Drosophila double-parked is sufficient to induce re-replication during development and is regulated by cyclin E/CDK2

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Summary

It is important that chromosomes are duplicated only once per cell cycle. Over-replication is prevented by multiple mechanisms that block the reformation of a pre-replicative complex (pre-RC) onto origins in S and G_2 phase. We have investigated the developmental regulation of Double-parked (Dup) protein, the *Drosophila* ortholog of Cdt1, a conserved and essential pre-RC component found in human and other organisms. We find that phosphorylation and degradation of Dup protein at G_1/S requires cyclin E/CDK2. The N terminus of Dup, which contains ten potential CDK phosphorylation sites, is necessary and sufficient for Dup degradation during S phase of mitotic cycles and endocycles. Mutation of these ten phosphorylation sites, however, only partially stabilizes the protein, suggesting that multiple mechanisms ensure Dup degradation. This regulation is important because increased Dup protein is sufficient to induce profound re-replication and death of developing cells. Mis-expression has different effects on genomic replication than on developmental amplification from chorion origins. The C terminus alone has no effect on genomic replication, but it is better than full-length protein at stimulating amplification. Mutation of the Dup CDK sites increases genomic re-replication, but is dominant negative for amplification. These two results suggest that phosphorylation regulates Dup activity differently during these developmentally specific types of DNA replication. Moreover, the ability of the CDK site mutant to rapidly inhibit BrdU incorporation suggests that Dup is required for fork elongation during amplification. In the context of findings from human and other cells, our results indicate that stringent regulation of Dup protein is critical to protect genome integrity.

Key words: DNA replication, Cyclin E, Double-parked, Cdt1, Chorion gene amplification

Introduction

During every cell cycle the entire genome must be copied, but it is important that each region replicates only once to maintain genome integrity. To prevent over-replication, the activity of origins of replication is tightly controlled. Beginning in late mitosis, origins are prepared for replication by binding of a pre-replicative complex (pre-RC), which is subsequently activated to initiate replication at the onset of S phase (reviewed by Bell and Stillman, 1992) (Bell and Dutta, 2002; Diffley et al., 1994). The building of the pre-RC onto origins in late mitosis/early G_1 is a stepwise process. The origin recognition complex (ORC) serves as a scaffold for subsequent association of Cdc6 and Cdt1, both of which are required to load the Minichromosome Maintenance (MCM) complex, the replicative helicase (Bell and Stillman, 1992; Cocker et al., 1996; Diffley et al., 1994) (reviewed by Diffley, 2001) (Liang et al., 1995; Maiorano et al., 2000; Nishitani et al., 2000; Nishitani and Nurse, 1995; Tada et al., 2001). Once MCMs are loaded, the origin is considered to be licensed for subsequent replication (Chong et al., 1995). Cdc7 kinase, with its activating subunit Dbf4, and CDK2 kinase, activated by cyclin E or cyclin A, are then required for initiation of replication (reviewed by Hengstschlager et al., 1999; Sclafani, 2000) (Dowell et al., 1994; Lei et al., 1997; Masumoto et al., 2002; Zou and Stillman, 1998). Initiation is associated with departure of Cdc6, Cdt1, MCMs, and, in multicellular eukaryotes, certain ORC subunits from the origin (reviewed by Bell and Dutta, 2002). Continued CDK activity in S, G_2, and early M phases inhibits reassembly of the pre-RC to block origin refiring (Dahmann et al., 1995; Hayles et al., 1994). Unique to multicellular eukaryotes is another inhibitor of pre-RC assembly, Geminin, which binds Cdt1 and renders it incapable of loading the MCM complex (McGarry and Kirschner, 1998; Quinn et al., 2001; Tada et al., 2001; Wohlschlegel et al., 2000). It is only after Geminin and cyclins are degraded at the subsequent metaphase that the pre-RC can reform, thereby restricting origin licensing, and DNA replication, to once per segregation of chromosomes (Dahmann et al., 1995; Diffley et al., 1994; Noton and Diffley, 2000; Piatti et al., 1996; Tada et al., 2001).

Although phosphorylation of pre-RC subunits appears to be important for initiation and to block pre-RC re-assembly, the biochemical mechanisms are not fully understood. In the yeasts *Saccharomyces cerevisiae* and *S. pombe*, CDKs block re-replication by phosphorylating several pre-RC targets including CDC6 and subunits of the ORC and MCM complex (Drury et al., 2000; Gopalakrishnan et al., 2001; Jallepalli et

al., 1997; Labib et al., 1999; Nguyen et al., 2000; Nguyen et al., 2001; Nishitani and Nurse, 1995; Vas et al., 2001). All three of these blocks must be abrogated before even partial re-replication is permitted in *S. cerevisiae* cells in G_2, suggesting that multiple reinforcing mechanisms have evolved to protect the integrity of the genome (Nguyen et al., 2001). In *S. pombe*, however, over-expression of Cdc18 (the Cdc6 homolog) alone, but not other pre-RC subunits, is sufficient to induce re-replication (Nishitani and Nurse, 1995). Thus, whether mis-regulation of a single protein can induce re-replication may differ among organisms. In higher eukaryotes, it also appears that CDKs block re-replication by targeting multiple pre-RC subunits to protect genome integrity (reviewed by Bell and Dutta, 2002) (Delmolino et al., 2001; Hua et al., 1997; Mihaylov et al., 2002; Pelizon et al., 2000; Petersen et al., 1999; Saha et al., 1998; Yamaguchi and Newport, 2003).

Despite the prevailing concept of redundant controls, recent evidence suggests that regulation of Cdt1 is especially important to inhibit re-replication. In a number of systems, over-expression of Cdt1, or inactivation of its inhibitor Geminin, causes partial, but not full, re-replication of the genome (Mihaylov et al., 2002; Quinn et al., 2001; Tada et al., 2001; Vaziri et al., 2003; Wohlschlegel et al., 2000). In all organisms examined, except *S. cerevisiae*, the majority of Cdt1 protein is rapidly degraded at the G_1/S transition. Evidence from several organisms suggests that Cdt1 is targeted for degradation at the proteasome by two ubiquitin ligase complexes, an SCF (Skp1, Cul1, F box) ubiquitin ligase that contains the specificity subunit Skp2, and an SCF-like ubiquitin ligase that is based on Cul4 (Higa et al., 2003; Li et al., 2003; Nishitani et al., 2001). This degradation is probably important because over-expression of Cdt1 in p53 mutant human cells in culture can lead to partial re-replication, and contributes to oncogenic transformation of mouse erythroid cells (Arentson et al., 2002; Vaziri et al., 2003). In *Caenorhabditis elegans*, RNAi of Cul4 leads to stabilization of Cdt1 protein and polyploidization (Zhong et al., 2003). It is unclear, however, whether Cul4 controls degradation of other proteins important for re-replication control. Therefore, two important remaining questions are whether increased Cdt1 protein is sufficient to induce genome reduplication in normal cells during development, and what coordinates the rapid degradation of Cdt1 with the initiation of DNA replication at the G_1/S transition.

The *Drosophila* ortholog of Cdt1, the *double-parked* (*dup*) gene, was initially identified as recessive embryonic lethal or female-sterile mutants that have defects in genomic replication or developmental amplification of eggshell (chorion) protein genes in the ovary (Underwood et al., 1990; Whittaker et al., 2000). In this report, we provide evidence that degradation of Dup is controlled in part by cyclin E/CDK2 phosphorylation, and that additional mechanisms also ensure Dup degradation. Control of Dup protein abundance is critical because increased expression of Dup in diploid cells is sufficient to induce polyploidization and cell death in developing tissues. Interestingly, over-expression of wild-type and mutant Dup derivatives have different effects on genomic replication than on amplification from chorion origins. These last results provide insight into how phosphorylation regulates Dup during these developmentally distinct replication programs, and suggest that Dup participates in replication fork elongation during amplification.

Materials and methods

Drosophila genetics

Standard techniques were used for culture of *Drosophila melanogaster*. Information about strains and genetic nomenclature can be found at http://www.flybase.harvard.edu. Myc-tagged Dup P elements were transformed into *y w* ^{67c23} by microinjection using standard methods (Spradling and Rubin, 1982).

Construction of Dup transgenes

P{w^{+mC}*, hsp70:Myc:FL-Dup}* was constructed by RT-PCR amplification of a Dup cDNA using primers that encompass the region from the start codon to the termination codon, subcloning into pBUM (provided by J. Sekelsky) resulting in an in frame N-terminal fusion of a new AUG and single Myc epitope, and finally into pP{CaSpeR-hs} (Thummel et al., 1988). *P{w+, hsp70:Myc:N-Dup}* was created similarly except that it contains a stop codon after amino acid 343. *P{w*$^{+mC}$*, hsp70:Myc:C-Dup}* was created similarly with primers that amplify from amino acid 344 E to the stop codon of the Dup coding region. The NLS from SV40 was inserted in frame between the Myc epitope and Dup coding region. *P{w*$^{+mC}$*, hsp70:Myc:Dup 10(A)}* was created by mutating the serines and threonines at putative phosphorylation sites to alanine using the Stratagene multi-mutagenesis kit, and then subcloning as above. The amino acid coordinates of the serines and threonines are: S37, S111, T158, S168, S226, S249, T256, T264, S285, S291. Detailed information on mutagenic oligonucleotides and methods are available upon request.

Immunofluorescent microscopy

Antibody and BrdU labeling were as previously described (Calvi and Lilly, 2003; Schwed et al., 2002). Guinea pig polyclonal Dup antibody was used at 1:1000 dilution (Whittaker et al., 2000). The affinity purified rabbit polyclonal Dup antibody (a gift from E. Beall and M. Botchan) was used at 1:500. Anti-cyclin E monoclonal 8B10 (Richardson et al., 1995) was used at 1:5, and anti-cyclin B monoclonal (Lehner and O'Farrell, 1990) was used at 1:4. Rabbit polyclonal anti-caspase 3 antibody (Cell Signaling, Beverly, MA) was used at 1:100. For Myc/BrdU stability experiments, cells were fixed and denatured with DNAse (Calvi and Lilly, 2003) and subsequently labeled with polyclonal sheep anti-BrdU (Research Diagnostics, Flanders, NJ) and monoclonal mouse anti-Myc (clone 9E10; Upstate Biotech, Lake Placid, NY), and Toto-3 as described previously (Schwed et al., 2002). Slides were analyzed using a Leica SP confocal microscope and TCS-NT software, or by using Openlab quantification software on non-confocal images (Image Processing and Vision Co. Ltd).

Antibody production, western blots and immunoprecipitation

The anti-Geminin antibody was raised in rabbits against a synthetic peptide (C)QQRQTLKPLQGNVNDKEN (ZYMED) corresponding to amino acid residues 24-41 predicted from the gene CG3183 in the *Drosophila* genomic sequence. Antibody was affinity purified using this peptide and the Sulfolink Kit (Pierce, Rockford, IL).

Standard methods were used for immunoprecipitation and western blot analysis of Dup and Geminin, which were detected using the ECL kit (Amersham Biosciences) (Harlow and Lane, 1999). For Fig. 4A, 0- to 16-hour *y w* embryos were homogenized in NP40 lysis buffer and spun for 5 minutes at 4°C to remove insoluble material. Guinea pig anti-Dup antibody was then used for standard immunoprecipitation. Approximately 1/100 total lysate (input) and 1/3 of the pellet samples were loaded. Dup was detected using the affinity purified polyclonal rabbit antibody at 1:5000.

For endogenous Dup, extracts were made as above from third instar larval brains from *P{hsp70:GAL4}*; *P{UAS:CycE}* and *P{hsp70:GAL4}*; *P{UAS:dap}* animals, some of which had received a 37°C, 30-minute heat shock followed by a 1-hour 22°C recovery.

Lambda protein phosphatase (Upstate Biotechnology, Lake Placid, NY) treatment was for 30 minutes, at 30°C. For Myc-tagged Dup, methods were as above except that a hsp70:CycE transgene was used (Lane et al., 1996). The Dup and hsp70:cyclin E transgenes were heterozygous in all animals. Westerns were quantified using a Bio-Rad Chemi Doc and Quantity One software, and were normalized against a linear regression curve generated from a western of extract serial dilutions.

Results

Dup protein declines rapidly at the G_1/S transition within cells of the *Drosophila* eye disc

Previous evidence suggested that Dup (Cdt1) protein levels oscillate during cell cycles in *Drosophila* (Whittaker et al., 2000). To determine whether this oscillation is due to Dup protein degradation at G_1/S, we examined its expression within the synchronized cell cycles of the larval eye primordium. Late in third instar, a wave of differentiation sweeps across the eye imaginal disc, which is visible as a morphogenetic furrow (MF). Cells are synchronized in G_1 upon entering the furrow. Specific cells posterior to the furrow then enter a synchronous S phase, which is visible as a stripe of BrdU labeling (Fig. 1A) (Thomas et al., 1994). Labeling with affinity-purified rabbit poly-clonal Dup antibody (kindly provided by L. Beall and M. Botchan) indicated that the protein was abundant in nuclei of late G_1 cells, but was undetectable in S phase cells incorporating BrdU (Fig. 1A). Labeling with a previously characterized guinea pig anti-Dup antibody gave identical results suggesting that immunolabeling reflects Dup protein in vivo (Fig. 4A and data not shown) (Whittaker et al., 2000). Double labeling for Dup and cyclin E indicated that both were abundant in nuclei of cells in late G_1, but then Dup rapidly declined while cyclin E persisted into S phase (Fig. 1B) (Richardson et al., 1995). Labeling for the G_2 and M phase marker cyclin B also indicated that Dup levels decline significantly before cells enter G_2 (Fig. 1C) (Lehner and O'Farrell, 1990). Similar results were obtained for the non-synchronized cell cycles in the eye and other imaginal discs (data not shown). This rapid decline in protein was primarily due to post-transcriptional regulation because in situ hybridization indicated that *dup* mRNA persists after G_1 (data not shown). Moreover, expression of a *dup* transgene from the strong hsp70 promoter did not result in detectable Dup protein during S phase (Fig. 5B and see below). The data suggest that, similar to Cdt1 in humans and other organisms, Dup protein is abundant in G_1 when origins are licensed, but is then rapidly degraded when cyclin E appears at G_1/S (Maiorano et al., 2000; Nishitani et al., 2000; Rialland et al., 2002; Wohlschlegel et al., 2000).

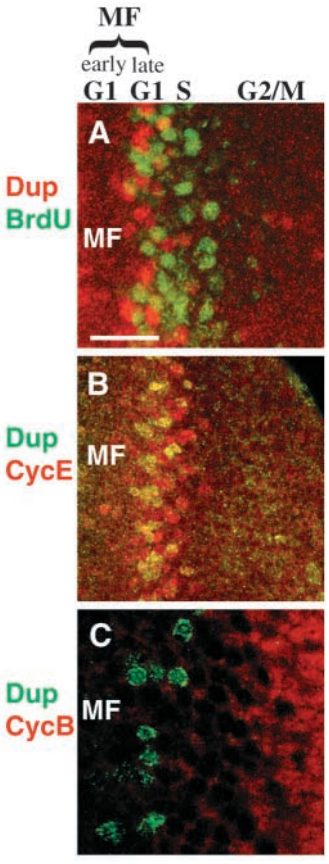

Fig. 1. Dup rapidly declines at G_1/S in the eye disc. Synchronized cell cycles of the eye morphogenetic furrow (MF) in a third instar larva are indicated above (posterior to the right). (A) Dup antibody labeling (red), BrdU (green). (B) Cyclin E (red) and Dup (green) (overlap appears yellow). (C) Cyclin B (red) Dup (green). Images are composites of confocal sections. Scale bar: 10 μm (A-C).

Degradation of Dup depends on cyclin E/CDK2 in vivo

To determine whether cyclin E/CDK2 is required for Dup degradation, we inhibited its activity with GMR:p21, which expresses the vertebrate p21 Cdk inhibitor posterior to the MF (de Nooij and Hariharan, 1995). GMR:p21 blocked Dup degradation resulting in high levels of Dup labeling in nuclei of cells posterior to the MF (compare Fig. 2A and B). This is not likely to be due to increased *dup* transcription because inhibition of CDK2 reduces the activity of the E2F1/Dp transcription factor which controls *dup* expression at G_1/S (Whittaker et al., 2000) (data not shown). Instead, the data suggest that cyclin E/CDK2 activity is required for Dup protein degradation in vivo, but does not resolve whether this regulation is direct.

Dup is associated with CDK2 protein and activity

To assess if CDK2 could directly regulate Dup, we investigated whether they co-immunoprecipitate from embryo extracts. We

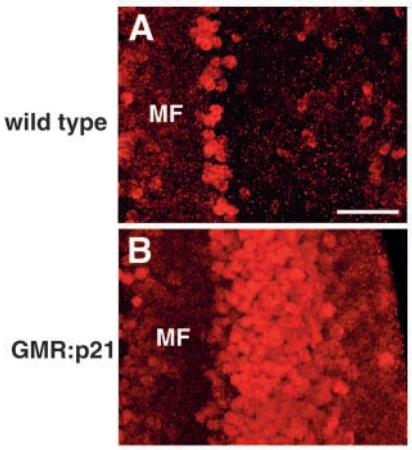

Fig. 2. Cyclin E/CDK2 is required for Dup degradation. Dup antibody labeling (red) in the morphogenetic furrow (MF) of (A) wild-type eye disc, (B) GMR:p21 expressing eye disc. Posterior is to the right. Image is a composite of confocal sections. Scale bar: 20 μm (A,B).

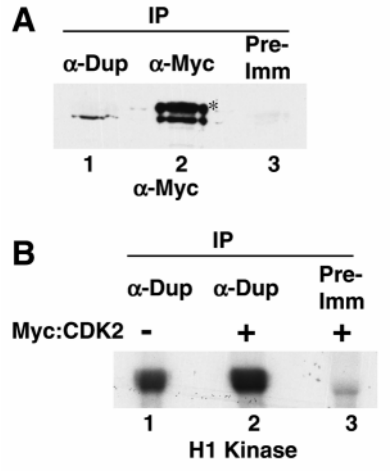

Fig. 3. Dup is associated with CDK2 protein and activity in embryo extracts. (A) Dup immunoprecipitates Myc:CDK2 from embryo extracts. Extracts from da:GAL4; UAS:6XMyc:CDK2 embryos were used for immunoprecipitation with antibodies to Dup (lane 1), Myc (lane 2) or with pre-immune sera (lane 3) and western blots were subsequently probed for Myc. The asterisk indicates the mouse immunoglobulin band. (B) Dup is associated with a CDK kinase activity. Dup antibody (lanes 1 and 2) or pre-immune serum (lane 3) was used for immunoprecipitation from wild-type embryos (lane 1) or those expressing 6XMyc:CDK2 (lanes 2 and 3), and the pellet was subsequently used in an in vitro kinase assay with ^{32}P[ATP] and histone H1 as substrate.

used a strain containing UAS:6XMyc:CDK2 that was expressed in embryos using the ubiquitous da:GAL4 driver (Meyer et al., 2000). Immunoprecipitation of Dup followed by western blotting for Myc gave evidence for association of 6XMyc:CDK2 with Dup (Fig. 3A). We did not detect Dup protein in reciprocal immunoprecipitations with anti-Myc antibodies probably because only a small fraction of the over-expressed 6XMyc:CDK2 protein is associated with endogenous Dup (data not shown).

To determine whether Dup is associated with a CDK activity, Dup was immunoprecipitated and the pellet was used as a source of kinase in a standard in vitro reaction using histone H1 as a substrate. The Dup pellet from the UAS:6XMyc:CDK2 strain contained robust H1 kinase activity that was not observed in pre-immune controls (Fig. 3B). Dup IP from a strain that lacked the UAS:6XMyc:CDK2 also was associated with H1 kinase activity, indicating that over-expression of CDK2 is not necessary (Fig. 3B). These results suggest Dup associates with a CDK kinase activity that is, at least in part, due to CDK2.

Phosphorylation of Dup depends on cyclin E/CDK2

To begin to address whether Dup is phosphorylated in vivo, we examined Dup for post-translational modification using western blotting. Immunoprecipitation of Dup from embryos indicated that there are at least three isoforms of the protein, a predicted full-length ~82 kDa form and a higher molecular weight doublet of ~105 kDa and ~106 kDa (Fig. 4A). To confirm that these isoforms are indeed Dup, we raised antibodies against *Drosophila* geminin protein, a tight binding inhibitor of Dup (Quinn et al., 2001). All three isoforms co-immunoprecipitated with Geminin, further suggesting that they represent Dup (Fig. 4A). Previous reports that used independently raised antibodies against *Drosophila* Geminin and Dup have also suggested the

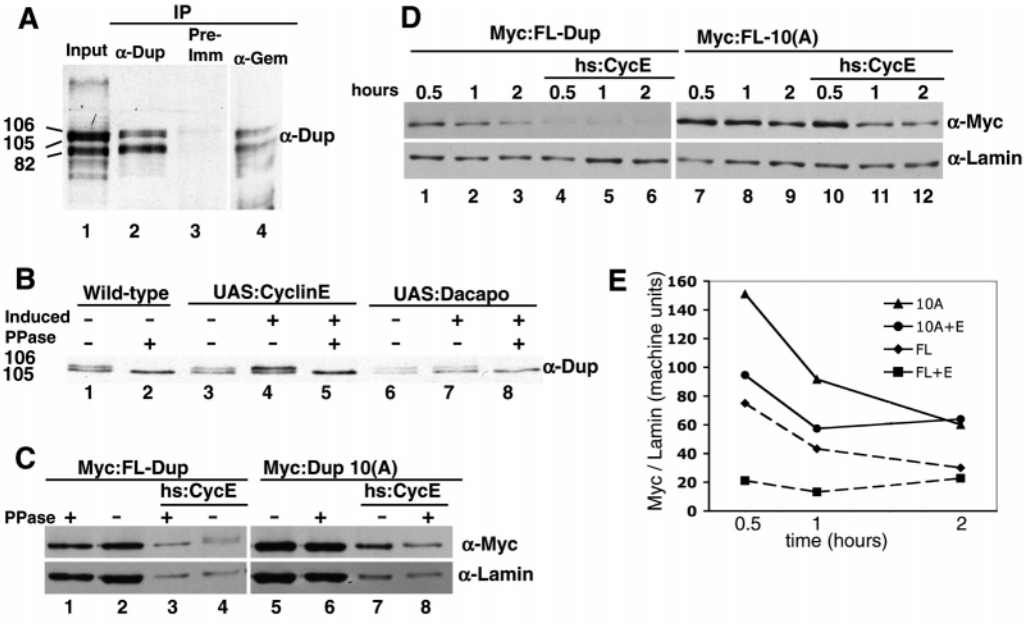

Fig. 4. Phosphorylation of the Dup N terminus in vivo depends on cyclin E/CDK2. (A) Immunoprecipitation (IP) of embryo extracts with polyclonal guinea pig Dup antibody followed by western blot with affinity-purified polyclonal rabbit Dup antibody detects 106 kDa, 105 kDa and 82 kDa isoforms (lane 2). (Lane 4) IP of these three isoforms with Geminin. (Lane 1) Input, (lane 3) pre-immune. (B) The 106 kDa isoform differs from the 105 kDa by a cyclin E/CDK2-dependent phosphorylation. Western blot labeled with Dup antibody of extracts from wild-type third instar larval brains (lanes 1, 2), *hsp70:GAL4; UAS:cyclin E* (lanes 3, 4, 5), or *hsp70:GAL4; UAS: Dacapo* (lanes 6, 7, 8). Lanes 4, 5, 7, 8: induced expression of UAS trangenes; lane 2, 5, 8: lambda phosphatase (PPase) treated. (C) Cyclin E/CDK2 alters migration of Myc:FL-Dup but not Myc:Dup 10(A). Transgene expression from Myc:FL-Dup (lanes 1-4) and Myc:Dup 10(A) (lanes 5-8) was detected with Myc antibodies. Over-expression of cyclin E (lanes 3, 4, 7, 8). Lanes 1, 3, 6, 8: phosphatase treated. Blots were reprobed for lamin C as a loading control. (D) Abundance of FL-Dup (lanes 1-6) and Dup 10(A) (lanes 7-12) at different times after a 30-minute heat pulse of expression without (lanes 1-3, 7-9) or with (lanes 4-6, 10-12) coexpression of cyclin E. (E) Quantification of Myc-tagged protein from the experiment shown in D. Each point shows the average value for three replicates normalized against the lamin C loading control and a linear regression curve.

existence of these higher molecular mass species (Higa et al., 2003; Quinn et al., 2001).

Treatment of extracts from larval brain and imaginal disc with lambda phosphatase resulted in the disappearance of the 106 kDa band and an increase in the intensity of the 105 kDa band, suggesting that they differ by phosphorylation (Fig. 4B). To ask if this phosphorylation is cyclin E dependent in vivo, we over-expressed cyclin E or its specific inhibitor, Dacapo, using the GAL4/UAS system and a heat inducible hsp70:GAL4 (Brand and Perrimon, 1993; de Nooij et al., 1996; Lane et al., 1996). One hour after heat induction, cyclin E increased the relative abundance of the 106 kDa relative to the 105 kDa isoform, whereas over-expression of Dacapo decreased the abundance of the 106 kDa phospho-isoform (Fig. 4B). The increase in the 106 kDa isoform by cyclin E was due to phosphorylation because it was completely reversed by treating the extracts with lambda phosphatase (Fig. 4B).

To further confirm and investigate Dup phosphorylation, we transformed flies with a full-length Dup cDNA tagged on the amino terminus with the Myc epitope, and under control of the heat-inducible hsp70 promoter (hsp70:Myc:FL-Dup). One hour after heat-induced expression, western blotting of larval brain extracts with Myc antibodies gave evidence for a single band that co-migrated with the endogenous 106 kDa isoform (Fig. 4C). Treatment of these extracts with lambda phosphatase did not alter this molecular mass to 105 kDa, but over-expression of cyclin E shifted Myc:FL-Dup to a higher molecular mass, which was completely reversed by lambda phosphatase (Fig. 4C). This suggests that the abundant Myc:Dup species represents the unphosphorylated isoform that migrates at 106 kDa, because of the addition of the Myc epitope. It also confirms that Dup phosphorylation responds to cyclin E/CDK2 activity in vivo.

Cyclin E/CDK2 induces phosphorylation of the Dup N terminus which is required for normal degradation in vivo

The amino-terminal half of the Dup protein contains ten sequences that resemble the consensus site for CDK phosphorylation ({S/T}PX{K/R}) and several appropriately spaced RXL sites, the consensus for cyclin binding (Fig. 5A) (Pearson and Kemp, 1991; Takeda et al., 2001). To determine if these sites are phosphorylated by cyclin E/CDK2, we transformed flies with a Dup mutant in which all ten of the serines or threonines at these sites were changed to alanine (hsp70:Myc:Dup 10(A)) (Fig. 5A).

Like Myc:FL-Dup, the migration of Myc:Dup 10(A) was unaffected by the addition of phosphatase to the extracts. Unlike Myc:FL-Dup, however, the migration of Myc:Dup 10(A) was not shifted upward by over-expression of cyclin E (Fig. 4C). Together with the CDK2 immunoprecipitation results, this suggests that one or more of the ten phosphorylation sites in the N terminus can be phosphorylated by cyclin E/CDK2 in dividing brain and disc cells.

To examine whether this phosphorylation alters Dup protein stability, we heat induced a pulse of expression of hsp70:Myc:FL-Dup and hsp70:Myc:Dup 10(A) and measured their abundance at different times thereafter. This showed that Myc:Dup 10(A) was somewhat more abundant than Myc:FL-Dup (Fig. 4D, lanes 1-3 and 7-9). Importantly, Myc:FL-Dup was more sensitive to over-expression of cyclin E than was

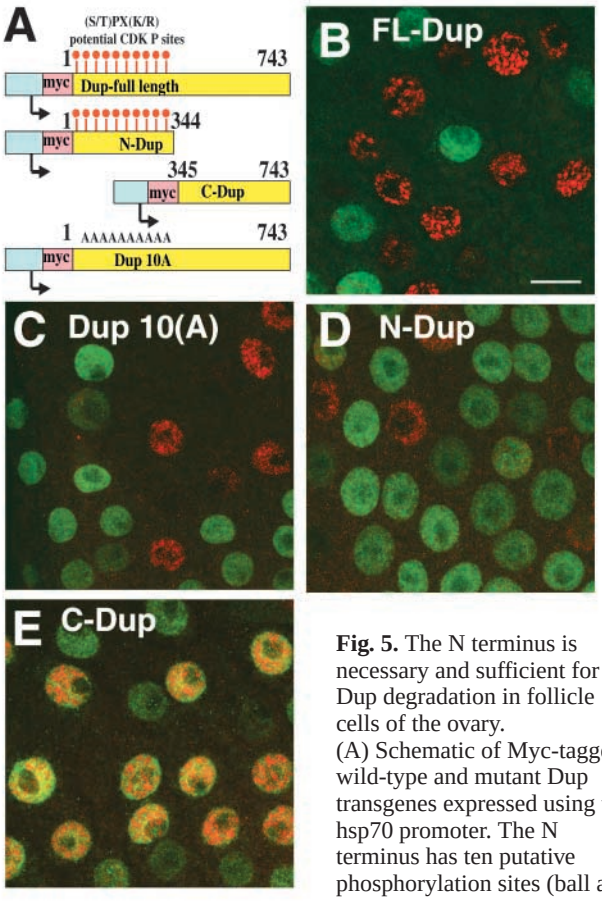

Fig. 5. The N terminus is necessary and sufficient for Dup degradation in follicle cells of the ovary. (A) Schematic of Myc-tagged wild-type and mutant Dup transgenes expressed using the hsp70 promoter. The N terminus has ten putative phosphorylation sites (ball and stick, consensus shown above). (B-E) Follicle cells of stage 8 egg chambers expressing different Myc-tagged Dup proteins double labeled for Myc (green) and BrdU (red). (B) Myc:FL-Dup, (C) Myc:Dup 10(A), (D) Myc:N-Dup, (E) Myc:C-Dup. Only C-Dup had significant overlap between Myc and BrdU labeling (yellow). Images are composites of confocal sections. Scale bar: 10 μm (B-E).

Myc:Dup 10(A) (Fig. 4D lanes 4-6 and 10-12). Nevertheless, Dup 10(A) stability was sensitive to over-expression of cyclin E, suggesting that mutation of the ten phosphorylation sites was not sufficient to completely block cyclin E-dependent degradation (Fig. 4D,E compare lanes 7-9 with 10-12). These results suggest that phosphorylation of Dup on one or more of the ten N-terminal sites is only partially responsible for its cyclin E/CDK2-dependent degradation.

The N terminus is required for Dup degradation during S phase in different types of cell cycles in the ovary

The combined evidence suggested that both phosphorylation and degradation of Dup at G_1/S depended on cyclin E/CDK2, but that phosphorylation of Dup protein could not fully account for its instability. To address this question by a different method, we examined the stability of the Myc-tagged proteins by immunofluorescence. To confirm the role of cyclin E, and expand this analysis to different types of cell cycles in development, we focused on follicle cells of the ovary. These cells originate from stem cells and transition from mitotic to endocycles during precise stages of oogenesis (Calvi et al.,

1998; Mahowald et al., 1979; Margolis and Spradling, 1995). The periodic S phases of the endocycle are controlled by cyclin E/CDK2, which is the only known oscillating CDK activity at that time (Lilly and Spradling, 1996). Previous immunolabeling had shown that Dup protein levels in the ovary oscillate during mitotic and endocycles (Whittaker et al., 2000).

hsp70:Myc:FL-Dup was induced at 37°C for 30 minutes, and after 1 hour recovery, ovaries were dissected and incubated in BrdU for 1 hour, followed by labeling for Myc and BrdU. The number of Myc/BrdU double-labeled cells was then counted as a measure of Dup stability during S phase. As a control for expression, in other animals an hsp70:Myc:P transposase gene was induced, which resulted in Myc labeling in 96% of cells (n=314) including many that labeled with BrdU (data not shown) (Xu and Rubin, 1993). Similar to endogenous Dup protein, Myc:FL-Dup protein was abundant in many nuclei in mitotically dividing and endocycling follicle cells, but there were none double labeled for Myc and BrdU (n=213), indicating Myc:FL-Dup is unstable in S phase (Fig. 5B and data not shown). Myc:Dup 10(A) was also abundantly expressed in many nuclei, and was never seen in cells in S phase (n=275) (Fig. 5C). This is in contrast to the western results which indicated that Myc:Dup 10(A) was at least partially stabilized.

To further explore this, we tested two other transgenes that expressed only the N-terminal half of Dup (hsp70:Myc:N-Dup), or only the C-terminal half (hsp70:Myc:C-Dup), which contain or lack the ten phosphorylation sites, respectively (Fig. 5A). The SV40 nuclear localization signal was included in Myc:C-Dup to compensate for deletion of the natural NLS (data not shown). Similar to Myc:FL-Dup, Myc:N-Dup was also highly expressed in many cells, and in only one cell was Myc labeling seen during S phase (n=344) (Fig. 5D). In contrast, deletion of the N terminus in Myc:C-Dup led to significant stability in S phase; 22% of the follicle cells (n=309) labeled for both Myc and BrdU (Fig. 5E). This indicates that the N terminus is necessary and sufficient for normal Dup degradation in S phase. Together with the western results, the data suggest that CDK2 phosphorylation contributes to Dup instability, but there must be other mechanisms that ensure Dup degradation during S phase.

Mis-expression of Dup is sufficient to induce re-replication and cell death during cell cycles in ovary and imaginal disc

We wished to test if degradation of Dup at G_1/S is essential, or whether other mechanisms would protect genome integrity in the presence of elevated Dup protein levels. Therefore, we heat-induced expression of Dup for 30 minutes twice a day, and examined follicle cells labeled with the fluorescent DNA dye Toto-3 in the confocal microscope at 30 hours (three heat pulses) and 54 hours (five heat pulses) thereafter. Heat shock alone or induction of hsp70:Myc:P-transposase had no consistent effects (Fig. 6A and data not shown). For Myc:FL-Dup, however, two phenotypes were observed by 30 hours: abnormally large nuclei, suggestive of re-replication, and small pycnotic nuclei, suggestive of cell death (Table 1) (Fig. 6B). All ovarioles examined (n>50) had these two phenotypes to varying extents, with ~10-80% of nuclei within mitotic stage follicle cells appearing enlarged, and ~5% appearing pycnotic.

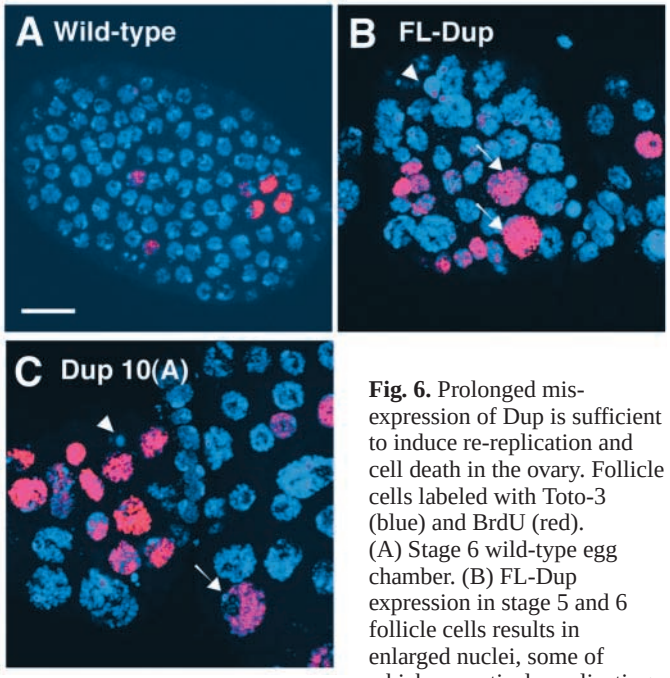

Fig. 6. Prolonged mis-expression of Dup is sufficient to induce re-replication and cell death in the ovary. Follicle cells labeled with Toto-3 (blue) and BrdU (red). (A) Stage 6 wild-type egg chamber. (B) FL-Dup expression in stage 5 and 6 follicle cells results in enlarged nuclei, some of which are actively replicating (two indicated by arrows), while others are pycnotic (arrowhead). The punctate BrdU incorporation is late replication of heterochromatin. (C) Myc:Dup 10(A) expression also results in enlarged nuclei that incorporate BrdU (arrow), and pycnotic nuclei (arrowhead). Images are composites of confocal sections. Scale bar: 10 μm.

Staining with the vital dye Acridine Orange or antibody against activated caspase-3 confirmed that follicle cells with pycnotic nuclei were in fact undergoing cell death (data not shown). Many stage 5 follicle cells, which are normally in the mitotic cycle with nuclei of ~3-4.5 μm in diameter, had nuclei that were enlarged up to 15 μm in diameter, twice the diameter (eight times greater volume) of wild-type stage 10B nuclei that have a DNA content of 16C (Fig. 6B arrows). Follicle cells in the endocycle also were unusually large, but cell death was less frequently observed than in mitotically dividing cells (data not shown). Some cells with large nuclei were actively undergoing DNA synthesis as evidenced by incorporation of BrdU (Fig. 6B). At 54 hours (five heat pulses) there was a notable increase in the number of dying follicle cells (data not shown). These

Table 1. The effect of mis-expression of wild-type and mutant Dup proteins

Transgene	Re-replication*	Cell death†	Amplification‡
None	–	–	+
FL-Dup	+	+	+
N-Dup	–	–	+
C-Dup	–	–	+++
Dup 10(A)	++	++	–

*–, Wild-type nuclei; +, enlarged nuclei, >4C DNA content; ++, many enlarged nuclei, increased fraction >4C DNA content.

†–, Wild-type nuclei; +, pycnotic nuclei, Acridine Orange and activated caspase staining; ++, many pycnotic nuclei, Acridine Orange and activated caspase staining.

‡+, Wild-type appearance of BrdU amplification foci; +++, increased; –, no BrdU.

results suggest that over-expression of Dup is sufficient to induce re-replication and cell death in follicle cells of the ovary.

We next examined the effect of over-expression of Dup mutants. Expression of the N-terminal or C-terminal half of the protein from Myc:N-Dup or Myc:C-Dup did not have noticeable effects on follicle cell cycles (Table I) (data not shown). Prolonged expression of Myc:Dup 10(A), however, resulted in enlarged and pycnotic nuclei (Fig. 6C). By 30 hours, Myc:Dup 10(A) induced more cell death (up to 50% of cells) than did Myc:FL-Dup, suggesting that CDK phosphorylation of sites in the N terminus influences Dup function. A subset of enlarged cells in Myc:Dup 10(A) incorporated BrdU, indicating that they were actively replicating (Fig. 6C).

We considered that the ability of Dup to induce re-replication may be peculiar to follicle cells because they normally undergo a developmental transition to endocycles. To test this idea, we over-expressed the various Dup transgenes in larval imaginal disc cells that have a canonical cell cycle (Fig. 7A,B). Larvae were subjected to heat induction twice a day and imaginal discs were examined after staining with the fluorescent DNA dye DAPI. As in the ovary, Myc:C-Dup and Myc:N-Dup over-expression had no obvious effects (data not shown). After 30 hours, about half of the Myc:FL-Dup imaginal discs had some nuclei that were greatly enlarged, while others were pycnotic and labeled positively for activated caspase-3 (Fig. 7C and data not shown). In some nuclei small polytene chromosomes could be seen (Fig. 7G). By 54 hours all discs displayed the enlarged and pycnotic nuclear phenotype, and disc morphology was extremely aberrant (data not shown). At 54 hours there was a decrease in the number of large nuclei with a concomitant increase in the number of dying cells, consistent with the idea that cell death is a consequence of re-replication. Similar results were obtained for Myc:Dup 10(A) over-expression except that enlarged and dying cells were more frequent (Fig. 7E).

To confirm that cells with larger nuclei were indeed polyploid, we examined the DNA content of wing disc cells by FACS (Fig. 7B,D,F) (Neufeld et al., 1998). Expression of Myc:C-Dup did not alter DNA content (data not shown). After three heat pulses over 30 hours, however, FL-Dup or Dup 10(A) expression resulted in a large fraction of wing disc cells with a greater than 4C DNA content with a modal value of 8C, consistent with a full reduplication of the 4C G_2 value (Fig. 7D,F). The cells of higher ploidy observed by microscopy were relatively rare, and therefore did not represent a significant population in the FACS. Expression of Myc:Dup 10(A) resulted in a larger fraction of cells with 8C DNA content than did FL-Dup (Fig. 7F). This implies that phosphorylation negatively regulates Dup activity. Furthermore, these results indicate that the ability of FL-Dup and Dup 10(A) to induce re-replication and cell death is not unique to the ovary, but that expression of Dup protein alone is sufficient to induce a full genome reduplication in a variety of diploid cells.

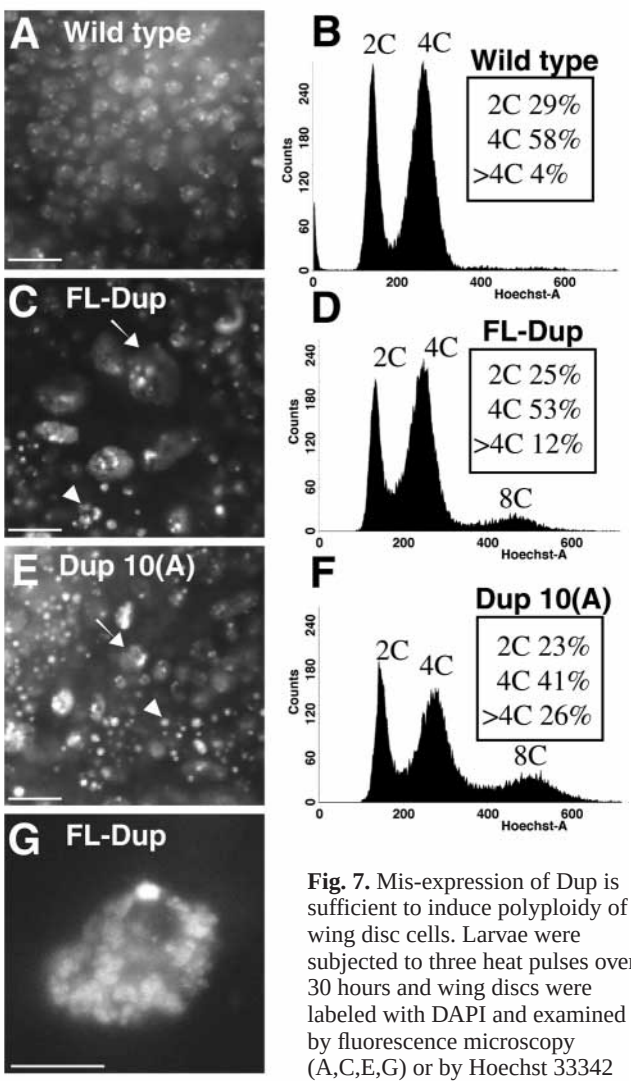

Fig. 7. Mis-expression of Dup is sufficient to induce polyploidy of wing disc cells. Larvae were subjected to three heat pulses over 30 hours and wing discs were labeled with DAPI and examined by fluorescence microscopy (A,C,E,G) or by Hoechst 33342 staining and analyzed by FACS (B,D,F). Arrows indicate giant polyploid cells and arrowheads, cell death. (A,B) Wild-type. (C,D) Myc:FL-Dup. (E,F) Myc:Dup 10(A). (G) High magnification of one polytene nucleus from a wing disc over-expressing FL-Dup. Polytene chromosome arms can be seen radiating from a heterochromatic chromocenter that labels brightly with DAPI. Scale bar: 10 μm.

Mis-expression of Dup enhances genomic replication during S phase

Although Dup mis-expression was sufficient to induce polyploidy, it was unclear in what stage of the cell cycle this occurred. We did not see evidence of chromosome mis-segregation after labeling with the mitotic chromosome marker anti-phosphohistone H3, suggesting that mitotic non-disjunction was not the cause of the 8C population (data not shown). To gain insight into the cell cycle stage affected, we induced three pulses of Dup over 30 hours and labeled third instar eye discs with BrdU for 1 hour. Mis-expression of Myc:FL-Dup or Myc:Dup 10(A) resulted in a greater number of the asynchronous cycling cells anterior to the MF labeling with BrdU (Fig. 8A-C). In contrast, Dup mis-expression did not induce an appreciable increase in BrdU incorporation in the differentiating cells posterior to the furrow, many of which are in G_2, despite having high levels of mis-expressed Dup protein (Fig. 8B,C and data not shown). Mis-expression of Dup did result in a low level of BrdU incorporation in some cells within the morphogenetic furrow, although the majority showed a normal G_1 arrest. Most strikingly, mis-expression of Myc:FL-Dup or Myc:Dup 10(A) resulted in a wider stripe of S-phase cells incorporating BrdU just posterior to the furrow.

In wild type, nucleus-wide incorporation of BrdU occurs for approximately four cell diameters behind the furrow, and an additional five cell diameters have focal BrdU over heterochromatin late in S phase (Fig. 8A). With FL-Dup mis-expression, nucleus-wide incorporation of BrdU incorporation was seen for approximately nine to ten cell diameters, with euchromatic replication continuing into late S phase (Fig. 8B). The BrdU stripe was wider in Dup 10(A), indicating that euchromatic replication was even more prolonged (Fig. 8C). Together with the FACS results, this enhanced genomic replication implies that Dup can induce re-replication during S phase.

Deletion or mutation of the CDK sites has different effects on amplification versus genomic replication

To further evaluate the effect of Dup on another developmental replication program, we turned to an examination of chorion gene amplification. In the last part of oogenesis, follicle cells enter an amplification phase during which most of the genome does not replicate but the origins at four loci repeatedly fire leading to an increase in their copy number (Calvi et al., 1998; Claycomb et al., 2004). We had previously shown that amplification can be detected as subnuclear foci of BrdU incorporation in follicle cells beginning in stage 10B of oogenesis (Fig. 9A) (Calvi et al., 1998; Calvi and Spradling, 2001). The two loci that amplify most highly encode clusters of eggshell protein (chorion) genes, which require a pre-RC-like amplification complex to be licensed (Austin et al., 1999; Landis et al., 1997; Schwed et al., 2002; Whittaker et al., 2000). The female sterile allele dup^{PA77} results in reduced amplification, and by immunofluorescence Dup protein was shown to localize to these amplification foci, indicating that Dup is a component of the amplification complex (Whittaker et al., 2000). The activity of this complex depends on cyclin E/CDK2 (Calvi et al., 1998).

Several hours after a single 30-minute heat induction of FL-Dup or N-Dup there was no observable effect on the intensity of BrdU incorporation into amplification foci (Table 1) (Fig. 9B and data not shown). Unlike the results for genomic replication, however, Myc:C-Dup and Myc:Dup 10(A) had dramatic effects on amplification soon after expression (Table 1). Within 3 hours of expression of Myc:C-Dup, incorporation of BrdU into amplification foci was greatly increased (Fig. 9C). The C-terminus was also better than full-length protein at rescuing the thin eggshell defect in flies homozygous for the dup^{PA77} amplification mutation (data not shown) (Whittaker et al., 2000). Thus the C-terminal half of Dup is a functional protein that hyperactivates amplification origins.

Expression of Myc:Dup 10(A) had the opposite effect; it severely inhibited amplification with no BrdU incorporation detected at amplification foci within 3 hours (Fig. 9D). We considered that the dominant-negative effect of the non-phosphorylatable Dup 10(A) could be due to tight binding of CDK2, thereby reducing its total cellular activity, which is required for chorion gene amplification (Calvi et al., 1998). To

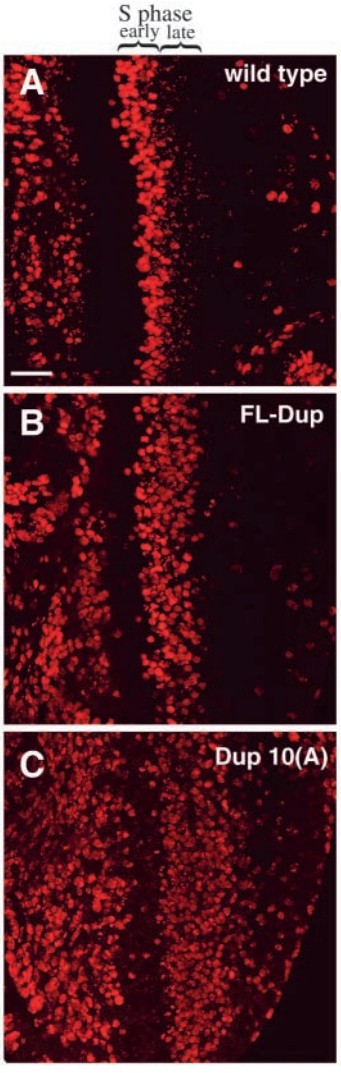

Fig. 8. Mis-expression of Dup enhances genomic replication within S phase. BrdU labeling in third instar eye imaginal discs. (A) Wild-type (B) FL-Dup mis-expression. (C) Dup 10(A) mis-expression. Anterior is to the left. Scale bar: 20 μm (A-C).

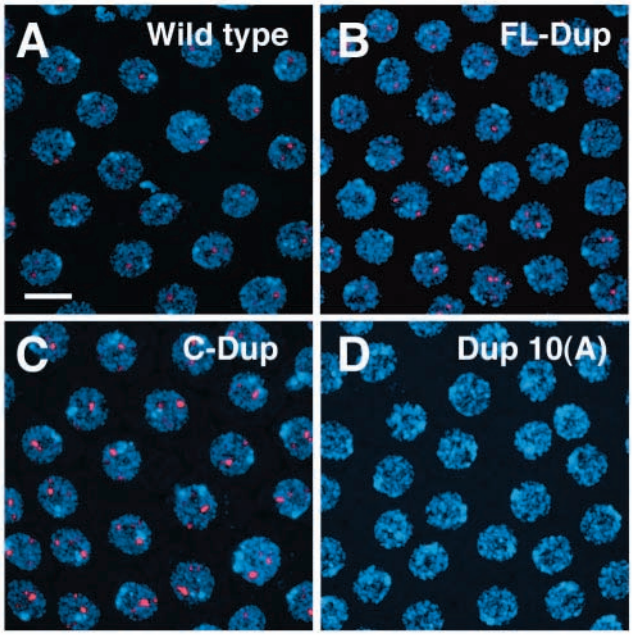

Fig. 9. Mis-expression of Dup has distinct effects on amplification of chorion genes. Confocal images of BrdU labeling (red) of amplification foci within mid-stage 10B follicle cell nuclei (Toto-3, blue) 3 hours after expressing different Dup proteins. (A) Wild-type, (B) FL-Dup, (C) C-Dup, (D) Dup 10(A). All images were taken at the same exposure. Scale bar: 10 μm (A-D).

test this, we used the MPM2 antibody, which we have previously shown detects a subnuclear phospho-epitope whose periodic cell cycle labeling is a reporter for cyclinE/CDK2 activity (Calvi et al., 1998). Although expression of Myc:Dup 10(A) completely inhibited BrdU incorporation at chorion foci within 3 hours, it had no effect on MPM2 labeling (data not shown). Therefore, the dominant negative effect of Dup 10(A) is not due to inhibition of CDK2. Rather, the results suggest that in the context of the full-length protein, phosphorylation of the N terminus is required for activity during amplification. Taken together, it appears that aspects of Dup regulation or function may differ between amplification and genomic replication.

Previous high resolution analysis of amplification foci showed that origin regions can be visually distinguished from migrating replication forks (Calvi et al., 1998; Calvi and Spradling, 2001). Dup antibody labeling co-localizes with BrdU and proteins at the replication fork, suggesting that, contrary to evidence from other cell cycles, it may have a role in elongation during amplification (Claycomb et al., 2002). Myc:Dup 10(A) inhibited BrdU incorporation at amplification foci as soon as 1 hour after expression in all stages, including stages 12-13 when only elongation of forks is occurring (data not shown) (Claycomb et al., 2002). This complete and rapid inhibition of BrdU incorporation suggests that Dup is required for fork elongation during amplification.

Discussion

We have investigated the cell cycle regulation of Dup, an essential component of the pre-RC that is required for loading the MCM complex to license origins. We have found that phosphorylation and stability of Dup depends on cyclin E/CDK2 activity. It is likely that part of this regulation is direct because Dup associates with CDK2 protein and activity in embryos. The results of the mutagenesis show that the N terminus of Dup is necessary and sufficient for degradation at G_1/S. Mutation of the CDK sites in the N terminus, however, only partially stabilized the protein, suggesting the existence of other CDK2-dependent mechanisms for degradation. We have shown that it is crucial to tightly regulate the abundance of Dup protein because its over-expression is sufficient to induce a full genome reduplication and cell death in the ovary and imaginal discs. The different effects on amplification and genomic replication suggest that phosphorylation of the N terminus of Dup protein may be required for replication fork elongation during amplification and provides insight into the mechanism of this developmentally specific replication program.

Regulation of Dup degradation at G_1/S

The results suggest that cyclin E/CDK2 phosphorylates the Dup N terminus contributing to its instability at G_1/S. Dup was degraded during periodic endocycle S phases that are solely regulated by oscillating cyclin E/CDK2, further supporting a link between this kinase and Dup degradation. Although the N terminus was necessary and sufficient for degradation, mutation of the ten N-terminal CDK sites within Dup 10(A) only partially stabilized the protein. This suggests that there are other cyclin E/CDK2-dependent mechanisms that trigger Dup degradation independent of these ten sites during S phase. It has been noted that the C terminus of Dup contains a PEST sequence (Whittaker et al., 2000), and there are several serines and threonines in the C terminus that are potential targets of phosphorylation (Gopalakrishnan et al., 2001). Although we have not directly tested the requirement for these sites, the stability of C-Dup indicates that they are not sufficient for degradation at G_1/S. To explain our results, we suggest a bi-phasic degradation model where cyclin E/CDK2 phosphorylation promotes Dup degradation in late G_1, whereas other fail-safe mechanisms become operative only during S phase. This would explain why inhibiting CDK2 and S phase entry with GMRp21 completely blocked Dup degradation.

A number of very recent publications describe results for Cdt1 in human cells that are similar to ours in flies (Kondo et al., 2004; Liu et al., 2004; Nishitani et al., 2004; Sugimoto et al., 2004). These results suggest that cyclin A/CDK2 phophorylates the human Cdt1 N terminus, which enhances its binding to the Skp2 subunit of the SCF ubiquitin ligase. Like Dup, non-phosphorylatable Cdt1 mutants were only partially stabilized, but simultaneously inhibiting CDK2 and S phase entry with p21 completely blocked degradation. Previous evidence in *C. elegans*, human and *Drosophila* cells suggested that destruction of Cdt1 may be mediated by two ubiquitin ligases, an SCF complex containing Skp2, and an SCF-like complex based on Cul4 (Higa et al., 2003; Li et al., 2003; Nishitani et al., 2001; Zhong et al., 2003). For many substrates of the SCF, prior phosphorylation is required for their subsequent recognition and ubiquitinylation, including substrates phosphorylated by CDK2 at G_1/S (reviewed by Jackson et al., 2000) (Montagnoli et al., 1999). It is not known whether prior phosphorylation is required for substrate recognition by Cul4-based ubiquitin ligases. It is tempting to speculate, therefore, that the bi-phasic degradation of Cdt1 that we suggest may reflect its modification by two distinct ubiquitin ligases: a phosphorylation-dependent ubiquitinylation by the SCF complex, and a phosphorylation-independent ubiquitinylation by a Cul4-based complex. Clearly, more experiments are needed to sort out the complexity of this regulation. Nonetheless, the similar results from flies and humans suggest that tight regulation of Cdt1 abundance is a generally conserved and important mechanism to protect genome integrity in eukaryotes.

Regulation of the pre-RC in re-replication control

CDK activity and Geminin play central roles in the block to re-replication (reviewed by Diffley, 2001). Our results show that Dup over-expression is sufficient to induce a full genome reduplication in normal cells in developing tissues, transforming diploid into polyploid cells. This phenotype is more profound than that of Geminin mutants, suggesting that degradation of Dup protein is of highest priority to protect genome integrity. An important caveat is that in our experiments Dup is over-expressed and therefore not equivalent to an absence of degradation. We have found, however, that even small, undetectable increases in Dup protein can have profound consequences (data not shown). Moreover, after multiple heat pulses, Dup protein was undetectable during S phase, yet it induced extensive re-replication in most cells. The prolonged genomic replication in the synchronized cells of the eye disc suggests that this small increase in Dup protein may permit origins to be relicensed and reinitiate within a single S phase. While the precise molecular mechanism for

how increased Dup promotes re-replication remains undefined, the results indicate that even a small increase in Dup protein is sufficient to compromise genome integrity.

The other phenotype associated with over-expression of Dup was cell death. Dup 10(A) caused more cell death than wild-type Dup, suggesting that phosphorylation of the Dup N terminus influences this phenotype. In human cells re-replication due to over-expression of Cdt1 is more easily detected when p53 is mutant, probably because they escape apoptosis triggered by re-replication (Vaziri et al., 2003). We therefore favor the model that Dup over-expression induces re-replication, which in turn can lead to the activation of checkpoints and apoptosis.

Dup function in chorion gene amplification

In recent years, the analysis of replication from the defined chorion amplification origins has been a prominent genetic and molecular model system for the regulation of DNA replication in metazoa. Chorion origins require pre-RC proteins, cyclin E/CDK2 and Dbf4/Cdc7 kinases, indicating that their regulation resembles that of genomic origins (reviewed by Bandura and Calvi, 2002). They clearly differ, however, in that they re-replicate at a time when no other origins are firing, and understanding this exception should provide insight into the rules of regulation of all origins. To our surprise, we found that the carboxyl-terminal half of Dup, although having no effect on genomic replication, is a hyperactive protein that causes over-amplification from chorion origins. The Dup 10(A) mutant gave the opposite result; it was dominant negative and strongly inhibited amplification. We propose that during amplification phosphorylation of the Dup N terminus abrogates its inhibition of the activity of the C terminus, explaining why deleting the N terminus results in a hyperactive protein, whereas blocking its phosphorylation results in an inactive protein. An important functional role for the C terminus is consistent with its binding to MCM proteins, and the fact that among Cdt1 family members the C-terminal half is much more highly conserved than the N-terminal half of the protein (Whittaker et al., 2000; Wohlschlegel et al., 2000; Yanagi et al., 2002). Most Cdt1 proteins have known or potential CDK phosphorylation sites in their N terminus despite its poor conservation, supporting the notion that its conserved function is to mediate regulation by CDKs.

The different effects on amplification versus genomic replication suggest a distinction in the regulation or function of Dup in these two processes. Claycomb et al. had previously proposed that Dup participates in fork elongation during amplification, based on immunolabeling at chorion foci (Claycomb et al., 2002). We show that in other cell cycles Dup is rapidly degraded at the onset of S phase and not present during fork elongation, similar to results from human and other cells. Moreover, *S. cerevisiae* cells experimentally depleted of Cdt1 within S phase are able to complete genomic replication, inconsistent with a role in elongation (Tanaka and Diffley, 2002). Expression of Dup 10(A), however, inhibited BrdU incorporation within 1 hour in all stages of amplification, including late stages when only elongation of forks is occurring. This rapid and complete inhibition of BrdU incorporation by Dup 10(A) cannot be an indirect effect of origin inhibition, and supports the proposed role for Dup at the replication fork. Furthermore, this suggests that phosphorylation is important for the function of Dup in elongation during amplification. The distinct activities of C-Dup and Dup 10(A) in genomic replication versus amplification provide a molecular handle on the mechanism by which these two developmental replication programs differ, possibly resulting from the activity of Dup at the fork. The function of Dup at the fork may be related to its known ability to load the MCM complex helicase onto chromatin. It also raises the possibility that Cdt1 family members may act at the fork under other special circumstances.

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