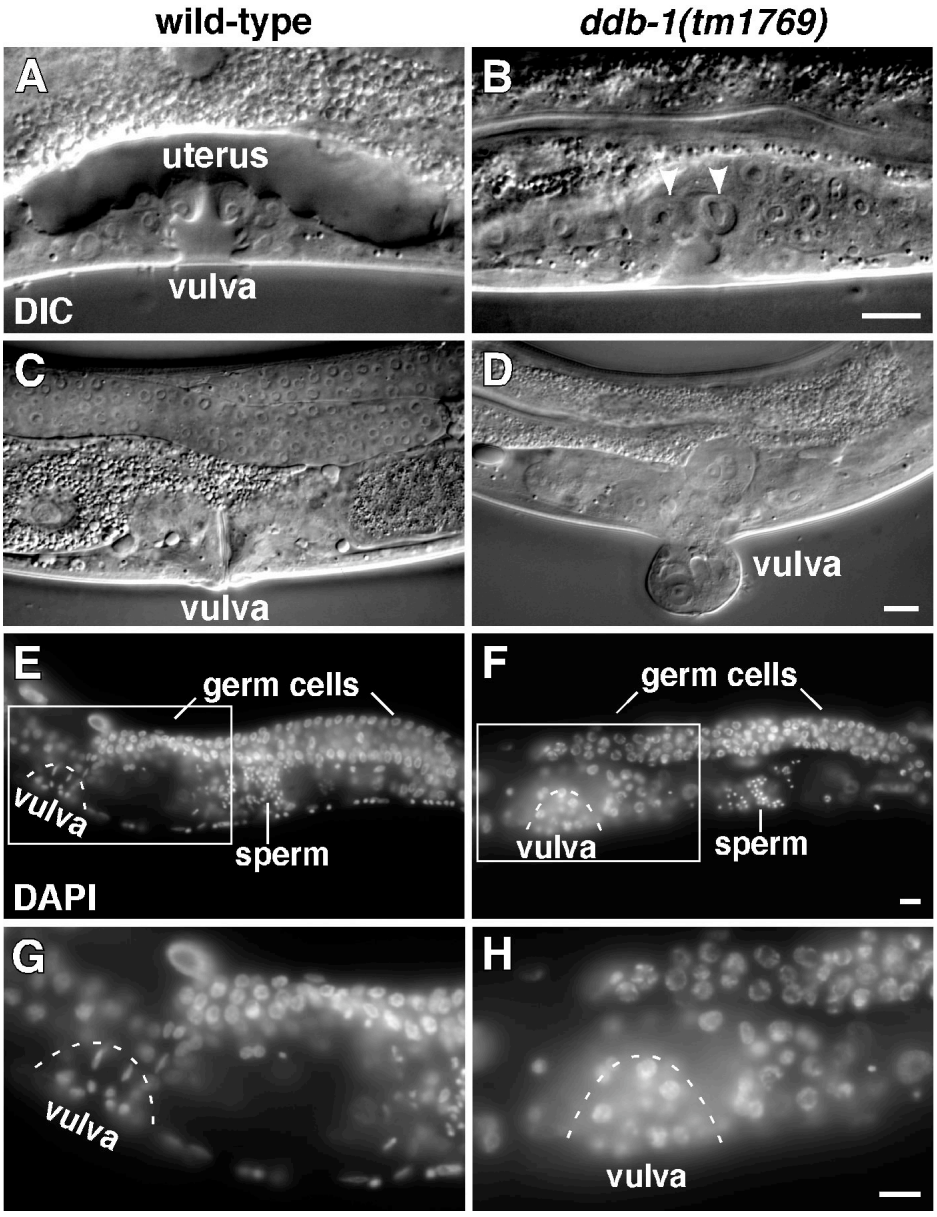

wild-type
ddb-1(tm1769)
A
uterus
vulva
DIC
B
C
vulva
D
vulva
E
germ cells
vulva
sperm
DAPI
F
germ cells
vulva
sperm
G
vulva
H
vulva

Figure 2.5. DDB-1 is required for CDT-1 degradation in S phase.

Wild-type, *ddb-1(RNAi)*, and *skpt-1(ok851)* larvae in G1 phase (90 min post-hatch) and S phase (180 min post-hatch), stained with anti-CDT-1 (green), DAPI (blue) and anti-AJM-1 (red overlay, highlighting seam cell boundaries). Note that CDT-1 is not present in S phase wild-type or *skpt-1(ok851)* homozygote seam cells, while CDT-1 remains in S phase *ddb-1(RNAi)* seam cells. Scale bar is 10 μm.

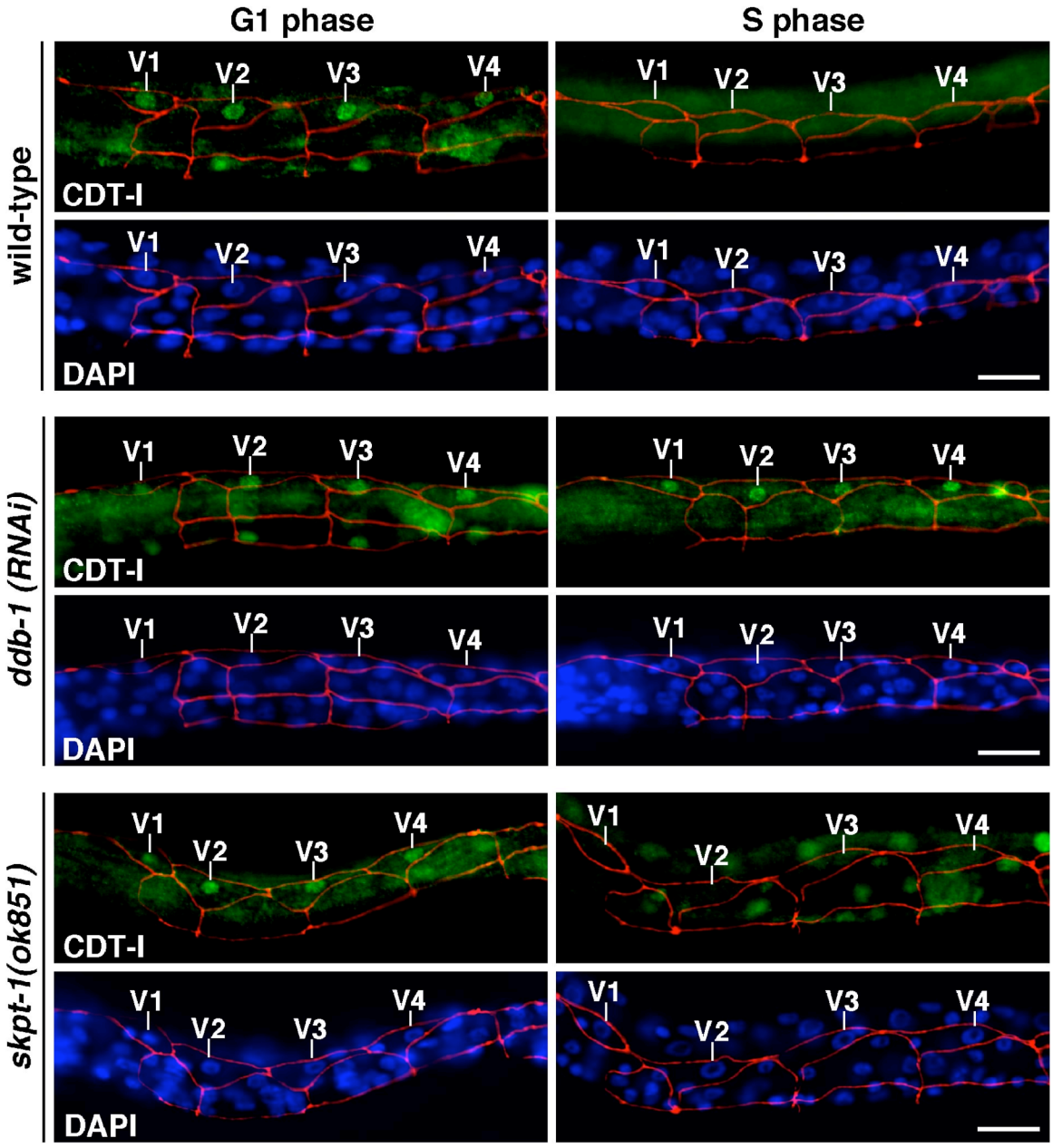

G1 phase
S phase
wild-type
ddb-1 (RNAi)
skpt-1(ok851)
V1
V2
V3
V4
CDT-I
DAPI

Figure 2.6. PIP box of CDT-1 is required for degradaton in S phase.

A, Representation of wild type and mutant CDT-1 proteins that were ectopically expressed as GFP fusions. The top panel is a diagram of wild-type CDT-1 with PIP box (green), cyclin-binding motif (red) and potential CDK phosphorylation sites (lines on top). In humans, cyclin-binding motif and CDK phosphorylation sites are required for recognition by SCF^{Skp2} (Liu et al., 2004; Sugimoto et al., 2004). The lower diagram is CDT-$1^{PIP(6A)}$, a mutant protein in which six conserved residues of the PIP box have been mutated to alanine (sequence below diagrams). **B-D**, Expression of CDT-1^{WT}::GFP with *ddb-1* RNAi treatment (B) or with no treatment (C), and CDT-$1^{PIP(6A)}$::GFP with no RNAi treatment (D). CDT-1::GFP epifluorescence image is on top and *Prnr-1*::RFP epifluorescence image is below. G1 phase seam cells (90 min post-hatch) are shown on the left, and S phase seam cells (180 min post-hatch) are shown on the right. Note that CDT-1^{WT}::GFP is absent from S phase seam cells, while CDT-$1^{PIP(6A)}$::GFP is present in S phase seam cells, similar to CDT-1^{WT}::GFP in *ddb-1(RNAi)* larvae. Seam cells are labeled. Scale bar is 10 μm.

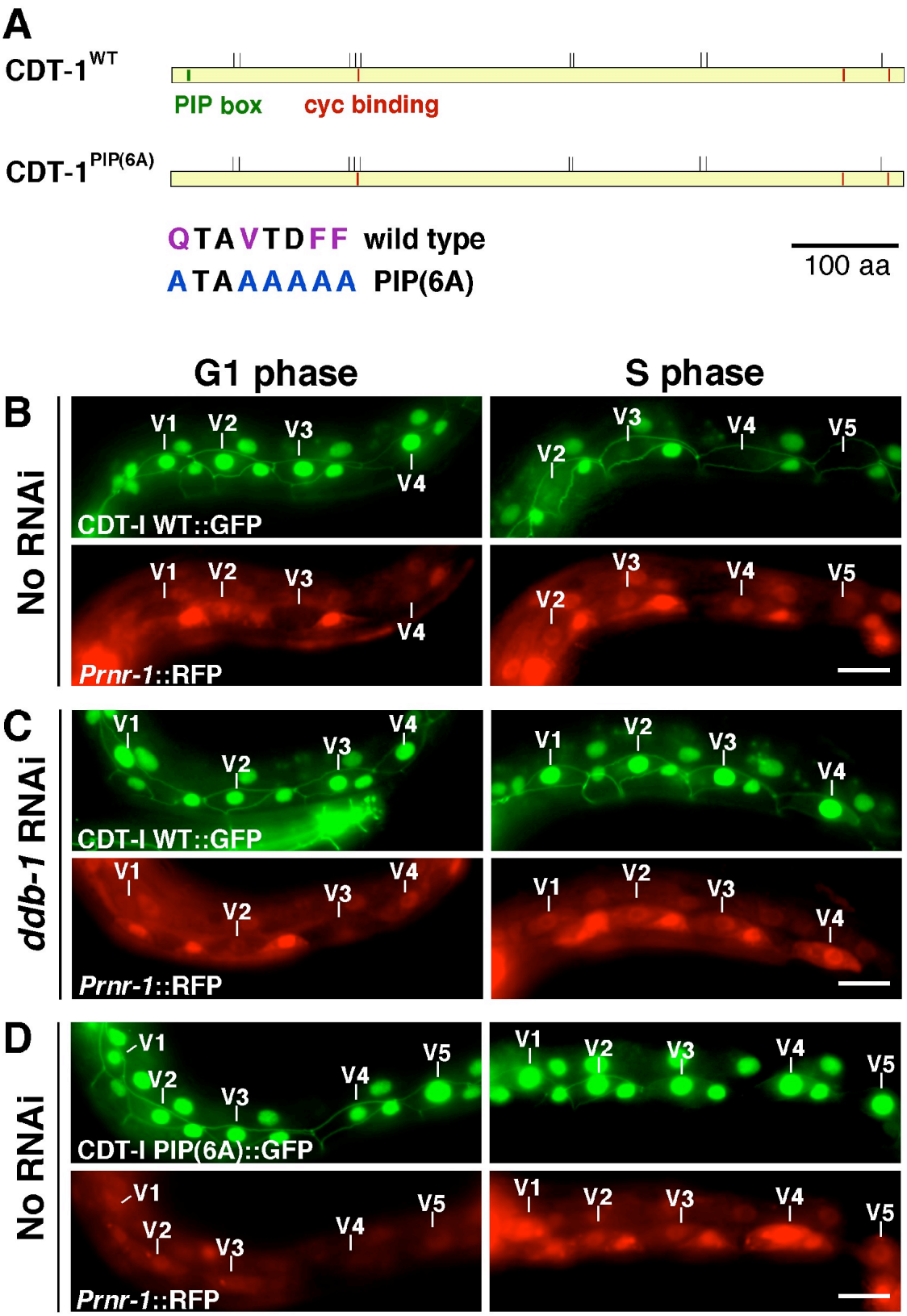
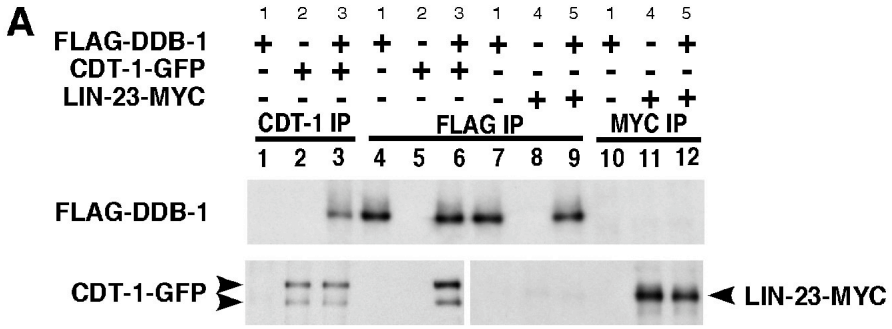

A
CDT-1WT
PIP box
cyc binding
CDT-1PIP(6A)
QTAVTDFF wild type
ATAAAAAA PIP(6A)
100 aa
G1 phase
S phase
B
No RNAi
V1
V2
V3
V4
V5
CDT-I WT::GFP
Prnr-1::RFP
C
ddb-1 RNAi
CDT-I WT::GFP
Prnr-1::RFP
D
No RNAi
CDT-I PIP(6A)::GFP
Prnr-1::RFP

Figure 2.7. DDB-1 physical interaction with CDT-1; coIP in mammalian cells. **A,** DDB-1 binding to CDT-1. FLAG-DDB-1, CDT-1-GFP and LIN-23-MYC were expressed in 293T cells as noted by plus symbols (+) above the lanes. Numbers on top indicate the combinations of expression/co-expression. Anti-CDT-1 immunoprecipitation (IP), anti-FLAG IP, or anti-MYC IP were analyzed by Western blot with anti-FLAG antibody (top panel), anti-CDT-1 antibody (bottom left panel) or anti-MYC antibody (bottom right panel). CDT-1-GFP expressed in 293T cells exhibited slow (120 kDa) and fast (100 kDa) migrating bands in SDS-PAGE, similar to what is observed for ectopic expression of human Cdt1 in 293T cells (Hu et al., 2004). **B,** SKPT-1-MYC binding to CDT-1 in 293T cells was examined similar to (**A**) with the IPs performed with anti-CDT-1 or monoclonal anti-MYC antibodies. Rabbit polyclonal anti-MYC antibody was used to detect SKPT-1-MYC in the MYC IP (lane 6-10, bottom panel) rather than the monoclonal anti-MYC antibody that is used in lanes 1-5, because SKPT-1-MYC runs at the same position as the mouse IgG heavy chain.


A
1 2 3 1 2 3 1 4 5 1 4 5
FLAG-DDB-1 + - + + - + + - + + - +
CDT-1-GFP - + + - + + - - - - - -
LIN-23-MYC - - - - - - - + + - + +
CDT-1 IP
FLAG IP
MYC IP
1 2 3 4 5 6 7 8 9 10 11 12
FLAG-DDB-1
CDT-1-GFP
LIN-23-MYC

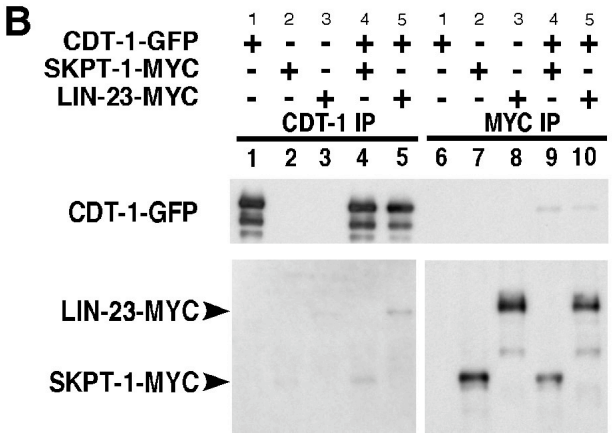
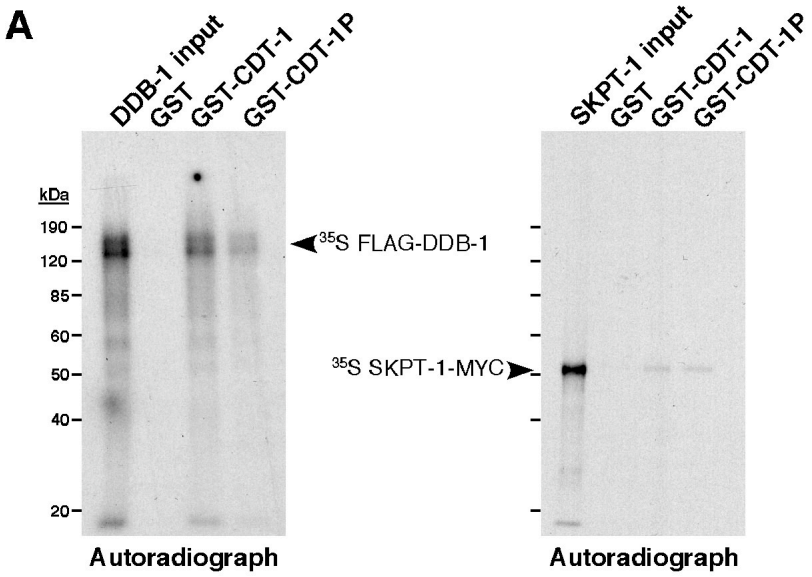

B
1 2 3 4 5 1 2 3 4 5
CDT-1-GFP + - - + + + - - + +
SKPT-1-MYC - + - + - - + - + -
LIN-23-MYC - - + - + - - + - +
CDT-1 IP
MYC IP
1 2 3 4 5 6 7 8 9 10
CDT-1-GFP
LIN-23-MYC
SKPT-1-MYC

Figure 2.8. DDB-1 physical interaction with CDT-1; DDB-1 and SKPT-1 binding to CDT-1 *in vitro*. ^{35}S-labeled FLAG-DDB-1 and SKPT-1-MYC were translated using a wheat germ extract system. DDB-1 was subsequently affinity purified using anti-FLAG antibody. **A,** Autoradiographs showing *in vitro* translated ^{35}S-labeled FLAG-DDB-1 or SKPT-1-MYC bound by GST, GST-CDT-1, or CDK-phosphorylated GST-CDT-1 (GST-CDT-1P). Input lanes are 10% of the *in vitro* translated protein that was added to each GST binding reaction. **B,** A Coomassie-stained gel of the GST, GST-CDT-1, and phosphorylated GST-CDT proteins that were used in the binding assay. On the right is a graph of the percentage of total input of ^{35}S-labeled FLAG-DDB-1 and MYC-SKPT-1 bound by GST-CDT-1 or GST-CDT-1P.


A
DDB-1 input
GST
GST-CDT-1
GST-CDT-1P
kDa
190
120
85
60
50
40
20
35S FLAG-DDB-1
35S SKPT-1-MYC
Autoradiograph
SKPT-1 input
GST
GST-CDT-1
GST-CDT-1P
Autoradiograph

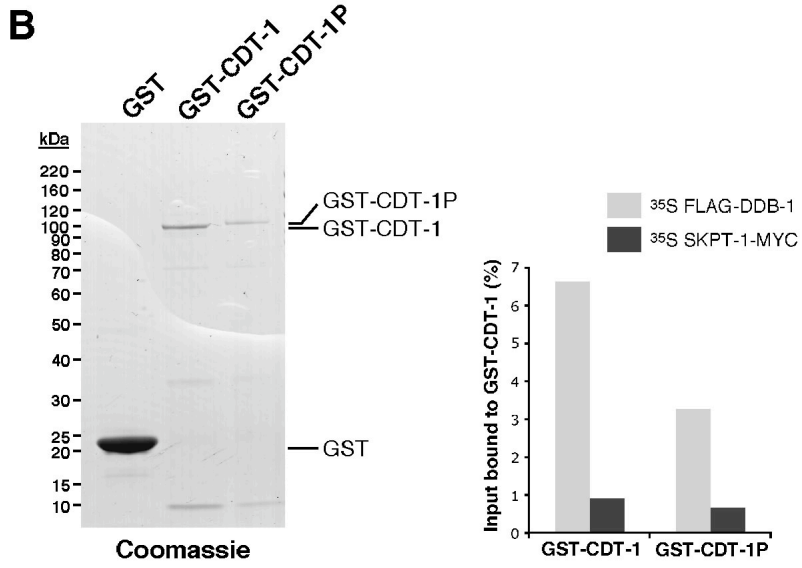
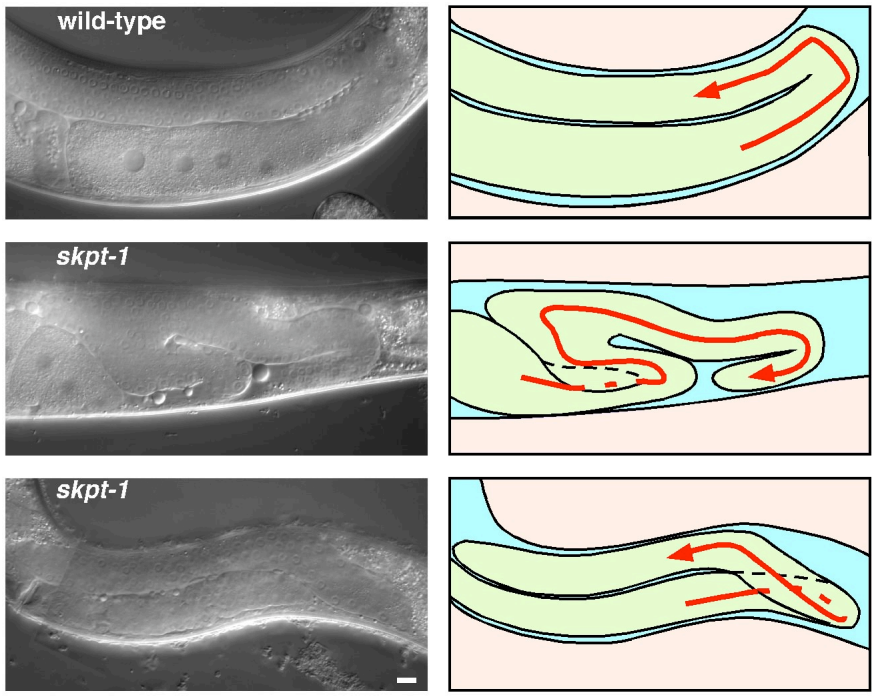

B
GST
GST-CDT-1
GST-CDT-1P
kDa
220
160
120
100
90
80
70
60
50
40
30
25
20
15
10
GST-CDT-1P
GST-CDT-1
GST
Coomassie
35S FLAG-DDB-1
35S SKPT-1-MYC
Input bound to GST-CDT-1 (%)
7
6
5
4
3
2
1
0
GST-CDT-1
GST-CDT-1P

Figure 2.9. *skpt-1* mutant phenotype and effect on CDT-1 degradation.

A, Gonad migration in wild type and *skpt-1(ok851)* mutants. DIC images (on left) of posterior gonads in wild-type (top) and *skpt-1* mutant adults. The second *skpt-1* mutant DIC image is a composite of two images of the same animal. Diagrams (on right) outline gonad migration in the animals represented in the DIC images. Dashed lines represent gonad outlines that are below the focal plane of the image. The red lines with arrows indicate the trajectory of gonad migration. **B,** Graph of the average number of V1-V6 seam cells with CDT-1 expression at specific times post-hatch for wild type (green squares) and *skpt-1(ok851)* mutants (red triangles).

A


wild-type
skpt-1
skpt-1

B

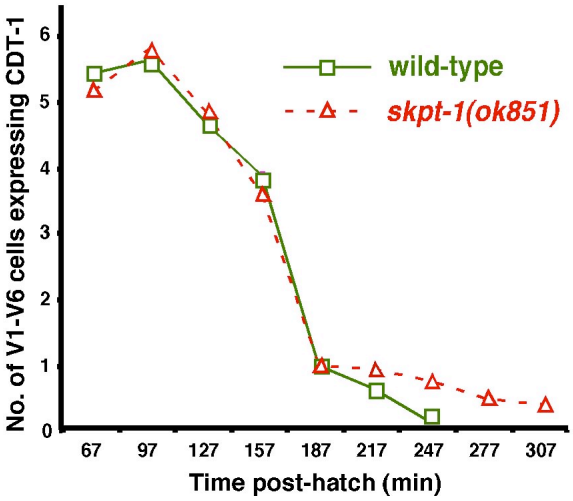
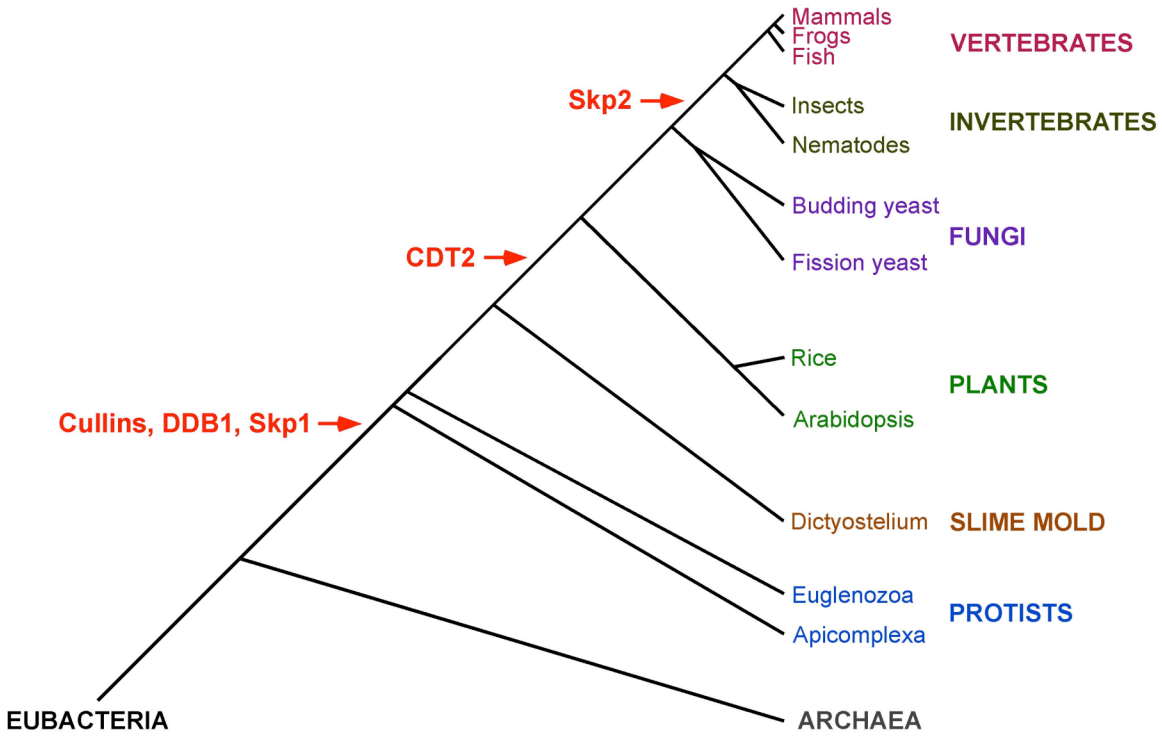

No. of V1-V6 cells expressing CDT-1
0
1
2
3
4
5
6
67
97
127
157
187
217
247
277
307
Time post-hatch (min)
wild-type
skpt-1(ok851)

Figure 2.10. The genesis of CUL4-DDB1^{CDT2} and SCF^{Skp2} E3 components. CUL4-DDB^{CDT2} and SCF^{Skp2} complex components were examined in representative organisms of diverse phyla (Table 2.1). A phylogenetic tree of the taxa analyzed, from eubacteria to mammals, is presented. Note that distances between branches are not to scale. Species and major classifications are color-coordinated, and the temporal locations of the presumed origins of E3 component genes are in red. CUL1-like and CUL4-like cullins, as well as their adaptor proteins DDB1 and Skp1, respectively, appear to have arisen early in eukaryotes, as they are absent from archaea and bacteria but are found in the eukaryotes examined. CDT2, the SRS for a CUL4-DDB1 E3 complex, appears to have arisen prior to the genesis of green plants. Skp2, the SRS for a CUL1 E3 complex, appears to have arisen after the genesis of fungi but prior to the genesis of metazoa. The branching order is based on a phylogenetic analysis using rRNA (Philippe and Germot, 2000). Note that other phylogenies, based on protein sequences, reverse the order of plants and slime molds (Baldauf et al., 2000). Combining our genomic data with this alternative branching of phyla (not shown) would imply that CDT2 was created prior to plants in the main eukaryotic lineage but then lost within the slime mold lineage.


Mammals
Frogs
Fish
VERTEBRATES
Skp2
Insects
INVERTEBRATES
Nematodes
Budding yeast
FUNGI
CDT2
Fission yeast
Rice
PLANTS
Cullins, DDB1, Skp1
Arabidopsis
Dictyostelium
SLIME MOLD
Euglenozoa
PROTISTS
Apicomplexa
EUBACTERIA
ARCHAEA

CHAPTER III

DTL/CDT2 FUNCTIONS AS THE SUBSTRATE RECOGNITION SUBUNIT IN A CUL-4/DDB-1 COMPLEX THAT PREVENTS RE-REPLICATION

BACKGROUND

Mechanisms to prevent re-replication

The faithful duplication of the genome is one of the principle missions of cell cycle regulation. Deregulation of DNA replication can cause genomic instability. Metazoan genomes have thousands of DNA replication origins (Bielinsky, 2003), and each of the origins are tightly controlled so that origin firing occurs only once per cell cycle. The mechanisms to prevent re-replication mainly focus on preventing replication origins from being reused in a single cell cycle (Blow and Dutta, 2005; Machida et al., 2005). As described in Chapter I, the origin recognition complex (ORC) complex, the DNA licensing factors Cdt1 and Cdc6, and the putative helicase Mcm2-7 complex are sequentially recruited to the replication origins to form active pre-replication complex during late M to G1 phases. In humans, it has been proposed that cyclin E facilitates Mcm2-7 loading in a CDK-independent fashion, through physical interaction with Cdt1 and Mcm2-7 (Geng et al., 2007). Eukaryotes employ redundant mechanisms to ensure that DNA replication occurs once per cell cycle and the members of pre-replication complex are the major targets of those regulations (Feng and Kipreos, 2003). In humans, three regulatory mechanisms act on pre-replication

complexes to prevent re-replication during S phase. First, the Orc1 subunit of the ORC complex is degraded through the SCF^{Skp2} and an unknown E3 ligase in S phase (Mendez et al., 2002). Second, the Cdc6 is exported from the nucleus in response to cyclin/CDK-mediated phosphorylation (Bell and Dutta, 2002; Nishitani and Lygerou, 2002). Finally, Cdt1 undergoes ubiquitin mediated proteolysis in S phase, which is mediated by the two E3 ligases, $CUL4/DDB1^{CDT2}$ and SCF^{Skp2} (Nishitani et al., 2006), or is inactivated by geminin (Kulartz and Knippers, 2004; Melixetian et al., 2004; Zhu et al., 2004).

Inactivation of CUL-4/DDB-1 causes a massive re-replication

Inactivation of CUL-4 or DDB-1 produces dramatic levels of re-replication that are associated with a failure to degrade CDT-1 ((Zhong et al., 2003) and chapter II). In *cul-4* or *ddb-1* RNAi seam cells, DNA level is increased up to *100C* in three-day post-hatch, compared to the *2C* of wild type cells. It is quite surprising as a single or combinational inactivation of mechanisms to prevent re-replication did produce only a limited extent of re-replication in yeast and other metazoa (Feng and Kipreos, 2003). The *cul-4* or *ddb-1* inactivated cells fail to degrade the DNA licensing factor CDT-1. The removal of one copy of *cdt-1* gene in the *cul-4* RNAi animals significantly abolishes re-replication, suggesting that the increased level of CDT-1 in *cul-4* or *ddb-1* inactivated cells is a causative agent to generate re-replication. In fission yeast and humans, however, overexpressing Cdt1 proteins do not induce re-replication and only modest re-replication in a subset of cells, respectively (Gopalakrishnan et al., 2001; Vaziri et

al., 2003; Yanow et al., 2001). Given the limited effects of greatly overexpressing Cdt1 in other organisms, it is likely that the substantial re-replication in *cul-4* or *ddb-1* mutants is associated with the loss of multiple mechanisms to prevent re-replication.

The role of CDT2 in the CUL-4/DDB-1 complex

It has been shown that in yeast, *Drosophila, Xenopus, Zebrafish* and humans, a 'WDXR' motif protein DTL/CDT2 (Denticleless/Cdc10 dependent transcript 2) is required to target Cdt1 for degradation in the context of CUL4/DDB1 complex (Higa et al., 2006a; Jin et al., 2006; Ralph et al., 2006; Sansam et al., 2006). Jin et al. found that CDT2 interacts with DDB1 and CUL4A following their cotransfection in human embryonic kidney cells (Jin et al., 2006). Endogenous DDB1 also interacts with endogenous CDT2. It has been shown that the 'WDXR' motif in CDT2 is required for DDB1 binding. In *Xenopus* egg extracts and human cells, CDT2 functions to degrade Cdt1 in S phase and after DNA damage (Higa et al., 2006a; Jin et al., 2006). In Zebrafish, depletion of CDT2 causes re-replication and checkpoint activation (Sansam et al., 2006). In *Xenopus* egg extract, it has been shown that CUL4/DDB1^{CDT2} complex is recruited to replication fork in response to the interaction between Cdt1 and PCNA (Proliferating Cell Nuclear Antigen) (Arias and Walter, 2006; Jin et al., 2006). Cdt1 binds PCNA through a PCNA-interacting protein (PIP) box motif in the Cdt1 N-terminus. PCNA forms a trimeric ring structure that is loaded onto DNA during both DNA replication and DNA repair (Barsky and Venclovas, 2005;

Maga and Hubscher, 2003). These findings suggest that CDT2 functions as the SRS for the CUL4/DDB1 complex to target Cdt1 with help of PCNA. However, this has not yet been formally established, as there are no reports that CDT2 can bind directly to Cdt1. It remains unclear how PCNA loading to DNA leads the recruitment of CUL4/DDB1^{CDT2} complex to the replication fork.

There is also a report that in humans, CDT2 is required to degrade the p53 tumor suppressor protein (Banks et al., 2006). Inactivation of CUL4A, DDB1, CDT2 or PCNA induces p53 stabilization and growth arrest in human cells. The isolated CUL4A complexes display robust polyubiquitination activity towards p53 in a CDT2 and PCNA dependent manner, which is similar to the mechanism to degrade Cdt1. In fission yeast, Cdt2 is also required to maintain genomic stability by targeting both Cdt1 and the 124 aa protein Spd1, a potential inhibitor of the small subunit of ribonucleotide reductase complex, for degradation (Liu et al., 2005; Ralph et al., 2006).

In this work, I characterized the loss-of-function phenotypes of DDB-1 to determine whether CUL-4/DDB-1 regulates other cell cycle regulators as well as CDT-1 to prevent re-replication. I found that CUL-4/DDB-1 is required for CIP/KIP family CDK inhibitor CKI-1 and cyclin E homolog CYE-1 degradation. The CKI-1 accumulation contributes to the occurrence of the massive re-replication in *ddb-1* or *cul-4* mutants. I also demonstrate that the *C. elegans* ortholog of CDT2 is required for CKI-1 degradation but not CYE-1. The CDT2

ortholog can interact with either of DDB-1, CKI-1 or CDT-1 in yeast hybrid assays, suggesting that it functions as the SRS in CUL-4/DDB-1 complex.

RESULTS

CUL-4/DDB-1 is required for CKI-1 and CYE-1 degradation

Zhong et al. reported that in *C. elegans*, depletion of *cul-4* by RNAi causes a massive re-replication in a subset of blast cells (Zhong et al., 2003). It has been shown that the re-replication is caused by the failure of CDT-1 degradation. In other systems, however, Cdt1 overexpression produces a limited extent of re-replication. Therefore we were seeking other cell cycle regulators that are controlled by CUL-4/DDB-1. Recently, it has been reported that CUL4/DDB1 promotes the degradation of the CDK-inhibitor $p27^{KIP1}$ and cyclin E in humans and *Drosophila* (Bondar et al., 2006; Higa et al., 2006c; Li et al., 2006a). To determine whether the *C. elegans* CUL-4/DDB-1 negatively regulates the CIP/KIP-family member CKI-1 and the cyclin E ortholog CYE-1, we analyzed CKI-1 and CYE-1 levels in *ddb-1(RNAi)* arrested larvae using immunofluorescence. Staining with anti-CKI-1 and CYE-1 antibodies showed accumulation of both proteins in the enlarged seam cell nuclei of *ddb-1(RNAi)* arrested larvae (Figure 3.1A). This suggests either that the CUL-4/DDB-1 complex directly regulates CKI-1 and CYE-1, or that the elevated levels are a secondary consequence of the S phase arrest/re-replication.

We tested whether CKI-1 accumulation contributes to the re-replication. RNAi was used to deplete CKI-1 in *cul-4(gk434)*/*mIn1* hermaphrodite adults. We found that *cki-1* RNAi did not suppress the L2 stage arrest of *cul-4* mutants. However, the size of seam cell nuclei in *cki-1(RNAi) cul-4(gk434)* larvae was reduced ~3-fold relative to *cul-4(gk434)* larvae (36.2 ± 14.0 μm^2 vs. 113.5 ± 52.7 μm^2, n = 20 each, respectively; wild type is 11.8 ± 0.7 μm^2, n = 6). Seam cell DNA level was reduced ~6.7-fold (3.8 ± 1.7 C for *cki-1(RNAi) cul-4(gk434)* vs. 25.6 ± 11.3 C for *cul-4(gk434)*, n = 6 each). This indicates that *cki-1* RNAi depletion suppresses the re-replication phenotype of *cul-4* mutants (Figure 3.1B, C). Significantly, the *cki-1(RNAi) cul-4(gk434)* larvae still accumulated CDT-1 protein in seam cells (Figure 3.1C), indicating that CKI-1 is not required for the accumulation of CDT-1 and that CDT-1 accumulation is not sufficient to induce extensive re-replication. We could not perform a similar test to determine if CYE-1 is required for re-replication, because *cye-1* RNAi produces an embryonic arrest that precludes analysis of larval cells (Fay and Han, 2000).

C. elegans CDT-2 loss-of-function phenotypes

In other systems, CDT2 has been shown to function in the control of DNA replication during S phase or in response to DNA damage. To clarify the role of CDT2 in CUL4/DDB1 complex functions, we analyzed the *C. elegans* CDT2 ortholog *cdt-2*. *C. elegans* has a single CDT2 ortholog, *cdt-2*, which is located on chromosome V. CDT-2 protein has significant sequence identity with orthologous CDT2 proteins, displaying 24% amino acid identity with *H. sapiens*

CDT2, 23% for *X. tropicalis*, 26% for *D. rerio*, 25% for *D. melanogaster*, and 24% for *S. pombe* CDT2.

To determine the *cdt-2* RNAi phenotype, wild-type L4 stage larvae were fed bacteria expressing *cdt-2* dsRNA, and progeny that were laid 20 hours after the start of the RNAi treatment were analyzed. The *cdt-2(RNAi)* embryos hatch normally and develop to the adult stage. The *cdt-2* RNAi phenotype contrasts with the phenotype of *cul-4 or ddb-1 RNAi*, most of which arrest at the L2 or L3 larval stages. In the *cdt-2(RNAi)* larvae, however, enlarged seam cells are detected throughout development with more obvious enlarged cells in older animals (Figure 3.2A). The large *cdt-2(RNAi)* cells show increased DNA levels, which are shown through either Histone H2::GFP epifluorescence or staining with DAPI (Figures 3.2A and 3.1A, respectively). These data suggest that *cdt-2* is required to prevent DNA re-replication, similar to CUL-4 or DDB-1.

CDT-2 is required for CDT-1 and CKI-1 degradation

It has been demonstrated that CDT2 is required for Cdt1 degradation in various systems. To determine whether the *C. elegans* CDT2 ortholog is required for CDT-1 degradation, we analyzed CDT-1 level in *cdt-2(RNAi)* L4 larvae that express CDT-1::GFP as a translational fusion. GFP epifluorescence showed accumulation of CDT-1 in the enlarged seam cell nuclei of *cdt-2(RNAi)* larvae (Figure 3.2B). This is consistent with observations in other systems,

suggesting that CDT2 functions to prevent re-replication by degrading CDT-1 in *C. elegans*.

As described above, the CDK inhibitor CKI-1 and the cyclin E homolog CYE-1 accumulate in re-replicating *ddb-1(RNAi)* cells. We wanted to identify the relevant SRS(s) of the CUL-4/DDB-1 E3 ligase for these substrates. To determine if the *C. elegans* CDT2 ortholog is required for CKI-1 and CYE-1 degradation, we analyzed CKI-1 and CYE-1 levels in *cdt-2(RNAi)* larvae. Notably, immunofluorescence staining of seam cells with anti-CKI-1 and CYE-1 antibodies showed accumulation of CKI-1 but not CYE-1 in the seam cell nuclei of *cdt-2(RNAi)* larvae (Figure 3.1A). This suggests that *cdt-2* is required for CKI-1 degradation in addition to CDT-1 degradation.

CDT-2 directly interacts with the substrates CDT-1 and CKI-1

In humans, *Xenopus*, zebrafish and fission yeast, CDT2 has been proposed to function as the SRS of CUL4/DDB1 complex to target Cdt1, p53 and Spd1 for degradation. These inferences are based on genetic evidence that CDT2 is required for the degradation of the substrates and the observation that CDT2 co-immunoprecipitates with CUL4/DDB1 and/or the substrates. However, the exact role of CDT2 in targeting these substrates (in the context of the CUL4/DDB1 complex) has not been completely elucidated. In particular, there is currently no evidence of direct interaction between CDT2 and the substrates. In most SCF type cullin-RING E3 ligase complexes, SRSs directly bind to the

adapter protein and to the substrate, but not to cullin nor RING H2 protein. The cullin binds to both adaptor protein and RING H2 protein but not to SRSs. Adaptor protein binds to cullin and SRSs but not to RING H2 protein.

The physical interactions between CDT-2 and CUL-4/DDB-1 complex components were analyzed through the yeast two-hybrid assay. We generated various combinations of the bait and prey to verify the proposed structural model (Figure 1.4). We found that CDT-2 positively interacts with DDB-1, CKI-1 and CDT-1, but not with CUL-4 (Figure 3.3A). CUL-4 interacts with DDB-1, but not with CDT-2 (Figure 3.3A). DDB-1 does not interact with CKI-1 or CDT-1 (Figure 3.3A and data not shown).

Further we determined whether CDT-2 can mediate an indirect interaction between DDB-1 and CKI-1 through yeast three-hybrid test, a variation of two-hybrid test. In this system, the third protein is expressed along with the bait and prey proteins that normally do not interact with each other (Licitra and Liu, 1996). The expression of the reporter gene is used to determine whether the third protein can mediate the interaction between the bait and prey proteins. To express the third protein, we used the pYES2 vector that contains GAL1 promoter that is induced in the presence of galactose but repressed in the presence of glucose as a carbon source (Giniger et al., 1985). We expressed DDB-1 and CKI-1 along with the pYES2/CDT-2 in a yeast strain. We found that this yeast grow in a galactose containing histidine selection plate but do not

survive in a glucose containing histidine selection plate, suggesting that CDT-2 can mediate the interaction between DDB-1 and CKI-1 (Figure 3.3B). We employed a similar experiment for DDB-1 and found that DDB-1 can mediate the interaction between CUL-4 and CDT-2 (Figure 3.3B). Overall, these data strongly suggest that CDT-2 functions as an SRS in CUL-4/DDB-1 E3 ligase complex.

DISCUSSION

The CIP/KIP family CKI-1 is regulated by CUL-4/DDB-1 in *C. elegans*

Mammalian and *Drosophila* CUL4 promote the degradation of cyclin E and the CKIs $p27^{KIP1}$ and Dacapo, respectively (Higa et al., 2006c; Li et al., 2006a). In $DDB1^{-/-}$ knock out mice, $p21^{CIP1}$ accumulates in epidermal cells, suggesting that CUL4/DDB1 is also involved in $p21^{CIP1}$ degradation (Cang et al., 2007). We observed that levels of both CKI-1 and cyclin E are increased in the re-replicating blast cells of *ddb-1(RNAi)* larvae. Significantly, *cki-1* RNAi depletion was able to rescue the re-replication phenotype of *cul-4* mutants, indicating that CKI-1 is essential for re-replication to occur. It is also important to note that *cki-1* RNAi did not rescue the L2-stage developmental arrest associated with inactivation of CUL-4 or DDB-1, indicating that the developmental arrest is not linked to re-replication or CKI-1 accumulation. Previously our lab showed that CKI-1 accumulation contributes to G1 arrest in *cul-2* mutant cells (Feng et al., 1999). In apparent conflict with this result, *cul-4* or *ddb-1* mutant cells do not arrest in G1 phase but instead enter S phase and undergo re-replication in the presence of

CKI-1 (Zhong et al., 2003); Figure 3.1). It is an open question why the failure to degrade CKI-1 is associated with different cell cycle outcomes in *cul-2* and *cul-4* mutant cells. It is possible that in *cul-4* or *ddb-1* mutant cells, the initial levels of CKI are not high enough to inhibit the G1-CDK/cyclin complex and prevent S phase entry, but are sufficient to block of S- and M-CDK/cyclin complexes to thereby promote DNA re-replication (Kim et al., 2007).

One report has indicated that human Skp2 can co-immunoprecipitate CUL4/DDB1, and that Skp2 is essential for CUL4/DDB1-mediated degradation of the CDK-inhibitor $p27^{KIP1}$ (Bondar et al., 2006). This work suggested that Skp2 can function as an SRS for the CUL4 complex. Our observation that CKI-1 accumulates in the enlarged seam cells of *ddb-1(RNAi)* larvae suggests that *C. elegans* CUL-4/DDB-1 negatively regulates a CKI. However, the *C. elegans* Skp2 homolog SKPT-1 and DDB-1 do not appear to interact in the 293T system beyond low background levels that are also observed between DDB-1 and the non-specific F-box protein LIN-23 (data not shown).

DTL/CDT2 functions as the SRS for CKI-1 and CDT-1 degradation in *C. elegans*

It has been shown that CDT2 is required for Cdt1 degradation in various systems. However its role in the CUL4/DDB1 complex was not clearly demonstrated. We found that the *C. elegans* CDT2 ortholog CDT-2 is required for CDT-1 and CKI-1 degradation, similar to the requirement for CUL-4 and DDB-

1. However *cdt-2* does not phenocopy *cul-4* or *ddb-1* in CYE-1 accumulation and developmental arrest. These results are consistent with CDT-2 functioning as a substrate specific factor of a Cullin-RING ligase, as SRSs exhibit only a subset of phenotypes of core complex components.

We analyzed the biochemical interactions between CUL-4/DDB-1 complex components to address whether CDT-2 functions as an SRS in the complex. CDT-2 directly binds to DDB-1 but not to CUL-4. However, CDT-2 can interact with CUL-4 in the presence of DDB-1, suggesting that all three proteins form a complex *in vivo*. We also analyzed interactions between CDT-2 and substrates. CDT-2 shows strong interactions with both CKI-1 and CDT-1, whereas DDB-1 does not show significant interactions with CKI-1 or CDT-1 in the yeast two-hybrid system. CDT-2 can mediate interaction between DDB-1 and CKI-1, suggesting that it recruits substrates to the CUL-4/DDB-1 complex through interaction with DDB-1.

The CUL-4/DDB-1^{CDT-2} complex is a master regulator of DNA replication

Eukaryotes employ multiple mechanisms to prevent re-replication (Feng and Kipreos, 2003). It has been shown that deregulating a single pathway that prevents re-replication is not sufficient to induce significant re-replication in either yeast or metazoa. In *C. elegans*, inactivation of *cul-4* or *ddb-1* causes massive re-replication with up to 50-fold increases in DNA levels, suggesting that CUL-4/DDB-1 is required for multiple pathways to prevent re-replication. In this study,

we showed that CUL-4/DDB-1 is required for both CDT-1 and CKI-1 degradation. It is well understood that deregulation of the DNA licensing factor CDT-1 contributes to re-replication, but how does the CDK-inhibitor CKI-1 impact the control of DNA replication?

Recently Kim et al. extended our findings on the regulation of CKI-1 by CUL-4/DDB-1 by showing how a failure to degrade CKI-1 promotes re-replication (Kim et al., 2007). In *C. elegans*, another DNA licensing factor, CDC-6, is exported from the nucleus during S phase in response to the phosphorylation of multiple CDK sites, which is presumably mediated by a CDK/cyclin complex. In *cul-4* mutant cells, CDC-6 fails to be phosphorylated and is exported from the nucleus during S phase. *cki-1(RNAi)* restores the phosphorylation and export of CDC-6 in *cul-4* mutants during S phase, suggesting that CUL-4 promotes CDC-6 nuclear export by negatively regulating CKI-1 levels. These results indicate that CKI-1 is essential for the prevention of CDC-6 phosphorylation in *cul-4* mutants. A model for how CKI-1 controls DNA replication can therefore be proposed (Figure 3.4). During G1 phase, CKI-1 activity allows CDC-6 to form pre-replication complex by restraining CDK-cyclin activity. As cells enter S phase, CUL-4 complex targets CKI-1 for degradation and then CDK-cyclin is released from the inhibitor and induces phosphorylation and translocation of CDC-6.

Currently we have not determined whether CKI-1 degradation by CUL-4/DDB-1^{CDT-2} occurs specifically during S phase. However it is interesting that

CKI-1 has at least one conserved PCNA-interaction motif, suggesting that PCNA loading onto chromatin during S phase can induce CKI-1 degradation similar to what has been observed for CDT-1 degradation (Arias and Walter, 2006). In this study, we showed that CUL-4/DDB-1^{CDT-2} regulates two important pathways to prevent DNA re-replication. We therefore propose that CUL-4/DDB-1^{CDT-2} E3 ligase is a master regulator that functions on multiple targets to prevent DNA re-replication.

MATERIALS AND METHODS

Strains and RNAi

The following *C. elegans* strains were used: N2, wild type; ET263, *ddb-1(tm1769)/dpy-20(e2017)*; VC1033, *cul-4(gk434)/mIn1* [*mIs14 dpy-10(e128)*]. *cdt-2* RNAi was performed by providing bacteria expressing *cdt-2* dsRNA to L4-stage larvae as a food source, as described (Kamath et al., 2001). The *cdt-2* feeding protocol employed *E. coli* strain HT115 containing plasmid pPD129.36/*cdt-2*, which contains a full-length *cdt-2* cDNA cloned into a multi-cloning site situated between double T7 primers. *cki-1* RNAi was performed by injection of dsRNA created with the MegaScript T7 and T3 kits (Ambion) using the cDNA clone yk490e9 as the template. Complementary single strand RNAs were annealed to create dsRNA and injected into adult hermaphrodites at a concentration of 0.5 – 1.0 mg/ml as described (Feng et al., 1999). *ddb-1* RNAi was performed using a modification of a described method (Chapter II). To

obtain *ddb-1(RNAi)* L4-stage larvae, wild-type L1 stage larvae were exposed to *ddb-1* RNAi feeding. These *ddb-1(RNAi)* larvae developed to the adult stage.

Two- and three-hybrid assay

To perform two-hybrid analysis among CUL-4 complex components and substrates, the full-length *cul-4, ddb-1, cdt-2, cki-1 and cdt-2* genes were cloned both in the pACTII (activation domain) and the pAS2 (DNA binding domain) vector (Clontech). Various combinations of the bait and prey were transformed into the *S. cerevisiae* strain pJ69-4A as described (James et al., 1996; Janssen, 1995). Interaction in the two-hybrid system was tested by growth on histidine-deficient selective media. For three-hybrid tests, *cdt-2* and *ddb-1* were cloned into the pYES2 vector (Invitrogen), which has the GAL1 promoter. The bait, prey and pYES2 constructs were transformed into the *S. cerevisiae* strain pJ69-4A with a same method as in the two hybrid assay. Interaction in the three-hybrid systems was tested by growth on histidine-deficient selective media in the presence of glucose or galactose.

Immunofluorescence

Affinity purified polyclonal anti-CKI-1 and monoclonal anti-CYE-1 (17C8) antibodies were as described (Brodigan et al., 2003; Feng et al., 1999; Zhong et al., 2003). Anti- CDT-1, anti-AJM-1 (MH27), secondary antibodies, and DNA staining were performed as described in Chapter II.

Microscopy

Animals were observed by differential interference contrast (DIC) and immunofluorescence microscopy. The processing microscopic images and DNA quantitation of DNA contents were as described in Chapter II.

ACKNOWLEDGEMENTS

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Figure 3.1. CKI-1 and CYE-1 accumulates in the re-replicating cells.

A, Seam cell nuclei of wild type, *ddb-1(RNAi)*, and *cdt-2(RNAi)* L4-stage larvae, stained with DAPI (blue), anti-CKI-1 (green), and anti-CYE-1 (red). **B,** DIC image of lateral hypodermis of *cul-4(gk434),* and *cul-4(gk434);cki-1(RNAi)* arrested L2-stage larvae (3 days post-hatch). Se, seam cell nuclei; H, hyp7 cell nuclei. **C,** Seam cell nuclei of *cul-4(gk434)* and *cul-4(gk434);cki-1(RNAi)* arrested L2-stage larvae (3 days post-hatch), stained with DAPI (blue) and anti-CDT-1 (green). Anti-AJM-1 staining (red), which highlights gap junctions (Koppen et al., 2001), was overlaid to mark seam cell boundaries. Scale bar is 10 μm.

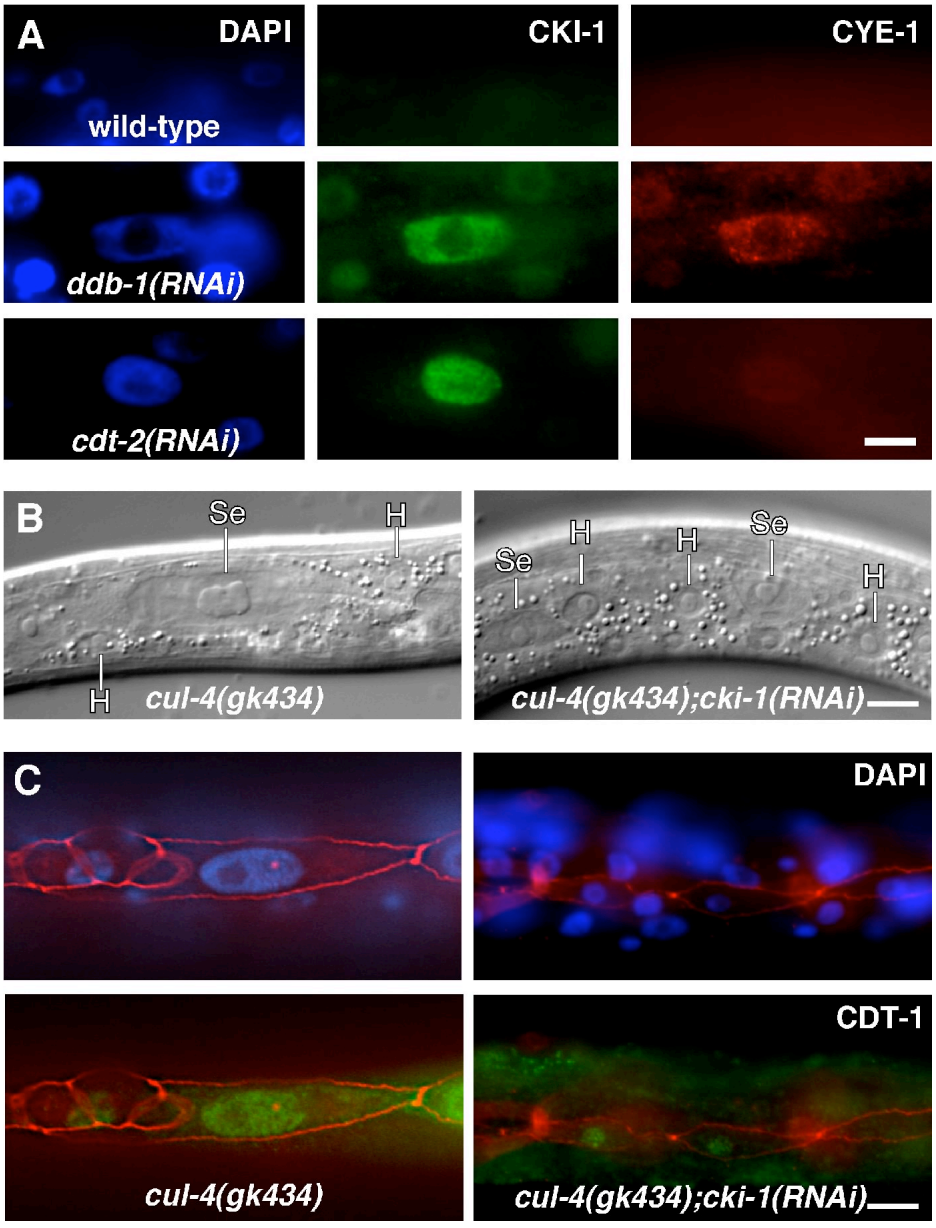

A
DAPI
CKI-1
CYE-1
wild-type
ddb-1(RNAi)
cdt-2(RNAi)
B
Se
H
H
cul-4(gk434)
Se
H
H
Se
H
cul-4(gk434);cki-1(RNAi)
C
DAPI
cul-4(gk434)
CDT-1
cul-4(gk434);cki-1(RNAi)

Figure 3.2. Loss of *cdt-2* is associated with re-replication.

A, Seam cell nuclei of wild type and *cdt-2(RNAi)* late L4-stage larvae expressing Histone H1::GFP. Upper panels are DIC images of seam cells, lower panels are GFP epifluorescence images. **B,** *cdt-2(RNAi)* late L4-stage larvae expressing CDT-1::GFP. Upper panels are DIC images of seam cells, lower panels are GFP epifluorescence images. Scale bar is 10 μm.

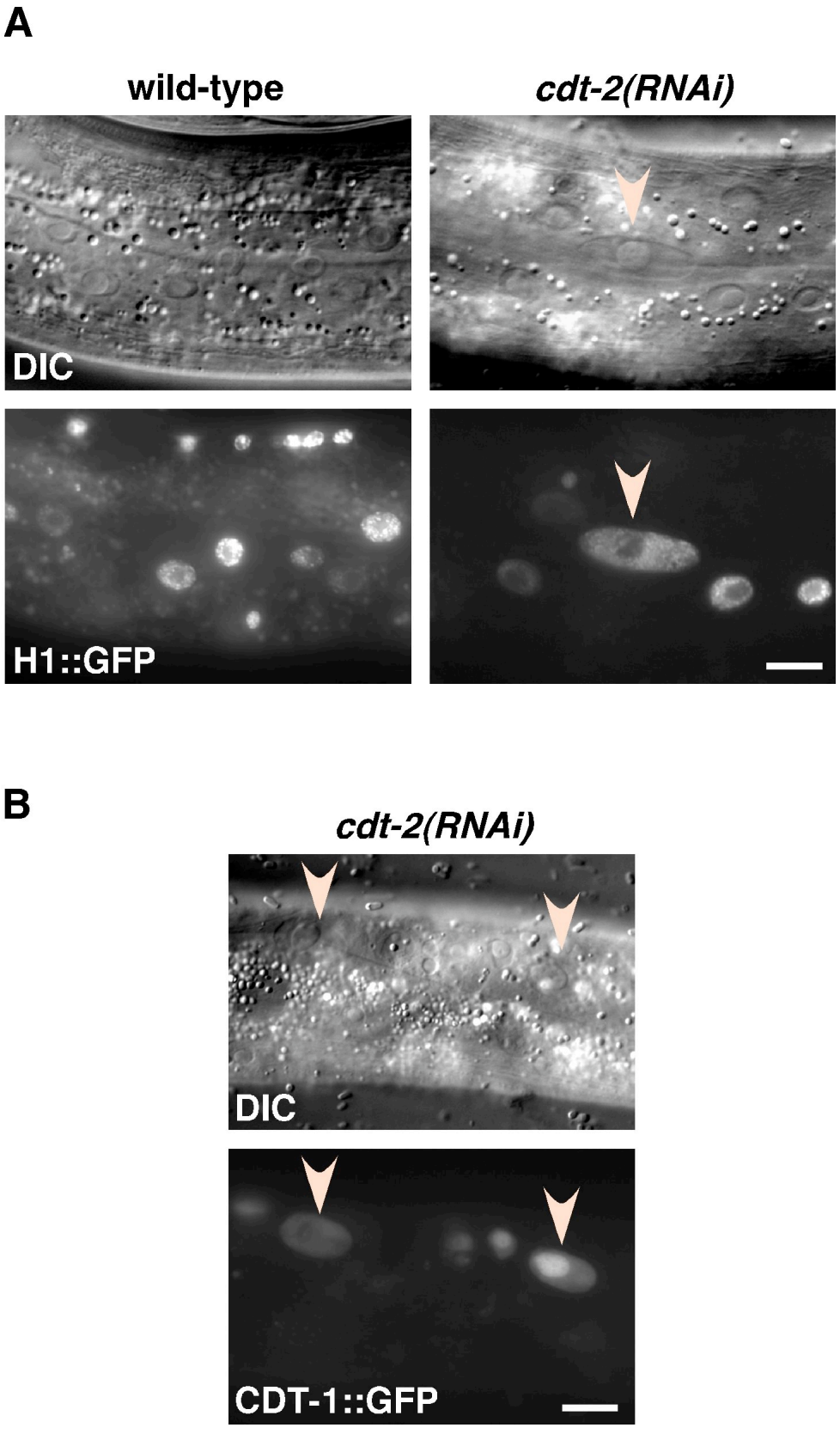
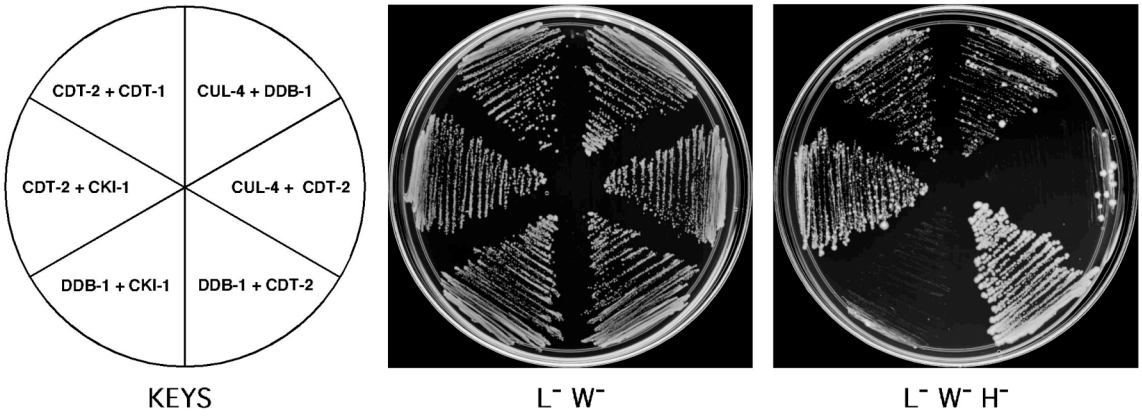

A
wild-type
cdt-2(RNAi)
DIC
H1::GFP
B
cdt-2(RNAi)
DIC
CDT-1::GFP

Figure 3.3. CKI-1 functions as an SRS of the CUL-4/DDB-1 complex.

A, Two-hybrid tests was employed to examine the direct interactions between CUL-4 complex components and substrates **B,** The abilities of DDB-1 or CDT-2 to mediate interactions between either CUL-4 and CDT-2 or DDB-1 and CKI-1, respectively, were examined through three-hybrid tests. Note that CUL-4 can interact with CDT-2 only in the presence of DDB-1 (left half of the plates), and DDB-1 can interact with CKI-1 only in the presence of CDT-2 (right half of the plates).

A

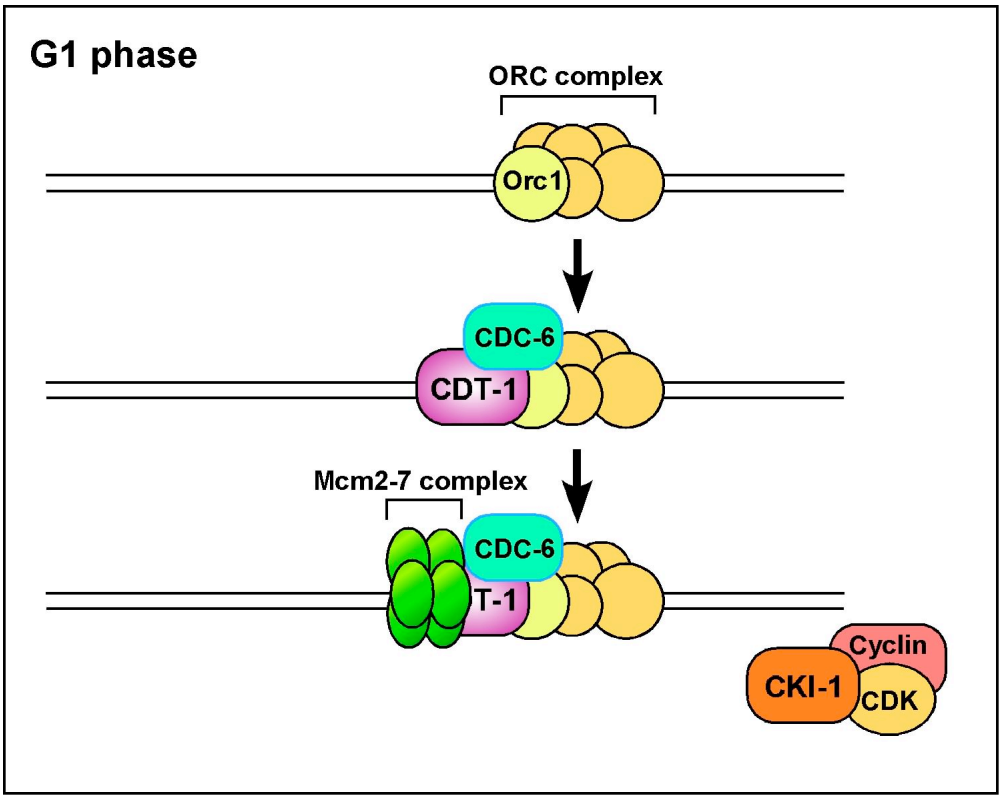

CDT-2 + CDT-1
CUL-4 + DDB-1
CDT-2 + CKI-1
CUL-4 + CDT-2
DDB-1 + CKI-1
DDB-1 + CDT-2
KEYS
L⁻ W⁻
L⁻ W⁻ H⁻

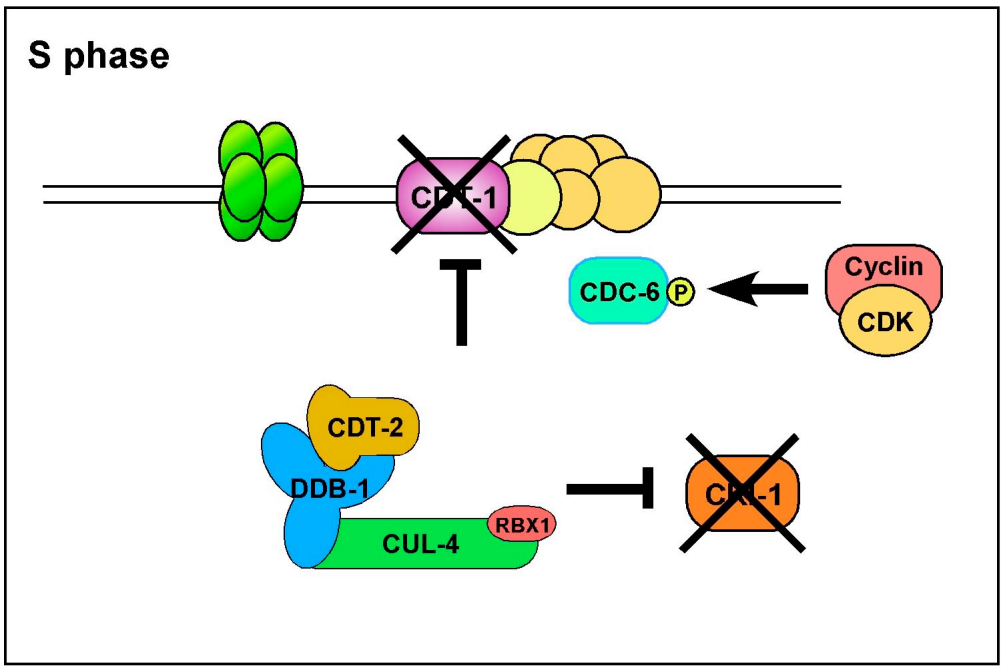
B


CUL-4 + CDT-2
and
pYES2 DDB-1
DDB-1 + CKI-1
and
pYES2 CDT-2
KEYS
L-W-U-H-
GLUCOSE
L-W-U-H-
GALACTOSE

Figure 3.4. A molecular model of CUL-4/DDB-1^{CDT-2} function

CUL-4/DDB-1^{CDT-2} directly targets the licensing factor CDT-1 and the CDK-inhibitor CKI-1 for degradation. CKI-1 prevents CDK/cyclin activation until cells enter S phase. CUL-4/DDB-1^{CDT-2} -mediated CKI-1 degradation release CDK/cyclin from the inhibitor and then the activated CDK/cyclin phosphorylates the licensing factor CDC-6. Phosphorylated CDC-6 is exported from the nucleus in S phase. Inactivation of CUL-4/DDB-1^{CDT-2} leads to the accumulation of both licensing factors CDT-1 and CDC-6 in the nucleus, producing extensive DNA re-replication.


G1 phase
ORC complex
Orc1
CDC-6
CDT-1
Mcm2-7 complex
CDC-6
T-1
Cyclin
CKI-1
CDK


S phase
CDT-1
CDC-6
P
Cyclin
CDK
CDT-2
DDB-1
CUL-4
RBX1
CKI-1

CHAPTER IV

THE *C. ELEGANS* CUL-4/DDB-1^{VprBP} E3 LIGASE COMPLEX IS REQUIRED FOR GERM CELL INTEGRITY

BACKGROUND

The structures of CUL4/DDB1 complexes vary based on the role of the core component DDB1 (damaged-DNA binding protein). It has been shown that DDB1 can either bind to substrates directly or bind to an SRS (substrate recognition subunit) that binds to the substrates. Recently a variety of proteomic approaches were used to identify human WD-repeat proteins containing a conserved 'WDXR' motif that binds to DDB1 through the 'WDXR' motif (Angers et al., 2006; He et al., 2006; Higa et al., 2006b; Jin et al., 2006). The 'WDXR' motif proteins contain known SRSs that have been shown to interact with DDB1 (Angers et al., 2006; Jin et al., 2006). In the previous study, we showed that a 'WDXR' motif protein CDT-2 can directly interact with the substrates CDT-1 and CKI-1, suggesting that 'WDXR' motif proteins functions as SRS in CUL4/DDB1 complexes. A genome analysis predicted that there may be at least 90 'WDXR' motif proteins in humans and 36 in *C. elegans* (He et al., 2006). These observations suggest that CUL4/DDB1 potentially forms a large family of distinct ubiquitin ligases with different 'WDXR' motif proteins, each of which target sets of substrate proteins for degradation. In humans, 23 'WDXR' motif proteins have been pulled-down in CUL4 or DDB1 affinity-purifications, and 12 of these are

conserved in the *C. elegans* genome. Therefore, it is expected that CUL-4/DDB-1 is involved in the regulation of multiple cellular pathways in *C. elegans* through interaction with various 'WDXR' motif proteins.

The 'WDXR' motif protein VprBP, which is associated with mammalian CUL4/DDB1 (Angers et al., 2006; He et al., 2006; Jin et al., 2006; Le Rouzic et al., 2007), was initially discovered as an interaction partner of Vpr, an auxiliary regulatory protein of the human immunodeficiency virus type 1 (HIV-1) (Zhang et al., 2001; Zhao et al., 1994). In humans, Vpr has been implicated in the generation of apoptosis, cell cycle arrest, differentiation, and immune suppression through the interaction with various cellular proteins (Muthumani et al., 2005). Recently it has been shown that Vpr triggers G2 arrest by hijacking the CUL4/DDB1^{VprBP} E3 ligase (Le Rouzic et al., 2007; Schrofelbauer et al., 2007). The Vpr-mediated G2 arrest requires the interaction with VprBP; and the cell cycle arrest can be abolished by siRNA of VprBP (Le Rouzic et al., 2007). VprBP has been shown to link Vpr and DDB1 in mammalian cells (Le Rouzic et al., 2007; Schrofelbauer et al., 2007). Overexpression of Vpr also leads to the degradation of a cellular protein UNG2 (Uracil DNA glycosylase), and siRNA of CUL4 or DDB1 abolishes the Vpr-mediated UNG2 degradation (Schrofelbauer et al., 2007). These data suggest that Vpr degrades proteins that are involved in cellular defense mechanisms by hijacking the CUL4/DDB1^{VprBP} ubiquitin ligase. However the endogenous cellular function of CUL4/DDB1^{VprBP} in cells with no viral infection has not yet been determined.

In this study, we found that CUL-4/DDB-1 is involved in germ cell development. Germ cells in *ddb-1* mutants exhibit vacuoles and corrupted cell- and nucleolar-morphology. Germ cells in *cul-4* or *ddb-1* mutants do not exhibit a re-replication phenotype. RNAi depletion of the *C. elegans* homolog of VprBP causes a germ cell defect that is identical to that observed upon inactivation of *cul-4* or *ddb-1*, suggesting that this gene functions as an SRS of a CUL-4/DDB-1 ubiquitin ligase complex to promote germ cell viability.

RESULTS

Germ cells are defective in *ddb-1* mutant

The adult hermaphrodite of *C. elegans* has two symmetric U-shaped gonad arms, each containing ~1000 germ cells (Kimble and Hirsh, 1979). The adult germ line exhibits distal-to-proximal polarity with a mitotic cell population located at the most distal end of the gonad, and meiotic cells extending proximally. The most distal end of the adult gonad contains a stem cell population, and is referred to as the mitotic zone. As germ cells move away from the influence of the distal tip cell (DTC), they enter meiosis I, proceeding through prophase I to diakinesis (Hansen et al., 2004). Germ cells in *ddb-1(tm1769)* mutants exhibit corrupted cell- and nuclear-morphology through development with more obvious defects in older animals, and often undergo necrosis in older adults (Figures 4.1A-D; data not shown). Spermatogenesis is observed in *ddb-1(tm1769)* mutants, and oogenesis occurs infrequently, suggesting that meiotic entry *per se* is not defective (Figures 4.1A, B). Similar germ cell defects are

observed when larvae are fed bacteria expressing *cul-4* dsRNA, indicating that CUL-4 is also required for germ cell viability (data not shown).

The germ cell defect is independent of re-replication

Previously we have shown that CUL-4/DDB-1 is required to prevent DNA re-replication by targeting CDT-1, CKI-1 and CYE-1 for degradation. There is, however, no clear link between the germ line defects and DNA replication. *ddb-1* mutant germ cells do not have DNA levels higher than the normal cell cycle range of 2n to 4n: the DNA level in wild-type germ cells is 2.2 ± 0.4 C while *ddb-1(tm1769)* germ cells have 2.6 ± 0.6 C, n = 19 each. Further, we did not observe marked differences in the level of CDT-1, CKI-1 or CYE-1, which are known to accumulate in re-replicating cells, within the mitotic germ cells of wild type or *ddb-1(tm1769)* mutants (data not shown). These data suggest that DDB-1 is required for germ cell integrity independently of its role in regulating DNA replication.

In vertebrates and *Drosophila*, Cdt1 is also inhibited by binding to geminin (Mihaylov et al., 2002; Quinn et al., 2001; Tada et al., 2001; Wohlschlegel et al., 2000). In *C. elegans*, RNAi depletion of the geminin homolog *gmn-1* is associated with the enlargement of some germ cell nuclei, which led to the proposal that GMN-1 prevents re-replication in germ cells (Yanagi et al., 2005). We determined whether geminin has a role to prevent re-replication in germ cells of *C. elegans*, and tested whether CUL-4/DDB-1 and geminin redundantly

function to prevent re-replication in germ cells. We analyzed a *gmn-1(tm2212)* allele that deletes 236 bp of the *gmn-1* locus, including conserved residues that are required for binding to CDT-1 (Lee et al., 2004) (Figure 4.2A). *gmn-1(tm2212)* is recessive and exhibits maternal-effect sterility: *gmn-1(tm2212)* homozygous progeny of heterozygotes are viable, but their progeny (which lack maternal product) are inviable or sterile. We observed that a subset of germ cells are enlarged in *gmn-1(tm2212)* homozygotes, similar to what was previously reported for *gmn-1* RNAi (Yanagi et al., 2005). Unexpectedly, DAPI staining of the *gmn-1(tm2212)* mutant gonad indicated that DNA levels are not increased in the enlarged germ cells, suggesting that re-replication is not occurring (Figure 4.2B, C). This phenotype of enlarged cells with normal DNA levels was also observed in homozygotes of the *ok1708* allele, which is a deletion that removes the entire *gmn-1* coding region and part of a nearby gene, Y75B8A.18 (data not shown). Inactivation of both DDB-1 and GMN-1 (by *ddb-1* RNAi depletion of *gmn-1(tm2212)* homozygotes) did not significantly increase germ cell DNA levels relative to that of *ddb-1(RNAi)* animals (Figure 4.2B, C). These data suggest that the germ cell defect in *ddb-1* mutants is not a secondary consequence of DNA re-replication.

The *C. elegans* VprBP homolog phenocoies *ddb-1* in germ cells

In humans, 23 different 'WDXR' motif proteins have been identified from the CUL4 or DDB1 affinity purification and proposed to function as SRSs of CUL4/DDB1 complexes. We found 12 different orthologs of the human 'WDXR'

motif proteins in the *C. elegans* genome (Table 4.1). To identify an SRS that is relevant to the germ cell defect, we employed RNAi depletion of the 12 genes. We found that RNAi depletion of the *C. elegans* VprBP ortholog *ZK1251.9* specifically produces germ cell defects, similar to that of *ddb-1* (Table 4.1; Figure 4.1E). The RNAi effect is fully penetrant with 100% occurrence of the germ cell defect phenotype in all examined animals. We also have characterized a *ZK1251.9* mutant deletion allele, *ZK1251.9 (ok1867)*. The *ok1867* allele is recessive and homozygotes are sterile, and thus the allele is maintained in a heterozygous strain. All of the hatched homozygous embryos develop to the adult stage but invariably have defects in germ cells, which are undistinguishable from *ZK1251.9(RNAi)* and *ddb-1(tm1769)* germ cells (data not shown). The timing of larval development is significantly slower for homozygous *ZK1251.9(ok1867)* mutants than for wild-type larvae (84.1 ± 2.6 hrs vs. 64.8 ± 3.9 hrs from laid eggs to adult at 20°C, n = 10 each; $p < 1 \times 10^{-5}$). These data indicate that the *C. elegans* VprBP homolog *ZK1251.9* functions as an SRS that is required for germ cell development.

qDf4, a deficiency chromosome can suppress the germ cell defect

We currently do not understand the molecular and cellular defect that is responsible for the germ cell phenotype associated with inactivation of CUL-4/DDB-1^{VprBP}. It is expected that the identification of the relevant substrate(s) or proteins that function in the same genetic pathway will provide an insight into the molecular pathways underlying the germ cell defects. We have taken a

candidate gene approach to test for genes whose inactivation can suppress the germ cell phenotype.

Programmed Cell Death (PCD) or apoptosis represents a major cell fate among adult germ cells. It is estimated that approximately one half of all potential oocytes are eliminated in the adult hermaphrodite during progression through prophase of meiosis I (Hengartner, 1997). All known apoptotic pathways in *C. elegans* converge on the caspase homolog *ced-3* (Gumienny et al., 1999). To test whether inactivation of *ced-3* can suppress the germ cell defect in *ZK1251.9,* we subjected *ced-3(n717)* homozygous mutants to feeding RNAi directed against *ZK1251.9*. We did not detected obvious differences between *ZK1251.9(RNAi)* and *ZK1251.9(RNAi); ced-3(n717)* germ cells (Table 4.2). We also tested whether inactivation of the *C. elegans* homologs of p53, ATM or ATR, which have known to activate checkpoint pathways in humans, can suppress the germ cell defects. However, the germ cell defects of *ZK1251.9 (RNAi)* animals were not rescued in those mutant alleles (Table 4.2). These results suggest that apoptosis or a p53-mediated cell cycle arrest are not required for the manifestation of the *ZK1251.9(RNAi)* germ cell phenotype.

Although we did not detect an obvious accumulation of CDT-1 protein in the *ddb-1* mutant germ cells, it is possible that non-detectable increases in CDT-1 level may lead to cell cycle defects in *ddb-1* mutant germ cells. To test this possibility we asked whether genetic inactivation of one copy of *cdt-1* can

suppress the germ cell phenotype associated with *ddb-1* or *ZK1251.9* inactivation. We used a deficiency chromosome *qDf4*, which deletes a genomic region including *cdt-1* gene locus (Figure 4.3). The *qDf4* allele is recessive and homozygotes are embryonic lethal, and thus the allele is maintained as a heterozygous strain. The *qDf4* heterozygous animals have been shown to suppress re-replication phenotype upon RNAi inactivation of *cul-4* (Zhong et al., 2003). Interestingly we found that *qDf4* heterozygous animals have significantly reduced germ cell defects upon RNA inactivation of *ddb-1* whereas wild type animals invariably have the germ cell defect (Table 4.2). Consistently *qDf4* heterozygous animals are also resistant to the *ZK1259.1* RNAi depletion (Table 4.2). *qDf4* is a relatively large genomic deletion that removes multiple genes (Table 4.3). We therefore tested the possibility that *cdt-1* is the relevant gene in the *qDf4* deletion region for the relevant substrate of the germ cell defect. We found that *cdt-1* RNAi inactivation does not rescue the germ cell defect either of *ddb-1(tm1769)* or *ZK1251.9(ok1867)* animals. The discrepancy between the effects of *qDf4* and *cdt-1* RNAi can be interpreted either that *cdt-1* RNAi is not effective enough to rescue the germ cell defect or that the deletion of a gene other than *cdt-1* in *qDf4* suppresses the germ cell phenotype.

DISCUSSION

In this study, we have described a new phenotype of *ddb-1* and identified a potential SRS for the phenotype. DDB-1 is required for germ cell integrity and shares the phenotype with *cul-4*. Inactivation of *ddb-1* produced a unique germ

cell phenotype. We previously showed that inactivation of *cul-4* or *ddb-1* causes a massive re-replication in somatic blast cells. However we have not detected indications of re-replication in *ddb-1* or *cul-4* germ cells. There were no detectable increases in CDT-1, CKI-1 or CYE-1 levels, suggesting that the germ cell defects are independent of re-replication.

It is possible that a redundant regulatory mechanism could be preventing re-replication in *ddb-1* mutant germ cells. In vertebrates and *Drosophila*, Cdt1 is inhibited by binding to geminin (Mihaylov et al., 2002; Quinn et al., 2001; Tada et al., 2001; Wohlschlegel et al., 2000). The *C. elegans* geminin ortholog, GMN-1, has been shown to bind CDT-1 in both the two-hybrid system and *in vitro* (Yanagi et al., 2005). *gmn-1* RNAi was reported to produce enlarged germ cells (Yanagi et al., 2005). We observed that *gmn-1* deletion allele homozygotes have similar large germ cells, but these enlarged cells did not contain increased DNA levels. Further, there was no increase in DNA content upon *ddb-1* RNAi depletion of *gmn-1* mutants. Therefore, the failure to observe re-replication in *cul-4* or *ddb-1* mutant germ cells is not attributable to redundant regulation by geminin.

We have screened putative SRS proteins for the germ cell defects, and found that the *C. elegans* VprBP homolog has similar phenotype of *ddb-1*. In humans, VprBP has been identified as an interacting partner of HIV-1 proteins Vpr (Zhang et al., 2001; Zhao et al., 1994). Recent reports suggest that Vpr controls its target proteins by hijacking the CUL4/DDB1^{VprBP} E3 ligase complex

(Le Rouzic et al., 2007; Schrofelbauer et al., 2007). HIV-1 protein Vpr binds to a list of cellular proteins, leading to various cellular defects (Muthumani et al., 2005). However it is not clear how many cellular targets of Vpr are regulated through CUL4/DDB1^{VprBP} E3 ligase. In a model of Vpr-mediated proteolysis, VprBP does not directly bind to substrates but instead Vpr brings the substrates to the CUL4/DDB1 complex through interaction with VprBP (Dehart et al., 2007). It would be interesting to address whether cellular CUL4/DDB1^{VprBP} complex requires an additional component to recruit its target proteins.

To identify the substrate for the germ cell defect, we randomly tested putative candidates. We initially asked whether inactivation of apoptotic or DNA damage response pathway can suppress the germ cell phenotype. Genetic data suggest that the germ cell defect is not related with either apoptosis or DNA damage response. Unexpectedly, however, we found that *qDf4*, a deficiency chromosome can suppress the germ cell phenotype. Considering that only *qDf4* heterozygotes are examined, ~40% suppression appears to be quite dramatic. 55 genes are located in the region deleted in the *qDf4*. Most of the 55 genes have currently known not to be involved in cell cycle or germ cell developments (Table 4.3). We will examine the 55 genes to find out the substrate that is relevant to the germ cell phenotype. It may provide an insight into the molecular mechanism of the germ cell defects in *cul-4* or *ddb-1* mutants.

MATERIALS AND METHODS

Strains and RNAi

The following *C. elegans* strains were used: N2, wild type; ET263, *ddb-1(tm1769)/dpy-20*(*e2017*); ET121, *qDf4/unc-11(e47)*; MT1522, *ced-3(n717)*; MT3002, *ced-3(n1286)*; DW101, *atl-1(tm853)/nT1*; VC381, *atm-1(gk186)*; JR1279*, cep-1(+)I;cep-1(w40)*. RNAi was performed by providing bacteria expressing dsRNA to L1- or L4-stage larvae as a food source, as described (Kamath et al., 2001). The *ZK1251.9* feeding protocol employed *E. coli* strain HT115 containing plasmid pPD129.36/*ZK125.19*, which contains a 1.3kb fragment of *ZK1251.9* cDNA cloned into a multi-cloning site situated between double T7 primers. Full length cDNAs of *D2030.9, R11D1.1, cdt-2* and *cdt-1* genes were cloned into pPD129.36, and 1.4kb fragment was cloned for *ZK43.7.* For *F47D12.9, F53C11.7* genes, we used RNAi feeding bacteria from a bacterial library of Julie Arhinger lab (Kamath et al., 2003).

Immunofluorescence, microscopy and quantitation of DNA content

Worms were analyzed for germ cell phenotype as described in chapter II.

ACKNOWLEDGEMENTS

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strains; J. Arhinger for RNAi feeding bacteria; T. Schedl for helpful discussion about germ cell defect.

Table 4.1. *C. elegans* homologs of 'WDXR' motif proteins

WDXR proteins	*C. e.* homologs	RNAi phenotype*
WDR21A	*F47D12.9*	WT
WDR68	*F53C11.7*	WT
WDR22	*R11D1.1b*	WT
WDR23	*D2030.9*	Egl, Gro
WDSOF1	*ZK430.7*	Lva, mild re-replication
VprBP	*ZK1251.9*	germ cell defect
CDT2	*T01C3.1*	re-replication
WDR26	*Y39H10A.6*	Ste, Emb, Age
GRWD1	*Y54H5A.1*	Emb
SMU1	*smu-1*	WT
TLE1		
TLE2	*unc-37***	Emb, Let, Unc
TLE3		
IQWD1		
WDR42A	*Y73E7A.9****	WT
WDTC1		

* RNAi phenotypes were experimentally determined or described based on genome wide RNAi analysis data; Age, long life span; Egl, egg laying defect; Emb, embryonic lethal; Gro, slow growth; Let, lethal; Lva, larval arrest; Ste, sterile; WT, wild type; Unc, uncoordinated

** TLE1~3 hit a single *C. elegans* gene *unc-37*

*** IQWD1, WDR42A and WDTC1 hit a single *C. elegans* gene *Y73E7A.9*

Table 4.2. Suppresser tests for the germ cell defect

Worm strain	RNAi	Germ cell defect (%)
ddb-1(tm1769)	-	100 (20/20*)
ZK1251.9(ok1867)	-	100 (20/20)
N2 (wild type)	*ddb-1*	100 (20/20)
N2 (wild type)	*ZK1251.9*	100 (20/20)
ddb-1(tm1769)	*cdt-1*	100 (10/10)
ZK1251.9(ok1867)	*cdt-1*	100 (10/10)
qDf4/unc-11	*ddb-1*	42 (5/12)
qDf4/unc-11	*ZK1251.9*	45 (18/44)
ced-3(n717)	*ZK1251.9*	100 (10/10)
ced-3(n1286)	*ZK1251.9*	100 (10/10)
atl-1(tm853)	*ZK1251.9*	100 (10/10)
atm-1(gk186)	*ZK1251.9*	100 (10/10)
cep-1(+)I;cep-1(w40)	*ZK1251.9*	100 0/10)

* Number of animals

Table 4.3. The list of genes deleted in *qDf4* deficiency chromosome

Sequence	Gene name	Concise Description
y54e10br.4		predicted RNA binding protein Nob1p involved in 26S proteasome assembly
y54e10br.3		predicted E3 ligase
y54e10br.5		signal peptidase
y54e10br.6		DNA-directed RNA polymerase subunit E
y54e10br.2		GTP-binding ADP-ribosylation factor-like protein
Y54E10BR.7	mod-5	Modulation Of locomotion Defective, a Na[+],Cl[-]-dependent serotonin transporter
y54e10br.1		Glycosylphosphatidylinositol anchor synthesis protein
m01b12.3	arx-7	ARp2/3 compleX component
m01b12.4		
m01b12.5		
m01b12.1		
c45e1.1	nhr-64	nuclear hormone receptor
c45e1.4		
k09h9.4		
k09h9.3	col-49	collagens
k09h9.5		
k09h9.6	lpd-6	rRNA-binding protein, required for ribosomal large subunit maturation
k09h9.2		
k09h9.1		
k09h9.7		
y54e10a.9	vbh-1	ATP-dependent RNA helicase
y54e10a.7		
y54e10a.6		Phenylalanyl-tRNA synthetase beta subunit
y54e10a.5		Dynactin subunit p27/WS-3, involved in transport of organelles along microtubules
y54e10a.10		Protein required for biogenesis of the ribosomal 60S subunit
y54e10a.11		Predicted E3 ubiquitin ligase
y54e10a.12		
y54e10a.13		

y54e10a.17		
y54e10a.4	fog-1	Feminization Of Germline
y54e10a.14	rom-5	RhOMboid (Drosophila) related
y54e10a.15	cdt-1	cdt-1
y54e10a.3		Thioredoxin-like protein
y54e10a.2	cogc-1	Conserved Oligomeric Golgi (COG) Component
y54e10a.16		
y44e3b.2		tyrosinase
y44e3b.1	zip-4	bZIP transcription factor family
w01b11.3		Ribosome biogenesis protein - Nop58p/Nop5p
w01b11.5	pqn-72	Prion-like-(Q/N-rich)-domain-bearing protein
w01b11.2	sulp-6	SULfate Permease family
w01b11.6		Thioredoxin
w01b11.1		
y44e3a.2	ace-2	abnormal ACEtylcholinesterase
y44e3a.3		Thioredoxin
y44e3a.4		Adaptor protein CMS/SETA
y44e3a.1		
y44e3a.6		
w05f2.7		
w05f2.2		
w05f2.3		
w05f2.6		
w05f2.5	fbxa-203	encodes a protein containing an F-box& FTH/DUF38 motif
w05f2.4		
k03e5.3	cdk-2	Protein kinase
k03e5.2		

Figure 4.1. Germ cells are defective in the *ddb-1* mutant, and RNAi of the VprBP homolog phenocopies the *ddb-1* mutant germ line defect.

A-D, DIC image of germ lines in wild-type (**A**) and *ddb-1(tm1769)* (**C**) young adults. The region of germ cells enclosed within the white boxes in **A** and **C** are shown magnified in **B** and **D**, respectively. **E,** DIC image of germ cells in *ZK1251.9(RNAi)* young adults. *ZK1251.9* is the *C. elegans* homolog of VprBP. Scale bars, 10 μm.

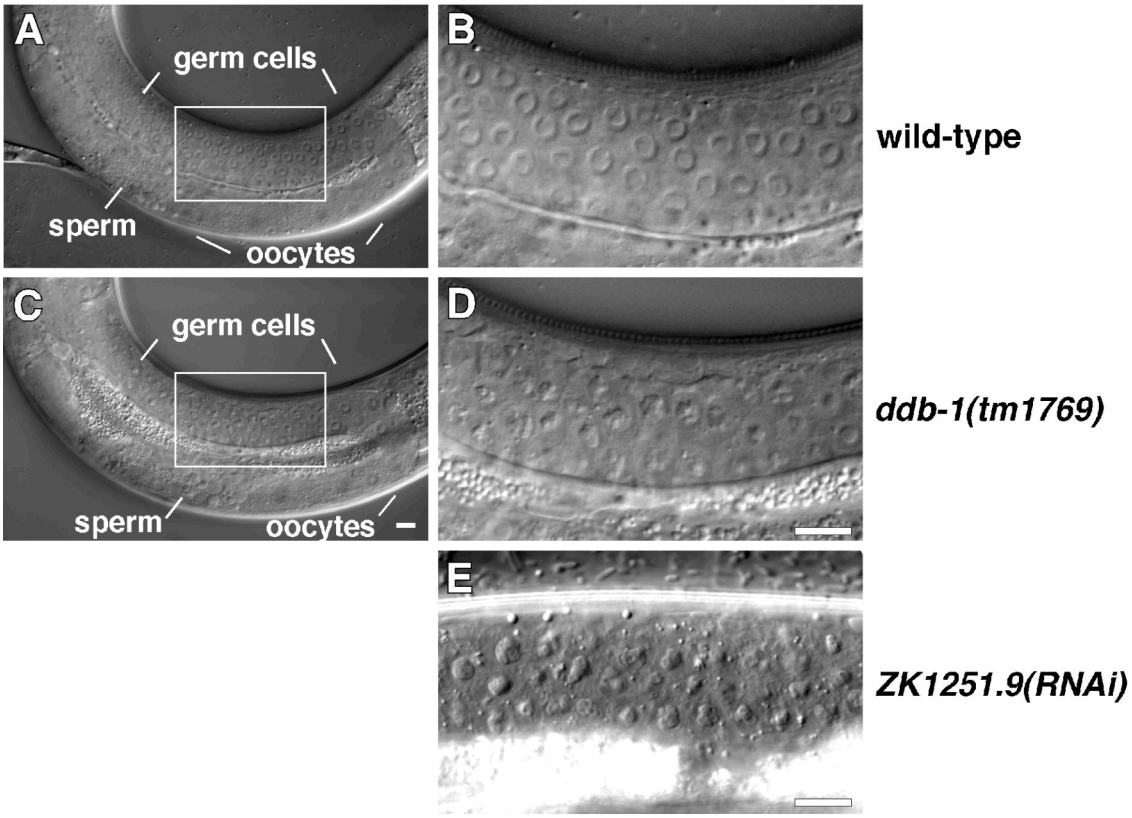

A
germ cells
sperm
oocytes
B
wild-type
C
germ cells
sperm
oocytes
D
ddb-1(tm1769)
E
ZK1251.9(RNAi)

Figure 4.2. The germ cell defect is not associated with re-replication.

A, Line drawing of the *gmn-1* genomic region on chromosome III. Exons are represented as boxes, and shaded areas represent coding regions. Arrow indicates the start point and direction of translation. Broken boxes with diagonal shading indicate the nearest exon of the closest upstream gene. The region deleted in the *gmn-1(tm2212)* deletion allele is represented below the gene as the missing region bounded by dashed lines. The *gmn-1(tm2212)* allele is a 236 bp deletion that removes 102 bp of intron 1 and 134 bp of exon 2. **B,** DAPI staining of wild-type, *ddb-1(RNAi), gmn-1(tm2212)*, and *ddb-1(RNAi); gmn-1(tm2212)* dissected gonads from young adults. The white boxes in **B**, which encompasses transition zone germ cells, are magnified in **C**. Arrowhead in *gmn-1(tm2212)* (**C**) indicates an enlarged germ cell. Scale bars, 10 μm.

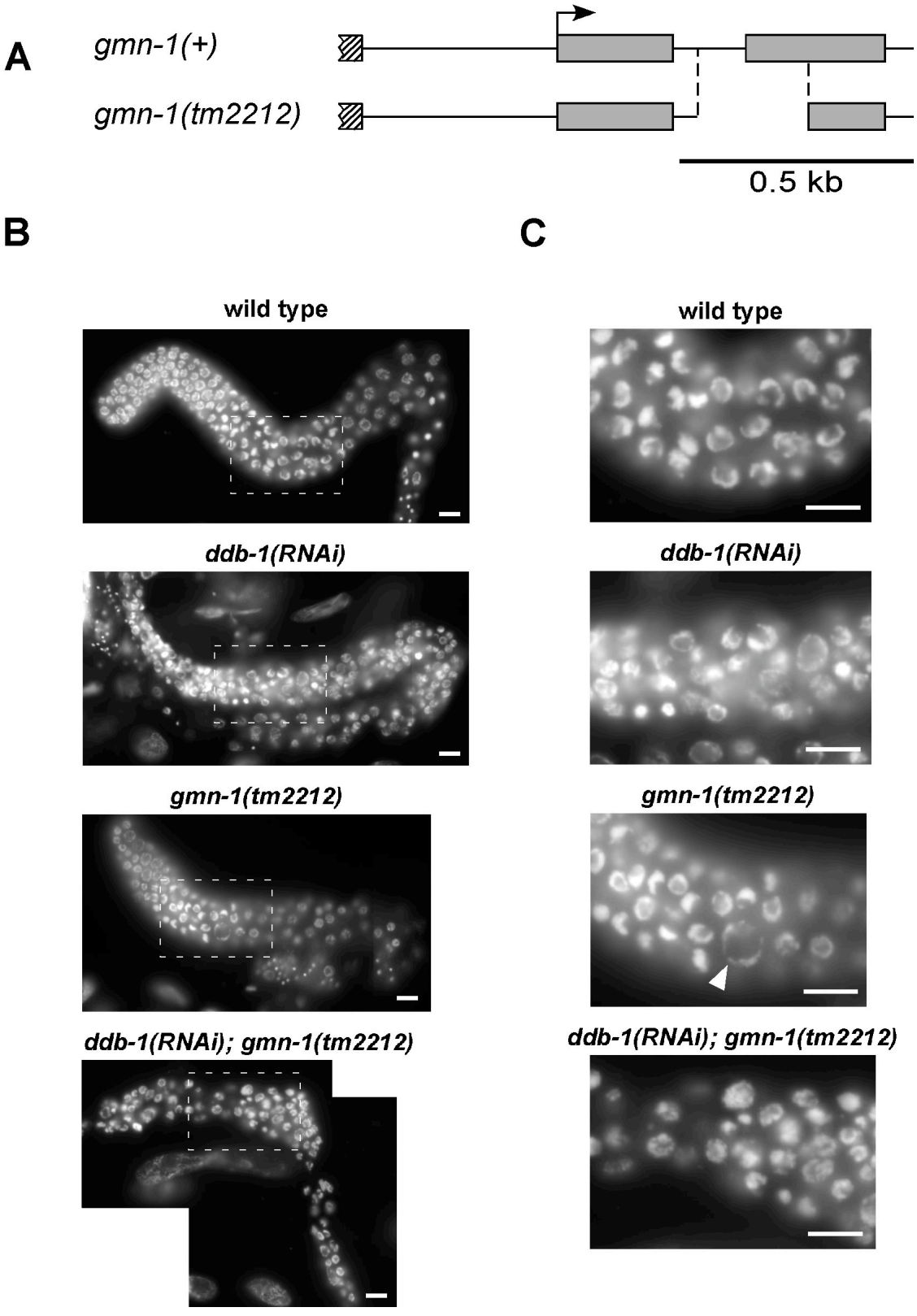
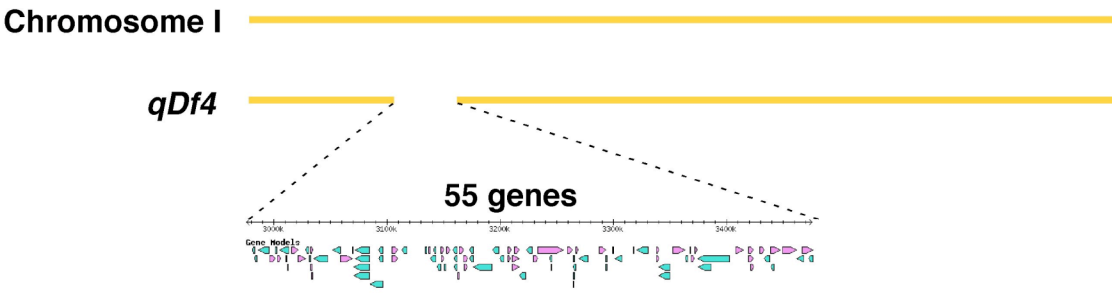

A
gmn-1(+)
gmn-1(tm2212)
0.5 kb
B
C
wild type
ddb-1(RNAi)
gmn-1(tm2212)
ddb-1(RNAi); gmn-1(tm2212)
wild type
ddb-1(RNAi)
gmn-1(tm2212)
ddb-1(RNAi); gmn-1(tm2212)

Figure 4.3. Line drawing of *qDf4* deficiency chromosome.

The region deleted in *qDf4* is represented below the gene as the missing regions. *qDf4* lacks a region of ~1.2 map unit that contains 55 gene models. The size of whole chromosome I is approximately 50 map units. Not in scale.


Chromosome I
qDf4
55 genes
3000k
3100k
3200k
3300k
3400k
Gene Models

CHAPTER V

GENERAL DISSCUSSION

The family of cullin/RING-H2 forms potentially the largest superfamily of E3 ubiquitin ligases. Each cullin member ubiquitinates a number of substrates through various SRSs (substrate recognition subunits). The number of putative SRSs, therefore, may be correlated with the potential number of cullin/RING-H2 E3 ligases. Analysis of the human genome revealed 109 F-box proteins, 51 VHL/SOCS box proteins, 439 BTB domain proteins and 90 WDXR motif proteins, which are potentially SRS or substrate specific adapter for CUL1, CUL2, CUL3 and CUL4 E3 ligases, respectively (He et al., 2006; Petroski and Deshaies, 2005). A great deal of information comes from the study of the CUL1 class of cullin/RING-H2 E3 ligase complexes, which function to ubiquitinate a variety of substrates (Petroski and Deshaies, 2005). In comparison to our knowledge about CUL1/RING-H2 E3 ligase complexes, information about the functions of CUL4 has been limited. The research presented in this dissertation has contributed to understanding the molecular mechanisms underlying the functions of CUL-4 E3 ligases using the model organism *C. elegans*. This should provide a framwork for analyzing uncharacterized functions of CUL4/DDB1 in the future. I will briefly discuss the major conclusions that have been described in Chapter 2 ~ 4.

The DNA licensing factor CDT-1 is degraded by CUL-4/DDB-1 E3 ligase

One central problem in eukaryotic DNA replication is to duplicate the entire genome precisely once per cell cycle. Eukaryotic DNA replication is initiated at hundreds of replication origins, and therefore these origins must be strictly regulated to fire only once per cell cycle. This high degree of regulation is achieved by the DNA replication licensing system. Licensing proteins are required for DNA replication initiation, and the removal of licensing proteins from replicated chromatin in S phase prevents DNA re-replication. *In C. elegans*, inactivation of *cul-4* leads to the failure to degrade the licensing factor CDT-1, causing a massive re-replication (Zhong et al., 2003). Whether different CUL4 E3 ligase complexes share an adaptor protein that links CUL4 and various SRSs had been a critical question. We showed that in *C. elegans,* DDB-1 shares all examined CUL-4 loss-of-function phenotypes, and DDB-1 can directly interact with CUL-4, suggesting that DDB-1 functions as the adaptor in CUL-4 E3 ligase complexes in *C. elegans*. We found that the CUL-4/DDB-1 complex is required for CDT-1 degradation, and DDB-1 can physically interact with CDT-1, indicating that CDT-1 is a direct substrate of the CUL-4/DDB-1 complex. In humans and *Xenopus*, additional components CDT2 and PCNA have been shown to be required for Cdt1 degradation as well (Arias and Walter, 2006; Jin et al., 2006). We confirmed that the requirement for CDT2 and PCNA for Cdt1 degradation is conserved in *C. elegans*.

In humans, two E3 ligases CUL4/DDB1^{CDT2} and SCF^{Skp2} are redundantly required for Cdt1 degradation (Nishitani et al., 2006). The single inactivation of

either CUL4/DDB1^{CDT2} or SCF^{Skp2} does not cause a failure of Cdt1 degradation in humans. However, the role of SCF^{Skp2} in Cdt1 degradation has not been directly shown in other systems. We examined the requirement of SCF^{Skp2} for Cdt1 degradation by analyzing *C. elegans* Skp2 homolog SKPT-1. SKPT-1 does not have an obvious role in CDT-1 degradation even in a sensitized *ddb-1* mutant background. This study provided the first direct evidence that SCF^{Skp2} is not involved in Cdt1 degradation in a metazoa. In the genomic analysis across phyla, we found that the SRS CDT2 has been evolveed earlier than Skp2, suggesting that CUL4/DDB1^{CDT2} is the ancient pathway to control DNA replication. We hypothesize that CUL4/DDB1 functions as the major E3 ligase to ubiquitinate Cdt1 in most examined organisms except humans.

CUL-4/DDB-1 E3 ligase negatively regulates the CIP/KIP family CKI-1 and cyclin E homolog CYE-1 in *C. elegans*

The scale of DNA re-replication observed in *cul-4* or *ddb-1* cells exceeds that of any other known re-replication phenotype resulting from inactivation of a single mechanism to prevent re-replication, suggesting that CUL-4/DDB-1 controls more than one pathway to prevent re-replication. We examined the level of cell cycle regulators CKI-1 and CYE-1 in the re-replicating cells. Both CKI-1 and CYE-1 levels are significantly higher in the *cul-4* or *ddb-1* cells. Significantly we found that CKI-1 and CDT-1 levels are independently regulated by CUL-4 or DDB-1, raising a possibility that CKI-1 is another direct target of CUL-4/DDB-1 E3 ligase. Currently we do not have evidence that CYE-1 is a direct substrate of

the CUL-4/DDB-1 E3 ligase. It has been shown that in humans, CDK-free cyclin E has a role in the formation of pre-replication complex (Geng et al., 2007). In the future, it would be rewarding to characterize CYE-1 and other pre-replication complex components for their interaction with CUL-4/DDB-1.

CDT2 functions as an SRS in a CUL-4/DDB-1 E3 ligase

It has been demonstrated that CDT2 is required for Cdt1 and p53 degradation (Banks et al., 2006; Higa et al., 2006a; Jin et al., 2006). However, the relative position of CDT2 in the CUL4/DDB1 complex has not been clearly demonstrated. Our genetic evidence indicates that *C. elegans* CDT2 homolog CDT-2 is required for CDT-1 and CKI-1 degradation. Currently we have not determined whether the *C. elegans* p53 homolog CEP-1 is regulated by CUL-4/DDB-1 complex. We showed that CDT-2 can function as an SRS in the CUL-4/DDB-1 complex. Our evidence of direct interaction between CDT-2 and either of CDT-1 or CKI-1 has not been shown before. It is interesting that the CDT2 substrates, CDT-1, CKI-1, and p53 have at least one conserved PIP (PCNA interacting protein) box motif. PCNA has been shown to be required for CDT-1 and p53 degradation. Therefore it is worthwhile to determine whether PCNA is also required for CKI-1 degradation, and how it interacts with the CUL-4/DDB-1 complex.

A function for CUL-4/DDB-1 E3 ligase in germ cells

In the course of the *ddb-1* mutant analysis, we found a unique phenotype in germ cells. Based on the published literature and our knowledge about *C. elegans* germ line, this phenotype has not been described before. Our genetic screening for the germ cell phenotype revealed that the *C. elegans* VprBP homolog ZK1251.9 may function as an SRS of CUL-4/DDB-1 to ensure the germ cell viability. We are currently investigating the molecular mechanism underlying this phenotype by analyzing putative suppressors.

The 'WDXR' motif proteins have been proposed to function as SRSs of CUL4/DDB1 complex. The presence of at least 36 'WDXR' motif proteins in the *C. elegans* genome suggests that there should be a number of uncharacterized phenotypes of *cul-4* or *ddb-1*. It is a promising avenue to analyze the 36 'WDXR' motif proteins and their genetic and biochemical interaction with CUL4/DDB-1 to provide new insights into the functions of CUL4/DDB1 E3 ligase complex. For example, RNAi depletion of *ZK430.7*, one of the 36 'WDXR' motif proteins, produces mild enlargement of seam cells, which may be an indication of re-replication or cell cycle arrest. Potentially, *ZK430.7* may have a role in the control of DNA replication by targeting other cell cycle regulator(s).

In summary, the research in this dissertation has provided mechanistic insights into how CUL-4/DDB-1 E3 ligase functions to precisely regulate DNA replication, a principle mission of the cell cycle. This work thereby contributes important pieces to a complete picture of cell cycle regulation. Furthermore, my

research provides a basis to understand uncharacterized functions of various CUL-4/RING-H2 E3 ligase complexes in *C. elegans*.

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