The *Caenorhabditis elegans* Replication Licensing Factor CDT-1 Is Targeted for Degradation by the CUL-4/DDB-1 Complex[▽]

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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. Two distinct ubiquitin ligase (E3) complexes, CUL4/DDB1 and SCF^{Skp2}, have been reported 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. Here, we show that *Caenorhabditis elegans* DDB-1 is required for the degradation of CDT-1 during S phase. DDB-1 interacts specifically with CUL-4 but not with other *C. elegans* cullins. A *ddb-1* null mutant exhibits extensive DNA rereplication in postembryonic BLAST cells, similar to what is observed in *cul-4(RNAi)* larvae. DDB-1 physically associates with CDT-1, suggesting that CDT-1 is a direct substrate of the CUL-4/DDB-1 E3 complex. In contrast, a deletion mutant of the *C. elegans* Skp2 ortholog, *skpt-1*, appears overtly wild type with the exception of an impenetrant gonad migration defect. There is no appreciable role for SKPT-1 in the degradation of CDT-1 during S phase, even in a sensitized *ddb-1* mutant background. We propose that the CUL-4/DDB-1 ubiquitin ligase is the principal E3 for regulating the extent of DNA replication in *C. elegans*.

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 (60). 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 (10, 34). DDB1 can function as an adaptor to link SRSs to the core CUL4 complex. In humans, DDB1 binds a heterodimeric SRS comprised of human DET1 (hDET1) and hCOP1 to target the degradation of the c-Jun transcription factor (80). Human DDB1 also binds the V proteins of paramyxoviruses, which act as SRSs in the degradation of STAT proteins (41, 75). 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 xeroderma pigmentosum protein XP-C; *Schizosaccharomyces 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 (20, 21, 44, 66, 79). 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 (26). In *C. elegans*, CUL-4 negatively regulates the Cdt1 ortholog, CDT-1, to prevent rereplication during S phase (84).

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 prereplicative complexes prior to S phase. Prereplicative complexes form on origins through the sequential binding of key DNA replication proteins: the six-member origin recognition complex, the replication licensing factors Cdt1 and Cdc6, and the presumptive replicative helicase, the Mcm2-7 complex (5, 47). Cdt1 and Cdc6 are essential loading factors for the Mcm2-7 complex, and they are regulated to ensure that the Mcm2-7 complex cannot rebind 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 (5, 14, 47).

In fission yeast and metazoa, Cdt1 is regulated by proteolysis at the onset of S phase and in response to DNA damage (3, 24, 26, 36, 57, 59, 73, 84). 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 rereplication (84). In mammals and *Drosophila*, expression of Cdt1 to high levels induces limited DNA rereplication (73, 77).

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 (36, 42). The binding of SCF^{Skp2} to Cdt1 is dependent on prior phosphorylation of Cdt1 by cyclin-

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dependent kinase (CDK)/cyclin complexes (46, 67). 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 (24, 26). 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 (3). 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 (58, 62). 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 nonviable 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 rereplication. 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.

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)*; ET281, *gmn-1(tm2212)/unc-64(e246)*; and VC1248, *gmn-1&Y75B8A18(ok1708)/hT2*. Plasmid pPD95.75/*ddb-1* was created by cloning genomic *ddb-1*, including 254 bp upstream of the translation start, into plasmid pPD95.75, which allows a C-terminal green fluorescent protein (GFP) fusion (primers used, GCCGCTGCA GTTCTTCTCGCTCATTTTAAAC and CGTCCCGGGGGTGCATTCT CGCCAAATCC). *ddb-1* RNA interference (RNAi) was performed by providing bacteria expressing *ddb-1* double-stranded RNA (dsRNA) to L4-stage larvae as a food source, as previously described (33). The *ddb-1* feeding protocol employed *Escherichia 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. *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 to 1.0 mg/ml as previously described (15).

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 *Saccharomyces cerevisiae* strain pJ69-4A (29) was performed as described previously (30). 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 and anti-CDK-inhibitor 1 (CKI-1) and monoclonal anti-CYE-1 (17C8) were as described previously (7, 15, 84). Anti-AJM-1 (MH27), which highlights gap junctions (37), 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 previously (53). For analysis of CDT-1 expression in L1 larvae at set times after hatching, 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 (84).

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, version 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 4',6'-diamidino-2-phenylindole (DAPI) staining (see Fig. 3B and 5) were deconvolved to equivalent extents to minimize background fluorescence using the multi-neighbor deconvolution program of Openlab.

Quantitation of DNA content. DNA content was measured using previously described methods (16, 22). In brief, single digital images were taken of nuclei stained with 1 µg/ml of Hoechst 33258. Every image included either 2n postmitotic 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 is equivalent to 1C (70).

Ectopic expression of CDT-1::GFP and Pmr-1::RFP. To generate extragenic transgenic lines, we microinjected into the strain PS3729 (which expresses AJM-1::GFP) N2 genomic DNA (200 µg/ml), the marker plasmid pRF4 containing *rol-6(su1006)* (5 µg/ml) (49), the S-phase marker plasmid pRED95.67/*Pmr-1* (5 µg/ml), and either pPD95.75/*Pwrt-2-cdt-1WT* (5 µg/ml) or pPD95.75/*Pwrt-2-cdt-1PIP(6A)* (5 µg/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 (52). 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 proliferating cell nuclear antigen (PCNA)-interacting protein (PIP) box was introduced using an overlap extension PCR method with the following primer pairs (lowercase letters indicate altered nucleotides): CCGCATGCTTA CGACGAATAATTTTATTGAATTTTG and GcagCAGCAGTCgcCGGAGT CTGGGACC and gcGACTGCTGctGtGcGcGtGctAAGGTAAATTGGAGTT TGAAG and CTTCACGAGATTCGGTC. Plasmid pRED95.67 was created by replacing the GFP cDNA sequence of pPD95.67 with red fluorescent protein (RFP) cDNA from plasmid pPD158.114. The *mr-1* promoter and simian virus 40 nuclear localization signal sequences, which came from pVT501, were introduced into SphI/AgeI sites of pRED95.67 to create plasmid pRED95.67/*Pmr-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 a 60-h incubation at 37°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-leucyl-L-leucinal-L-norleucinal (Sigma). To minimize proteasome-mediated degradation, transfected cells were treated with 50 µM *N*-acetyl-L-leucyl-L-leucinal-L-norleucinal 10 h 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 (84). The secondary antibodies used were anti-rabbit-horseradish peroxidase (Pierce) and anti-mouse-horseradish peroxidase (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 (64). 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 previously described method (17). Briefly, bacteria were induced with 1 mM isopropyl-β-D-thiogalactopyranoside for 4 h at 37°C, pelleted, and resuspended in STE medium (10 mM Tris, pH 8.0, 150 mM NaCl, and 1 mM EDTA) containing 100 µg/ml of lysozyme. *N*-Laurylsarcosine (sarcosyl) was added to a final concentration 0.5% to solubilize the recombinant CDT-1 proteins. After a 1-h 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/cyclin B (Upstate) in phosphorylation buffer (40 mM Tris, pH 7.4, 5 mM MgCl₂, 1 mM dithiothreitol, 500 µM ATP, and 1 µM okadaic acid) for 90 min at 30°C. ³⁵S-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 a TNT T7/T3 Coupled Wheat Germ Extract System (Promega). ³⁵S-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 ³⁵S-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 a washing step, proteins associated with the beads were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis followed by autoradiography. Ten percent of the ³⁵S-labeled FLAG-DDB-1 or SKPT-1-MYC proteins that were used for each binding reaction was loaded in input lanes.

RESULTS

C. elegans has a single DDB1 ortholog, *ddb-1*, which is located on chromosome IV (Fig. 1A). The DDB-1 protein has significant sequence identity across its entire length with orthologous DDB1 proteins, displaying 37% amino acid identity with *Homo sapiens* DDB1, 37% for *Xenopus laevis*, 36% for *Drosophila 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 (2, 21, 26, 45, 76, 80). Consistent with this, we observed that DDB-1 interacts specifically with CUL-4, but not with other cullins, in the yeast two-hybrid assay (Fig. 1D).

DDB-1 developmental expression pattern. To determine the DDB-1 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 (Fig. 2A to D and 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 DDB-1::GFP in newly hatched L1 larvae; however, expression was observed later in the L1 stage as the cells began to proliferate (Fig. 2B). During the L2 stage, the P-cell descendants 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 descendants that create the vulva (the vulval precursor cells [VPCs]), as they move from quiescence to proliferation (12), and expression continues through the L4 stage (Fig. 2C and D). In adults, all P cell descendants 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 (Fig. 2F and G). Additionally, adult hermaphrodites exhibit high-level expression in the spermatheca (Fig. 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 (35). 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 postembryonic blast cells. DDB-1 is also expressed in a subset of nonproliferative cells, suggesting that it may have functions unrelated to cell cycle progression.

FIG. 1. Deletion alleles of the *ddb-1*, *cul-4*, and *skpt-1* genes and two-hybrid interaction between DDB-1 and CUL-4. (A to C) Line drawings of the *ddb-1*, *cul-4*, and *skpt-1* genomic regions on chromosomes 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. 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 (A). The *cul-4(gk434)* allele is a 580-bp deletion that removes 305 bp of the promoter region and the complete exons 1 and 2 (B). 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 (C). 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 medium. DDB-1 was fused to the GAL4 activation domain, and the cullins were fused to the GAL4 DNA binding domain.

***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 (Fig. 1A). *ddb-1(tm1769)* is recessive, and homozygotes are nonviable, 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 (see Fig. 4D). The timing of larval development is significantly slower for homozygous *ddb-1(tm1769)* mutants than for wild-type larvae (81.3 ± 5.6 h versus 64.8 ± 3.9 h from laid eggs to adult at 20°C; $n = 10$ for each group; $P < 1 \times 10^{-6}$).

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 h after the start of the RNAi treatment were analyzed. A small percentage of *ddb-1(RNAi)* progeny (7/149, 5%) arrested as late, pretzel-stage embryos. The remaining *ddb-1(RNAi)* embryos hatched normally but then

FIG. 2. Developmental expression of *ddb-1*. For all panels A to H, the DIC image is on top, and the GFP epifluorescence image of the 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 descendants expressing DDB-1::GFP. (D) L4-stage larva showing expression in vulval cells after the completion of VPC divisions. (E to 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; Sp, spermathecal cells; RG, rectal gland cells; RE, rectal epithelial cell; and I, intestinal cells. Scale bar, 10 μ m.

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 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 rereplication. Loss of *cul-4* causes proliferating blast cells to undergo DNA rereplication, resulting in dramatically enlarged nuclei and increased DNA content (84). We observed that arrested *ddb-1(tm1769)* homozygotes have similarly enlarged seam cells (Fig. 3A). Other blast cell lineages also exhibit increased size; however, nonproliferating cells are not affected (data not shown). The enlarged *ddb-1* mutant cells show markedly increased DNA levels (25.9 ± 15.4 versus 2.0 ± 0.2 C in wild type; $n = 20$ for each group; 1 C is equivalent to $1n$ DNA content; $P < 1 \times 10^{-7}$) (Fig. 3B), suggesting that DDB-1 prevents DNA rereplication, 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 (84). 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 allele (Fig. 1B). The *cul-4(gk434)* allele is recessive and homozygotes are nonviable. *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 (Fig. 3A). 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 and germ line development is defective in *ddb-1*-deficient animals. To investigate whether *ddb-1* mutants that develop to the adult stage have defects associated with rereplication, we focused on the late-dividing vulval lineage. The vulva is composed of 22 cells that are generated from three progenitor VPCs during the L3 stage (68). *ddb-1(tm1769)* homozygotes have a significantly reduced number of vulval cells (11.0 ± 1.2 ; $n = 23$ versus 22 in wild type) (68), and these cells are enlarged (Fig. 4A and B). *ddb-1* mutants exhibit abnormal vulval morphology during larval development and protruding vulvae in adults (Fig. 4A to D), similar to defects associated with inadequate vulval cell numbers (11, 63). DNA levels in *ddb-1* mutant vulval cells are significantly increased (7.2 ± 1.4 versus 2.1 ± 0.4 C in wild type; $n = 14$ for each group; $P < 1 \times 10^{-9}$), suggesting that the enlargement of vulval cells results

FIG. 3. Loss of DDB-1 and CUL-4 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)*, *cul-4(gk434)*, and *cul-4(gk434);cki-1(RNAi)* arrested L2-stage larvae (3 days posthatching). Se, seam cell nuclei; H, hyp7 cell nuclei. (B) Seam cell nuclei of wild-type L2-stage larvae, and *ddb-1(tm1769)*, *cul-4(gk434)* and *cul-4(gk434);cki-1(RNAi)* arrested L2-stage larvae (3 days posthatching), stained with DAPI (blue) and anti-CDT-1 (green). Anti-AJM-1 staining (red), which highlights gap junctions (37), was overlaid to mark seam cell boundaries. (C) Seam cell nuclei of wild-type and arrested *ddb-1(RNAi)* L3-stage larvae, stained with DAPI (blue), anti-CKI-1 (green), and anti-CYE-1 (red). Scale bar, 10 μ m.

from DNA rereplication (Fig. 4I to L). Somatic gonadal cells also divide during the L3 stage, and we observed that these cells are also larger and contain increased DNA levels (Fig. 4A, B, and I to L).

Germ cells in *ddb-1(tm1769)* mutants exhibit vacuoles and corrupted cell and nuclear morphology during the L4 stage and

FIG. 4. Loss of zygotically expressed *ddb-1* causes defects in late developmental stages. (A to 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 panel B indicate enlarged vulval cells in *ddb-1(tm1769)* vulva. (E to H) DIC image of germ line in wild-type (E) and *ddb-1(tm1769)* (F) young adults. The region containing germ cells enclosed within the white boxes in panels E and F are shown magnified in panels G and H, respectively. (I to L) DAPI staining of wild-type (I) and *ddb-1(tm1769)* mutant (J) young adults, mid to posterior region shown. The vulval regions encompassed in the white boxes in panels I and J are magnified in panels K and L, respectively. Scale bars, 10 μ m.

often undergo necrosis in older adults (Fig. 4E to H; also data not shown). Spermatogenesis is observed in *ddb-1(tm1769)* mutants, and oogenesis occurs infrequently, suggesting that meiotic entry per se is not defective (Fig. 4E, F, I, and J). 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). There is,

FIG. 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 posthatching) and S phase (180 min posthatching), 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, 10 μ m.

however, no clear link between these 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 $2.2 \pm 0.4 C$ while *ddb-1(tm1769)* germ cells have $2.6 \pm 0.6 C$ ($n = 19$ for each group). This suggests that DDB-1 is required for germ cell viability independently of its role in regulating DNA replication.

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 rereplication does not occur (5, 47). In *C. elegans*, CUL-4 is required for CDT-1 degradation during S phase (84). We asked whether DDB-1 is similarly required for CDT-1 degradation during S phase. The V1 to V6 seam cells enter S phase at approximately 120 min posthatching at 20°C (84). Immunofluorescence staining with anti-CDT-1 antibody shows that CDT-1 is present in late G₁ phase nuclei (90 to 110 min posthatching) in both wild-type and *ddb-1(RNAi)* seam cells (Fig. 5A and 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 to 200 min posthatching (Fig.

5A) (84). 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 to 200 min posthatching, indicating that DDB-1 is required for CDT-1 degradation during S phase (Fig. 5B).

We observed a similar requirement for DDB-1 in the degradation of a CDT-1::GFP fusion protein during S phase (Fig. 6B and 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 (*Pmr-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 (Fig. 6B). The observed requirement of DDB-1 for CDT-1 degradation during S phase is similar to that observed for CUL-4 (84), strongly suggesting that DDB-1 functions with CUL-4 to negatively regulate DNA replication in vivo.

In humans and *Xenopus*, PCNA is required for CUL4/DDB1-mediated CDT-1 degradation at the onset of S phase or

FIG. 6. PIP box of CDT-1 is required for degradation 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 sites (red), and potential CDK phosphorylation sites (lines on top). 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 to D) Expression of CDT-1^{WT}::GFP with no treatment (B) or with *ddb-1* RNAi treatment (C), and CDT-1^{PIP(6A)}::GFP with no RNAi treatment (D). CDT-1::GFP epifluorescence image is on top and the *Pmr-1*::RFP epifluorescence image is below. G₁ phase seam cells (90 min posthatching) are shown on the left, and S-phase seam cells (180 min posthatching) are shown on the right. Seam cells are labeled. 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. The cells surrounding the seam cells are hypodermal hyp7 cells. In hyp7 cells, *Pmr-1*::RFP expression arises during embryonic cell divisions and perdures through 180 min posthatching, due to the stability of RFP. Therefore, because of the perdurance, *Pmr-1*::RFP expression cannot be used as an S-phase marker in hyp7 cells at 90 or 180 min posthatching. Scale bar, 10 μ m.

in response to DNA damage (2, 27, 58, 62). PCNA interacts with Cdt1 through a PIP box in Cdt1. Cdt1 mutant proteins that lack the PIP box are unable to be degraded by the CUL4/DDB1 complex (2, 58, 62). 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 (Fig. 6B and D), indicating that *C. elegans* CDT-1 degradation requires an intact PIP box.

In vertebrates and *Drosophila*, Cdt1 is also inhibited by binding to geminin (51, 61, 71, 81). 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 rereplication in germ cells (82). We investigated the possibility that *ddb-1* mutant germ cells failed to undergo rereplication because of redundant regulation by GMN-1. We analyzed a *gmn-1*(*tm2212*) allele that deletes 236 bp of the *gmn-1* locus, including conserved residues that are required for binding CDT-1 (38). *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 nonviable or sterile (data not shown). We observed that a subset of germ cells is enlarged in *gmn-1*(*tm2212*) homozygotes, similar to what was previously reported for *gmn-1* RNAi (82). Unexpectedly, DAPI staining of the *gmn-1*(*tm2212*) mutant gonad indicated that DNA levels are not increased in the enlarged germ cells, suggesting that rereplication is not occurring (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 (data not shown). Further, we did not observe marked differences in the level of CDT-1 within the mitotic germ cells of wild type or *ddb-1*(*tm1769*) mutants (data not shown). This suggests that a CDT-1-independent pathway prevents rereplication in germ cells.

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 (26, 75, 80). 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 coimmunoprecipitated each other (Fig. 7A). We also tested for interaction between DDB-1 and CDT-1 using an in vitro system. ³⁵S-labeled FLAG-tagged DDB-1, which was in vitro translated using a wheat germ extract and then was affinity purified, specifically bound to purified, recombinant GST-tagged CDT-1 in vitro (Fig. 7C). 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 (26). It also has been proposed that cyclin E/CDK2 phosphorylation of the *Drosophila* Cdt1 ortholog promotes its degradation at the G₁/S-phase transition (73). 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 ³⁵S-labeled FLAG-DDB-1 in vitro (Fig. 7C), suggesting that CDK phosphorylation of CDT-1 is not essential for CUL-4/DDB-1 mediated degradation.

CUL-4/DDB-1 negatively regulates CKI-1 and CYE-1 protein levels. Recently, it has been reported that CUL4/DDB1 promotes the degradation of the CDK-inhibitor p27^{KIP1} and cyclin E in humans and *Drosophila* (6, 25, 40). To determine whether *C. elegans* CUL-4/DDB-1 negatively regulates the

FIG. 7. DDB-1 physical interaction with CDT-1. (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/coexpression. Anti-CDT-1 immunoprecipitation (IP), anti-FLAG IP, or anti-MYC IP were analyzed by Western blotting 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 sodium dodecyl sulfate-polyacrylamide gel electrophoresis, similar to what is observed for ectopic expression of human Cdt1 in 293T cells (26). (B) SKPT-1-MYC binding to CDT-1 in 293T cells was performed similar to the method in panel A with the immunoprecipitations (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 immunoprecipitation (lanes 6 to 10, bottom panel) rather than the monoclonal anti-MYC antibody that is used in lanes 1 to 5, because SKPT-1-MYC runs at the same position as the mouse immunoglobulin G heavy chain. (C) DDB-1 and SKPT-1 binding to CDT-1 in vitro. ³⁵S-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. On the left are autoradiographs showing in vitro translated ³⁵S-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. On the right is a Coomassie-stained gel of the GST, GST-CDT-1, and phosphorylated GST-CDT proteins that were used in the binding assay. On the far right is a graph of the percentage of total input of ³⁵S-labeled FLAG-DDB-1 and MYC-SKPT-1 bound by GST-CDT-1 or GST-CDT-1P.

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 anti-CYE-1 antibodies showed accumulation of both proteins in the enlarged seam cell nuclei of *ddb-1(RNAi)*-arrested larvae (Fig. 3C). 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/rereplication.

We tested whether CKI-1 accumulation contributes to the rereplication. 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 approximately threefold relative to *cul-4(gk434)* larvae ($36.2 \pm 14.0 \mu\text{m}^2$ versus $113.5 \pm 52.7 \mu\text{m}^2$ [$n = 20$ for each group], respectively; wild type is $11.8 \pm 0.7 \mu\text{m}^2$; $n = 6$). The seam cell DNA level was reduced ~ 6.7 -fold [3.8 ± 1.7 C for *cki-1(RNAi) cul-4(gk434)* versus 25.6 ± 11.3 C for *cul-4(gk434)*; $n = 6$ each]. This indicates that *cki-1* RNAi depletion

suppresses the rereplication phenotype of *cul-4* mutants (Fig. 3A and B). Significantly, the *cki-1(RNAi) cul-4(gk434)* larvae still accumulated CDT-1 protein in seam cells (Fig. 3B), indicating that CKI-1 is not required for the accumulation of CDT-1 and that CDT-1 accumulation is not sufficient to induce extensive rereplication. We could not perform a similar test to determine if CYE-1 is required for rereplication, because *cye-1* RNAi produces an embryonic arrest that precludes analysis of larval cells (13).

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 (42, 46, 58, 67). *C. elegans* has only one *Skp2* ortholog, *skpt-1* (*Skp-two* related protein) (Fig. 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 sug-

FIG. 8. *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. Bar, 10 μ m (B) Graph of the average number of V1 to V6 seam cells with CDT-1 expression at specific times posthatching for wild type (green squares) and *skpt-1(ok851)* mutants (red triangles).

gesting that it is a null allele. *skpt-1(ok851)* homozygotes are viable and produce similar numbers of eggs compared to wild type (277 ± 26 versus 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 (Fig. 8A). *skpt-1* RNAi produces a similar level of gonad migration defects (7%; 13/180) (H. Jin and E. T. Kipreos, 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 the *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 (Fig. 5A and C and 8B). 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 rereplication, we asked whether the coelimination 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 rereplicating seam cells ($107.2 \pm 48.6 \mu\text{m}^2$ and 112.6 ± 48.0

μm^2 , respectively; $n = 34$ for each group), their DNA levels (25.9 ± 15.4 and 21.7 ± 20.0 C, respectively; $n = 20$ each), and their CDT-1 levels (data not shown) were comparable. 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 their 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 nonspecific F-box protein, LIN-23 (Fig. 7B). In contrast, the interaction of CDT-1 and DDB-1 was considerably stronger than the interaction between DDB-1 and LIN-23 (Fig. 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 (Fig. 7C). Skp2 recognizes only CDK-phosphorylated substrates (60). Significantly, phosphorylation of CDT-1 by CDK did not increase interaction with SKPT-1 (Fig. 7C), 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.

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 ex-

pression correlates with cell cycle progression in the P blast cell lineage. The P blast cells and their descendants express DDB-1 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 nonproliferative 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 rereplication with large, polyploid blast cells, similar to what we have previously described for *cul-4*(RNAi) larvae (84) 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.

We had previously observed rereplication in a wide range of somatic blast cells in arrested *cul-4*(RNAi) larvae (84). Our current data suggest 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. Interestingly, we did not observe rereplication defects in germ cells or embryos upon loss of *ddb-1* or *cul-4* function. However, germ cells in *ddb-1* mutants or *cul-4*(RNAi) animals have defects in proliferation and aberrant morphology, suggesting that the CUL-4/DDB-1 complex plays a critical role in these cells independently of restraining DNA replication.

It is possible that a redundant regulatory mechanism could be preventing rereplication in *ddb-1* mutant germ cells. In vertebrates and *Drosophila*, Cdt1 is inhibited by binding to geminin (51, 61, 71, 81). The *C. elegans* geminin ortholog, GMN-1, has been shown to bind CDT-1 in both the two-hybrid system and in vitro (82). *gmn-1* RNAi was reported to produce enlarged germ cells (82). 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 rereplication in *cul-4* or *ddb-1* mutant germ cells is not attributable to redundant regulation by geminin.

Mammalian and *Drosophila* CUL4 promote the degradation of cyclin E and the CKIs p27^{Kip1} and Dacapo, respectively (25, 40). We observed that levels of both CKI-1 and cyclin E accumulate in the rereplicating blast cells of *ddb-1*(RNAi) larvae. Significantly, *cki-1* RNAi depletion was able to rescue the rereplication phenotype of *cul-4* mutants, indicating that CKI-1 is essential for rereplication to occur. The role that CKI-1 plays in promoting rereplication is currently unknown but may involve arresting the cells in S phase. *C. elegans cul-4* mutant rereplicating cells are characterized by both an S-phase arrest and dramatic increases in ploidy (84); however, an S-phase arrest is not observed in rereplicating mammalian cells, which have only limited increases in ploidy (77). It is significant

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 rereplication or CKI-1 accumulation.

CDT-1 is a direct target of CUL-4/DDB-1. We had previously shown that CUL-4 is required for the degradation of CDT-1 in S phase (84). We also reported that the deletion of one copy of genomic *cdt-1* can significantly suppress the rereplication phenotype of *cul-4*(RNAi) larvae, suggesting that CDT-1 perdurance is a decisive factor in causing the rereplication. 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.

The crystal structure of DDB1 reveals a multidomain structure consisting of three β -propellers that have multiple potential protein-protein interaction sites that could, in principle, allow DDB1 to bind to many substrates or SRSs (1, 41). A number of proteins have been identified as DDB1 interactors in the context of the CUL4 complex (1, 20, 21, 32, 44, 66, 75, 80), suggesting that in many instances DDB1 functions as an adaptor to bind SRSs. Two proteins that bind mammalian DDB1 (hDET1-COP1 and paramoxyvirus V proteins) have been shown to function as SRSs that directly bind the substrate (75, 80). In more cases, potential SRSs have been linked to specific CUL4/DDB1 functions (20, 21, 23, 32, 44, 66, 79).

In humans, *Xenopus*, and *Drosophila*, CDT2/L2DTL binds to DDB1 and has been shown to be required for CDT1 degradation in response to DNA damage (23, 32). It is possible that CDT2/L2DTL is the SRS that binds CDT1 to the complex. However, this has not yet been completely established, as there is no report that CDT2/L2DTL can bind CDT1 directly. Another possibility is that CDT2/L2DTL is a cofactor for the CUL4/DDB1 complex. A well-studied cofactor for a cullin-RING complex is Cks1, which facilitates the binding of the substrate, p27^{Kip1}, to the SCF complex SRS Skp2 (18, 65). Intriguingly, in the absence of CDT2/L2DTL, DDB1 binding to CUL4 in human cells is significantly reduced (23), suggesting that CDT2/L2DTL may regulate the formation of the CUL4/DDB1 complex.

We observed that *C. elegans* DDB-1 and CDT-1 can physically associate with each other when coexpressed 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 cell 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 (26), indicating that in humans, the two proteins can interact directly. Our data in two different expression systems are consistent with this result.

The *C. elegans* Skp2 homolog is not required for CDT-1 degradation. It has been reported that the human SCF^{Skp2} E3 complex targets Cdt1 for degradation and that depletion of

Skp2 by treatment with small interfering RNA increases Cdt1 levels, particularly in S phase (42). SCF^{Skp2} binds substrates that are phosphorylated by CDK/cyclin kinases, and the mutation of CDK phosphorylation sites on Cdt1 has been reported to partially stabilize Cdt1 in S phase (46, 67). In contradiction, two other studies report that mutation of CDK phosphorylation sites on Cdt1 does not affect S-phase degradation (58, 72). Recently, it has been reported that both SCF^{Skp2} and CUL4/DDB1 E3 ligases are redundantly required for the complete degradation of Cdt1 in S phase (58, 62). Taken together, these results suggest that in humans, both SCF^{Skp2} and CUL4/DDB1 E3 ligases contribute to Cdt1 degradation.

One report has indicated that human Skp2 can coimmunoprecipitate CUL4/DDB1 and that Skp2 is essential for CUL4/DDB1-mediated degradation of the CKI p27^{KIP1} (6). 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, *C. elegans* SKPT-1 and DDB-1 do not appear to interact in the 293T system beyond low background levels that are also seen between DDB-1 and the nonspecific F-box protein LIN-23 (data not shown).

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 migrates in response to extracellular guidance cues and leads the developing gonad into its final shape (39). It is presently unclear whether SKPT-1 functions cell autonomously in the distal tip cell 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, nonredundant 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 rereplicating cells, their DNA content, or CDT-1 levels. This suggests that SKPT-1 has no discernible redundant function with DDB-1 to degrade CDT-1. Coimmunoprecipitation 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 nonspecific interaction between LIN-23 and CDT-1.

It is an open question to what extent Skp2-dependent Cdt1 degradation is conserved across phyla or even among species of the same evolutionary class. The degradation of Cdt1 by Skp2 has only been reported in human cells. Small interfering RNA depletion of human Skp2 produces an approximately threefold increase in the level of Cdt1 (42, 58). In contrast, depletion of Skp2 in mouse cells has no effect on Cdt1 levels (56, 58). Human Skp2 has been reported to degrade a host of proteins, including Cdt1, Orc1, p27^{KIP1}, p21^{CIP1}, cyclin E, cyclin D, cyclin A, c-Myc, b-Myb, p130, E2F-1, p57, MKP-1, RAG-2, FOXO1, and possibly Cdk9, although this has been disputed (4, 8, 9, 19, 28, 31, 43, 48, 50, 69, 74, 78, 83). Nevertheless, Skp2^{-/-} mice are completely viable and fertile (55).

Skp2^{-/-} mice exhibit a minor defect of polyploidy and extra centrosomes in the cells of a few tissues, but this is attributable to a failure of those cells to enter mitosis due to an inability to degrade p27^{KIP1} (54, 56). The lack of phenotypes associated with a failure to degrade other substrates suggests either that many Skp2 functions are not conserved between humans and mice or that mammalian Skp2 functions are largely redundant with other degradation pathways. Our findings demonstrate that Skp2-mediated degradation of Cdt1 is not conserved in nematodes, thereby highlighting the question of the pathway's conservation in other metazoa.

Overall, our study indicates that the requirement for two distinct ubiquitin ligases to degrade CDT-1 is not conserved in *C. elegans*; rather, CUL-4/DDB-1 is the paramount E3 for CDT-1 degradation during S phase. The conservation between nematodes and humans in the degradation of the replication licensing factor CDT-1 by a CUL4/DDB1 complex suggests that this is an ancient pathway for regulating the extent of DNA replication.

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