Cell Type–dependent Requirement for PIP Box–regulated Cdt1 Destruction During S Phase

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DNA synthesis–coupled proteolysis of the prereplicative complex component Cdt1 by the CRL4Cdt2 E3 ubiquitin ligase is thought to help prevent rereplication of the genome during S phase. To directly test whether CRL4Cdt2-triggered destruction of Cdt1 is required for normal cell cycle progression in vivo, we expressed a mutant version of Drosophila Cdt1 (Dup), which lacks the PCNA-binding PIP box (Dup^APIP) and which cannot be regulated by CRL4Cdt2. Dup^APIP is inappropriately stabilized during S phase and causes developmental defects when ectopically expressed. Dup^APIP restores DNA synthesis to dup null mutant embryonic epidermal cells, but S phase is abnormal, and these cells do not progress into mitosis. In contrast, Dup^APIP accumulation during S phase did not adversely affect progression through follicle cell endocycles in the ovary. In this tissue the combination of Dup^APIP expression and a 50% reduction in Geminin gene dose resulted in egg chamber degeneration. We could not detect Dup hyperaccumulation using mutations in the CRL4Cdt2 components Cul4 and Ddb1, likely because these cause pleiotropic effects that block cell proliferation. These data indicate that PIP box–mediated destruction of Dup is necessary for the cell division cycle and suggest that Geminin inhibition can restrain Dup^APIP activity in some endocycling cell types.

INTRODUCTION

Accurate genome duplication during cell cycle progression requires assembly of a prereplicative complex (pre-RC) at origins of DNA replication. Pre-RCs contain the origin recognition complex (ORC), Cdc6, and Cdc10-dependent transcript1 (Cdt1) proteins, which assemble at origins during late mitosis/G1 and recruit the minichromosome maintenance complex (MCM2–7), a core component of the replicative DNA helicase (Bell and Dutta, 2002). After DNA synthesis is initiated, pre-RC components are displaced from the chromatin and prevented from reassembling until the next G1 via multiple mechanisms including nuclear export, inhibitory phosphorylation, and ubiquitin-mediated proteolysis (Arias and Walter, 2007).

Preventing pre-RC assembly and reloading of the MCM complex within S phase is crucial to prevent rereplication, which can cause DNA damage and genomic instability that may contribute to cancer (Petropoulou et al., 2008). Negative regulation of Cdt1 is a key aspect of pre-RC assembly in metazoans, as increased Cdt1 activity is sufficient to trigger rereplication in many situations (Zhong et al., 2003; Arias and Walter, 2005; May et al., 2005; Arias and Walter, 2006; Sansam et al., 2006). Moreover, recent experiments in mice suggest that Cdt1 overexpression may promote tumor formation or progression (Arentson et al., 2002; Seo et al., 2005; Lioniots et al., 2007; Petropoulou et al., 2008). Metazoan Cdt1 activity is negatively regulated by two mechanisms: regulated proteolysis and binding to the protein Geminin (Arias and Walter, 2007). Geminin blocks the ability of Cdt1 to load the replicative helicase at origins, most likely because the Geminin and MCM2–7 binding domains of Cdt1 overlap (Yanagi et al., 2002; Cook et al., 2004; Lee et al., 2004; Saxena et al., 2004; De Marco et al., 2009). Studies in mammalian and Drosophila cells have shown that the loss of Geminin function can cause rereplication, indicating that this inhibitory mechanism is required for normal genome duplication in some cell types (Melixetian et al., 2004; Zhu et al., 2004; Hall et al., 2008).

After origins are licensed, Cdt1 is rapidly destroyed upon the onset of DNA replication via ubiquitin-mediated proteolysis (Kim and Kipreos, 2007b). Cdt1 proteolysis is controlled by two members of the Cullin-RING family of E3 ubiquitin ligases (CRL): CRL1 (aka SCF) and CRL4 (Deshayes and Joazeiro, 2009). These two ligases utilize different mechanisms for targeting Cdt1. Phosphorylation of Cdt1 by S phase cyclin-dependent kinases (e.g., cyclin E/Cdk2) is mediated by a conserved cyclin binding (Cy) motif and triggers ubiquitylation by CRL1SKp2 (Nishitani et al., 2001, 2006; Li et al., 2003; Kondo et al., 2004; Liu et al., 2004). CRL4CaR2 directs replication-coupled destruction of Cdt1 through a degron at the Cdt1 NH2-terminus containing a motif called a PIP (PCNA-interacting polypeptide) box. The PIP box confers direct binding to PCNA at replication forks after the initiation of S phase, and the PIP box–containing degron recruits CRL4CaR2 for ubiquitylation and subsequent destruction of Cdt1 (Higa et al., 2003, 2006a; Hu et al., 2004;
Arias and Walter, 2006; Hu and Xiong, 2006; Jin et al., 2006; Ralph et al., 2006; Senga et al., 2006; Hall et al., 2008; Havens and Walter, 2009). In human cells these pathways act redundantly, as mutations in both the PIP box and Cy domains are necessary to stabilize Cdt1 in S phase (Nishitani et al., 2006). In other situations there appears to be no redundancy between these ligases. For instance, Cul4 loss of function in Caenorhabditis elegans causes Cdt1 hyperaccumulation and rereplication (Zhong et al., 2003; Kim and Kipreos, 2007a). Cdt1 is also destroyed after DNA damage, and CRL4 deple- tion or mutations in the PIP box block this destruction in fission yeast, Drosophila, and mammalian cells (Higa et al., 2003, 2006a; Hu et al., 2004; Hu and Xiong, 2006; Ralph et al., 2006; Hall et al., 2008).

The degree of redundancy or cell-type specificity between CRL- and Geminin-mediated inhibition of Cdt1 during animal cell development is not completely understood. For instance, Geminin is sufficient for Cdt1 regulation in all cell types, cell cycle progression should not be affected when Cdt1 destruction is inhibited. To test the significance of Cdt1 destruction during development, we studied the Drosophila melanogaster homolog of Cdt1, double parked (Dup). Dup is required to initiate DNA replication (Whittaker et al., 2000) and is degraded promptly upon S phase entry (Thomer et al., 2004; May et al., 2005). Dup contains a Cy domain that is important for its normal function and mediates regulation by cyclin E/Cdk2 (Thomer et al., 2004) as well as a conserved PIP box whose function has yet to be specifically studied (Figure 4).

Although many previous studies have focused on the molecular mechanisms of Cdt1 regulation, they have not directly addressed whether loss of CRL4(Cdt2) regulation of Cdt1 disrupts cell cycle progression in vivo. We took advantage of the well-characterized dup null mutant phenotype (Whittaker et al., 2000) to test whether a mutant version of Dup protein lacking the PIP box could provide normal function in the absence of endogenous Dup. Our results indicate that PIP box-dependent regulation is necessary for rapid Dup destruction during S phase and for normal progression of the embryonic cell division cycle, but not for normal endocycle progression in a cell type whereGem function can compensate for Dup stabilization in S phase. Thus, specific cell types depend on different modes of Cdt1 regulation during normal animal development.

MATERIALS AND METHODS

Fly Stocks

Stocks carrying Cul4 mutant alleles EP2518 and KG02900, and Ddb1/Hpc10 mutant alleles FY01408, pic2, and pic2/2 in the ’10874 line were obtained from the Bloomington Stock Center (Bloomington, IN). The Dbl1/pic2/2 line was obtained from the Szeged Stock Center (Szeged, Hungary). gem2/H9262 was a gift from Helen Richardson (University of Melbourne, Australia; Quinn et al., 2001). pic2/2 resulted from an x-ray-induced rearrangement, leaving a large segment of genomic DNA inserted within the Dbl1 locus (Scott et al., 1985; Clark and Chovnick, 1986). Publicly available sequence flanking the SO26316 P element insertion corresponds to the 5′ UTR of Dbl1 (Flybase ID FBti025527; Deak et al., 1997). The pic x-ray allele contains an Asp substitution for the well-conserved Gly21 (Hu et al., 2008) positioned at a turn in propeller A of the WI-38 p53 C terminus (Krokan et al., 1987).

P-Element Excision–mediated Mutagenesis

The EP2518 P-element in the 3′ UTR of Cul4 was mobilized by crossing to w0; w P-element excision. In Df(2R) X299B/1TM6 flies, resulting males were crossed to P[w1]cyO flies, and three EP2518 excision events were identified from ~400 w0 progeny as novel Cul4 mutant alleles by failure to complement Cul4/H11001, Cul4/H11002 lethality was reverted after precise excision of the KG02900 P-element. The breakpoints of Cul4HG02900, Cul4HG02900, and Cul4HG02900 were determined by sequencing. Note that in Hu et al. (2008) the amount of truncation in Cul4HG02900 allele was incorrectly indicated as that of Cul4HG02900. The EY01408 P-element in the 5′ UTR of Dbl1 was similarly mobi-
fixed in 5% formaldehyde/PBS, and permeabilized in 0.5% Triton-X for 30 min. To expose BrdU epitope, dissected ovaries were treated with 30 U/µl DNaseI (Fermentas, Hanover, MD). Stained tissues were analyzed using a Zeiss 510 confocal microscope (Thornwood, NY).

**Microscopic Quantification of Dup-GFP Expression**

DupFL or Dup6AP were expressed in embryos and ovaries using prd-Gal4 and 323a-Gal4, respectively, stained as described above, and imaged at the same time. Adobe Photoshop was used to measure DAPI and GFP intensity in a single confocal section from five randomly chosen cells from five different embryos or five different egg chambers. GFP values were normalized to DAPI intensity with average and SD reported. p values were derived using a paired Student’s t test.

**RESULTS**

**Isolation and Molecular Characterization of Drosophila Cul4 and Ddb1 Mutants**

We began testing whether CRL4Cdt2 regulates Dup accumulation during S phase by analyzing mutant alleles of the single Drosophila Cul4 and Ddb1 genes that we had previously identified (Hu et al., 2008). We first characterized these alleles molecularly. For Cul4, we generated three lethal alleles by imprecise excision of the viable Cul4EP2518 P-element insertion: Cul46AP, Cul411L, and Cul411R (Figure 1A). All three Cul4 excision mutants arrested during development as first instar larvae, either as homozygotes, in trans to each other, or over a deficiency (Df(2R)CA53) that deletes Cul4. The Cul46AP lethal allele is less severe, and Cul411R/Df(2R)CA53 mutants arrest as second instar larvae. Although Cul4 mutants display early developmental arrest, they do not die and can survive for at least a week without growing (Hu et al., 2008).

We generated an antibody specifically recognizing the NH2-terminus of fly Cul4 and detected full-length Cul4 and neddylated Cul4 in cultured S2 cells and wild-type (WT) first instar larvae (Figure 1B, lanes 1 and 2), but not in Cul411L, Cul411R, or Cul46AP mutant larvae (Figure 1B, lanes 4–7). Cul4KG02900 mutants expressed reduced levels of full-length Cul4, although the ratio of neddylated to unneddylated Cul4 was increased relative to WT larvae (Figure 1B, lane 3). Sequencing of the breakpoints of each excision mutant predicts open reading frames encoding a C-terminal deletion of 18 residues in Cul411L, 65 residues in Cul46AP, and 82 residues in Cul411R (Figure 1A). Truncated proteins corresponding to the predicted molecular weights were detected in both Cul411R and Cul46AP mutants as a single species (Figure 1B, lanes 6 and 7), whose stability may be partly attributable to an inability to be neddylated (Wu et al., 2005). The Cul411R allele produced very little if any protein as assessed by Western blot and is likely null (Figure 1B, lanes 4 and 5). All three truncation mutants retain the Roc1a binding site, but lack a highly conserved C-terminal domain that is also required for the function of Drosophila Cul3 (Mistry et al., 2004).

Coimmunoprecipitation analysis using cultured S2 cells demonstrated that Drosophila Cul4 and Ddb1 physically interact either when ectopically expressed (Figure 1C) or as endogenous proteins (Figure 1D). The Ddb1EV01308 P-element allele (Figure 1A) causes developmental arrest early during second larval instar when homozygous or when

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**Figure 1.** Molecular Analysis of Drosophila Cul4 and Ddb1 mutants. (A) The Drosophila Cul4 locus is located on chromosome 2R at 44A and contains 12 exons (black and gray boxes). The P-elements KG02900 and EP2518 are located in the 5’ UTR and 3’ UTR, respectively (gray boxes). Open arrowheads indicate the breakpoints within the open reading frame (black boxes) of P-element excision alleles Cul411R, Cul46AP, and Cul411L. The Drosophila Ddb1piccolo locus is located on chromosome 3R at 87D and contains seven exons. The P-elements EY01408 or SO26316 are located in the 5’ UTR, and the pic2 missense mutation is located at the 5’ end of exon 2. (B) S2 cells or first instar larvae of the indicated genotypes (Df = Df(2R)CA53) were homogenized and analyzed by Western blot with anti-Cul4 or anti-Dup antibodies. The asterisk (∗) indicates a cross-reacting protein that comigrates with the truncated Cul46AP protein. (C) HA-Ddb1 was ectopically expressed in S2 cells, immunoprecipitated, and analyzed by Western blot using anti-Cul4 and anti-HA antibodies. (D) Extracts from S2 cells were immunoprecipitated with increasing concentrations of anti-Cul4 antibodies specific for the NH2- or COOH-terminus and analyzed by Western blot using anti-Dup, anti-Cul4, or anti-Dup antibodies. (E) Second instar larvae of the indicated genotypes (Df = Df(3R)ry75) were homogenized and analyzed by Western blot with anti-Ddb1, anti-Dup, or anti-Tubulin antibodies. Several lower molecular weight Dup species hyperaccumulated in the mutants.
placed in trans with deficiencies Df(3R)Eox6167 or Df(3R)ry75. Precise excision of EY01408 reverted the lethality of Ddb1EY01408, indicating that Ddb1 is an essential gene as previously reported (Takata et al., 2004; Lin et al., 2009). We isolated multiple additional Ddb1 alleles with a range of severity resulting from imprecise repair of EY01408 excision events. The most severe Ddb1EY01408 excision alleles caused second instar lethality, whereas the least severe resulted in adult flies with reduced viability and fertility that displayed growth defects including missing and thin thoracic bristles (Hu et al., 2008). These morphological phenotypes led us to establish (Hu et al., 2008) that Ddb1 is allelic to the piccolo (pic) locus (Hilliker et al., 1980; Rushlow and Chovnick, 1984; Clark and Chovnick, 1986; Deak et al., 1997). We found that flies carrying Ddb1pic alleles cause second (pic5026316 and pic5293) or third (pic7) instar lethality and fail to complement the lethality caused by Ddb1EY01408.

By Western blot analysis, picDros, pic5026316, and Ddb1EY01408 appear to be Ddb1 null alleles (Figure 1E, lanes 3–5). pic2 mutants express reduced amounts of Ddb1 (Figure 1E, lane 2), consistent with this Gly216 Asp missense allele being a hypomorph (Hu et al., 2008). The pic2 allele combined with other weak Ddb1EY01408 excision alleles (i.e., Ddb1picDros,12C) results in viable flies that are piccolo in phenotype (Hu et al., 2008). Similar to previous observations in which Ddb1 was silenced by RNAi in Drosophila larvae (Takata et al., 2004), we observed melanotic masses in Ddb1 mutant larvae, as well as in hypomorphic Ddb1 mutant adults and Cul4D1L/ KcG02980 mutant larvae. Melanotic masses are thought to result from abnormal hemocyte development that elicits an auto-immune response (Rizki and Rizki, 1983; Dearolf, 1998) suggesting that CRL4 may be involved in hemocyte development.

Cul4 and Ddb1 Mutant Cells Proliferate Poorly

To assess the effect of Cul4 or Ddb1 disruption on cell proliferation and Dup expression, we generated mutant imaginal disk clones via FLP-FRT–mediated mitotic recombination (Xu and Rubin, 1993). Mitotic recombination was induced in first instar larvae, and the resulting clones were analyzed as adjacent groups of GFP-positive and -negative cells (i.e., twin spots) in wing and eye-antennal discs dissected from third instar larvae. Wild-type controls yielded twin spot clones that were roughly equal in size (Figure 2A). The area of Ddb1 mutant cell clones was on average four times smaller than wild type, indicating that the growth of Ddb1 mutant cells is defective (Figure 2, A and C). In contrast to the Ddb1 clones, Cul4 mutant clones were undetectable when generated in first instar larvae and analyzed during third instar. When mitotic recombination was induced at late second instar, however, small Cul4 mutant clones were visible (Figure 2B). These results suggest that Cul4 mutant cells proliferate poorly and are consequently eliminated from the disk epithelium by cell–cell competition, a well-known phenomenon in Drosophila whereby faster growing cells actively induce apoptosis in adjacent slower-growing cells during larval development (Adachi-Yamada and O’Connor, 2004). These results are essentially indistinguishable to the Cul4 and Ddb1 mutant cell clone analysis recently described by Lin et al. (2009). In addition, disruption of pcu4 or ddb1 in fission yeast causes proliferation defects (Osaka et al., 2000; Zolezzi et al., 2002; Bondar et al., 2003; Liu et al., 2003), as does mutation of mouse Ddb1 (Cang et al., 2006; Liu et al., 2009).

Developmental defects consistent with reduced growth and proliferation were also apparent in tissues dissected from Cul4 or Ddb1 mutant larvae. Hypomorphic Ddb1 mutant animals (pic2/Df(3R)ry75) develop until the third larval instar, but contain imaginal discs that are smaller in size relative to wild type (Figure 3, A and B). Eye imaginal discs from these animals displayed a reduced and irregular pattern of BrdU incorporation within the second mitotic wave, a group of cells just posterior to a wave of differentiation that sweeps across the eye disk epithelium and synchronously enter a final mitotic cell division cycle before differentiating (Figure 3, A and B, arrows). Similarly, the CNS dissected from Cul4 null mutant first instar larvae contained very few if any BrdU-positive cells compared with WT controls (Figure 3, C and D). These data indicate that Cul4 and Ddb1 are necessary for normal cell proliferation in Drosophila.

**Cdt1**mut Does Not Hyperaccumulate in Cul4 or Ddb1 Mutant Imaginal Cells

Using S2 cell extracts, we detected Dup in Cul4 immunoprecipitates (Figure 1D), suggesting that a CRL4 E3 ubiquitin ligase may act to regulate the abundance of Cdt1 in Drosophila as occurs in other species (Higa et al., 2003; Hu et al., 2004; Ralph et al., 2006; Kim and Kipreos, 2007a). Consistent with this possibility, Western analysis of extracts made from whole first instar larvae indicated an elevated level of Dup in Cul4 or Ddb1 mutants relative to WT controls (Figures 1, B and E). To more specifically test whether Dup is regulated by CRL4 during cell proliferation, we measured Dup levels by immunostaining wing imaginal discs containing Cul411L or Ddb1EY01408 mutant clones (Figure 2, B and C). Other proteins have previously been shown to inappropriately accumulate in mitotic clones mutant for components of CRL E3 ubiquitin ligases (Jiang and Struhl, 1998; Nouredine et al., 2002; Ou et al., 2002). In WT imaginal cells, Dup is primarily nuclear and most abundant in G1 and then rapidly destroyed as cells enter S phase (Thomer et al., 2004). However, we could neither detect Dup hyperaccumulation in Cul4 or Ddb1 mutant clones (Figure 2, B and C), nor did we observe an overlap between Dup staining and BrdU incorporation, as would be expected if CRL4 were required for destruction of Dup during S phase. This result was not due to redundancy between CRL4 and CRL1 ligases, as was observed in human cells (Nishitani et al., 2006), because Cul4 Cul1 double mutant cells also failed to show evidence of Dup misregulation (Figure 2D). Similar results were obtained with Cul1 single mutant clones.

Although one interpretation of this clonal analysis is that CRL4 does not regulate Dup, there are several caveats to consider. Most importantly, because CRL4 complexes regulate the degradation of many substrates, phenotypic pleiotropy may have masked our ability to detect alterations to the normal accumulation of Dup. For instance, G1 arrest is known to occur after RNAi depletion of Cul4 in cultured S2 cells (Rogers et al., 2002; Bjorklund et al., 2006; Higa et al., 2006b; Li et al., 2006; Rogers and Rogers, 2008). G1 arrest, which is consistent with the proliferation defect we observed, would preclude our ability to detect inappropriate Dup accumulation during S phase. The few BrdU-positive cells in Cul4 and Ddb1 mutant clones may not have yet been sufficiently depleted of Cul4 and Cul1 protein to observe an effect on Dup. Likewise, the hyperaccumulation of Dup in Cul4 and Ddb1 mutant whole larval extracts may result from an increase in the number of G1-arrested cells throughout the animal. To test this, we extended our analysis of BrdU incorporation in Cul4 mutant first instar larvae to include endoreplicating cells, which constitute most larval tissues and which accumulate in G1 under conditions of growth arrest (Britton and Edgar, 1998). We did not detect BrdU-labeled nuclei in midguts dissected from Cul4 mutant ani-
mals, whereas we could readily detect them in sibling controls (Figure 3, E and F). Thus, widespread G1 arrest could account for the overall increase in Dup protein measured by Western blotting of Cul4 mutant animals. Because of the caveats in interpreting CRL4 mutant phenotypes at the cellular and whole animal level, we developed an alternative strategy to specifically test the requirement for CRL4Cdt2 regulation of Dup during the cell cycle.

**PIP Box Deletion Blocks Dup Degradation at the Onset of S Phase**

To specifically test the contribution of CRL4-dependent Dup regulation to S phase and cell cycle progression in vivo, we generated a mutant version of Dup (Dup<sup>ΔPIP</sup>) lacking the NH<sub>2</sub>-terminal PIP box (Figure 4, A and B). Previous studies have shown that mutating the PIP box abolishes CRL4 binding to Cdt1 (Arias and Walter, 2006; Higa <i>et al</i>., 2006a; Hu and Xiong, 2006; Senga <i>et al</i>., 2006). Both full-length WT Dup (Dup<sup>FL</sup>) and Dup<sup>ΔPIP</sup> were tagged with GFP at their COOH-termini and were expressed using various ubiquitous or tissue-specific Gal4 drivers. Ubiquitous Dup<sup>ΔPIP</sup> expression using da-Gal4 and act-Gal4 caused embryonic lethality, whereas animals expressing Dup<sup>FL</sup> with the same drivers developed until adulthood (five independent UAS-Dup<sup>ΔPIP</sup> and UAS-Dup<sup>FL</sup> transgenic lines were examined). Eye-specific expression of Dup<sup>ΔPIP</sup> using GMR-Gal4 resulted in

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**Figure 2.** Analysis of Cul4 and Ddb1 mutant imaginal disk clones. (A) Histogram of the size measured in pixel area of twin spot clones analyzed in imaginal discs of third instar larvae. Twin spots are ordered on the X axis by GFP<sup>+</sup> clone size. (B–D) Wing imaginal discs containing Cul4 (B), Ddb1 (C), or Cul1Cul4 (D) mutant clones generated during second instar and analyzed 1 d later. Multiple GFP-negative mutant cell clones (outlined in white) resulting from multiple independent mitotic recombination events are apparent in B and C (a single clone is shown in D). Brightly stained, WT GFP-positive cells adjacent to the GFP-negative mutant cell clones are likely sister clones (i.e., the “twin spot”). Because of the density of twin spots, it is not always possible to unambiguously assign the WT clones with the corresponding mutant sister clone. Clones containing cells with (arrows) or without (arrowheads) Dup staining is outlined in white.
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massive tissue malformation, whereas DupFL caused mildly rough eyes (Figure 4C). These data indicate that DupAPTTP behaves distinctly from DupFL and suggest that with these drivers our DupFL transgenes do not produce the level of overexpression previously shown to cause rereplication after heat-shock production of WT Dup (Thomer et al., 2004).

One possibility for the severe developmental defects observed after DupAPTTP expression is disruption to cell cycle progression because of stabilization of Dup during S phase, which may cause rereplication and DNA damage that results in cell cycle arrest or cell death. To determine whether or not DupAPTTP is degraded correctly at the onset of S phase, we expressed DupFL and DupAPTTP in alternating segments of the embryo using paired (prd)-GAL4 and detected S phase cells with BrdU pulse labeling and exogenous Dup with anti-GFP antibodies. We did not detect DupFL staining in BrdU-positive cells, indicating that DupFL is correctly degraded very early in S phase (Figure 4D). In contrast, 48% of S phase cells within the prd-GAL4–expressing domains also expressed DupAPTTP, indicating that the PIP motif is required for Dup destruction at the onset of S phase (Figure 4E, open arrows).

Although the absence of DupAPTTP in ~50% of the S phase cells does not formally demonstrate regulated proteolysis, this observation is consistent with the possibility of PIP box–independent mechanisms of inducing Cdt1 destruction during S phase. One possibility is that DupAPTTP may still be recognized by CRL4Cdt2, but much more poorly than WT Dup, resulting in slower destruction during S phase. Another possibility is the activity of a different E3 ubiquitin ligase. Cdk-directed phosphorylation triggers CRL1-mediated destruction of mammalian Cdt1 (Li et al., 2003; Liu et al., 2004; Takeda et al., 2005; Nishitani et al., 2006). Thomer et al. (2004) showed that the SDS-PAGE mobility of a Dup mutant containing 10 consensus (IS/T)PX(K/R) CycE/Cdk2 phosphorylation sites (Figure 4A) mutated to alanine (Dup10A) was not slowed after ectopic cyclin E/Cdk2 induction as WT Dup’s mobility was and also that Dup10A was somewhat more stable than WT Dup after heat-shock–induced production. We therefore hypothesized that the 10A mutations would augment the stability of DupAPTTP in the embryo. To test this hypothesis, we generated UAS-Dup10A-GFP and UAS-DupAPTTP/10A-GFP transgenes and expressed them with prd-GAL4. Dup10A was degraded normally during S phase because we could not detect cells that were positive for both BrdU and GFP (Figure 4F). The same observation was made by Thomer et al. (2004) in ovarian follicle cells. Similar to our observations using DupAPTTP ~45% of BrdU-positive cells in the prd-GAL4 stripe also contained DupAPTTP/10A (Figure 4G). These data indicate that DupAPTTP stability during S phase cannot be further increased by mutating the 10 previously identified consensus CycE/Cdk2 phosphorylation sites within Dup. Whereas it is possible that there are additional Cdk phosphorylation sites remaining on Dup10A, these data suggest that Cdk mediated destruction is not a major contributor to Dup regulation during S phase.

DupAPTTP Supports DNA Replication But Not Completion of the Cell Division Cycle

Many studies have reported that overexpression of Cdt1 leads to rereplication (Zhong et al., 2003; Arias and Walter, 2003; May et al., 2005; Arias and Walter, 2006; Sansam et al., 2006). However, these studies did not directly test whether PIP-dependent destruction of Cdt1 is required for normal cell cycle progression in vivo. Moreover, the redundancy between CRL1 and CRL4 for S phase destruction of human Cdt1 and the inhibition of Cdt1 by Geminin raise the possibility that CRL4-mediated destruction of Cdt1 may not be essential for cell cycle progression. We therefore determined if DupFL-GFP and DupAPTTP-GFP could rescue the lack of S phase and consequent cell cycle arrest in dup null mutant embryos. DupFL mutant embryos develop normally through the first 15 cell cycles, presumably because of maternal stores of Dup protein, but fail to incorporate BrdU in S phase of the 16th cell cycle (Figure 5, A and B; Whittaker et al., 2000). Both DupFL and DupAPTTP expression driven by prd-GAL4 restored BrdU incorporation in dup null ectodermal cells (Figure 5, C and D), indicating that these transgenic proteins were capable of assembling pre-RC complexes and supporting the initiation of DNA replication. However,
close inspection revealed an unusual BrdU incorporation pattern in Dup<sup>ΔPIP</sup>-expressing cells (Figure 5F): the staining appeared less uniform and more punctate than when Dup<sup>FL</sup> was expressed (Figure 5E).

We therefore asked if <i>dup</i> null cells expressing Dup<sup>ΔPIP</sup> could complete mitosis and divide, which would be indicative of normal completion of S phase (Figure 6A). A curious feature of the <i>dup</i> mutant phenotype is that although the epidermal cells fail to undergo S16, they nonetheless enter and arrest in mitosis with condensed chromosomes that can be detected with anti-phospho histone H3 (pH3) antibodies (Figure 6B; Whittaker <i>et al.</i>, 2000). The entry into and arrest in mitosis likely occurs because of an inability to activate a checkpoint response to aberrant or incomplete replication (Kelly <i>et al.</i>, 1993; Piatti <i>et al.</i>, 1995). We hypothesized that if Dup<sup>FL</sup> or Dup<sup>ΔPIP</sup> expression could support a complete cell cycle, then this aberrant accumulation of pH3-positive cells throughout the epidermis would be eliminated. Indeed, both Dup<sup>FL</sup> and Dup<sup>ΔPIP</sup> expression eliminated pH3 staining in <i>prd</i>-GAL4 stripes (Figure 6, C and D). However, this result could be obtained in two very different ways: 1) a normal S phase and completion of mitosis, or 2) an aberrant S phase caused by Dup<sup>FL</sup> and Dup<sup>ΔPIP</sup> that triggered a checkpoint response resulting in the cells arresting in interphase prior to entry into mitosis.

To distinguish between these two possibilities, we assessed whether cell division occurred by first examining cell size. Each epidermal cell division during <i>Drosophila</i> embryogenesis results in a reduction in cell size (Lehner and O’Farrell, 1989). Thus, if the Dup transgenes were able to...
BrdU-labeled embryos. (A) dupa1/Gal4 pattern in the embryos. Note the restoration of BrdU incorporation in the prd-null neighbors (Figures 6D and E). DupFL-expressing cells remained the same size as their neighbors (Figures 6D and E). This finding suggests that DupFL can rescue the dup null cell phenotype and support completion of the cell cycle, whereas DupΔPIP-expressing dup null cells remain in interphase and do not enter mitosis. To test this assertion, we detected cyclin A protein, which should accumulate in cells arrested in interphase of cycle 16 but not in cells that divide and enter the following G1 phase of cycle 17 (Lehner and O’Farrell, 1989). The DupΔPIP-expressing cells accumulate high levels of cyclin A (Figure 7B), whereas the DupFL-expressing cells do not (Figure 7A). Together these data indicate that DupFL transgenic protein provides normal Dup function and rescues the replication and cell cycle defect of dup null cells, whereas DupΔPIP does not.

Why do DupΔPIP-expressing cells fail to enter mitosis? One possibility is that these cells rereplicate, due to the failure to degrade Dup, resulting in DNA damage that induces a cell cycle checkpoint. However, we were unable to detect a difference in γ-H2aV staining between DupΔPIP-expressing and -nonexpressing cells, although we could detect and increase in γ-H2aV staining after irradiation (Figure S1). These data suggest that either DupΔPIP does not induce rereplication or that the level of rereplication-induced DNA damage is low enough not to be detected by the γ-H2aV antibody. In addition, DupΔPIP does not induce continuous rereplication or a slow S phase, because we did not detect BrdU incorporation in dup mutant cells expressing DupΔPIP at the time when the neighboring dup mutant cells (which are not expressing DupΔPIP) have arrested in mitosis 16. We found no difference in cleaved Caspase-3 staining within and outside of the DupΔPIP transgene expression domain, suggesting that DupΔPIP-expressing cells do not apoptose. Taken together, our data suggest that dup mutant epidermal cells expressing DupΔPIP enter but do not complete S phase of cell cycle 16 and arrest in interphase before mitosis.

**DupΔPIP Causes Cell Cycle Arrest in a Wild-Type Background**

Our data indicate that DupΔPIP cannot support cell division in a dup null background. Because endogenous Dup is promptly degraded at the onset of S phase, ectopic expression of DupΔPIP in a WT background should create a situation in which DupΔPIP is the only active Dup present in S phase. If the cell cycle arrest we see in dup null embryos is due to having active Dup in S phase, DupΔPIP expression in WT embryos should also cause the cells to arrest in interphase. This prediction was confirmed by the presence of large undivided, cyclin A–positive cells expressing DupΔPIP (Figure 7D, Figure S2). In contrast, these phenotypes did not arise after DupFL expression in WT embryos (Figure 7C, Figure S2). The DupΔPIP-expressing cells are not simply delayed in cell cycle progression, as anti-pH3 staining does not reveal mitosis in later embryonic stages (not shown). Together, our data indicate that stabilization of Dup in S phase causes cell cycle arrest.

**Follicle Cell Endocycle Progression Is Not Affected by DupΔPIP**

Our data show that PIP box–mediated destruction of Cdt1 is required for progression through the cell division cycle. We next wished to determine if there was a similar requirement in a replicating cell type that does not divide. Certain animal cells and much of plant growth and development rely on endoreplication, the process by which cells in certain tissues become polyploid as part of their terminal differentiation.

Figure 5. DupΔPIP can support DNA replication. All panels show BrdU-labeled embryos. (A) dupa1/CyO control. (B) dupa1 homozygous mutant embryo. (C and D) prd-Gal4–driven expression of DupFL–GFP (C) or DupΔPIP–GFP (D) in dupa1 homozygous mutant embryos. Note the restoration of BrdU incorporation in the prd-null mutant embryos. (E and F) Higher magnification images of the BrdU incorporation pattern after prd-Gal4 expression of DupFL–GFP (E) and DupΔPIP–GFP (F) in dupa1 homozygous mutant embryos.
program (Lee et al., 2009). Endoreplication in Drosophila occurs via endocycles, which consist of alternating S and G phases without cell division. Current models of replication control in endocycles suggest that individual origins of DNA replication fire once and only once as they do in mitotic cycles and that cycles of low (G phase) and high (S phase) CDK activity permit and prevent pre-RC assembly, respectively. Follicle cells of the Drosophila ovary become 16C polyploid via developmentally controlled endocycles that occur between stages 6–9 of oogenesis (Lilly and Duronio, 2005). To test the requirement for Dup degradation in endocycle progression, we expressed DupFL and DupATP in endocycling follicle cells using c323a-Gal4, which drives expression in all follicle cells of stages 8–14 (Figure 8A). More follicle cells expressed DupATP than DupFL, suggesting that DupATP was stabilized (Figure 8B and C). We then determined whether Dup degradation during endo S phase is PIP-box dependent by quantifying the number of BrdU pulse-labeled S phase cells that also express DupFL or DupATP. We found that 43% of endocycle S phase cells retained DupATP (Figure 8E, open arrows), whereas DupFL is degraded at the onset of endocycle S phase (Figure 8D).

Figure 6. DupATP cannot support a full cell division cycle. (A–D) Anti-pH3 (red, A’–D’) and discs large (Dlg) (white, A’–D’) staining of dupa1 null (B–D) or sibling control (A) embryos expressing DupFL-GFP (green, C and C’) or DupATP-GFP (green, D and D’) with prd-Gal4. The enlarged area of the merged image in C’ and D’ is indicated by the box in C and D. The enlarged area of the Dlg panel is indicated by the box in A’–D’. Note the ~50% smaller size of the DupFL-expressing cells on the left side of the C’ panel, whereas the DupATP-expressing cells are similar in size to control (D’ and E). (E) Quantification of relative cell size in DupFL or DupATP-expressing cells compared with that of their dupa1 null neighbors. Error bars, SD.
The pattern of BrdU incorporation and total number of BrdU-labeled cells (Figure 9G) was similar between Dup FL and Dup\textsuperscript{APIP}-expressing follicle cells. We did not observe an increase in either apoptosis (Figure 9, A–C) or γ-H2aV staining (Figure 9, D–F) of follicle cells after Dup\textsuperscript{FL} and Dup\textsuperscript{APIP} expression. To test whether stabilizing Dup during follicle cell S phase adversely affected oogenesis, we determined the rate of hatching of eggs laid by Dup FL or Dup\textsuperscript{APIP}-expressing females. About 94% of eggs laid by Dup FL or Dup\textsuperscript{APIP}-expressing females hatched into viable larvae, similar to WT (Figure 9G). Together these data suggest that Dup\textsuperscript{APIP} expression with c323a-Gal4 does not disrupt follicle cell function or oogenesis. This result is in contrast to the defects caused by Dup\textsuperscript{APIP} in the proliferating embryonic cells. One possibility is that we achieved a higher level of Dup\textsuperscript{APIP} expression in the embryo than in the follicle cells and that this higher level of expression triggers cell cycle arrest. However, we did not detect any significant difference in expression of Dup\textsuperscript{APIP} between embryonic cells and follicle cells, as assessed by measuring Dup\textsuperscript{APIP}-GFP fluorescence by confocal microscopy of individual nuclei (Figure S3). We conclude that proliferating cells are more sensitive to Dup\textsuperscript{APIP} expression than endocycling follicle cells.

**Follicle Cell Gene Amplification Is Not Inhibited by Dup\textsuperscript{APIP}**

Beginning in stage 10A and after the completion of endoreplication, several specific follicle cell loci begin a program of gene amplification that increases the copy number, and thus the biosynthetic capacity, of genes encoding proteins necessary for chorion synthesis and vitellogenesis (Calvi and Spradling, 1999; Tower, 2004; Claycomb and Orr-Weaver, 2005). Gene amplification occurs by repeated firing of specific origins of replication, whereas the remainder of the origins throughout the genome stays quiescent. This phenomenon can be detected as distinct foci of BrdU incorporation within each follicle cell nucleus (Figure 9H). Although the precise mechanism of this regulation is unknown, it likely involves cycles of pre-RC assembly/disassembly because virtually all the known pre-RC components, including Dup, are required for gene amplification (Tower, 2004).

To determine whether PIP-mediated regulation of Dup was required for this process, we examined BrdU incorporation...
in stage 10A follicle cells expressing DupFL or Dup\textsuperscript{ATIP}. Our results indicate that this pattern of BrdU incorporation is largely unaffected by Dup\textsuperscript{FL} (Figure 9I), whereas expression of Dup\textsuperscript{ATIP} caused slightly enlarged BrdU foci (Figure 9J) as previously described for an allele of Dup lacking the first 46% of the protein (including the PIP box; Thomer et al., 2004). Importantly, no ectopic BrdU incorporation throughout the nucleus was observed, indicating that the normal inactivation of genomic replication is retained in the presence of Dup\textsuperscript{ATIP}.

**Figure 8.** Dup\textsuperscript{ATIP} is stabilized in follicle cell endocycles. (A–C) Confocal images of follicle cells from stage 9 egg chambers expressing GFP (A), Dup\textsuperscript{FL}-GFP (B) or Dup\textsuperscript{ATIP}-GFP (C) using the c323a-Gal4 driver and stained with anti-GFP (green, A’–C’) and DAPI (blue, A”–C”). (D and E) Confocal images of follicle cells from stage 9 egg chambers expressing Dup\textsuperscript{FL}-GFP (D) or Dup\textsuperscript{ATIP}-GFP (E) with c323a-Gal4 and stained with anti-GFP (green, D’ and E’) and anti-BrdU (red, D” and E”). Arrows and arrowheads as in Figure 4.

**Geminin Function Restrains Dup\textsuperscript{ATIP} Activity in Follicle Cells**

Our findings indicate that the absence of PIP box–dependent degradation of Dup does not adversely affect follicle cell function. Because this result is different from what we obtained in mitotic embryonic cells, we asked whether Geminin function acts to restrain Dup\textsuperscript{ATIP} activity in endocycling follicle cells. To test this hypothesis, we reduced the gene dose of \textit{geminin} in half together with c323a-Gal4-driven expression of Dup\textsuperscript{FL} or Dup\textsuperscript{ATIP} and compared the results to WT and \textit{geminin} heterozygote ovaries. Although ova-
ries from \textit{geminin}/+ heterozygous control flies and \textit{geminin}/+ flies expressing Dup\textsuperscript{FL} appeared WT (Figure 9, K, L, K', and L'), \textit{geminin}/+ flies expressing Dup\textsuperscript{ΔPIP} contained ovaries lacking normal stage 9 and older egg chambers due to massive degeneration (Figure 9, M and M'). This phenotype occurred soon after the initiation of

\begin{figure}
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\includegraphics[width=\textwidth]{Figure9}
\caption{Dup\textsuperscript{ΔPIP} expression in follicle cells disrupts oogenesis only when Gem gene dose is reduced. (A–F) Confocal images of follicle cells from WT stage 8 egg chambers (A and D) or from those expressing Dup\textsuperscript{FL}-GFP (B and E), or Dup\textsuperscript{ΔPIP}-GFP (C and F) using the c323a-Gal4 driver and stained with anti-GFP (green), DAPI (blue), and anti-cleaved caspase 3 (red, A–C) or anti-\textit{αH2aV} (red, D–F). (G) Percent hatching of eggs laid by female flies expressing GFP, Dup\textsuperscript{FL}-GFP, or Dup\textsuperscript{ΔPIP}-GFP in follicle cells with c323a-Gal4. Average and SD from five independent experiments (n = 100). Average and SD of the total number of BrdU-labeled follicle cells expressing GFP, Dup\textsuperscript{FL}-GFP, or Dup\textsuperscript{ΔPIP}-GFP with c323a-Gal4. N = 5 stage 8 egg chambers. (H–J) Confocal images of follicle cells undergoing chorion gene amplification expressing GFP (H), Dup\textsuperscript{FL}-GFP (I), or Dup\textsuperscript{ΔPIP}-GFP (J) with c323a-Gal4 and stained with anti-BrdU (red) and DAPI (blue). (K–M) \textit{gem\textsuperscript{2010Lcd2010}}/CyO stage 9 follicle cells expressing GFP (K), Dup\textsuperscript{FL}-GFP (L), or Dup\textsuperscript{ΔPIP}-GFP (M) with c323a-Gal4 and stained with anti-GFP antibodies (green) and DAPI (blue). (K'–M') Enlarged images. The arrowheads in L and L' indicate cells expressing Dup\textsuperscript{FL}-GFP. The brackets in M and M' indicate degenerated stage 9 egg chambers.
\end{figure}
Dup\textsuperscript{ATF} expression around stage 8–9. This strong genetic interaction suggests that Geminin and PIP-mediated destruction cooperate to control Dup activity during follicle cell endocycles.

**DISCUSSION**

Although several regulatory mechanisms of Cdt1 have been described, how they work together and when they are required in different tissues during animal development is not well understood. Here we show that regulation of *Drosophila* Dup via an NH\textsubscript{2}-terminal PIP box is required for progression through the cell division cycle in embryonic epidermal cells but is dispensable for progression through follicle cells endocycles.

**PIF Box–dependent Degradation of Dup**

Our results indicate that deletion of the PIF box prevents the rapid destruction of Dup at the beginning of S phase. Before discovery of the PIF degron/CRL4 mechanism of replication-coupled proteolysis, Thomer *et al.* (2004) reported a similar result with a mutant version of Dup lacking the NH\textsubscript{2}-terminal 46% of the protein, including the PIF box. Thus, our results suggest that the Thomer *et al.* (2004) observation is due to deletion of the PIF degron. Biochemical and genetic experiments from a number of species suggest that the PIF degron recruits proteins to chromatin-bound PCNA at replication forks during S phase. These proteins are subsequently ubiquitylated by CRL4\textsubscript{Cdt2} and proteolized (Arias and Walter, 2006; Higa *et al.*, 2006a; Hu and Xiong, 2006; Senga *et al.*, 2006; Abbas *et al.*, 2008; Kim and Michael, 2008; Kim *et al.*, 2008; Nishitani *et al.*, 2008; Shibutani *et al.*, 2008; Havens and Walter, 2009). Although we did not detect hyperaccumulation of Dup in embryonic cells mutant for components of CRL4\textsubscript{Cdt2}, the PIF degron mechanism is conserved in *Drosophila* (Shibutani *et al.*, 2008), and CRL4\textsubscript{Cdt2} is required for Dup destruction after DNA damage in cultured S2 cells (Higa *et al.*, 2006a). As discussed above, phenotypic pleiotropy resulting from abrogation of CRL4\textsubscript{Cdt2} function may have masked our ability to detect effects on Dup protein.

Interestingly, deletion of the PIF box resulted in inappropriate Dup accumulation in only about half of BrdU-positive S phase cells. CRL1 and CRL4 act redundantly in triggering human Cdt1 destruction during S phase (Higa *et al.*, 2006a; Hu and Xiong, 2006; Senga *et al.*, 2006; Abbas *et al.*, 2008; Kim and Michael, 2008; Kim *et al.*, 2008; Nishitani *et al.*, 2008; Shibutani *et al.*, 2008; Havens and Walter, 2009). Although we did not detect hyperaccumulation of Dup in embryonic cells mutant for components of CRL4\textsubscript{Cdt2}, the PIF degron mechanism is conserved in *Drosophila* (Shibutani *et al.*, 2008), and CRL4\textsubscript{Cdt2} is required for Dup destruction after DNA damage in cultured S2 cells (Higa *et al.*, 2006a). As discussed above, phenotypic pleiotropy resulting from abrogation of CRL4\textsubscript{Cdt2} function may have masked our ability to detect effects on Dup protein.

Interestingly, deletion of the PIF box resulted in inappropriate Dup accumulation in only about half of BrdU-positive S phase cells. CRL1 and CRL4 act redundantly in triggering human Cdt1 destruction during S phase (Nishitani *et al.*, 2006). In contrast, our results suggest that cyclin E/Cdk2-dependent phosphorylation and CRL1 ubiquitylation of Cdt1 do not contribute significantly to Dup destruction during S phase and thus likely do not account for the disappearance of Dup\textsuperscript{ATF} from BrdU-positive cells. One recently proposed possibility is that CRL1-dependent regulation of Cdt1 arose in higher metazoans (Kim and Kipreos, 2007b).

**A Requirement for Dup Degradation in Mitotic Cycles**

By using the rescue of *dup* embryonic mutant phenotypes as an assay, our data clearly demonstrate that Dup\textsuperscript{ATF} is unable to support progression through the cell division cycle. Similarly, Dup\textsuperscript{ATF} expression in WT embryos caused cell cycle arrest in interphase. In these experiments there was no obvious large increase in DNA content, as occurs from re-replication in other cell types after overexpression of Cdt1 or depletion of Cdt1 regulatory mechanisms (e.g., CRL4 or Geminin; Arias and Walter, 2007). We also did not detect extensive DNA damage or apoptosis. We propose that the near physiological levels of Dup\textsuperscript{ATF} expression achieved in our experiments, as suggested by our ability to phenotypically rescue *dup* mutant cells using transgenic WT Dup, causes a small number of replication origins to reinitiate. This situation results in a low level of DNA damage that activates a checkpoint and arrests cells in interphase. Alternatively, Dup\textsuperscript{ATF} may block DNA synthesis more directly, as a recent study reported that excess Cdt1 prevents nascent DNA strand elongation (Tsuyama *et al.*, 2009).

**Mechanisms of Dup Regulation in Endocycling Cells**

Previous studies reported that heat-shock driven overexpression of Dup in endocycling follicle cells causes rereplication (Thomer *et al.*, 2004), and that Cul\textsubscript{4} mutant follicle cells hyperaccumulate Dup and exhibit replication defects during gene amplification (Lin *et al.*, 2009). We found that Gal4-driven expression of Dup\textsuperscript{ATF} did not cause either of these phenotypes and did not dramatically alter endocyte S phase or chorion gene amplification. As in the embryo, we propose that the lack of large increases in DNA content seen in our experiments with Dup\textsuperscript{ATF} is due to lower expression levels of Dup than that obtained by Thomer *et al.* (2004). Also, a small amount of DNA damage might not disrupt the endocyte (Mehrotra *et al.*, 2008). Lin *et al.* (2009) showed that ectopic genomic BrdU incorporation during gene amplification stages occurs in Cul\textsubscript{4} or Ddb1 mutant follicle cells. We did not observe the same phenotype after Dup\textsuperscript{ATF} expression, suggesting that these replication defects may be due to misregulation of another CRL4 target.

Several observations suggest the possibility that Cdt1 is regulated in a cell-type-specific manner. In *Drosophila* S2 cells and mammalian cells, RNAi against Gem but not Cul1 or Cul4 results in rereplication (Melixetian *et al.*, 2004; Zhu *et al.*, 2004; Hall *et al.*, 2008). In contrast, *Drosophila* Gem is not required for proliferation of imaginal discs or endoreplication in salivary glands (Quinn *et al.*, 2001). Null mutations of *C. elegans* Cul4 or Ddb1 cause overreplication primarily in seam cells (Zhong *et al.*, 2003; Kim and Kipreos, 2007a). Finally, ectopic expression of *Arabidopsis* Cdt1-induced over-replication only in endocycling cells (Castellano Mdel *et al.*, 2004). The basis for these cell type differences is not known.

We showed that reduction of Gem gene dose in combination with Dup\textsuperscript{ATF} expression in follicle cells causes deterioration of egg chambers during oogenesis. We favor the possibility that Dup inhibition by Gem can compensate for the loss of PIP-mediated destruction of Dup in this cell type. In proliferating embryonic ectodermal cells, loss of PIP-mediated Dup destruction was sufficient to block the cell cycle, suggesting that Gem activity is unable to provide compensatory inhibition of Dup in this situation. Cell type specific differences in Gem expression or activity could explain why cells are differently sensitive to stabilized Dup. For instance, the *C. elegans* Gem homolog, GMN-1, is expressed at higher levels in the germ line (Yanagi *et al.*, 2005), suggesting that this tissue might be buffered against disruption of Dup destruction as we observed in *Drosophila* follicle cells. May *et al.* (2005) reported that in some cell types Gem levels increase concomitantly with increased levels of Dup after DNA replication is compromised. Determining the mechanisms by which certain cell types are more sensitive to mis-regulation of Cdt1 destruction than others will be necessary for a complete understanding of replication control in developing organisms.

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