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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 CRL4^{Cdt2} E3 ubiquitin ligase is thought to help prevent rereplication of the genome during S phase. To directly test whether CRL4^{Cdt2}-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$^{\Delta PIP}$) and which cannot be regulated by CRL4^{Cdt2}. Dup$^{\Delta PIP}$ is inappropriately stabilized during S phase and causes developmental defects when ectopically expressed. Dup$^{\Delta PIP}$ 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$^{\Delta PIP}$ accumulation during S phase did not adversely affect progression through follicle cell endocycles in the ovary. In this tissue the combination of Dup$^{\Delta PIP}$ expression and a 50% reduction in Geminin gene dose resulted in egg chamber degeneration. We could not detect Dup hyperaccumulation using mutations in the CRL4^{Cdt2} 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$^{\Delta PIP}$ 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; Liontos *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 (Deshaies 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 CRL1^{Skp2} (Nishitani *et al.*, 2001, 2006; Li *et al.*, 2003; Kondo *et al.*, 2004; Liu *et al.*, 2004). CRL4^{Cdt2} directs replication-coupled destruction of Cdt1 through a degron at the Cdt1 NH_2-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 CRL4^{Cdt2} for ubiquitylation and subsequent destruction of Cdt1 (Higa *et al.*, 2003, 2006a; Hu *et al.*, 2004;

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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 depletion 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 development is not completely understood. For instance, if 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 where Gem 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/piccolo* mutant alleles *EY01408*, pic^2, and pic^{Drv3} were obtained from the Bloomington Stock Center (Bloomington, IN). The $Ddb1/pic^{SO26316}$ line was obtained from the Szeged Stock Center (Szeged, Hungary). $gem^{l(2)k03202}$ was a gift from Helena Richardson (University of Melbourne, Australia; Quinn *et al.*, 2001). Pic^{Drv3} resulted from an x-ray–induced rearrangement, leaving a large segment of genomic DNA inserted within the *Ddb1* locus (Scott *et al.*, 1983; Clark and Chovnick, 1986). Publicly available sequence flanking the SO26316 P-element insertion corresponds to the 5′ UTR of *Ddb1* (Flybase ID FBrf0125057; Deak *et al.*, 1997). The pic^2 x-ray allele contains an Asp substitution for the well-conserved Gly21 (Hu *et al.*, 2008) positioned at a turn in propeller A of Ddb1 (Li *et al.*, 2006).

P-Element Excision–mediated Mutagenesis

The *EP2518* P-element in the 3′ UTR of *Cul4* was mobilized by crossing to w^*; $wg^{Sp\text{-}1}/CyO$; ry^{506} Dr^1 $P[ry^{+t7.2} = Delta2\text{-}3]99B/TM6$ flies. Resulting mosaic males were crossed to Pin^{88k}/CyO flies, and three EP2518 excision events were identified from ~400 w^- progeny as novel *Cul4* mutant alleles by a failure to complement $Cul4^{KG02900}$. $Cul4^{KG02900}$ lethality was reverted after precise excision of the KG02900 P-element. The breakpoints of $Cul4^{6AP}$, $Cul4^{11L}$, and $Cul4^{11R}$ were determined by sequencing. Note that in Hu *et al.* (2008) the amount of truncation in $Cul4^{11R}$ allele was incorrectly indicated as that of $Cul4^{11L}$. The *EY01408* P-element in the 5′ UTR of *Ddb1* was similarly mobilized, and resulting w^- progeny were tested for complementation with the $Ddb1^{S026316}$ allele.

Western Blot Analysis

S2 cells were cultured in Schneider's/10% FBS at 25°C and were transfected using Effectene (Qiagen, Chatsworth, CA). Larval and cell lysates were made in RIPA (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1 mM EDTA, 0.1% SDS, 0.1% Triton X-100, 0.5% sodium deoxycholate), supplemented with 1 mM DTT, 1 mM PMSF, 1 mM sodium vanadate, 2 μg/ml aprotinin, 2 μg/ml leupeptin, 10 μg/ml trypsin inhibitor, and 150 μg/ml benzamidine, and cleared by high-speed centrifugation. Larval lysate were further clarified through 0.65-μm centrifugal low-binding Durapore membrane filters (Ultrafree-MC, Millipore, Bedford, MA). Lysates were resolved by SDS-PAGE and analyzed by Western blot.

Mitotic Recombination and Clonal Analysis

Mitotic recombination was carried out using the (FLP)/FLP recognition target (FRT) technique (Xu and Rubin, 1993) using *hs-FLP; FRT42B Ubi-GFP/FRT42B* $Cul4^{11L}$ or *hs-FLP; FRT82B Ubi-GFP/FRT82B* $Ddb1^{EY01408}$ or *hs-FLP; FRT42D Ubi-GFP/FRT42D* $Cul1^{EX}$ $Cul4^{11L}$. Larvae were heat-shocked for 45 min at 37°C, 48–80 h after egg deposition, and dissected as third instar larvae.

Transgenic Flies

Dup^{FL}, $Dup^{\Delta PIP}$, Dup^{10A}, and $Dup^{\Delta PIP10A}$ cDNAs were cloned into pENTR (Invitrogen, Carlsbad, CA) and recombined into a Gateway compatible set of UASp vectors that permitted a COOH-terminal green fluorescent protein (GFP) fusion and that were provided by Terence Murphy (Carnegie Institution, Baltimore, MD; http://www.ciwemb.edu/labs/murphy/Gateway%20vectors.html). The Dup^{10A} open reading frame (Thomer *et al.*, 2004) was kindly provided by Brian Calvi (Indiana University). Transgenic flies were generated by Rainbow Transgenic Flies (Newbury Park, CA) and BestGene (Chino Hills, CA). The *prd*- (Treisman *et al.*, 1991), c323a- (Manseau *et al.*, 1997), and GMR- (Moses and Rubin, 1991) Gal4 driver lines were obtained from the Bloomington Stock Center.

dup^{a1} Rescue

Staged embryo collections from dup^{a1}/+; *prd*-gal4/+ and dup^{a1}/+; UAS-Dup-GFP/+ parents were fixed and stained with various combinations of antibodies (see below). dup^{a1}/dup^{a1}; *prd*-Gal4/UAS-Dup-GFP embryos were identified by a combination of GFP expression and the *dup* mutant phenotype, which is obvious because there are fewer DAPI-staining nuclei. Relative cell size was determined using confocal images of anti-Dlg staining, which detects the cortex of cells. We measured the distance across individual cells in two perpendicular axes using Photoshop (Adobe Systems, San Jose, CA). The product of these two measurements produced an area in square inches that was used to compare the relative size of different cells.

Geminin Reduction Schemes

$gem^{l(2)k03202}$/CyO; UAS-Dup-GFP/+ females were crossed to 323a-Gal4/Y; *Sco/CyO* males, and ovaries dissected from 323a-Gal4/+; $gem^{l(2)k03202}$/CyO; UAS-Dup-GFP/+ female progeny were compared with those from 323a-Gal4/+; *Sco/CyO*; UAS-Dup-GFP/+ as control.

Antibodies

A synthetic peptide (MSAAKKYKPMDTTELHEN) derived from the NH_2-terminus of *Drosophila* Cul4 was coupled to keyhole limpet hemocyanin and used to generate antibodies in rabbits (Pocono Rabbit Farm and Laboratory, Canadensis, PA) that were subsequently affinity-purified (Hu *et al.*, 2004). A COOH-terminal anti-Cul4 antibody was a gift from Dr. Hui Zhang (Yale University). Mouse antibodies generated using a GST fusion protein containing the NH_2-terminal 2/3 of human Ddb1 (Zymed Laboratories, South San Francisco, CA) were used to recognize *Drosophila* Ddb1. Guinea pig anti-Dup was kindly provided by Dr. Terry Orr-Weaver (MIT, Cambridge, MA; Whittaker *et al.*, 2000), Rabbit anti-γH2aV was kindly provided by Dr. Kim McKim (Rutgers University; Mehrotra and McKim, 2006), and mouse anti-*Drosophila* cyclin A was obtained from the Developmental Studies Hybridoma Bank (University of Iowa, Iowa City, IA). Mouse anti-HA (12CA5, NeoMarkers, Fremont, CA), mouse anti-tubulin (NeoMarkers) mouse anti-BrdU (BD Biosciences, San Jose, CA), rabbit anti-GFP (Abcam, Cambridge, MA), and rabbit anti-cleaved caspase 3 (Cell Signaling, Beverly, MA) were obtained commercially.

Immunohistochemistry

Dissected larval tissues were fixed in 4% formaldehyde/phosphate-buffered saline-Tween (PBS-T) for 20 min and blocked in 5% normal goat serum (NGS) for 1 h. Dissected larvae were incubated with 10 μM bromodeoxyuridine (BrdU) in Schneider's media for 1 h before fixation. Embryos were BrdU labeled as described (Shibutani *et al.*, 2008) and fixed in 5% formaldehyde. For BrdU and GFP costaining, embryos were stained for GFP and fixed again in 5% formaldehyde, before 2 N HCl treatment and anti-BrdU staining. Ovaries were incubated with 0.1 mg/ml BrdU in Schneider's medium for 45 min,

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

Dup^{FL} or $Dup^{\Delta PIP}$ 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 $CRL4^{Cdt2}$ 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 $Cul4^{EP2518}$ P-element insertion: $Cul4^{6AP}$, $Cul4^{11L}$, and $Cul4^{11R}$ (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 $Cul4^{KG02900}$ lethal allele is less severe, and $Cul4^{KG02900}$/*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 NH_2-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 $Cul4^{11L}$, $Cul4^{11R,}$ or $Cul4^{6AP}$ mutant larvae (Figure 1B, lanes 4–7). $Cul4^{KG02900}$ 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 $Cul4^{11L}$, 65 residues in $Cul4^{6AP}$, and 82 residues in $Cul4^{11R}$ (Figure 1A). Truncated proteins corresponding to the predicted molecular weights were detected in both $Cul4^{11R}$ and $Cul4^{6AP}$ 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 $Cul4^{11L}$ 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 $Ddb1^{EY01408}$ P-element allele (Figure 1A) causes developmental arrest early during second larval instar when homozygous or when

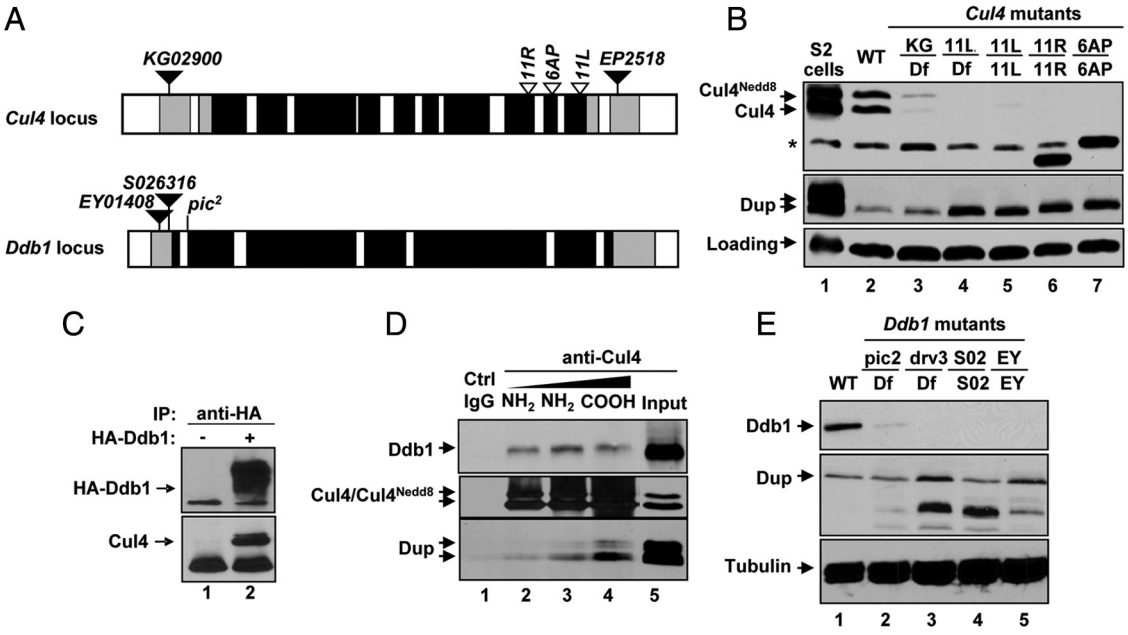


Figure 1. Molecular Analysis of *Drosophila Cul4* and *Ddb1* mutants. (A) The *Drosophila Cul4* locus is located on chromosome 2R at 44A and contains 12 exons (black and gray boxes). The P-elements *KG02900* and *EP2518* are located in the 5′ UTR and 3′ UTR, respectively (gray boxes). Open arrowheads indicate the breakpoints within the open reading frame (black boxes) of P-element excision alleles $Cul4^{11R}$, $Cul4^{6AP}$, and $Cul4^{11L}$. The *Drosophila Ddb1/piccolo* 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 pic^2 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 $Cul4^{6AP}$ 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 NH_2- or COOH-terminus and analyzed by Western blot using anti-Ddb1, 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)Exel6167* or *Df(3R)ry*^{75}. Precise excision of EY01408 reverted the lethality of *Ddb1*^{EY01408}, 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 *Ddb1*^{EY01408} 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 *Ddb1*^{pic} alleles cause second (*pic*^{S026316} and *pic*^{Drv3}) or third (*pic*^{2}) instar lethality and fail to complement the lethality caused by *Ddb1*^{EY01408}.

By Western blot analysis, *pic*^{Drv3}, *pic*^{S026316}, and *Ddb1*^{EY01408} appear to be *Ddb1* null alleles (Figure 1E, lanes 3–5). *pic*^{2} mutants express reduced amounts of Ddb1 (Figure 1E, lane 2), consistent with this Gly^{21} Asp missense allele being a hypomorph (Hu *et al.*, 2008). The *pic*^{2} allele combined with other weak *Ddb1*^{EY01408} excision alleles (i.e., *Ddb1*^{PL12C}) 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 *Cul4*$^{11L/KG02900}$ 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 (*pic*^{2}/*Df(3R)ry*^{75}) 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^{Dup} *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 *Cul4*^{11L} or *Ddb1*^{EY01408} 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 cells (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-

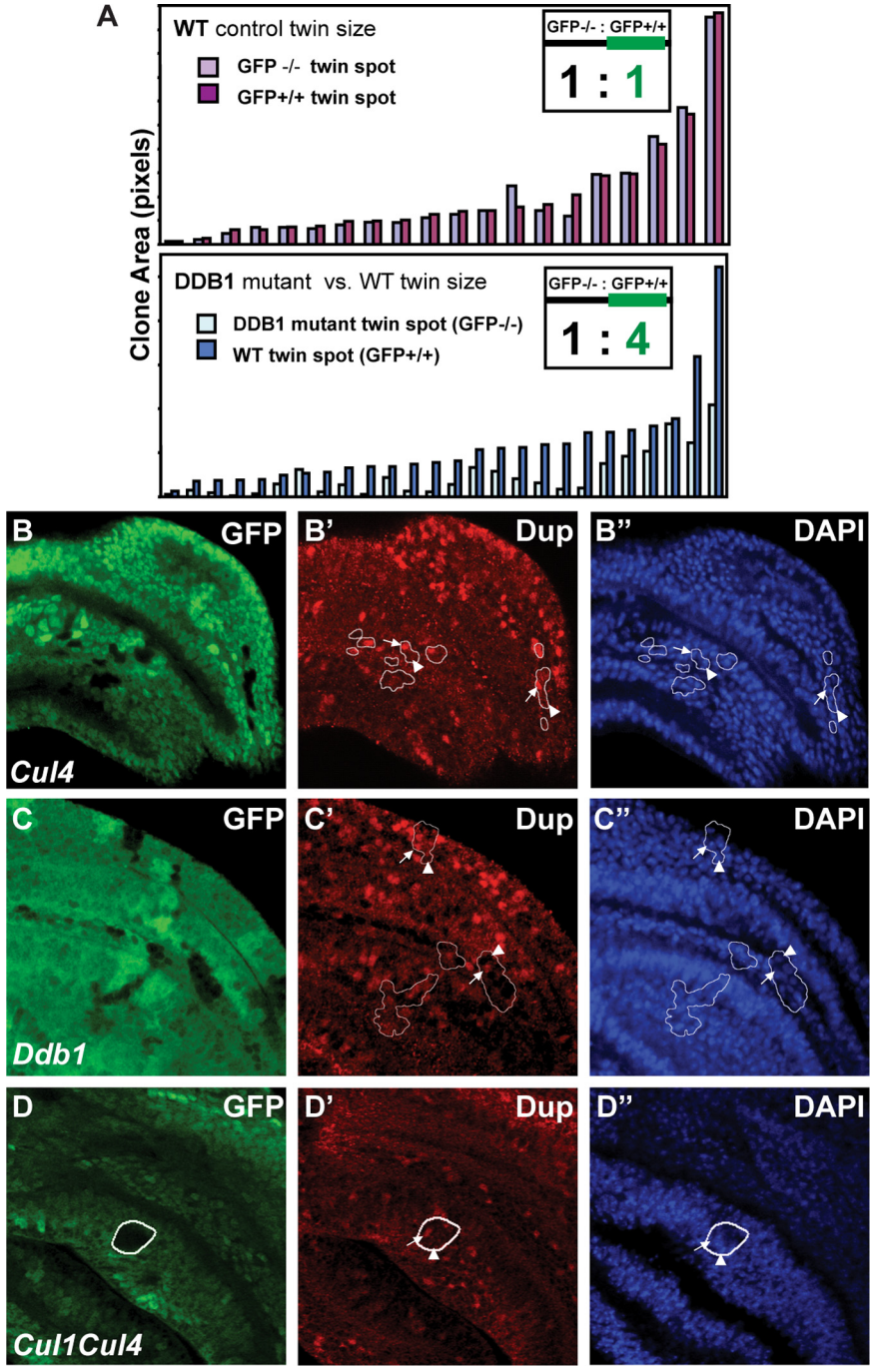


Figure 2. Analysis of *Cul4* and *Ddb1* mutant imaginal disk clones. (A) Histogram of the size measured in pixel area of twin spot clones analyzed in imaginal discs of third instar larvae. Twin spots are ordered on the X axis by GFP^+ clone size. (B–D) Wing imaginal discs containing *Cul4* (B), *Ddb1* (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.

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 $CRL4^{Cdt2}$ 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 ($Dup^{\Delta PIP}$) lacking the NH_2-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 *et al.*, 2006a; Hu and Xiong, 2006; Senga *et al.*, 2006). Both full-length WT Dup (Dup^{FL}) and $Dup^{\Delta PIP}$ were tagged with GFP at their COOH-termini and were expressed using various ubiquitous or tissue-specific Gal4 drivers. Ubiquitous $Dup^{\Delta PIP}$ expression using *da*-Gal4 and *act*-Gal4 caused embryonic lethality, whereas animals expressing Dup^{FL} with the same drivers developed until adulthood (five independent UAS-$Dup^{\Delta PIP}$ and UAS-Dup^{FL} transgenic lines were examined). Eye-specific expression of $Dup^{\Delta PIP}$ using GMR-Gal4 resulted in

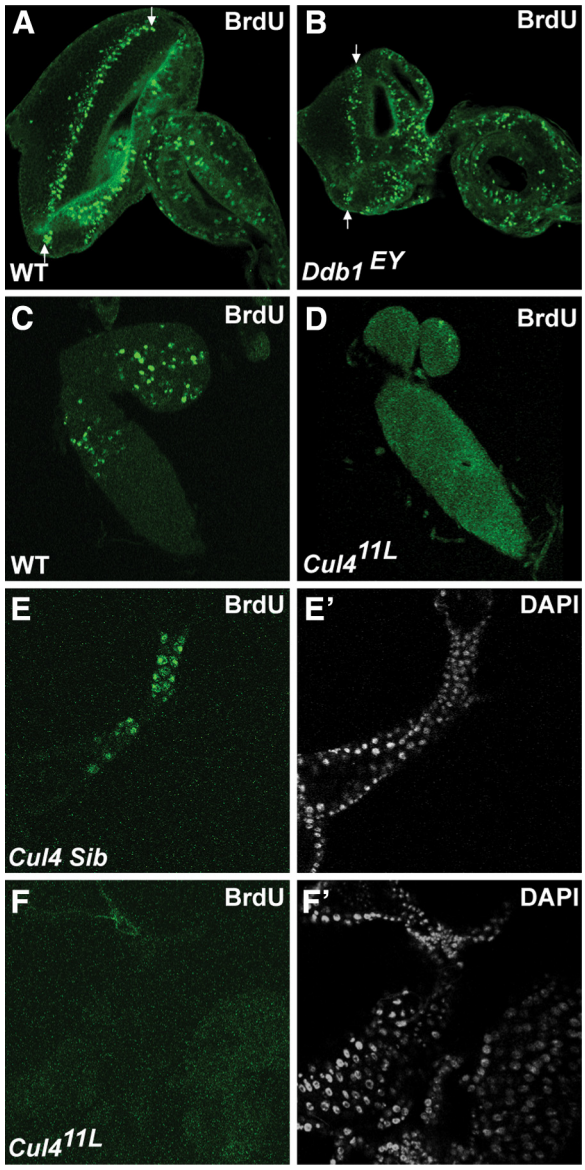


Figure 3. Replication defects in proliferating and endoreplicating *Cul4* mutant larval tissues. All green panels show BrdU-labeled larval tissues. (A and B) Eye imaginal discs dissected from WT (A) or $Ddb1^{pic2}/Df(3R)CA53$ (B) third instar larvae. Arrows indicate BrdU incorporation in the synchronous S phase of the second mitotic wave. (C and D) Brain and CNS dissected from WT (C) or $Cul4^{11L}$ mutant (D) first instar larvae. (E and F) Larval midgut dissected from $Cul4^{11L}/+$ sibling control (E) or $Cul4^{11L}/Cul4^{11L}$ mutant (F) larvae and also stained with DAPI (E′ and F′).

massive tissue malformation, whereas Dup^{FL} caused mildly rough eyes (Figure 4C). These data indicate that $Dup^{\Delta PIP}$ behaves distinctly from Dup^{FL} and suggest that with these drivers our Dup^{FL} 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 $Dup^{\Delta PIP}$ 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 $Dup^{\Delta PIP}$ is degraded correctly at the onset of S phase, we expressed Dup^{FL} and $Dup^{\Delta PIP}$ 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 Dup^{FL} staining in BrdU-positive cells, indicating that Dup^{FL} 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 $Dup^{\Delta PIP}$, indicating that the PIP motif is required for Dup destruction at the onset of S phase (Figure 4E, open arrows).

Although the absence of $Dup^{\Delta PIP}$ 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 $Dup^{\Delta PIP}$ may still be recognized by $CRL4^{Cdt2}$, 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 ([S/T]PX[K/R]) CycE/Cdk2 phosphorylation sites (Figure 4A) mutated to alanine (Dup^{10A}) was not slowed after ectopic cyclin E/Cdk2 induction as WT Dup's mobility was and also that Dup^{10A} was somewhat more stable than WT Dup after heat-shock–induced production. We therefore hypothesized that the 10A mutations would augment the stability of $Dup^{\Delta PIP}$ in the embryo. To test this hypothesis, we generated UAS-Dup^{10A}-GFP and UAS-$Dup^{\Delta PIP/10A}$-GFP transgenes and expressed them with *prd*-GAL4. Dup^{10A} 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 $Dup^{\Delta PIP}$, ~45% of BrdU-positive cells in the *prd*-GAL4 stripe also contained $Dup^{\Delta PIP/10A}$ (Figure 4G). These data indicate that $Dup^{\Delta PIP}$ 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 Dup^{10A}, these data suggest that Cdk mediated destruction is not a major contributor to Dup regulation during S phase.

$Dup^{\Delta PIP}$ 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, 2005; 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 Dup^{FL}-GFP and $Dup^{\Delta PIP}$-GFP could rescue the lack of S phase and consequent cell cycle arrest in *dup* null mutant embryos. Dup^{a1} 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 Dup^{FL} and $Dup^{\Delta PIP}$ 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,

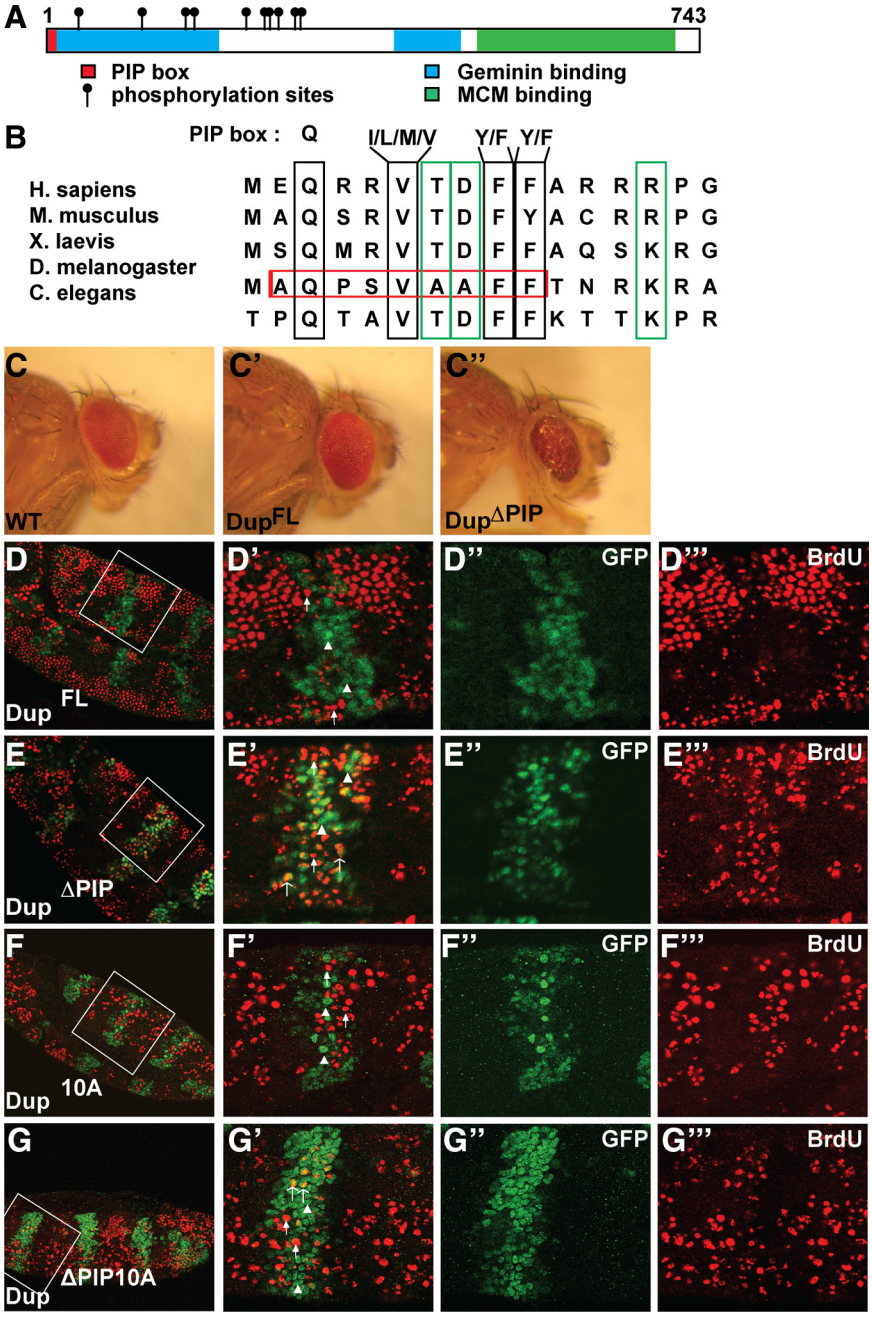


Figure 4. Stabilization of Dup during S phase after deletion of the PIP box. (A) Schematic of the *Drosophila* Dup protein. The 10 consensus CycE/Cdk2 phosphorylation sites changed in the 10A allele are S37, S111, T158, S168, S226, S249, T256, T264, S285, and S291. Gem and MCM binding domains taken from Lee *et al.* (2004) and Saxena *et al.* (2004). (B) Alignment of the Cdt1 $CRL4^{Cdt2}$ degron from several species. Highly conserved residues within the PIP box are located in the black boxes, and the conserved residues necessary for PIP degron function are boxed in green (Havens and Walter, 2009). The red box indicates the residues deleted in $Dup^{\Delta PIP}$. (C–C″) Image of a WT adult eye (C) and eyes expressing Dup^{FL}-GFP (C′) or $Dup^{\Delta PIP}$-GFP (C″) driven by GMR-Gal4. Twenty independent UAS-$Dup^{\Delta PIP}$-GFP and UAS-Dup^{FL}-GFP lines were examined. (D–G) Confocal micrographs of proliferating embryonic ectodermal cells expressing the indicated Dup-GFP transgenes using the *paired* (*prd*)-Gal4 driver. Dup-GFP is visualized by staining with anti-GFP antibodies (green; D″–G″), and S phase cells are marked by BrdU incorporation (red; D‴–G‴). Closed arrows, BrdU-positive cells; arrowheads, Dup-GFP–expressing cells; open arrows, BrdU-positive cells also expressing Dup-GFP. The rectangles indicate the area that was magnified for the images shown in D′–G‴.

close inspection revealed an unusual BrdU incorporation pattern in $Dup^{\Delta PIP}$-expressing cells (Figure 5F): the staining appeared less uniform and more punctate than when Dup^{FL} was expressed (Figure 5E).

We therefore asked if *dup* null cells expressing $Dup^{\Delta PIP}$ could complete mitosis and divide, which would be indicative of normal completion of S phase (Figure 6A). A curious feature of the *dup* 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 *et al.*, 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 *et al.*, 1993; Piatti *et al.*, 1995). We hypothesized that if Dup^{FL} or $Dup^{\Delta PIP}$ expression could support a complete cell cycle, then this aberrant accumulation of pH3-positive cells throughout the epidermis would be eliminated. Indeed, both Dup^{FL} and $Dup^{\Delta PIP}$ expression eliminated pH3 staining in *prd*-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^{FL} and $Dup^{\Delta PIP}$ 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 *Drosophila* embryogenesis results in a reduction in cell size (Lehner and O'Farrell, 1989). Thus, if the Dup transgenes were able to

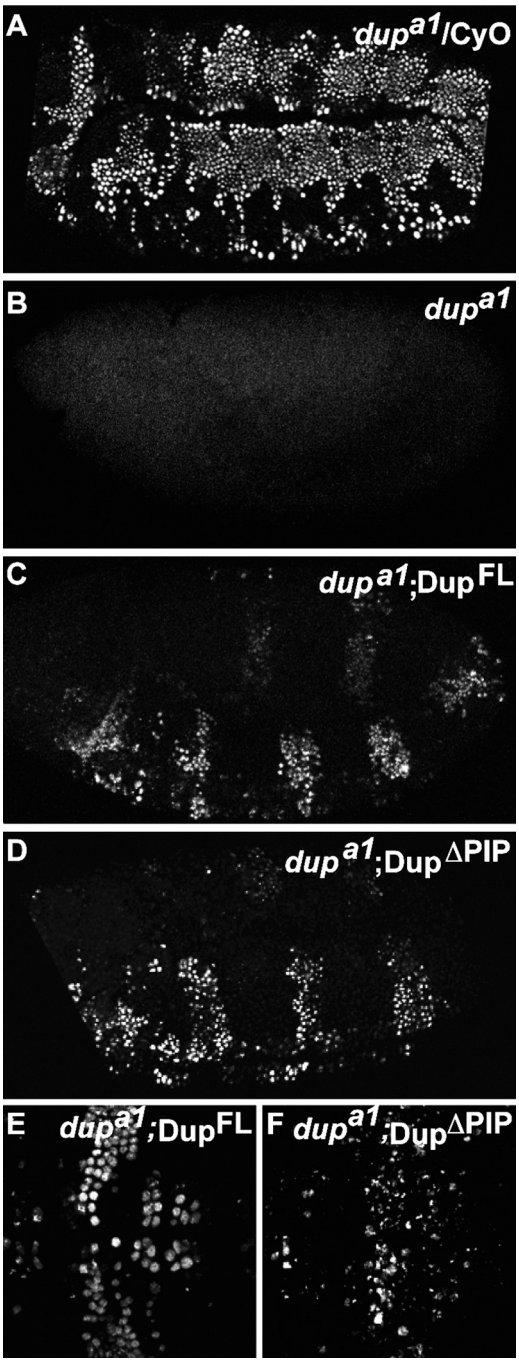


Figure 5. Dup$^{\Delta PIP}$ can support DNA replication. All panels show BrdU-labeled embryos. (A) *dup*^{a1}/CyO control. (B) *dup*^{a1} homozygous mutant embryo. (C and D) *prd*-Gal4–driven expression of Dup^{FL}-GFP (C) or Dup$^{\Delta PIP}$-GFP (D) in *dup*^{a1} homozygous mutant embryos. Note the restoration of BrdU incorporation in the prd-Gal4 pattern in the *dup*^{a1} mutant embryos. (E and F) Higher magnification images of the BrdU incorporation pattern after *prd*-Gal4 expression of Dup^{FL}-GFP (E) and Dup$^{\Delta PIP}$-GFP (F) in *dup*^{a1} homozygous mutant embryos.

support progression through mitosis and cell division, then the cells would be smaller than the *dup* null neighbors. To assess cell size, embryos were stained for the membrane protein Discs large (Dlg), and the size of the cells within and outside the domain of Dup transgene expression was quantified. Although Dup^{FL}-expressing cells were approximately half the size of their *dup* mutant neighbors (Figure 6, C″ and E), Dup$^{\Delta PIP}$-expressing cells remained the same size as their neighbors (Figures 6D″ and E). This finding suggests that Dup^{FL} can rescue the *dup* null cell phenotype and support completion of the cell cycle, whereas Dup$^{\Delta PIP}$-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 Dup$^{\Delta PIP}$-expressing cells accumulate high levels of cyclin A (Figure 7B), whereas the Dup^{FL} cells do not (Figure 7A). Together these data indicate that Dup^{FL} transgenic protein provides normal Dup function and rescues the replication and cell cycle defect of *dup* null cells, whereas Dup$^{\Delta PIP}$ does not.

Why do Dup$^{\Delta PIP}$-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 Dup$^{\Delta PIP}$-expressing and -nonexpressing cells, although we could detect and increase in γ-H2aV staining after irradiation (Figure S1). These data suggest that either Dup$^{\Delta PIP}$ 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, Dup$^{\Delta PIP}$ does not induce continuous rereplication or a slow S phase, because we did not detect BrdU incorporation in *dup* mutant cells expressing Dup$^{\Delta PIP}$ at the time when the neighboring *dup* mutant cells (which are not expressing Dup$^{\Delta PIP}$) have arrested in mitosis 16. We found no difference in cleaved Caspase-3 staining within and outside of the Dup$^{\Delta PIP}$ transgene expression domain, suggesting that Dup$^{\Delta PIP}$-expressing cells do not apoptose. Taken together, our data suggest that *dup* mutant epidermal cells expressing Dup$^{\Delta PIP}$ enter but do not complete S phase of cell cycle 16 and arrest in interphase before mitosis.

Dup$^{\Delta PIP}$ Causes Cell Cycle Arrest in a Wild-Type Background

Our data indicate that Dup$^{\Delta PIP}$ cannot support cell division in a *dup* null background. Because endogenous Dup is promptly degraded at the onset of S phase, ectopic expression of Dup$^{\Delta PIP}$ in a WT background should create a situation in which Dup$^{\Delta PIP}$ 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, Dup$^{\Delta PIP}$ 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 Dup$^{\Delta PIP}$ (Figure 7D, Figure S2). In contrast, these phenotypes did not arise after Dup^{FL} expression in WT embryos (Figure 7C, Figure S2). The Dup$^{\Delta PIP}$-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 Dup$^{\Delta PIP}$

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

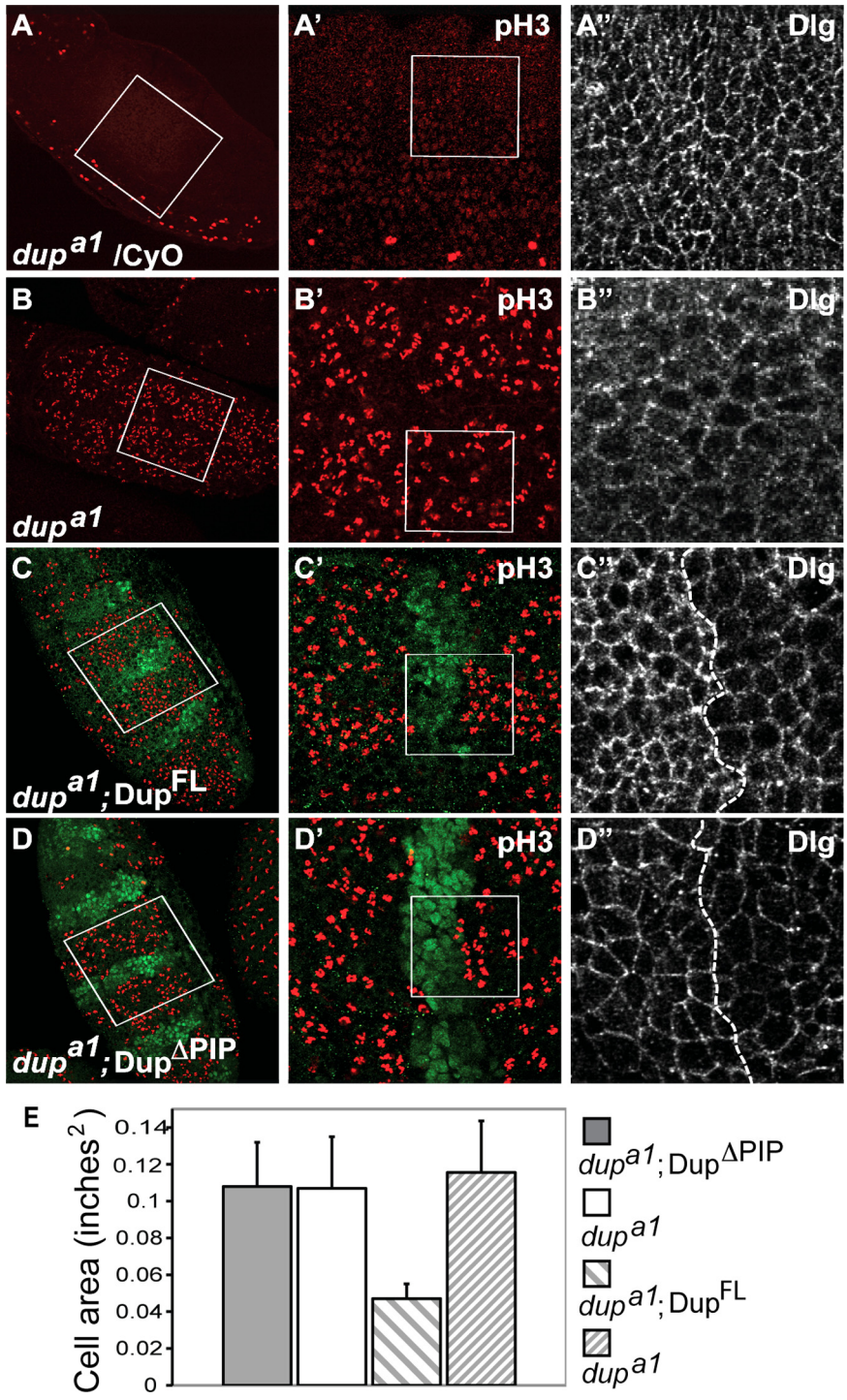


Figure 6. Dup$^{\Delta PIP}$ cannot support a full cell division cycle. (A–D) Anti-pH3 (red A′–D′) and discs large (Dlg) (white, A″–D″) staining of *dup*^{a1} null (B–D) or sibling control (A) embryos expressing Dup^{FL}-GFP (green, C and C′) or Dup$^{\Delta PIP}$-GFP (green, D and D′) with *prd*-Gal4. 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% (E) smaller size of the Dup^{FL}-expressing cells on the left side of the C″ panel, whereas the Dup$^{\Delta PIP}$-expressing cells are similar in size to control (D″ and E). (E) Quantification of relative cell size in Dup^{FL} or Dup$^{\Delta PIP}$-expressing cells compared with that of their *dup* null neighbors. Error bars, SD.

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 Dup^{FL} and Dup$^{\Delta PIP}$ in endocycling follicle cells using c323a-Gal4, which drives expression in all follicle cells of stages 8–14 (Figure 8A). More follicle cells expressed Dup$^{\Delta PIP}$ than Dup^{FL}, suggesting that Dup$^{\Delta PIP}$ was stabilized (Figure 8, B 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 Dup^{FL} or Dup$^{\Delta PIP}$. We found that 43% of endocycle S phase cells retained Dup$^{\Delta PIP}$ (Figure 8E, open arrows), whereas Dup^{FL} is degraded at the onset of endocycle S phase (Figure 8D).

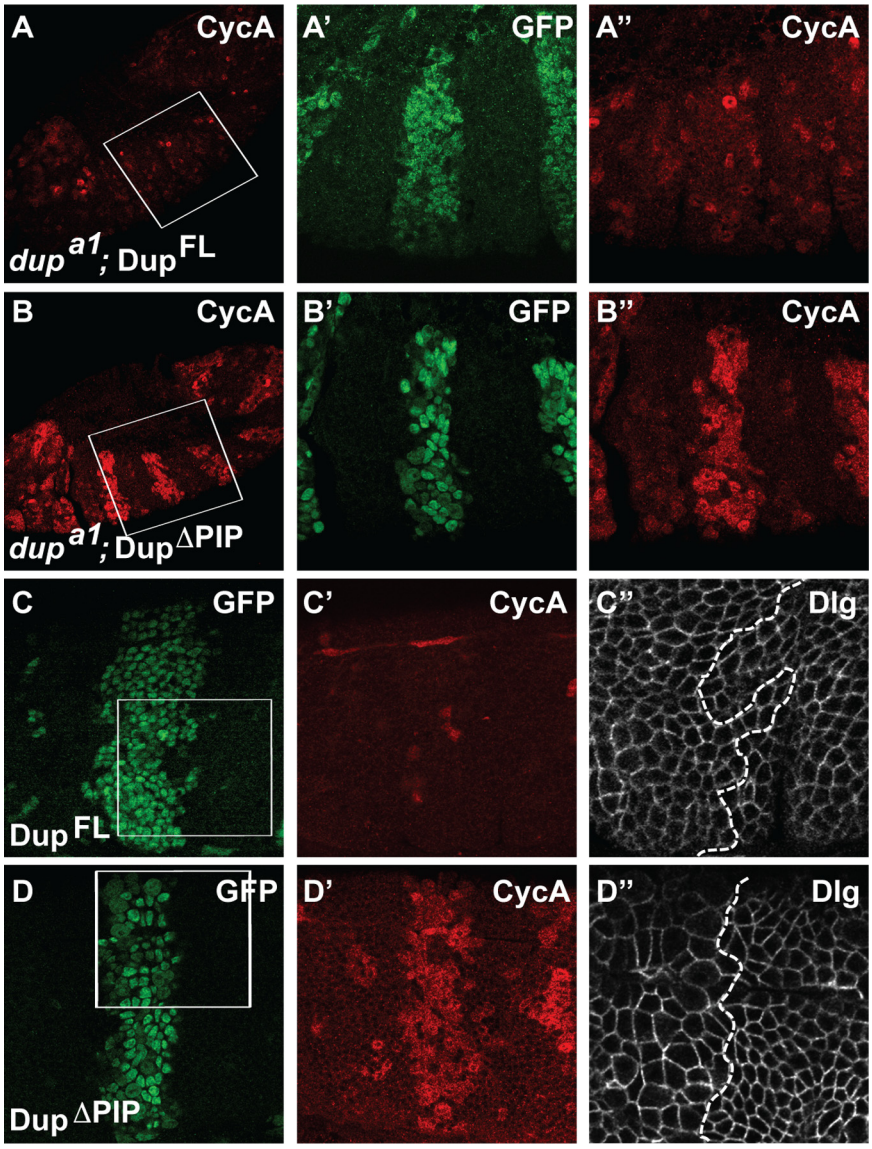


Figure 7. Dup$^{\Delta PIP}$ arrests the cell cycle in interphase. (A and B) Dup^{FL}-GFP (A) or Dup$^{\Delta PIP}$-GFP (B)-expressing *dup*^{a1} null cells stained with anti-CycA (red) and anti-GFP (green). (C and D) Dup^{FL}-GFP– (C) or Dup$^{\Delta PIP}$-GFP– (D) expressing WT cells stained with anti-GFP (green, C and D), anti-CycA (red, C′ and D′), and anti-Dlg (white, C″ and D″). Note the larger cell size in the left side of D″ relative to the right side, indicating cell cycle arrest caused by Dup$^{\Delta PIP}$ expression.

The pattern of BrdU incorporation and total number of BrdU-labeled cells (Figure 9G) was similar between Dup^{FL} and Dup$^{\Delta PIP}$-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^{FL} and Dup$^{\Delta PIP}$ 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} and Dup$^{\Delta PIP}$-expressing females. About 94% of eggs laid by Dup^{FL} or Dup$^{\Delta PIP}$-expressing females hatched into viable larvae, similar to WT (Figure 9G). Together these data suggest that Dup$^{\Delta PIP}$ expression with c323a-Gal4 does not disrupt follicle cell function or oogenesis. This result is in contrast to the defects caused by Dup$^{\Delta PIP}$ in the proliferating embryonic cells. One possibility is that we achieved a higher level of Dup$^{\Delta PIP}$ 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$^{\Delta PIP}$ between embryonic cells and follicle cells, as assessed by measuring Dup$^{\Delta PIP}$-GFP fluorescence by confocal microscopy of individual nuclei (Figure S3). We conclude that proliferating cells are more sensitive to Dup$^{\Delta PIP}$ expression than endocycling follicle cells.

Follicle Cell Gene Amplification Is Not Inhibited by Dup$^{\Delta PIP}$

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

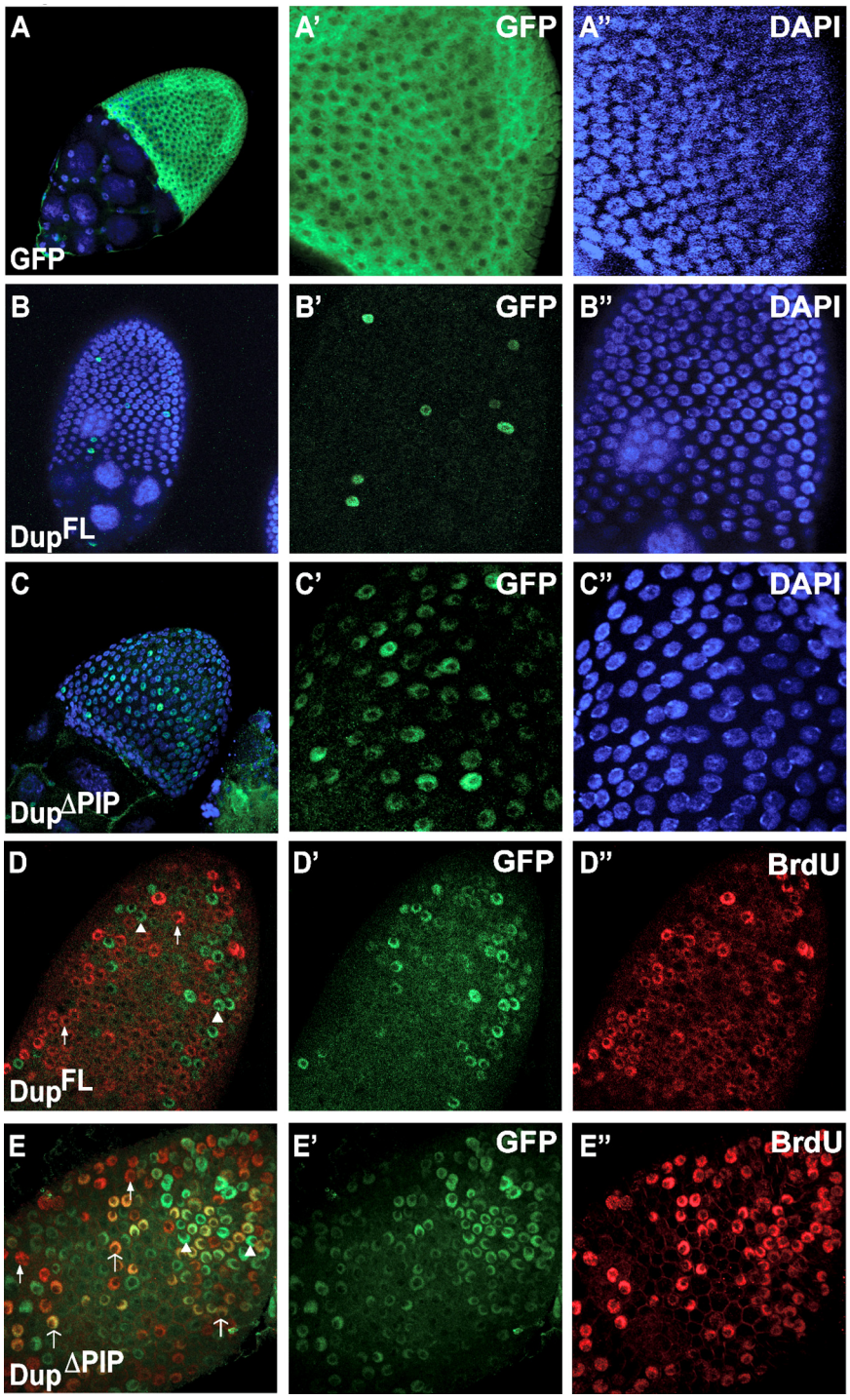
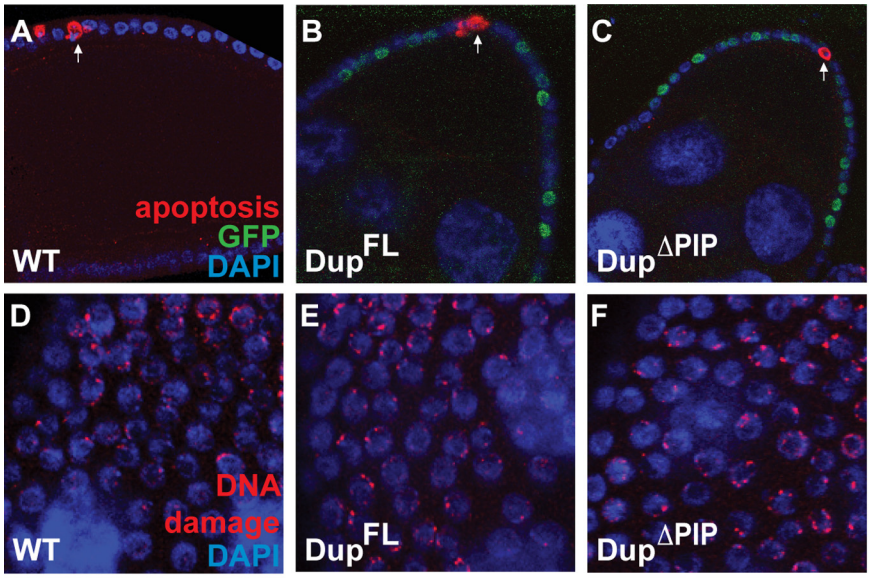


Figure 8. Dup$^{\Delta PIP}$ is stabilized in follicle cell endocycles. (A–C) Confocal images of follicle cells from stage 9 egg chambers expressing GFP (A), Dup^{FL}-GFP (B) or Dup$^{\Delta PIP}$-GFP (C) using the c323a-Gal4 driver and stained with anti-GFP (green, A′–C′) and DAPI (blue, A″–C″). (D and E) Confocal images of follicle cells from stage 9 egg chambers expressing Dup^{FL}-GFP (D) or Dup$^{\Delta PIP}$-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.

in stage 10A follicle cells expressing Dup^{FL} or Dup$^{\Delta PIP}$. Our results indicate that this pattern of BrdU incorporation is largely unaffected by Dup^{FL} (Figure 9I), whereas expression of Dup$^{\Delta PIP}$ 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$^{\Delta PIP}$.

Geminin Function Restrains Dup$^{\Delta PIP}$ 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$^{\Delta PIP}$ activity in endocycling follicle cells. To test this hypothesis, we reduced the gene dose of *geminin* in half together with c323a-Gal4-driven expression of Dup^{FL} or Dup$^{\Delta PIP}$ and compared the results to WT and *geminin* heterozygote ovaries. Although ova-



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	% hatching	S phase index
WT	94.1 +/- 1.63	33.6 +/- 4.41
Dup^{FL}	93.8 +/- 2.15	35.4 +/- 3.61
Dup$^{\Delta PIP}$	93.6 +/- 1.96	36.8 +/- 4.53

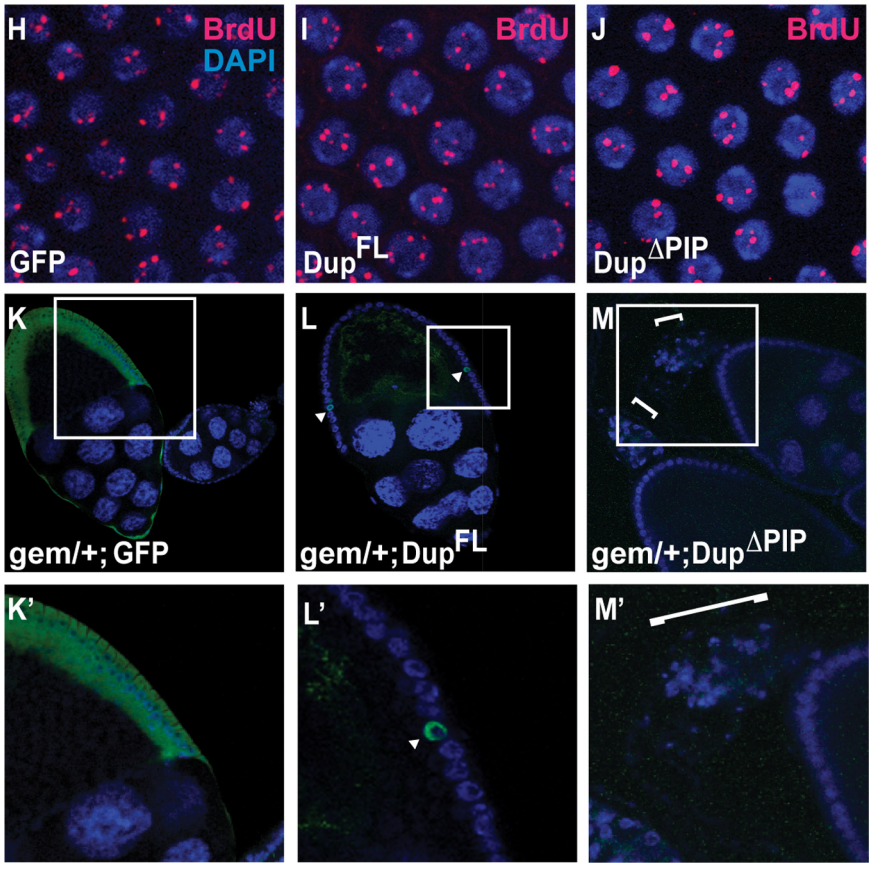

Figure 9. Dup$^{\Delta PIP}$ expression in follicle cells disrupts oogenesis only when Gem gene dose is reduced. (A–F) Confocal images of follicle cells from WT stage 8 egg chambers (A and D) or from those expressing Dup^{FL}-GFP (B and E), or Dup$^{\Delta PIP}$-GFP (C and F) using the c323a-Gal4 driver and stained with anti-GFP (green), DAPI (blue), and anti-cleaved caspase 3 (red, A–C) or anti-γH2aV (red, D–F). (G) Percent hatching of eggs laid by female flies expressing GFP, Dup^{FL}-GFP, or Dup$^{\Delta PIP}$-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^{FL}-GFP, or Dup$^{\Delta PIP}$-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^{FL}-GFP (I), or Dup$^{\Delta PIP}$-GFP (J) with c323a-Gal4 and stained with anti-BrdU (red) and DAPI (blue). (K–M) *gem*$^{l(2)03202}$/CyO stage 9 follicle cells expressing GFP (K), Dup^{FL}-GFP (L), or Dup$^{\Delta PIP}$-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^{FL}-GFP. The brackets in M and M′ indicate degenerated stage 9 egg chambers.

ries from *geminin*/+ heterozygous control flies and *geminin*/+ flies expressing Dup^{FL} appeared WT (Figure 9, K, L, K′, and L′), *geminin*/+ flies expressing Dup$^{\Delta PIP}$ 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

$Dup^{\Delta PIP}$ 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_2-terminal PIP box is required for progression through the cell division cycle in embryonic epidermal cells but is dispensable for progression through follicle cell endocycles.

PIP Box–dependent Degradation of Dup

Our results indicate that deletion of the PIP box prevents the rapid destruction of Dup at the beginning of S phase. Before discovery of the PIP degron/CRL4 mechanism of replication-coupled proteolysis, Thomer *et al.* (2004) reported a similar result with a mutant version of Dup lacking the NH_2-terminal 46% of the protein, including the PIP box. Thus, our results suggest that the Thomer *et al.* (2004) observation is due to deletion of the PIP degron. Biochemical and genetic experiments from a number of species suggest that the PIP degron recruits proteins to chromatin-bound PCNA at replication forks during S phase. These proteins are subsequently ubiquitylated by $CRL4^{Cdt2}$ and proteolyzed (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 imaginal cells mutant for components of $CRL4^{Cdt2}$, the PIP degron mechanism is conserved in *Drosophila* (Shibutani *et al.,* 2008), and $CRL4^{Cdt2}$ 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 $CRL4^{Cdt2}$ function may have masked our ability to detect effects on Dup protein.

Interestingly, deletion of the PIP 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 $Dup^{\Delta PIP}$ 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 $Dup^{\Delta PIP}$ is unable to support progression through the cell division cycle. Similarly, $Dup^{\Delta PIP}$ 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 rereplication in other cell types after overexpression of Cdt1 or depletion of Cdt1 regulatory mechanisms (e.g., CRL4 or Gem; Arias and Walter, 2007). We also did not detect extensive DNA damage or apoptosis. We propose that the near physiological levels of $Dup^{\Delta PIP}$ 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, $Dup^{\Delta PIP}$ 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 cause rereplication (Thomer *et al.,* 2004), and that *Cul4* mutant follicle cells hyperaccumulate Dup and exhibit replication defects during gene amplification (Lin *et al.,* 2009). We found that Gal4-driven expression of $Dup^{\Delta PIP}$ did not cause either of these phenotypes and did not dramatically alter endocycle 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 $Dup^{\Delta PIP}$ 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 endocycle (Mehrotra *et al.,* 2008). Lin *et al.* (2009) showed that ectopic genomic BrdU incorporation during gene amplification stages occurs in *Cul4* or *Ddb1* mutant follicle cells. We did not observe the same phenotype after $Dup^{\Delta PIP}$ 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 overreplication 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 $Dup^{\Delta PIP}$ 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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