Levels of the origin-binding protein Double parked and its inhibitor Geminin increase in response to replication stress

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Summary

The regulation of a pre-replicative complex (pre-RC) at origins ensures that the genome is replicated only once per cell cycle. Cdt1 is an essential component of the pre-RC that is rapidly degraded at G1-S and also inhibited by Geminin (Gem) protein to prevent re-replication. We have previously shown that destruction of the *Drosophila* homolog of Cdt1, Double-parked (Dup), at G1-S is dependent upon cyclin-E/CDK2 and important to prevent re-replication and cell death. Dup is phosphorylated by cyclin-E/Cdk2, but this direct phosphorylation was not sufficient to explain the rapid destruction of Dup at G1-S. Here, we present evidence that it is DNA replication itself that triggers rapid Dup destruction. We find that a range of defects in DNA replication stabilize Dup protein and that this stabilization is not dependent on ATM/ATR checkpoint kinases. This response to replication stress was cell-type specific, with neuroblast stem cells of the larval brain having the largest increase in Dup protein. Defects at different steps in replication also increased Dup protein during an S-phase-like amplification cell cycle in the ovary, suggesting that Dup stabilization is sensitive to DNA replication and not an indirect consequence of a cell-cycle arrest. Finally, we find that cells with high levels of Dup also have elevated levels of Gem protein. We propose that, in cycling cells, Dup destruction is coupled to DNA replication and that increased levels of Gem balance elevated Dup levels to prevent pre-RC reformation when Dup degradation fails.

Key words: DNA replication, Double parked, Cdt1, Geminin, Genome stability

Introduction

In dividing cells, the entire genome must be copied but each region of the genome must be replicated only once. Either under- or over-replication compromises genome integrity and can result in cell death or cancer. Therefore, several regulatory mechanisms have evolved to ensure that the genome is completely replicated with high fidelity exactly once each cell cycle. Over the past ten years, a picture has emerged of how over-replication of the genome is prevented (for review, see Bandura and Calvi, 2002). The targets of this regulation are origins of DNA replication, which are restricted to initiate replication only once per cell cycle. In late M and early G1 phases, a pre-replicative complex (pre-RC) assembles onto origin DNA which prepares, or 'licenses', them for replication (for reviews, see Bell and Dutta, 2002; Chong et al., 1995; Diffley et al., 1994). The assembly of the pre-RC onto origins is a stepwise process with the origin-recognition complex (ORC) serving as a scaffold for subsequent association of Cdc6 and Cdt1 proteins, both of which are required to load the mini-chromosome maintenance (MCM) complex replicative helicase (for review, see Diffley, 2001). At the onset of S phase, cyclin-dependent kinases (CDKs) and CDC7 kinase are then required for the initiation of replication (for reviews, see Hengstschlager et al., 1999; Sclafani, 2000). Importantly, upon initiation, the pre-RC is remodeled with CDC6, Cdt1, MCMs and, in higher eukaryotes, the Orc1 subunit, leaving the origin. The pre-RC is then inhibited from reassembling onto origins until after the next mitosis, thereby restricting the initiation of DNA replication to once per segregation of chromosomes (Diffley et al., 1994; Piatti et al., 1996).

In recent years, there have been significant insights into the mechanisms that restrict pre-RC assembly to once per cell cycle. CDK activity is required for the initiation of DNA replication, but CDKs also prevent reassembly of the pre-RC during S, G2 and early M phases (Broek et al., 1991; Dahmann et al., 1995). In the yeasts *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe*, CDKs phosphorylate ORC, CDC6 and MCMs, resulting in their inactivation, degradation and exclusion from the nucleus (Drury et al., 2000; Jallepalli et al., 1997; Labib et al., 1999; Nguyen et al., 2000; Nguyen et al., 2001; Tanaka and Diffley, 2002; Vas et al., 2001). In multicellular animals (metazoa), it appears that CDKs also prevent re-replication, perhaps by regulating multiple pre-RC subunits, although the mechanism is less well-defined than it is in yeast (Ballabeni et al., 2004) (reviewed in Bell and Dutta, 2002; Coverley et al., 2000; Ekholm-Reed et al., 2004; Hua et al., 1997; Itzhaki et al., 1997). There is another inhibitor of re-

replication that is unique to metazoa, Geminin (Gem), which is expressed during S, G2 and early M phases. (McGarry and Kirschner, 1998; Tada et al., 2001; Wohlschlegel et al., 2000). Gem binds directly to Cdt1 to inhibit its ability to load the MCM complex onto origins (Lee et al., 2004; Saxena et al., 2004; Tada et al., 2001; Wohlschlegel et al., 2000). Through several CDK targets and Gem inhibition, it appears that the cell has taken a multipronged approach to preventing the catastrophic mistake of unbalanced genomic replication.

Although multiple pre-RC subunits are targeted by CDKs, the requirement for the Gem inhibitor, and other recent evidence, suggests that regulation of Cdt1 is especially important to prevent re-replication in metazoa (for review, see Saxena and Dutta, 2005). Mutation or inactivation of Gem can result in at least partial re-replication suggesting that it plays an important role to restrain Cdt1 (Melixetian et al., 2004; Mihaylov et al., 2002; Quinn et al., 2001; Tada et al., 2001; Vaziri et al., 2003; Zhu et al., 2004). Cdt1 is also rapidly destroyed at the onset of S phase, and several ubiquitin-ligase complexes have been implicated in targeting Cdt1 for proteasome degradation (Hu et al., 2004; Li and Blow, 2005; Li et al., 2003; Liu et al., 2004) (for a review, see Saxena and Dutta, 2005; Sugimoto et al., 2004; Zhong et al., 2003). This destruction is crucially important because increased levels of Cdt1 are sufficient to induce re-replication and cell death in a range of organisms, despite the presence of Gem (Castellano Mdel et al., 2004; Gopalakrishnan et al., 2001; Li and Blow, 2005; Maiorano et al., 2005; Nishitani et al., 2004) (for reviews, see Saxena and Dutta, 2005; Thomer et al., 2004; Vaziri et al., 2003). Cdt1 also appears to be the target of a checkpoint response to genotoxic damage and is rapidly degraded in G1 phase in response to ultraviolet light or ionizing irradiation via a ubiquitin-mediated pathway (Higa et al., 2003; Hu et al., 2004; Kondo et al., 2004). Cdt1 expression is elevated in several human cancers and, in mice, overexpression of Cdt1 in erythroid cells increases their oncogenic potential (Arentson et al., 2002; Karakaidos et al., 2004; Xouri et al., 2004). The emerging picture is that tight regulation of Cdt1 is crucial for protecting genome integrity. It remains unclear, however, what coordinates the rapid destruction of Cdt1 with the onset of S phase.

We have previously shown that the *D. melanogaster* ortholog of Cdt1, Double-parked (Dup) (Whittaker et al., 2000), is rapidly destroyed beginning at the G1-S transition and that this degradation is important because elevated levels of Dup induced profound re-replication and cell death during development (Thomer et al., 2004). We also showed that Dup is a direct target of cyclin-E/CDK2. Although inhibition of cyclin-E/CDK2 completely blocked Dup degradation, mutation of the phosphorylation sites within Dup did not block its degradation at the onset of S phase. This suggested that other CDK2-dependent mechanisms ensure degradation of Dup in S phase, independent of direct phosphorylation by CDK2. Here, we present evidence that it is DNA replication itself that is the CDK2-dependent mechanism that triggers Dup degradation. Defects in different steps of DNA replication result in stabilization of Dup protein in developing tissues, with Dup accumulating to highest levels in stem cells. Replication stress also results in a concomitant increase in Gem protein in cells with high levels of Dup. We propose a model wherein Dup destruction is coupled to DNA replication, and that when problems with DNA replication are encountered, Gem balances Dup accumulation to prevent origin relicensing.

Materials and Methods

Drosophila genetics and drug treatment

Standard techniques were used for culture of *Drosophila melanogaster*. Information about strains and genetic nomenclature can be found at http://flybase.bio.indiana.edu/. Homozygous mutant larvae were identified by the absence of appropriate green fluorescent protein (GFP)-marked balancers. For hydroxyurea (HU) treatment before microscopy, 200 μl 640 mM HU (Sigma) was added to vials containing second- to third-instar larvae and incubated at 25°C for a minimum of 27 hours before fixation. For caffeine feeding, vials were supplemented with 200 μl 100 mM caffeine and larvae were fed for 27-30 hours before addition of caffeine and HU together for an additional 27 hours (Boyd and Setlow, 1976). In all cases, food coloring was also added to the medium, and larvae were selected that had a colored digestive tract as a guarantee that they had ingested the drug(s).

Immunolabeling and microscopy

Antibody and BrdU labeling were performed as previously described (Calvi and Lilly, 2004; Schwed et al., 2002; Thomer et al., 2004). In most cases, we used an affinity-purified guinea-pig antibody against Dup (1:1000) provided by Terry Orr-Weaver, Whitehead Institute, Cambridge, MA (Whittaker et al., 2000). Results were confirmed with an affinity-purified rabbit polyclonal anti-Dup antibody (1:500) kindly provided by Eileen Beall and Michael Botchan (University of California, Berkeley, CA). Anti-Gem antibody was raised and purified as described (Thomer et al., 2004) and used at 1:500. Other antibodies and their concentrations were as follows: anti-cyclin-E monoclonal 8B10 (1:5) (Richardson et al., 1995); anti-cyclin-B monoclonal (1:4) (University of Iowa Hybridoma Bank, Iowa City, IA) (Lehner and O'Farrell, 1990); anti-phosphorylated histone H3 (1:500; Upstate Biotechnology) (Hendzel et al., 1997). Alexa 488 and Alexa 568 (Molecular Probes) or Cy3 (Jackson ImmunoResearch) secondary antibodies (1:400) of appropriate specificity were used for detection. Dup immunofluorescence was quantified using a Leica SP Confocal and TCS-NT quantification software, and by using Openlab quantification software on non-confocal images. All images used for quantification were matched for exposure between wild-type and mutant or drug-treated, and not saturated for pixel intensity.

RNA in-situ hybridization

For in-situ hybridization probe, a *dup* cDNA was isolated by reverse-transcription PCR (RT-PCR) and subcloned into the pCR2.1-Topo plasmid (Invitrogen). This template was used to generate antisense or sense (control) digoxigenin-labeled RNA probes by in-vitro transcription (Roche). The probes were used for in-situ hybridization according to standard methods (Tautz and Pfeifle, 1989). Third-instar wild-type and $Mcm6^{3}$ larval brains were processed in parallel in three separate experiments.

Results

Dup levels are increased in *Mcm6* mutant cells

To address what is required for Dup destruction during S phase, we examined Dup protein levels in cells of developing tissues that have specific S-phase defects. To do this, we used an affinity-purified guinea-pig anti-Dup antibody for immunofluorescence (Whittaker et al., 2000). We first examined larval tissues defective in origin licensing and

replication initiation caused by mutation of *Mcm6*, a subunit of the MCM helicase complex, which is loaded onto origins by Dup. Animals homozygous for the null mutation *Mcm6*^{3} survive to metamorphosis on maternally supplied protein but die as pupae with no imaginal discs and small brains owing to progressively worsening defects in cell proliferation (Schwed et al., 2002). Labeling of brains from living *Mcm6*^{3} third-instar larvae revealed that many cells had large increases in Dup levels compared with wild-type (Fig. 1A,B). Dup immunofluorescence was increased at least two- to threefold in 100-200 cells per brain, with a few cells having tenfold increases compared with the wild type.

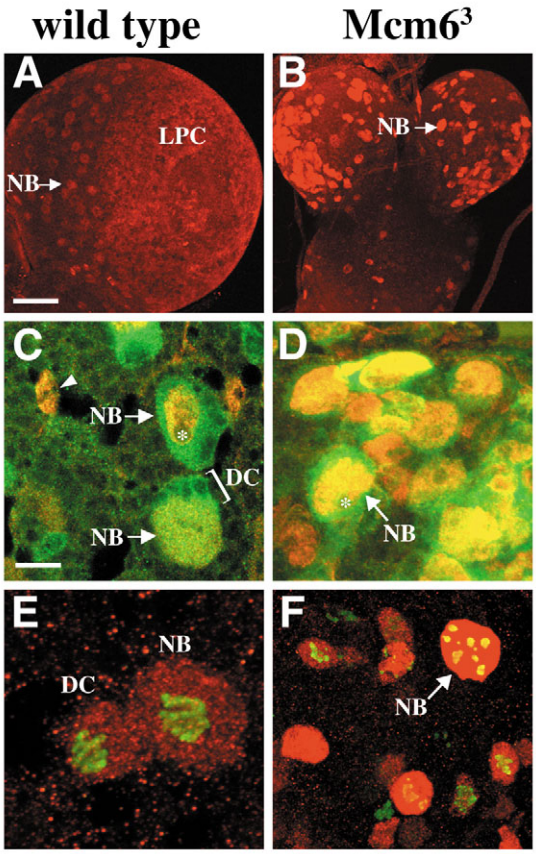

Fig. 1. Dup persists past the G1-S transition and accumulates to high levels in *Mcm6*^{3} mutant brains, (A,C,E) Wild type. (B,D,F) *Mcm6*^{3}. (A) One lobe of a wild-type third-instar brain labeled with anti-Dup antibody (red) highlights dividing lamina-precursor cells (LPCs) in the optic lobe and neuroblast stem cells (NB) in the mid-brain (arrow). (B) A brain from an *Mcm6*^{3} mutant third-instar larva has many cells with high levels of Dup and is smaller than the wild type owing to defects in cell proliferation. The Dup increase is especially pronounced in the large neuroblast stem cells. (C,D) Double labeling for Dup (red) and cyclin B (green). (C) Most neuroblasts in G2 (top arrow) have Dup in the nucleus and cyclin B in the cytoplasm, whereas those in mitosis (bottom arrow) have Dup and cyclin B in the cytoplasm and nucleus. By contrast, the smaller, cyclin-B-positive daughter cells (DC, extent of one shown with bracket) surrounding the neuroblast have no detectable Dup during S, G2 or M phase. Cells in G1 phase have Dup in the nucleus but no cyclin B (arrowhead). Asterisk indicates the nucleolus in the stem cell. (D) An *Mcm6*^{3} mutant brain has many cells with high levels of Dup (red) in the nucleus or nucleus and cytoplasm. Many of these cells are in late S, G2 or early M, as evidenced by cyclin-B expression (green; overlap is yellow). (E,F) Labeling for Dup (red) and PH3 (green). (E) Wild-type neuroblast-stem-cell division in anaphase shows that there is a low but detectable level of Dup during mitosis. (F) Some *Mcm6*^{3} mutant cells with elevated Dup levels enter M phase and have PH3-positive chromosomes that are abnormal in number and structure. The wild-type and mutant images are matched for exposure, resulting in pixel saturation for some mutant cells owing to intense Dup labeling. Images are composites of confocal sections. Scale bar, 50 μm (A,B), 10 μm (C-F).

Interestingly, the accumulation of Dup protein was cell-type specific. Highest levels of Dup were consistently seen in neuroblast stem cells (NB) of the brain and ventral ganglion, which were identified by their large size, position and absence of labeling for prospero protein (Fig. 1B and data not shown) (Ceron et al., 2001; Ito and Hotta, 1992; Vaessin et al., 1991). Dup was also increased, but to a much lesser extent, in the surrounding NB daughter cells and some cells of the optic-lobe proliferation centers (Fig. 1B). An increase in Dup levels was observed as early as day 2 of development in brains of *Mcm6*^{3} mutant first-instar larvae when replication defects are first manifest because of depletion of maternal Mcm6 protein stores (data not shown). This suggested that replication defects in *Mcm6*^{3} result in increased levels of Dup.

Dup protein persists into G2 and M phase

If the increase in Dup levels is due to a failure to degrade Dup during S phase, one prediction is that Dup should persist past the G1-S transition, when it is normally rapidly destroyed. To address this, we used antibodies against cyclin B, which labels cells from late S phase until metaphase, and against phosphorylated histone H3 (PH3), which labels condensed chromosomes in mitosis (Hendzel et al., 1997; Lehner and O'Farrell, 1990). Many cells with increased Dup also labeled for cyclin B (74%, n=54) (Fig. 1D). Many of these cells had cyclin B restricted to the cytoplasm, which is indicative of G2 phase, whereas others had cyclin B in cytoplasm and nucleus, suggesting that they had entered M phase. This also showed that some cells had high levels of Dup restricted to the nucleus, whereas, in others, Dup was distributed throughout the cell, which was confirmed by labeling nuclear envelope for lamin C and cell membranes for α-spectrin (Fig. 1D and data not shown). Many of the *Mcm6*^{3} cells with high levels of Dup also labeled with PH3 (30%, n=27) (Fig. 1F). The morphology and number of these mitotic chromosomes was highly abnormal, suggesting that genome integrity was compromised in these cells (Fig. 1F and data not shown). This suggests that some *Mcm6*^{3} mutant cells do not permanently maintain a checkpoint arrest and enter mitosis despite severe defects in DNA replication.

Unexpectedly, all wild-type stem cells also had very low, but detectable, levels of Dup protein in G2 and M phase (Fig. 1C,E). This labeling in G2 and M phase was not observed in the primary stem-cell daughter [ganglion mother cell (GMC)] or cells that arise from subsequent divisions of the GMC, despite the fact that these stem cells and many of their daughters are actively dividing at this developmental time (Fig. 1C,E). This suggests that the mechanisms controlling Dup steady-state levels differ somewhat in stem cells. Similar results for wild-type and *Mcm6* mutant stem cells were

obtained with an independently raised Dup antibody (E. Beall and M. Botchan), which did not label *dup* mutant cells, confirming that the labeling represents Dup (data not shown). These results suggest that Dup persists and accumulates past the G1-S transition in *Mcm6*^{3} mutant cells, and that this effect is enhanced in stem cells.

Replication defects increase Dup protein primarily by a post-transcriptional mechanism

The persistence of Dup protein past the G1-S transition suggested that its normal degradation does not occur when DNA replication is defective. We previously showed that degradation of Dup is robust; overexpression of a Myc-epitope-tagged Dup from a strong *hsp70* promoter did not result in detectable Dup protein during S phase (Thomer et al., 2004). It remained possible, however, that an increase in Dup transcription during replication stress could contribute to the observed rise in protein levels. To examine this, we performed in-situ hybridization to *dup* mRNA in wild-type and *Mcm6*^{3} mutant brains. In the wild type, *dup* mRNA was most abundant in stem cells of the mid-brain and ventral ganglion, and in dividing lamina precursor cells in the optic lobe (Fig. 2A). In *Mcm6*^{3} mutant brains, *dup* mRNA abundance was not increased but was instead clearly less abundant than in wild-type brains in three separate experiments in which hybridization was performed in parallel (Fig. 2B).

During normal cell cycles, Dup expression responds to the E2F1/DP transcription factor (data not shown) (Whittaker et al., 2000). Using a Myc-tagged reporter based on the E2F1/DP-responsive *ORC1* promoter, we found that E2F1/DP activity was not increased (<1.5 times control) in the same cells that had elevated Dup (two to ten times control) in response to replication stress (Fig. 2C,D and see below) (Asano and Wharton, 1999; Whittaker et al., 2000). Labeling for the E2F-responsive proteins Orc1 and Humpty dumpty also showed that E2F/DP activity is not increased (data not shown) (Bandura et al., 2005). We conclude that the dramatic increase in Dup protein levels during replication stress is not mediated primarily by enhanced E2F1/DP-dependent transcription. Instead, this increase is probably caused by a defect in Dup protein degradation combined with continued translation from a low level of Dup mRNA.

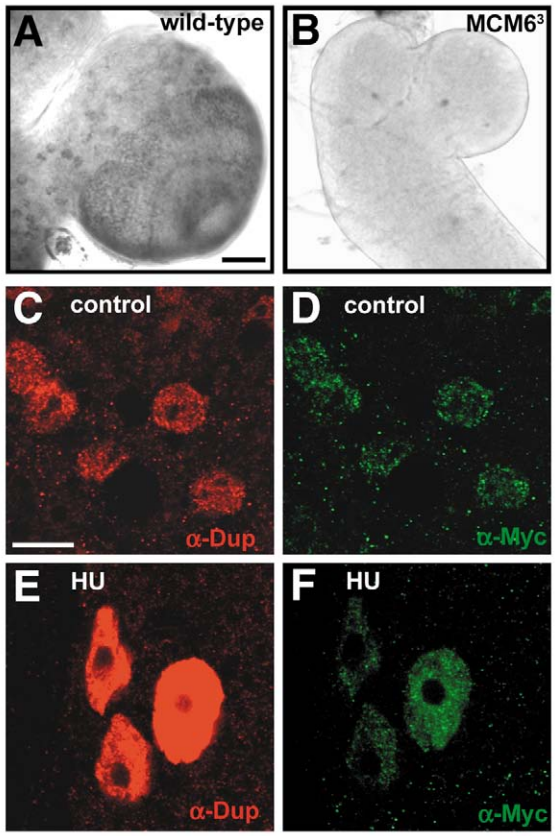

Fig. 2. *Dup* mRNA levels and E2F1/DP-dependent transcription are not increased during replication stress. (A,B) In-situ hybridization to *Dup* mRNA in a third-instar brain from wild-type (A) and *Mcm6*^{3} mutant (B) larvae. (C-F) E2F1/DP transcription-factor activity is not increased in brain cells with elevated Dup levels. Dup labeling (red) and anti-Myc labeling (green) to detect expression of the E2F1/DP-sensitive reporter ORC1p:ftz-GFP-Myc in control (C,D) or HU-treated (E,F) brain cells. (E) In HU-treated cells, Dup was often abundant in the nucleus and cytoplasm but was excluded from the nucleolus, which appears as an absence of staining in a spherical area in the nucleus. HU-treated cells did not have significantly elevated E2F/DP reporter activity (F) despite having high levels of Dup in the same cells (E). Images in A,B are bright field and those in C-F are composites of confocal sections. Scale bar, 50 μm (A,B), 10 μm (C-F).

Accumulation of Dup is not specific to defects in *Mcm6*

Given that Dup is required to load the MCM complex into the pre-RC, one hypothesis was that the failure to degrade Dup could represent a feedback mechanism that senses defects in origin licensing. Alternatively, Dup degradation might be sensitive to other defects in DNA replication. To address this, we fed *Drosophila* larvae the replication inhibitor HU, which causes replication-fork arrest. After 27 hours on medium containing HU, S phase was inhibited in brain cells, as evidenced by greatly reduced BrdU incorporation (data not shown). HU also resulted in greatly increased levels of Dup and, like *Mcm6*^{3} cells, stem cells often had higher levels of Dup than their surrounding daughter cells (Fig. 2E, Fig. 3A, and data not shown). Typically ~50-100 cells in the brain lobes and ventral nerve cord (VNC) had levels of Dup that were two to three times the wild-type levels (n=20 brains), although the intensity of labeling and number of cells were variable among different brains, presumably owing to differences in HU delivery. HU also increased Dup in some diploid cells of imaginal discs and polyploid cells of the gut, but to a lesser extent than that seen for stem cells of the brain (data not shown). Cyclin-B labeling of brains from HU-treated animals indicated that 100% cells with abnormally high levels of Dup also labeled with cyclin B, indicating that HU results in Dup persisting past the G1-S transition (data not shown). Similar results were obtained when origin licensing or DNA-polymerase processivity was impaired in mutants for Orc2 (*orc2*^{1}) and PCNA (*mus209*^{02448}), respectively (data not shown) (Landis et al., 1997; Spradling et al., 1999). Because only a subset of cells in the brain had increased Dup levels, we were unable to detect an increase in Dup abundance by western blotting

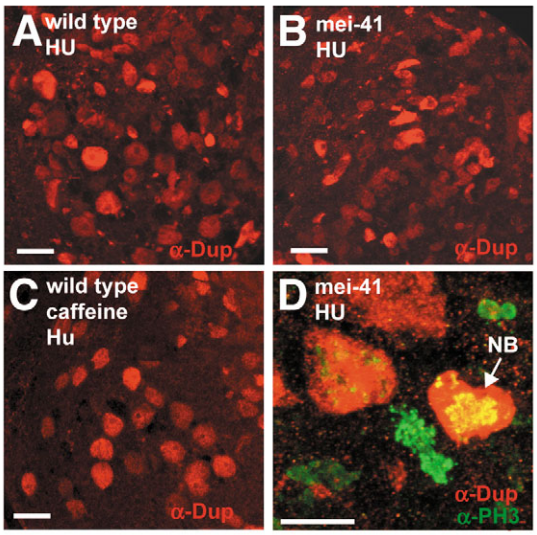

Fig. 3. The increase in Dup levels in response to replication stress is not sensitive to ATM/ATR-checkpoint-kinase activity. Dup labeling in a single third-instar brain lobe from HU-fed wild-type (A), HU-fed *mei-41*^{29D} mutant (B) and HU- plus caffeine-fed wild-type (C) larvae. (D) High-power image of cells from an HU-treated *mei-41*^{29D} brain labeled for Dup (red) and PH3 (green). Some *mei-41*^{29D} cells proceeded into mitosis in the presence of HU and had chromosomes that were highly aberrant in morphology. Arrow indicates a neuroblast stem cell (NB). Scale bars, 10 μm.

of whole-brain lysates (data not shown). These results indicate that a failure to degrade Dup is not specific to mutation of *Mcm6* but that Dup is also stabilized when other steps in DNA replication are defective.

Stabilization of Dup is not sensitive to ATM/ATR activity

Stabilization of Dup during replication stress raised the possibility that this could be the result of a replication checkpoint. To test this idea, we examined Dup levels in checkpoint-compromised animals. The *Drosophila mei-41* gene resembles the ATR (ATM- and RAD3-related) kinase from humans and mediates the DNA-replication and -damage checkpoint in flies (Boyd et al., 1976; Brodsky et al., 2000; Hari et al., 1995; Sekelsky et al., 2000; Sibon et al., 1999). Flies homozygous for the null allele *mei-41*^{29D} are viable but sensitive to HU and other mutagens owing to a defective checkpoint response (Brodsky et al., 2000). To determine whether *mei-41* signaling is required for the increase of Dup, we examined brains from *mei-41*^{29D} homozygotes fed HU. Labeling for PH3 indicated that many *mei41*-mutant brain cells proceeded into mitosis despite inhibition of DNA replication by HU, consistent with an impaired checkpoint in these cells. These mutants also had an enhanced abnormal mitotic chromosome phenotype after HU treatment (Fig. 3D). Nonetheless, ~50-100 cells in *mei-41*^{29D} brains still displayed intense Dup labeling after HU treatment (n>20 brains), comparable in intensity and number of cells to wild-type animals fed HU in parallel (Fig. 3A,B). These results indicate that the ATR homolog *mei41* is not required for the accumulation of Dup protein.

The *Drosophila tefu* gene resembles ATM from human cells and is required for the maintenance of telomere structure and the checkpoint response to double-strand DNA breaks (Bi et al., 2004; Oikemus et al., 2004; Sekelsky et al., 2000; Silva et al., 2004; Song et al., 2004). It remained possible, therefore, that *tefu* mediates the stabilization of Dup in response to DNA damage incurred during replication stress. To address this and to eliminate the possibility of partial redundancy between *mei-41* and *tefu*, we inhibited both kinases by feeding larvae the ATM/ATR kinase inhibitor caffeine (Boyd and Setlow, 1976; Sarkaria et al., 1999). Like *mei-41* animals, the brain cells of larvae fed caffeine for 27 hours followed by HU and caffeine for an additional 27 hours proceeded to metaphase at a higher frequency and had more severe chromosomal abnormalities than those fed HU alone (data not shown). Despite this evidence for an impaired checkpoint, the increases in Dup levels in response to HU were similar with or without caffeine (Fig. 3C). Similar results were obtained when *mei-41*^{29D} animals were fed caffeine and HU (data not shown). It is possible, however, that even these caffeine-treated animals retain a low level of ATM/ATR activity. Nonetheless, the large increase in Dup levels suggests that the canonical genotoxic stress checkpoints might not be required to stabilize Dup in response to replication stress.

Various replication defects alter Dup abundance and localization in follicle cells

One possible cause for the accumulation of Dup protein is that cells arrest in a cell-cycle phase during which Dup degradation does not occur. This seemed unlikely given that Dup levels were increased in both wild-type and checkpoint-defective cells that failed to maintain cell-cycle arrest. To examine this question further, and to gain insight into the requirements for Dup degradation, we turned to the follicle cells of the ovary. During stage 10B of oogenesis, these terminally differentiated cells enter a final extended S phase during which only a few origins repeatedly initiate, resulting in the developmental amplification of eggshell protein and other genes (Calvi et al., 1998; Claycomb et al., 2004; Spradling and Mahowald, 1980). The activity of these amplification origins can be visualized as distinct subnuclear foci by BrdU or fluorescent in-situ hybridization during stages 10B-13 of oogenesis (Calvi et al., 1998; Calvi and Spradling, 2001). Antibodies against several replication proteins also label the amplified regions as distinct foci (Fig. 4A,B) (for review, see Tower, 2004).

We examined replication-protein labeling in follicle cells from females homozygous for the hypomorphic mutation *Mcm6*^{K1214}, an allele that, as previously shown by us, reduces amplification severely but has no observable effect on earlier cell cycles in the ovary (Komitopoulou et al., 1983; Schwed et al., 2002). As a control, we first labeled with antibodies against the pre-RC subunit Orc2, which localizes to amplification foci (Fig. 4A). In *Mcm6*^{K1214} mutant follicle cells, Orc2 was still localized to amplifying foci but much less than in the wild type, consistent with the reduced DNA copy number at the amplified loci in this mutant (Fig. 4B). By contrast, Dup labeling was dramatically altered in the *Mcm6*^{K1214} cells. Unlike its focal labeling in the wild type, in 75% of *Mcm6*^{K1214} stage 10B egg chambers (n=100), 100% of the follicle cells had Dup labeling throughout the nucleus, and total fluorescent intensity was

increased up to twofold over the wild type (Fig. 4C,D). These results indicate that, similar to cycling cells of the larval brain, decreased Mcm6 activity results in an increase in Dup levels in terminally differentiated follicle cells, suggesting that this increase is not an indirect effect of a cell-cycle arrest.

To gain further insight into the requirements for normal Dup regulation, we analysed other hypomorphic mutants that impair amplification in the ovary. All these mutants also increased Dup levels in amplification-stage follicle cells, although the increase was, in some cases, not as great as that seen for *Mcm6*^{K1214} (Fig. 4E-H). The product of the *mus101* gene is similar to Dpb11 in *S. cerevisiae* and TopBP1 in humans, and acts downstream of pre-RC assembly for the initiation and elongation phases of replication, and also participates in a checkpoint response (Araki et al., 1995; Makiniemi et al., 2001; Wang and Elledge, 1999; Yamamoto et al., 2000). Dup staining was delocalized and increased in follicle cells homozygous for the hypomorphic amplification mutant allele *mus101*^{K451} (Fig. 4E) (Komitopoulou et al., 1983). Dup protein was also delocalized and increased in *chiffon*^{0233} mutants (Fig. 4F) (Calvi et al., 1998). The *chiffon* gene is similar to DBF4, the activating subunit of the essential S-phase kinase CDC7, whose targets include the MCM proteins (Landis and Tower, 1999) (for a review, see Sclafani, 2000). Finally, mutation of two members of the pre-RC increased the levels of Dup: *Orc2*^{293} and *dup*^{PA77}, a mis-sense mutant of *dup* itself (Fig. 4G,H) (Royzman et al., 1999; Whittaker et al., 2000). Together with the results from mutant brain cells, the data suggest that the regulation of Dup stability is sensitive to several steps in DNA replication. These steps range from activation by kinases upstream of initiation to the polymerase processivity factor PCNA at the replication fork.

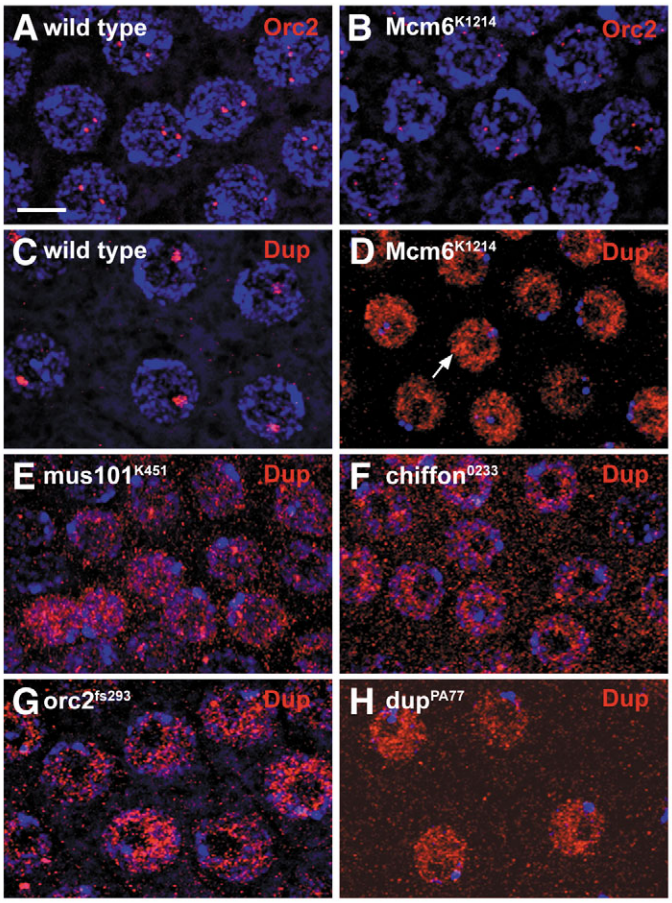

Fig. 4. Replication defects delocalize and increase Dup levels in follicle cells. (A,B) Immunolabeling for Orc2 (red) indicates that it is localized to amplification foci in wild-type (A) and *Mcm6*^{K1214} mutant (B) early-stage 10B follicle cell nuclei (TOTO-3, blue). (C-H) Immunolabeling for Dup (red). Dup is localized to chorion foci in wild-type stage 10B follicle cells (C), whereas in *Mcm6*^{K1214} (D) Dup protein is largely dispersed and more abundant, although some nuclei had detectable concentrations of Dup protein at chorion loci (arrow). Because late-stage 10B follicle cells are shown in C and D, one focus of Dup staining at the chorion locus on the third chromosome locus predominates. The bright blue foci are heterochromatin. (E-H) Dup localization and abundance are altered in the other amplification mutant strains *mus101*^{K451} (E), *chiffon*^{0233} (F), *Orc2*^{fs293} (G) and *dup*^{PA77} (H). Images are composites of confocal sections. Scale bar, 5 μm.

Gem levels are increased during replication stress

We previously showed that an increase in Dup levels is sufficient to induce re-replication in *Drosophila*, and similar results have been obtained for other Cdt1 family members in a range of organisms (Arias and Walter, 2004; Castellano Mdel et al., 2004; Li and Blow, 2004; Maiorano et al., 2005; Thomer et al., 2004; Vaziri et al., 2003). The increase in Dup levels in response to replication stress therefore represents a vulnerable condition for the cell during which inappropriate origin relicensing may occur. However, Gem, the inhibitor of Dup, is present from S phase until mid-M phase and could potentially restrain relicensing when defects in Dup degradation are encountered (McGarry and Kirschner, 1998; Mihaylov et al., 2002; Nishitani et al., 2001; Quinn et al., 2001; Tada et al., 2001; Wohlschlegel et al., 2000). Our previous results had indicated, however, that normal levels of Gem are insufficient to prevent relicensing when Dup is only mildly overexpressed from a transgene (Thomer et al., 2004).

To investigate the levels of Gem protein in replication-stressed cells, we used antibodies that we raised against Gem to label normal and replication-stressed brain cells (Thomer et al., 2004). In normal brain and disc cells, double labeling with antibodies against cell-cycle markers indicated that Gem was abundant in the nucleus during S, G2 and early M phase, similar to previous reports in *Drosophila* and other organisms (Fig. 5A and data not shown) (Quinn et al., 2001) (for review, see Saxena and Dutta, 2005). This included the neuroblast stem cells in the mid-brain, suggesting that Gem might be important to balance the low level of Dup protein that is present in these cells during G2 (Fig. 5A,B).

In *Mcm6*-mutant and HU-treated wild-type animals, Gem levels were dramatically increased in many cells of the brain. Similar to the increase in Dup levels, 20-100 cells per brain typically had per-cell Gem labeling after HU treatment that was two- to fivefold the intensity of the wild-type, with some cells having higher levels of Gem. Almost all cells with increased Dup levels also had correspondingly high levels of Gem, whereas others had elevated Gem levels only (Fig. 5C-E). Also similar to Dup, after HU treatment, Gem levels were increased most in neuroblast stem cells, which consistently had the highest levels of Gem, even compared with their surrounding daughter cells (Fig. 5D,E). These results indicate that neural

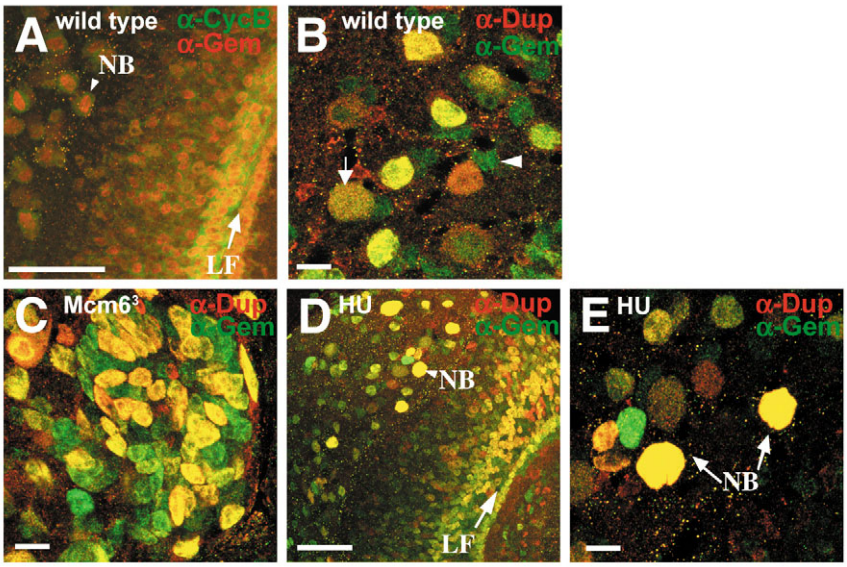

Fig. 5. Gem levels increase during replication stress. (A) Cyclin B (green) and Gem (red) labeling in third-instar brain lobe. Gem is abundant in late-S-, G2- and early M-phase nuclei, whose cytoplasm labels for cyclin B. This is most evident in the lamina-precursor cells (LPCs), which undergo synchronized cell cycles as they migrate (left to right) towards the lamina furrow (LF, arrow). The cells immediately to the left of the LF are in G2 phase and have high levels of Gem in the nucleus and of cyclin B in the cytoplasm. Gem is also expressed during S and G2 phase in the nuclei of neuroblast stem cells (NB) (e.g. arrowhead). (B) Dup (red) and Gem (green) are both present in the nucleus in mid-brain neuroblast stem cells during G2 phase (yellow overlap). During mitosis, stem cells have Dup and Gem distributed throughout the cell (arrow). By contrast, the smaller daughter cells have nuclear Gem (green) during S and G2 phase but little Dup (arrowhead). Cells in G1 are positive for Dup (red) but not Gem. (C) One lobe of an *Mcm6*^{3} mutant brain. Most cells with high levels of Dup (red) also have elevated levels of Gem (green; overlap is yellow), whereas other cells have only high levels of Gem (green). (D,E) HU increases levels of Dup and Gem in neuroblasts and LPCs. (D) A lower-power micrograph shows that the mid-brain NBs have greater increases in Dup and Gem than other cells in response to replication stress. (E) Higher magnification of mid-brain stem cells from D. Neuroblast stem cells (arrows) have very high levels of Dup and Gem (bright yellow), whereas surrounding cells have high levels of Gem only (green) or normal levels of either protein. Scale bar, 50 μm (A,D), 10 μm (B,C,E).

stem cells normally have both Dup and Gem during G2, and that problems with DNA replication result in increased levels of both proteins in these cells. This suggests that the activity of Gem to restrain Dup might be more important in certain cycling cells than others during development, and also when Dup degradation fails during replication stress.

Discussion

During a normal cell cycle, Dup is rapidly degraded as cells enter S phase, but the mechanism that coordinates this ubiquitin-mediated process with S-phase entry is unknown. We have found that defects at different steps in DNA replication increase levels of Dup, the *D. melanogaster* homolog of the origin-licensing protein Cdt1. Our data suggest that the normal degradation of Dup protein that begins at G1-S is impaired in replication-stressed cells, resulting in the persistence and accumulation of Dup. The magnitude of this response was cell-type specific, with stem cells of the brain having the highest levels of Dup during replication stress. Low levels of Dup were also detected during G2 phase in unchallenged stem cells, suggesting that these cells differ in the cell-cycle expression of this origin-licensing protein. There are two important implications of these findings. First, they suggest that, in an unperturbed cell cycle, Dup degradation during S phase is linked to DNA replication. Second, they suggest that the replication checkpoint is a vulnerable time when increased Dup could lead to inappropriate origin relicensing. Our observation that Gem protein also increases in response to replication stress suggests that it might play a prominent role under these conditions to balance Dup protein and guard against re-replication.

Destruction of Dup is coupled to DNA replication

We had previously shown that Dup degradation required cyclin-E/CDK2 and that Dup is a direct substrate of this kinase. However, mutation of the phosphorylation sites in Dup only partially stabilized it and this phosphorylation-site mutant of Dup was still rapidly degraded as cells entered S phase. We therefore suggested that CDK2 might regulate Dup stability in two ways: directly by phosphorylation and indirectly through another CDK2-dependent mechanism in S phase. Here, we find that cells of the brain and ovary have high levels of Dup protein when DNA replication is defective. This was not due to increased transcription and did not depend on the canonical checkpoint pathways mediated by ATM/ATR kinases. Instead, we propose that impaired degradation of Dup, combined with continued translation from a low level of persistent mRNA, leads over time to accumulation of high levels of Dup during S and G2 phase (Fig. 6C). Importantly, this suggests that, in the unstressed cell, the destruction of Dup during S phase is coupled to ongoing DNA replication (Fig. 6B). Because DNA replication depends on CDK2 activity, this provides a mechanism by which this kinase can regulate Dup stability independently of direct phosphorylation. We found that defects in different steps of DNA replication elicited an increase in Dup levels, from impaired pre-RC activation by the CDC7/Dbf4 kinase (*chiffon* mutants) to inhibition of replication fork elongation in PCNA mutants (*mus101* mutants) or upon HU treatment (Fig. 6A). The triggering of Dup destruction by replication itself would be an efficient way for the cell to ensure that origin relicensing does not occur during S phase.

It remains unclear, however, at what step in DNA replication Dup destruction is triggered, and what downstream activities are required for its ultimate degradation. Evidence from a range of systems suggests that Cdt1 can be targeted for degradation by three different ubiquitin ligases: a Cul1-based Skp1/Cullin/F-box (SCF) complex that contains the Skp2 specificity subunit (SCF^{Skp2}); an SCF-like Cul4-based ligase

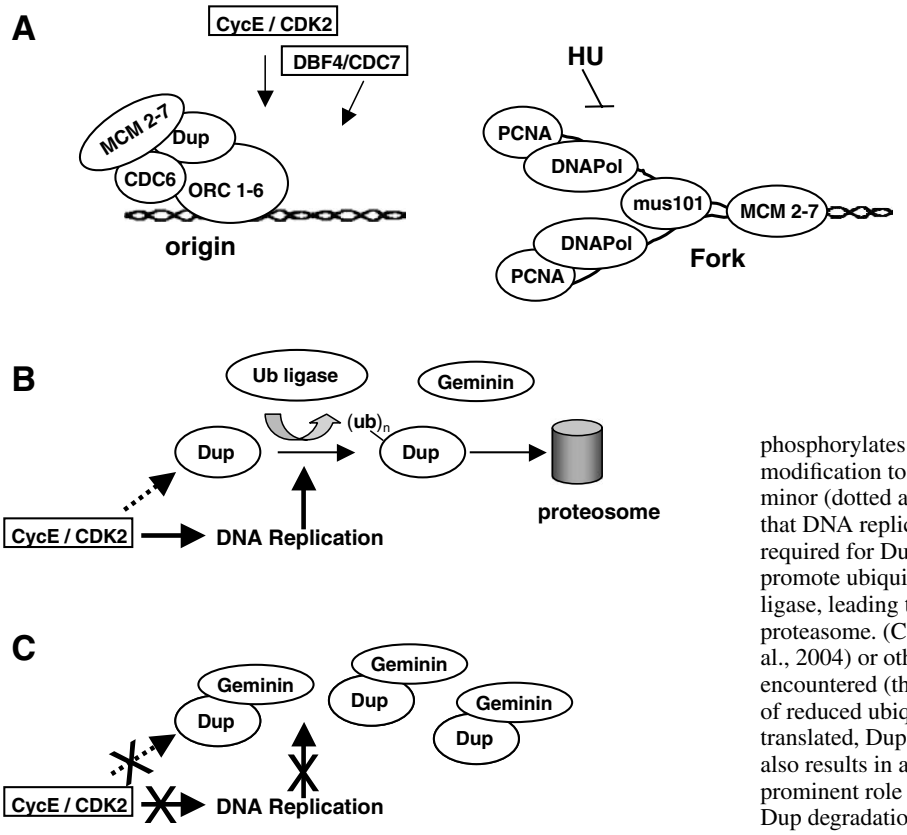

Fig. 6. A model for Dup degradation during S phase. (A) Summary of proteins and activities at origins and forks that were tested and found to be required for normal Dup degradation. Only the origin and replication-fork proteins that were tested are shown, except for the pre-RC protein CDC6, which was not tested. HU inhibits polymerase indirectly by inhibiting ribonucleotide reductase, which results in depletion of dNTPs. (B) Dup degradation during a normal S phase. We had previously shown that cyclin-E/CDK2 is indirectly required for Dup degradation (Thomer et al., 2004). Cyclin-E/CDK2 phosphorylates Dup but the relative contribution of this modification to the instability of Dup appears to be relatively minor (dotted arrow). Based on current evidence, we propose that DNA replication is the CDK2-dependent activity that is required for Dup degradation. This might be required to promote ubiquitylation of Dup by an unknown ubiquitin (Ub) ligase, leading to the rapid destruction of Dup at the proteasome. (C) When CDK2 activity is inhibited (Thomer et al., 2004) or other problems with DNA replication are encountered (this study), Dup is not degraded, perhaps because of reduced ubiquitylation. Because *Dup* mRNA continues to be translated, Dup accumulates to high levels. Replication stress also results in an increase in Gem levels, which probably plays a prominent role in preventing the relicensing of origins when Dup degradation fails

that contains DDB1 as a specificity subunit ($Cul4^{DDB1}$); and the anaphase-promoting complex (APC) (Higa et al., 2003; Hu et al., 2004; Kondo et al., 2004; Li and Blow, 2005; Li et al., 2003; Zhong et al., 2003). Evidence from human cells suggested that phosphorylation of Cdt1 by CDK2 targets it for ubiquitination by SCF^{Skp2} and subsequent proteasome degradation (Li et al., 2003; Sugimoto et al., 2004). It appears, however, that cyclin binding and Skp2 are not required for Cdt1 degradation in human cells (Takeda et al., 2005) and that, similar to our results and those from other organisms, direct phosphorylation by CDK2 does not fully account for the rapid destruction of Cdt1 at G1-S (Arias and Walter, 2004; Thomer et al., 2004). Evidence from *Caenorhabditis elegans* and *D. melanogaster* suggests that Cul4 complexes might be primarily responsible for degradation of Cdt1 and Dup during S phase of an unperturbed cell cycle (Higa et al., 2003; Zhong et al., 2003). Our results here suggest that the activity of one or more of these ubiquitin ligases towards Dup during S phase is dependent upon normal DNA replication (Fig. 6B).

While this manuscript was in preparation, Arias and Walter reported results from a *Xenopus* in-vitro replication system that are very similar to our results in *Drosophila* (Arias and Walter, 2004). They showed that depletion of several different replication proteins from embryo extracts coordinately impaired DNA synthesis and the ubiquitination and destruction of Cdt1. They found that steps as far downstream as polymerase-α, which synthesizes the DNA primer, were required for normal Cdt1 degradation, and here we extend that to the downstream step of loading PCNA onto those primers. Similar to Dup in flies, they also found that this stabilization of *Xenopus* Cdt1 in response to replication stress was independent of ATM/ATR-checkpoint-kinase activity and provided evidence that Cdt1 degradation in *Xenopus* is not solely regulated by direct phosphorylation by CDK2.

The dependence of Cdt1 degradation on replication is reminiscent of previous observations from *Xenopus*, in which ubiquitination and degradation of the p27-like inhibitor protein Xic1 at origins by the SCF ligase also depends on initiation of DNA replication but is independent of cyclin-E/CDK2 phosphorylation of Xic1 (Furstenthal et al., 2001; You et al., 2002). It is also reminiscent of SCF-mediated degradation of human Orc1 in S phase, which might be independent of its direct phosphorylation by CDK2 (Mendez et al., 2002). Thus, the mechanism that links the destruction of Dup/Cdt1 to DNA replication might target other proteins for destruction to promote S-phase progression and prevent re-replication. The similarity of our findings to those in vertebrates further suggests that the mechanism for Cdt1 degradation is conserved in multicellular eukaryotes.

Developmental modulation of Dup cell-cycle expression

Our previous results suggested that, in most cells, Dup is abundant in the nucleus in late G1 phase and disappears rapidly upon S-phase entry. Unexpectedly, we found that, in the absence of replication stress, stem cells and lamina-precursor cells in the brain have a low but detectable level of Dup during G2 and M phase. Stem cells clearly had the highest levels of Dup during replication stress, even compared with their immediate daughter cells. In the absence of stress, in-situ hybridization showed that *Dup* mRNA is most abundant in stem cells and lamina-precursor cells, and that some mRNA

persisted into S and G2 phase, similar to the cell-cycle distribution of human *Cdt1* mRNA (Fig. 2A and data not shown) (Nishitani et al., 2001). In the context of our model, the relatively high levels of Dup in stem cells during replication stress are caused by a defect in protein degradation combined with continued translation from a relatively higher steady-state level of *Dup* mRNA. It is possible, however, that other aspects of Dup regulation differ in stem cells. Interestingly, unchallenged germ-line stem cells also had high levels of Dup relative to other cells (data not shown). Investigation into Dup regulation in stem cells might provide clues to how their cell cycles differ to support their function in tissue homeostasis.

Despite the presence of Dup during S and G2 phase in normally cycling stem cells, there is no evidence that they undergo re-replication. We found that Gem protein is present in these cells during S, G2, and M phase, which probably prevents origin relicensing. An unexpected implication of our observations is that Gem might be more important in some cells than in others during development to protect genome integrity.

Dup, Gem and genome integrity

Evidence from a range of organisms indicates that Cdt1 must be tightly regulated by proteolysis and Gem to prevent re-replication. The high levels of Dup in the replication-stressed cell therefore represents a vulnerable condition when origin re-licensing could occur. We found, however, that the Dup inhibitor Gem was also elevated during replication stress. This suggests that Gem might play a prominent role to prevent re-replication when Dup degradation is impaired during replication stress (Fig. 6C). We could not directly test this model here because replication was acutely blocked by the same defects that led to elevated Dup and Gem. In a cell with mild defects and transiently arrested in the checkpoint, however, Gem might be crucial to prevent origin relicensing so that, when cells eventually resume normal cycling, they duplicate their genome only once. This fail-safe role for Gem during the checkpoint is consistent with previously suggested models (for a review, see Saxena and Dutta, 2005) (Tada et al., 2001; Wohlschlegel et al., 2000). It was reported previously that overexpression of Cdt1 in human cells results in a concomitant rise in Gem levels, suggesting there might be a conserved mechanism that actively maintains the proper balance of these two proteins (Vaziri et al., 2003). Given that CDKs might play other roles in preventing re-replication, inhibition of CDKs during a checkpoint arrest, together with elevated levels of Cdt1, puts the cell especially at risk for inappropriate origin re-licensing. This has important medical implications because elevated Cdt1 protein is found in several human cancers, is sufficient to induce re-replication in human cells and increases the oncogenic potential of mouse erythroid cells (Arentson et al., 2002; Karakaidos et al., 2004; Melixetian et al., 2004; Vaziri et al., 2003; Xouri et al., 2004; Zhu et al., 2004). It is therefore possible that, under certain conditions, such as replication stress, defects in Cdt1 degradation might contribute to genome instability and transformation of the cancer cell.

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