An essential role for Cdk1 in S phase control is revealed via chemical genetics in vertebrate cells

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In vertebrates Cdk1 is required to initiate mitosis; however, any functionality of this kinase during S phase remains unclear. To investigate this, we generated chicken DT40 mutants, in which an analog-sensitive mutant *cdk1as* replaces the endogenous Cdk1, allowing us to specifically inactivate Cdk1 using bulky ATP analogs. In cells that also lack Cdk2, we find that Cdk1 activity is essential for DNA replication initiation and centrosome duplication. The presence of a single Cdk2 allele renders S phase progression independent of Cdk1, which suggests a complete overlap of these kinases in S phase control. Moreover, we find that Cdk1 inhibition did not induce re-licensing of replication origins in G2 phase. Conversely, inhibition during mitosis of Cdk1 causes rapid activation of endoreplication, depending on proteolysis of the licensing inhibitor Geminin. This study demonstrates essential functions of Cdk1 in the control of S phase, and exemplifies a chemical genetics approach to target cyclin-dependent kinases in vertebrate cells.

Introduction

Cyclin-dependent kinases (Cdks) and their regulatory cyclin subunits play a crucial role in cell cycle control (Hunt and Murray, 1993). In budding and fission yeast, a single Cdk, bound to different sets of cyclins, initiates DNA synthesis and centrosome duplication, suppresses re-replication of already duplicated DNA, and triggers entry into mitosis once replication is complete (Nasmyth, 1993; Stern and Nurse, 1996). Higher eukaryotes have evolved a group of specialized Cdks, each of which is active in a different phase of the cell cycle (Malumbres, 2005). Cdk1 together with cyclin A and B forms the maturation-promoting factor, and is required for entry into mitosis. Cdk2 bound to cyclin E and A was considered to be essential for initiation and completion of DNA replication, and the control of centrosome duplication, until several groups found that mice lacking Cdk2 develop normally (Berthet et al., 2003; Ortega et al., 2003). This raises the question of which Cdk controls the initiation and completion of S phase in the absence of Cdk2. Although Cdk1 is an apparent candidate for this redundant S phase Cdk, as Aleem et al. (2005) proposed, an essential function for vertebrate Cdk1 during G1 and S phase has not been directly demonstrated. In fact, Cdk4 has also been implicated recently as a back up kinase for Cdk2 in G1 phase (Berthet et al., 2006). Hence, we do not know to what extent different Cdks overlap in the initiation of S phase in vertebrate cells.

In addition to the initiation of replication, the inhibition of endoreplication is another essential S phase function of yeast Cdk1, which ensures that each replication origin fires only once per cell cycle by inhibiting the untimely assembly of pre-replication complexes (pre-RCs) (Diffley, 2004). At the exit from mitosis, Cdk1 activity is shut down by the anaphase promoting complex, also known as cyclosome (APC/C), which triggers cyclin destruction (Zachariae et al., 1998). This inactivation of Cdk1 by cyclin proteolysis seems sufficient for the re-licensing of origins in the next G1 phase (Noton and Diffley, 2000). This idea is supported by the observation that artificial inactivation and reactivation of yeast Cdk1 are sufficient to reset the cell cycle and induce endoreplication (Hayles et al., 1994). Several studies also implicate Cdk1 in the inhibition of endoreplication in flies and human cells (Hayashi, 1996; Itzhaki et al., 1997; Coverley et al., 1998). However, higher eukaryotes, but not yeast, contain an additional licensing inhibitor, Geminin, which binds to and inactivates the pre-RC assembly factor Cdt1 (McGarry and Kirschner, 1998; Wohlschlegel et al., 2000; Tada et al., 2001).

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Abbreviations used in this paper: APC/C, anaphase-promoting complex/cyclosome; cdk1as, Cdk1 analogue sensitive mutant; cdk1-WT, Cdk1-wild type; CHX, cycloheximide; pre-RC, pre-replication complex.

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Moreover Cdk-dependent and -independent proteolysis pathways regulate the stability of the licensing factor, Cdt1 during S phase (Arias and Walter, 2007). It remains elusive how Geminin, Cdk1 activity, and proteolysis of Cdt1 are coordinated to suppress endoreplication in human cells.

The following two questions arise regarding the contribution of Cdk1 to the control of S phase: Is Cdk1 involved in the initiation of DNA replication and centrosome duplication? Is Cdk1 inhibition sufficient to induce endoreplication in vertebrate cells, despite the presence of Geminin? These questions have not been sufficiently addressed, owing to the difficulty to specifically, rapidly, and effectively inactivate Cdk1. In fact, a conditional deletion of the Cdk1 promotor in a human cell line has been achieved, but the levels of the kinase drop only very slowly and incompletely (Itzhaki et al., 1997). A mouse cell line (FT210) that carries a temperature-sensitive mutation has also been isolated, but this cell line appears to maintain about 25% kinase activity at the restrictive temperature (Th'ng et al., 1990). A variety of chemical inhibitors of Cdk1, such as Roscovitine and Olomoucine, have been used to explore Cdk1 function (Fischer et al., 2003; Vassilev et al., 2006). However, these inhibitors are likely to affect other kinases within and possibly outside of the Cdk family. To increase the specificity of chemical inhibition, Shokat and coworkers recently developed a chemical genetics approach to sensitize kinases to bulky ATP analogs by mutating a conserved bulky residue in the active site (Bishop et al., 2001; Shokat and Velleca, 2002). This strategy has been successfully applied to Cdk1 and other kinases in yeast (Bishop et al., 2000), and a similar approach has been exploited to study Jun and Trk kinase in mouse models (Chen et al., 2005; Jaeschke et al., 2006; Ventura et al., 2006) and in human cells to analyze Cdk7 (Larochelle et al., 2007).

We have taken advantage of the high gene-targeting frequencies in chicken DT40 cells to disrupt the endogenous chicken *CDC2* gene, encoding the Cdk1 kinase, and ectopically express a mutant Cdk1 cDNA (*cdk1as)* that is selectively sensitive to inhibition by the ATP analog 1NM-PP1. Using this system, we have investigated S phase functions of vertebrate Cdk1. We found that Cdk1 activity is essential for triggering DNA replication and centrosome duplication in cells lacking Cdk2. Conversely, if Cdk2 is present, Cdk1 inhibition does not delay S phase or block centrosome duplication. We also show that whereas inhibition of Cdk1 in G2 phase, before entry into mitosis, does not induce endoreplication, inhibition of Cdk1 during prometaphase does stimulate origin licensing and endoreplication. This depends on the proteolysis of Geminin. These results clarify the role of vertebrate Cdk1 in controlling replication and endoreplication.

Results

Selective inhibition of Cdk1 by chemical genetics results in a reversible G2 arrest

We initiated this study by establishing DT40 cell lines, in which we disrupted the endogenous chicken Cdk1 by gene targeting and exogenously expressed either an analog sensitive F80G mutant (*cdk1as) or* wild-type (*cdk1WT*) cDNA of *Xenopus laevis* Cdk1 (Fig. S1, A–E; available at http://www.jcb.org/cgi/content/full/jcb.200702034/DC1). We isolated two independent *cdk1as* cell lines, with slightly different levels of Cdk1as expression. The *cdk1as1* cell line grew with similar kinetics as DT40 WT cells, whereas *cdk1as2* cells that expressed less Cdk1 transgene showed a slight growth retardation. (Fig. S1 F). In both cases the activity of the Cdk1 kinase was reduced when compared with WT Cdk1, probably due to the F80G mutation in the active site. We continued to work with the *cdk1as1* cell line, hereafter called *cdk1as* cells.

To confirm that the mutant Cdk1 was indeed selectively sensitive to the bulky ATP analog inhibitor 1NM-PP1, we immunoprecipitated Cdk1 from extracts of Cdk1-deficient cells reconstituted with WT *Xenopus* Cdk1 (*cdk1WT* cells) or *cdk1as* cells, and measured the Cdk1 kinase activity of the immunoprecipitates in the presence or absence of 1NM-PP1, using Histone H1 as a substrate. Although the amount of Cdk1 was comparable in the immunoprecipitates (unpublished data), the kinase activity of *cdk1as* cells was reduced to about 20% of WT *Xenopus* Cdk1 activity (Fig. 1, A and B). Addition of 10 μM 1NM-PP1 inhibited phosphorylation of Histone H1 by the mutant kinase but had no effect on the WT Cdk1. Likewise, 10 μM 1NM-PP1 had no effect on the growth of *cdk1WT* cells, but abolished proliferation of *cdk1as* cells (Fig. 1 C).

To investigate the effects of Cdk1 inhibition on the cell cycle, we isolated the G1 fraction of *cdk1WT* and *cdk1as* cells by elutriation, added 10 μM 1NM-PP1 to the media, and took samples every 2 h for FACS analysis of the DNA content. Fig. 1 D shows that both cell lines initiated and completed S phase with very similar kinetics. The *cdk1WT* cells subsequently completed mitosis and re-entered the next cell cycle, whereas *cdk1as* cells remained arrested in the 4N state (Fig. 1, D and E). This arrest was maintained for several days without further division and DNA synthesis (Fig. 1 E).

To analyze the activity of the APC/C in the arrested cells, we measured cyclin levels in arrested *cdk1as* cells, in which de novo protein synthesis was inhibited by cycloheximide (CHX). Both cyclins were stable during the G2 arrest for more than 6 h after CHX treatment (Fig. 1 F, left). CHX did not interfere with cyclin destruction after the release from a Nocodazole block, excluding the possibility that CHX itself affects the proteolysis of cyclins (Fig. 1 F, right). We also found that cyclin B2 localized predominantly in the cytoplasm throughout this prolonged arrest (Fig. 1 G). These observations indicate that Cdk1 inhibition blocks the cell cycle in the G2 phase before APC/C activation and translocation of cyclin B to the nucleus.

To explore whether the arrest induced by Cdk1 inhibition was reversible, we removed 1NM-PP1 after 8 h incubation of *cdk1as* cells with the inhibitor. This resulted in rapid entry into M phase, as evidenced by Histone H3 phosphorylation, and cyclin destruction, during a synchronous passage through mitosis (Fig. S2, available at http://www.jcb.org/cgi/content/full/jcb.200702034/DC1). These findings suggest that the active state of Cdk1, including association of cyclin B, is unaltered during the 1NM-PP1 mediated inhibition of *cdk1as*, allowing the rapid activation of Cdk1 upon removal of the inhibitor during the G2 arrest.

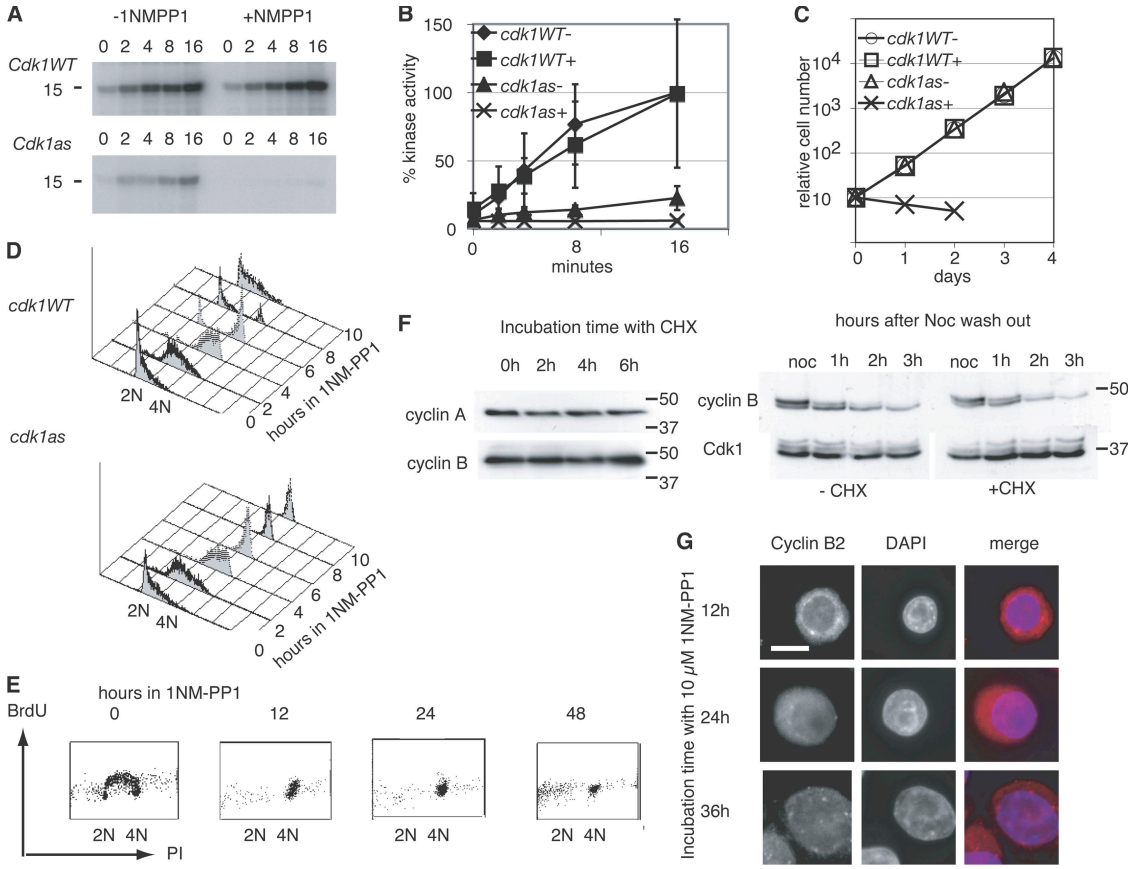

Figure 1. **Inhibition of Cdk1 by chemical genetics in DT40 cells results in a G2 arrest.** (A) Histone H1 kinase assays with 9E10 immunoprecipitates from *CDC2* gene-disrupted DT40 cells, reconstituted with myc-tagged *Xenopus cdk1WT* or *cdk1as* cDNAs. Each immunoprecipitate was split and washed with buffer containing equal amounts of either DMSO or 10 μM 1NM-PP1. Samples from the kinase assay were taken at the indicated incubation times and analyzed by SDS-PAGE and autoradiography to detect ^{32}P-labeled Histone H1. For a comparison of Cdk1 levels in the immunoprecipitates, see Fig. S1 G. (B) Quantification of the Histone H1 kinase activity of *cdk1WT* and *cdk1as* immunoprecipitates in the presence (+) or absence (−) 10 μM 1NM-PP1. Shown are values relative to the activity of *cdk1WT* kinase, 16 min after initiation of the reaction. The mean values and SD of four independent experiments are shown. (C) Proliferation kinetics of Cdk1-deficient DT40 cells reconstituted with either *cdk1WT* or *cdk1as* cDNAs in the presence (+) or absence (−) of 10 μM 1NM-PP1. (D) Cell cycle profile of *cdk1WT* and *cdk1as* cells. Cells were synchronized in G1 phase by elutriation at time zero and cultured for 10 h in medium containing 10 μM 1NM-PP1. Samples were taken every 2 h and analyzed by PI staining and FACS. (E) Cell cycle analysis of BrdU pulse-labeled *cdk1as* cells incubated with 10 μM 1NM-PP1 for 0, 12, 24, and 48 h. The X-axis of the dot blot shows the content of DNA on a linear scale; the Y-axis shows the BrdU uptake on a log scale. (F) Cyclin stability in 1NM-PP1 arrested *cdk1as* cells. The cells arrested in G2 phase by a 12-h incubation with 10 μM 1NM-PP1. 100 μg/ml CHX were added and whole-cell extracts were analyzed by SDS-PAGE and immunoblotting at the indicated times. To rule out an interference of CHX with proteolysis *cdk1as* cells were released from a 4-h Nocodazole arrest in the absence or presence of 100 μg/ml CHX. Cyclin B or Cdk1 amounts were measured by immunoblotting at the indicated times after the release. (G) Localization of cyclin B2 in the cytoplasm of *cdk1as* cells treated with 10 μM 1NM-PP1. Samples were taken at the indicated times, fixed, and analyzed by immunofluorescence (bar, 10 μm), using the anti–chicken B2 antibody (red color), and *DAPI* staining of DNA (blue color).

Cdk1 and Cdk2 have overlapping functions in both S phase progression and centrosome duplication

To explore the redundancy of Cdk1 and Cdk2 during S phase, we disrupted the chicken *CDK2* gene to generate *cdk1as/cdk2*$^{-/-}$ mutants (Fig. S3, available at http://www.jcb.org/cgi/content/full/jcb.200702034/DC1). Ablation of Cdk2 had little effect on DT40 WT cells, but retarded the cellular proliferation in the *cdk1as* background even in the absence of 1NM-PP1 (Fig. 2, A and B). Accordingly, *cdk1as/cdk2*$^{-/-}$ cells that were synchronized in G1 phase by elutriation took approximately 2 h longer than *cdk1as* cells to initiate S phase (compare the FACS histogram in Fig. 2 C with Fig. 1 D). Nonetheless, the double-mutant cells were still able to complete S phase and accumulated in G2 phase even in the presence of a low dose (1 μM) of 1NM-PP1 (Fig. 2 D). A tenfold higher dose of the inhibitor blocked the asynchronous cell cycle both in G1 and G2, as judged by the histogram of DNA content (Fig. 2 D). This suggests that Cdk1 is responsible for S phase control in the absence of Cdk2.

We next analyzed the centrosomes by γ-tubulin staining in arrested *cdk1as* and *cdk1as/cdk2*$^{-/-}$ cells. 8 h after Cdk1 inhibition, *cdk1as* cells contained two separated centrosomes, and the centrosome number appeared to double subsequently during 8-h intervals (Fig. 2, E and F). A similar separation and doubling of the centrosomes occurred in *cdk1as/cdk2*$^{-/-}$ cells that were treated with 1 μM 1NM-PP1 (Fig. 2 E). However, the centrosomes no longer duplicated in *cdk1as/cdk2*$^{-/-}$ treated with 10 μM 1NM-PP1 (Fig. 2, E and F). This phenotypic difference between *cdk1as* and *cdk1as/cdk2*$^{-/-}$ cells indicates that Cdk1

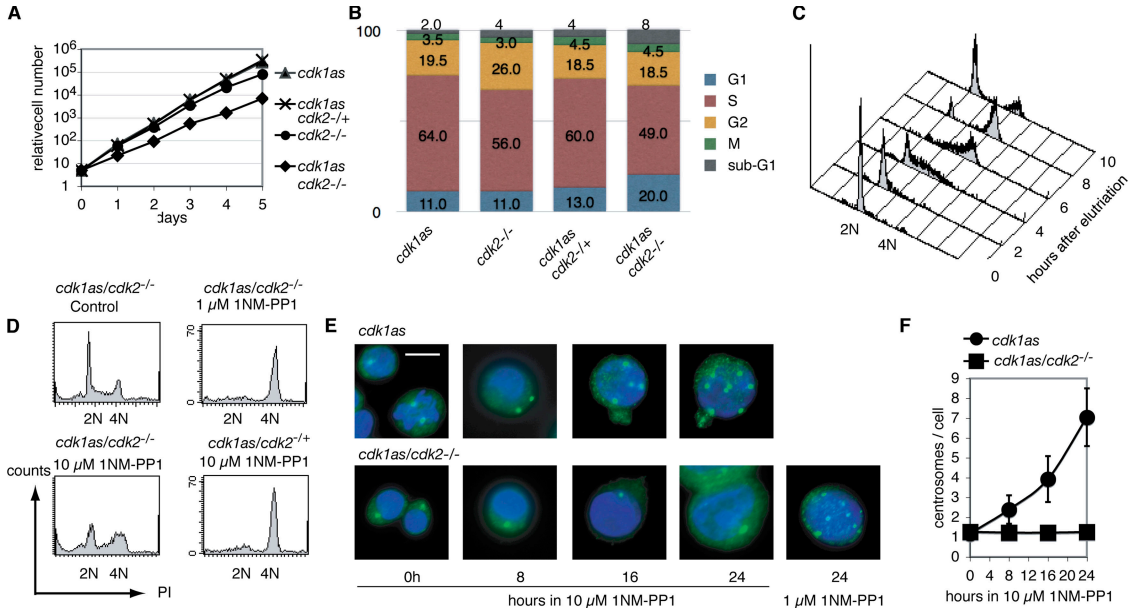

Figure 2. **Effects of a Cdk2 deletion on *cdk1as* cells.** (A) Growth curve of *cdk1as/cdk2*$^{-/-}$ cells compared to the indicated controls. (B) Cell cycle distribution of the indicated cell lines, calculated from BrdU pulse labeling, phosphohistone H3 staining, and PI staining experiments. (C) Cell cycle analysis of *cdk1as/cdk2*$^{-/-}$ cells, following synchronization in G1 phase by elutriation. (D) Cell cycle analysis of *cdk1as/cdk2*$^{-/-}$ and *cdk1as/cdk2*$^{-/+}$ cells treated for 8 h with the indicated concentration of 1NM-PP1. The control was not exposed to 1NM-PP1. (E) Centrosomes detected by γ-tubulin immunofluorescence (green color) and counterstained with *DAPI* (blue color) in fixed *cdk1as* and *cdk1as/cdk2*$^{-/-}$ cells (bar, 10 μm), at the indicated times after 1 μM or 10 μM 1NM-PP1 addition. (F) Average number of centrosomes per cell (n = 50) plotted against time after 10 μM 1NM-PP1 addition.

and Cdk2 share overlapping functions in the control of both DNA replication and centrosome duplication.

Cdk1 activity is required to initiate but not to sustain DNA synthesis throughout S phase, in cells lacking Cdk2

We aimed to analyze the S phase functions of Cdk1 in *cdk1as/cdk2*$^{-/-}$ cells in closer detail, by examining cell cycle progression. We collected the G1 fraction of cells, treated them with 10 μM 1NM-PP1, and analyzed the subsequent progression through the cell cycle (Fig. 3, A and B). The G1 fraction of *cdk1as/cdk2*$^{-/+}$ cells was able to increase their DNA content (Fig. 3 A), and uptake BrdU (Fig. S4, available at http://www.jcb.org/cgi/content/full/jcb.200702034/DC1) in the presence of 1NM-PP1. In contrast, the same dose of the inhibitor completely abolished the cell cycle progression and BrdU uptake in *cdkas/cdk2*$^{-/-}$ cells (Fig. 3 B; Fig. S4). These observations indicate functional redundancy between Cdk1 and Cdk2 for the initiation of S phase.

To confirm this notion, we examined the cell cycle progression in asynchronous populations of *cdk1as/cdk2*$^{-/+}$ and *cdk1as/cdk2*$^{-/-}$ cells using pulse-chase BrdU labeling. After 10 min of BrdU exposure, G1, S, and G2 fractions of the cells were clearly distinguishable by dot blot analysis of BrdU/PI double staining (Fig. 3 C). BrdU was subsequently removed from the medium and the cells were further incubated in the presence of 10 μM 1NM-PP1. Both the G1 and S phase population of *cdk1as/cdk2*$^{-/+}$ cells were able to complete DNA synthesis, and the entire population of the cells was blocked in the G2 phase. Conversely, the G1 fraction of the homozygous *cdk1as/cdk2*$^{-/-}$ mutants did not initiate DNA synthesis, whereas the mid S phase cells were shifted toward G2 (Fig. 3 C). Thus, although ongoing DNA synthesis during S phase does not appear to require Cdk1/2 activity, either Cdk1 or Cdk2 activity is essential to initiate DNA replication.

To further explore this G1 arrest induced by Cdk1 inhibition in *cdk1as/cdk2*$^{-/-}$ cells, we measured the levels of cyclin A and B in cells synchronized in G1 by elutriation (Fig. 3 D). Both cyclins accumulated even after addition of 10 μM 1NM-PP1, suggesting that Cdk1/2 inhibition does not interfere with the expressions of genes required for the G1/S transition.

Inhibition of Cdks in G2 phase does not result in endoreplication in DT40 or HeLa cells

Hayles et al. (1994) demonstrated that artificial inactivation and reactivation of *Schizosaccharomyces pombe* Cdc2 in G2 phase is sufficient to reset the cell cycle and to initiate a further round of DNA replication, without previous chromosome segregation. To investigate whether a similar reversible inhibition of Cdk activity is also sufficient to induce endoreplication in vertebrate cells, we synchronized *cdk1as/cdk2*$^{-/-}$ cells in G2 phase by addition of 1 μM 1NM-PP1 for 6 h, and subsequently increased the inhibitor concentration to 10 μM for an additional hour to completely block Cdk1 activity. Afterward, we washed the inhibitor out with excess medium, and monitored the cell cycle progression by measuring DNA content by using FACS analysis (Fig. 4 A). Surprisingly, unlike *S. pombe*, this inhibition and reactivation of Cdk1 in G2 phase did not result in endoreplication, and all cells initiated the next round of replication only after mitosis (Fig. 4 A; note the absence of cells containing DNA >4N). This observation is in marked contrast with a previous study by Itzhaki et al. (1997), who showed that a human fibroblast

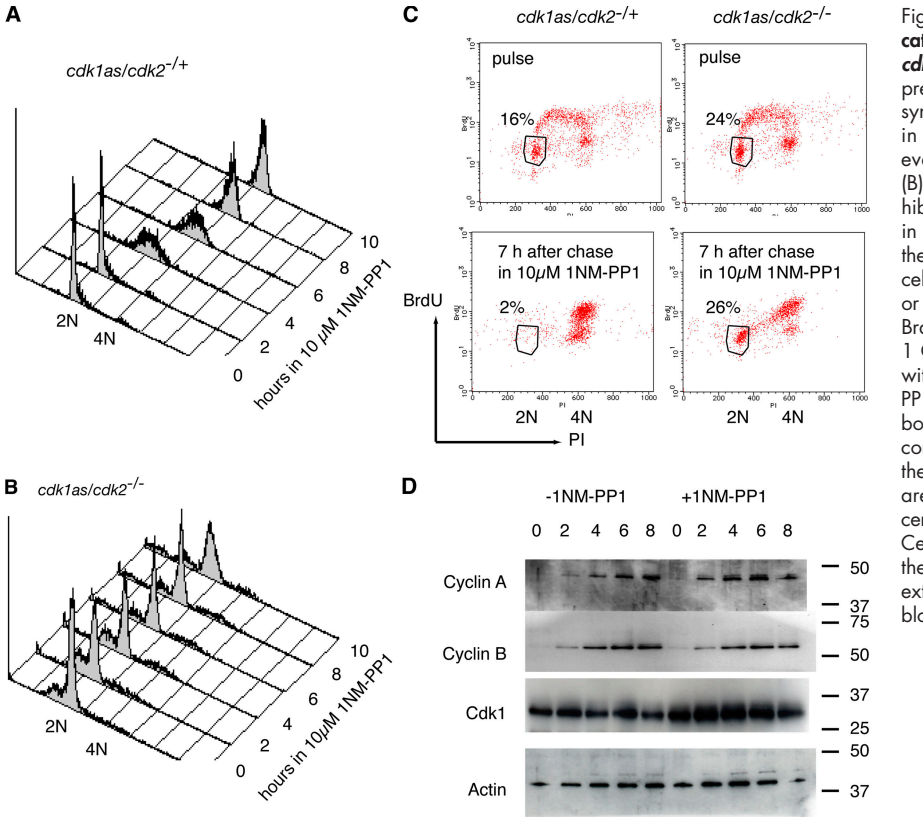

Figure 3. **Effects of Cdk1 inhibition on DNA replication and cyclin synthesis in *cdk1as/cdk2*$^{-/-}$ and *cdk1as/cdk2*$^{-/+}$ cells.** (A) Cdk1 inhibition does not prevent S phase in *cdk1as/cdk2*$^{-/+}$ cells. Cells synchronized in G1 by elutriation were incubated in 10 μM 1NM-PP1, and samples were taken every 2 h for cell cycle analysis, as in Fig. 1 D. (B) Cdk1 inhibition in *cdk1as/cdk2*$^{-/-}$ cells inhibits entry into S phase. The same experiment as in A was performed. (C) Inhibition of Cdk1 blocks the initiation of DNA synthesis in *cdk1as/cdk2*$^{-/-}$ cells. Asynchronous cultures of *cdk1as/Cdk2*$^{-/+}$ or $^{-/-}$ cells were exposed to a 10-min pulse of BrdU and subjected to cell cycle analysis as in Fig. 1 C (top panels). BrdU labeled cells were chased with BrdU-free medium containing 10 μM 1NM-PP1 for a further 7 h (pulse-chase analysis shown in bottom panel). The X-axis of the dot blot shows the content of DNA on a linear scale; the Y axis shows the BrdU uptake on a log scale. Cells in the gate area represent the G1 fraction, of which the percentage is shown. (D) Time course of cyclin synthesis. Cells synchronized in G1 phase were incubated in the presence or absence of 10 μM 1NM-PP1. Cell extracts were analyzed by SDS-PAGE and immunoblotting at the indicated time points.

cell line continued to increase in ploidy, after conditional inhibition of Cdk1 expression.

To confirm that Cdk1 inactivation does not activate replication origins of *cdk1as/cdk2*$^{-/-}$ cells, we analyzed the recruitment of Mcm proteins, essential components of the pre-RC, to chromatin. In G2 phase, Mcm2–7 are excluded from chromatin, and need to be loaded as a hexameric ring structure onto the DNA to license origins for a new round of DNA replication. In correlation with the results in Fig. 4 A, we could not detect Mcm2, 3, and 4 in the chromatin fraction of *cdk1as/cdk2*$^{-/-}$ cells that were synchronized in G2 by1 μM 1NM-PP1, and then further treated with 10 μM 1NM-PP1 to fully block Cdk1 (Fig. 4 B, lane 5). Moreover, additional inhibition with the general Cdk inhibitor Roscovitine also failed to induce Mcm2–4 binding to chromatin (Fig. 4 B, lane 6). Thus, inhibition of both Cdk1 and Cdk2 during G2 phase may not be sufficient for origin licensing in chicken DT40 cells.

Previous studies suggested that the general kinase inhibitor DMAP induces Mcm loading onto chromatin in the G2 phase of HeLa cells (Coverley et al., 1996). We repeated the same experiments by inhibiting Cdks more specifically by treating HeLa cells with Roscovitine. For this purpose, we synchronized the cells in early S phase by a double thymidine block, released them, and analyzed the chromatin binding of both the licensing factor Cdt1 and Mcms, as cells progressed through S phase. After 7 h after release, when most cells were in G2 phase (Fig. 4 C), Mcms had been largely displaced from the chromatin (Fig. 4 D, lane 8). At this time, we added Roscovitine to the cells, while a control sample was left without Cdk inhibition (Fig. 4, C and D; compare "9" with "9+Ros"). Both samples were treated with Nocodazole, an inhibitor of spindle formation, to avoid the entry in the next G1 phase. In accordance with the results obtained with chemical genetics in DT40 cells (Fig. 4, A and B), Roscovitine did not appear to induce Mcm loading onto chromatin in the G2 population of HeLa cells (Fig. 4 D, lane 10). In conclusion, Cdk inhibition in G2 phase is not necessarily sufficient to induce origin licensing in chicken and human cells.

Inhibition of Cdk1as in mitotic cells causes endoreplication

Vassilev et al. (2006) recently observed an induction of endoreplication in Nocodazole arrested human cells treated with a Cdk1 inhibitor. We wished to know whether Cdk1 inhibition by the chemical genetics method also triggers endoreplication in mitotic DT40 cells. To avoid a prolonged treatment with Nocodazole, we first synchronized *cdk1as* cells in G2 using 1NM-PP1, and then released them into mitosis by removing the inhibitor, while adding Nocodazole to obtain cells that were briefly arrested in prometaphase (see diagram in Fig. 5 A). In contrast to the cells arrested in G2 (Fig. 4 C), the mitotic *cdk1as* cells exhibited DNA replication without completing mitosis, after addition of 10 μM 1NM-PP1 (Fig. 5 B). Cdk1 inhibition also led to rapid dephosphorylation of Histone H3, indicative of decondensation of chromosomes (Fig. 5 C). We also observed the quick dephosphorylation of the APC/C subunit Cdc27 (Fig. 5 D), which is hyperphosphorylated by Cdk1 in mitosis (Rudner and Murray, 2000). Moreover, we found that the APC/C was

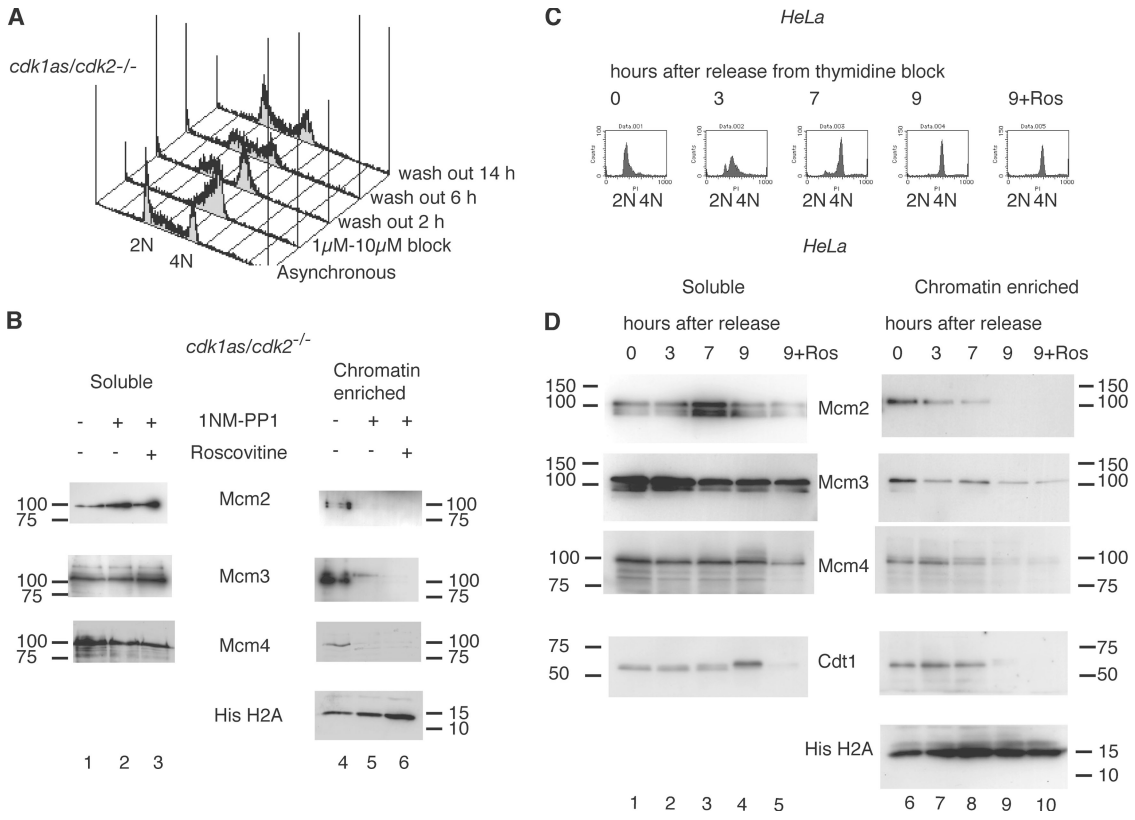

Figure 4. **Analysis of DNA endoreplication and Mcm binding to chromatin after Cdk1 and Cdk1/2 inhibition in DT40 and HeLa cells.** (A) Inhibition of Cdk1 does not trigger endoreplication in *cdk1as/cdk2*$^{-/-}$ cells. The cells were treated with 1 μM 1NM-PP1 for 6 h to allow accumulation in G2 phase, followed by addition of 10 μM 1NM-PP1 for an additional hour (1–10 μM block). The inhibitor was washed out in excess medium and the cells were further incubated without the inhibitor for 14 h. At 2, 6, and 14 h after wash out, cell cycle analysis was performed. (B) Evaluation of origin licensing by analyzing chromatin binding of Mcm2-4 in *cdk1as/cdk2*$^{-/-}$ cells. Asynchronous populations (lanes 1 and 4), and cells synchronized in G2 by a 12-h incubation in 1 μM 1NM-PP1, and then further treated with 10 μM 1NM-PP1 (lanes 2 and 5), or with 10 μM 1NM-PP1, and 50 μM Roscovitine (lanes 3 and 6) are shown. The indicated samples were subjected to chromatin fractionation and immunoblot analysis of the soluble (left) and chromatin (right) fraction. (C) Cell cycle analysis of HeLa cells released from a double thymidine block. At 7 h after release, 100 μM Roscovitine or DMSO was added to the cells, which were incubated for additional 2 h (9 and 9+Ros). Progression into the next G1 phase was inhibited by simultaneous addition of 100 ng/ml Nocodazole. (D) Lack of origin licensing in HeLa cells following Roscovitine treatment in G2. Cells were prepared as in C. Samples were analyzed by SDS-PAGE and immunoblotting with the indicated antibodies. Note the absence of Cdt1 and Mcms in the chromatin fraction after Cdk1 inhibition in lane 9.

activated in response to mitotic Cdk1 inhibition, as judged by the rapid degradation of cyclin A upon addition of the inhibitor (Fig. 5 E). Nocodazole arrested *cdk1WT* cells showed no such response to 1NM-PP1 and remained unchanged in mitosis (unpublished data). These data suggest that inhibition of endoreplication may be carried out differently in G2 and M phase.

Endoreplication induced by Cdk1 inhibition in mitosis depends on proteolysis

A possible explanation for the differential effects of Cdk1 inhibition on DNA synthesis in G2 and M phase could be the activation of APC/C mediated proteolysis during mitosis but not G2 phase (compare cyclin stability in Fig. 1 F and Fig. 5 E). To test this hypothesis, we treated *cdk1as* cells that were synchronized in mitosis as shown in Fig. 5 A with the proteasome inhibitor MG132 before Cdk1 inhibition. We found that MG132 prevented the induction of DNA synthesis by 1NM-PP1 (compare Fig. 5 F with Fig. 5 B). Furthermore, MG132 suppressed the induction of Mcm loading on chromatin after Cdk1 inhibition (Fig. 5 G, compare lane 5 with lane 6). These results suggest that Cdk1 inhibition is not sufficient to allow origin licensing and endoreplication, unless proteolysis is activated.

Geminin needs to be degraded in human cells to allow origin licensing

To verify these results from DT40 cells in a human cell line, we analyzed Nocodazole-arrested HeLa cells treated with Roscovitine. We found that this Cdk inhibitor initiated origin licensing, as judged by Cdt1 and Mcm2 loading onto chromatin, as previously described by Ballabeni et al. (2004). Inhibition of the proteasome by MG132 abolished the Roscovitine induced chromatin binding of Mcm2 and Cdt1 in HeLa cells (Fig. 6 A, lane 3 and lane 6), confirming our previous results with DT40 cells (Fig. 5, F and G).

The proteolysis dependence of origin licensing and endoreplication induced by Cdk1 inhibition, suggests that proteins other then cyclins need to be degraded to allow pre-RC formation. Geminin, which is an APC/C substrate and a licensing inhibitor (McGarry and Kirschner, 1998; Wohlschlegel et al., 2000), is a good candidate to account for this proteolysis requirement after

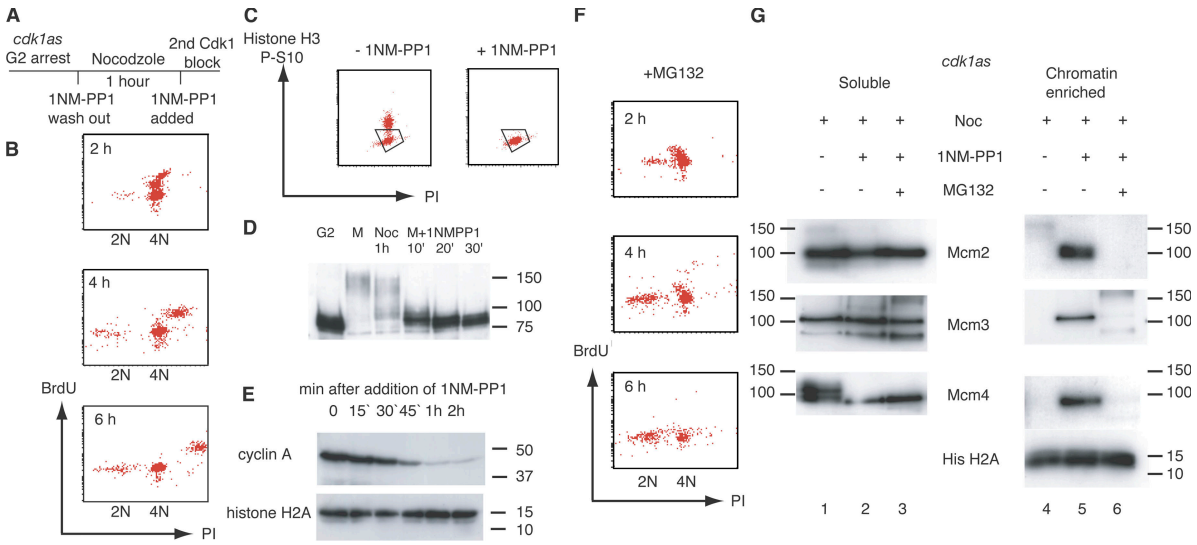

Figure 5. **Inhibition of Cdk1 in mitosis causes endoreplication depending on proteolysis.** (A) Experimental design. After a 7-h exposure to 10 μM 1NM-PP1 we released the cells from a G2 arrest into medium containing Nocodazole. After a 1-h incubation in Nocodazole, Cdk1 was again inhibited by the addition of 10 μM 1NM-PP1. (B) Endoreplication after inhibition of Cdk1 in mitosis. *cdk1as* cells arrested in prometaphase as described in A were incubated with 10 μM 1NM-PP1 and pulse labeled with BrdU at the indicated time points. Dot blot analysis is displayed as in Fig. 1 E. (C) Analysis of Histone H3 phosphorylation in samples treated as described in A. Cells were fixed at 10 min after the second Cdk1 inhibition or without inhibition, and stained with both PI (X-axis, linear scale) and anti phospho-Ser10 Histone H3 antibodies (Y-axis, log scale). (D) Analysis of Cdc27 phosphorylation by immunoblotting in cells synchronized in G2 phase or mitosis as described in A. 15 min after release from the 1NM-PP1 block into mitosis (lane M) Cdc27 shifts upwards due to hyper-phosphorylation by Cdk1. It remains phosphorylated after 1 h in Nocodazole (lane 1, Noc 1h). Following Cdk1 inhibition by addition of 10 μM 1NM-PP1, Cdc27 is rapidly de-phosphorylated and shifts back to its G2 form within minutes (see lane 10′). (E) Immunoblot analysis of cyclin A and Histone H2A levels in Nocodazole arrested *cdk1as* cells at the indicated times after the second Cdk1 block. (F) Endoreplication initiated by the second Cdk1 inhibition is blocked by MG132. The same experiment as in B in the presence of the proteasome inhibitor MG132 is shown. (F) MG132 blocks origin licensing induced by the second Cdk1 inhibition. Chromatin fractionation of *cdk1as* cells arrested in mitosis as shown in A, before (lane 1 and 4) and 2 h after 1NM-PP1 addition (lane 2 and 5). To inhibit the proteasome, cells had been incubated for 1 h with MG132 before the 1NM-PP1 inhibitor was added (lane 3 and 6). Mcm and Histone H2A levels were measured by SDS PAGE and immunoblotting.

Cdk inhibition. Accordingly, we found that Roscovitine triggered the destruction of both cyclin B1 and Geminin (Fig. 6 A, lane 2), whereas MG132 treatment stabilized these proteins (Fig. 6 A, lane 3). Fig. 6 B shows that the APC/C targets Aurora kinase A and Cdc20 are also degraded upon Cdk inhibition during mitosis.

To test if the inhibition of Geminin degradation is sufficient to prevent Roscovitine-induced origin licensing, we investigated the effects of ectopic expression of a Geminin mutant that is resistant to APC/C dependent degradation, on mitotic HeLa cells treated with Roscovitine (Benjamin et al., 2004). We transiently expressed this stable Geminin mutant in HeLa cells, after release from a double thymidine block. As a control experiment, we expressed GFP or an APC/C-resistant mutant of mouse cyclin B1 in HeLa cells treated in the same manner. The transfected cells were arrested in prometaphase by Nocodazole treatment. In the control samples, licensing was initiated by the addition of Roscovitine, as judged by the chromatin loading of Mcms and Cdt1 (Fig. 6 C, lane 8 and lane 10). In contrast, expression of the stable Geminin mutant inhibited loading of Cdt1 and Mcm onto chromatin in response to Roscovitine treatment (Fig. 6 C, lane 12). In fact, we observed that even overexpression of WT Geminin in mitosis partially inhibited Mcm loading (unpublished data). We conclude that Geminin needs to be targeted for degradation by the APC/C, even after Cdk inactivation, to allow for origin licensing and endoreplication in vertebrate cells.

Geminin and proteolysis suppress endoreplication during G2 phase independently of Cdk activity

To clarify the relationship of Geminin and Cdks during G2 phase, we tested if Geminin depletion and Cdk inhibition is sufficient to induce origin licensing and endoreplication in HeLa cells, synchronized in G2 phase as shown in Fig. 7 B. Surprisingly, Roscovitine treatment during the G2 phase did not cause Mcm loading onto chromatin even after Geminin depletion (Fig. 7 A, lane 7). We noticed that in these cells Cdt1 was hardly detectable, suggesting the Cdk inhibition was not sufficient to stabilize this licensing factor during the G2 phase (note that the Cdt1 antibody used in this experiment and in bottom lane of Fig. 7 D, produces a cross-reacting band, marked with an asterisk). Conversely, inhibition of proteolysis by MG132 caused an increase in Cdt1 levels, and resulted in origin licensing, however, only after Geminin depletion. This origin licensing in G2 occurred with similar efficiency with or without Cdk inhibition (compare Fig. 7, lanes 11 and 12).

To test if these prematurely licensed origins were able to trigger re-replication we incubated cells synchronized in G2 with MG132 and/or Roscovitine. After 2 h we removed the inhibitors from the medium and measured the DNA content of the cells after further 12 h of incubation (see experimental outline in Fig. 7 B). The FACS histograms in Fig. 7 C show that Geminin depletion and MG132 treatment but not Cdk inhibition was sufficient to induce endoreplication in the majority of these G2 cells.

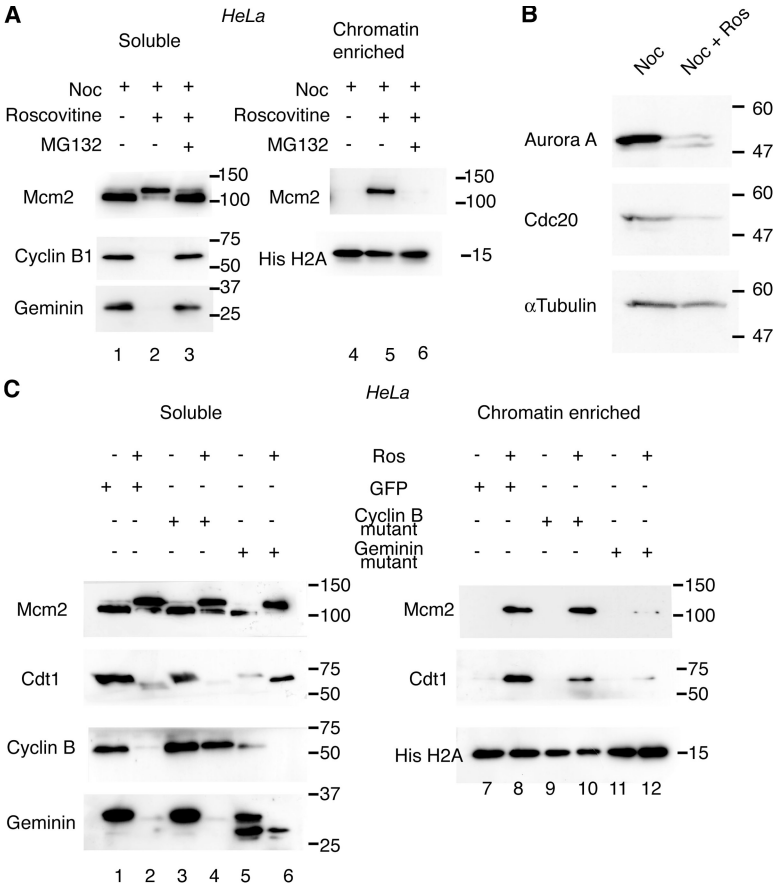

Figure 6. **Causal relationship of Geminin destruction and origin licensing in mitotic HeLa cells after Cdk1 inhibition.** (A) Chromatin fractionation of Nocodazole (Noc) arrested cells with the indicated additions. MG132 was added to mitotic cells after a 12-h incubation in Nocodazole. 50 μM Roscovitine was added 1 h later, and the cells were incubated for an additional 2 h. Shown are the immunoblots of soluble and chromatin fractions with the indicated antisera. (B) Degradation of APC/C substrates Cdc20 and Aurora kinase A after Roscovitine treatment of Nocodazole arrested HeLa cells. Cell lysates before or 2 h after 50 μM Roscovitine treatment were analyzed by immunoblotting with the indicated antibodies. (C) Chromatin fractionation of Nocodazole-arrested HeLa cells after transient expression of the indicated cDNAs. The cells were pre-synchronized by a double thymidine block, and transient transfections of either GFP or the destruction box deficient mutants of cyclin B or Geminin were performed, after release from the second thymidine block. Nocodazole was added 7 h after the release, and samples were taken after 12 h further incubation. The soluble and chromatin fractions were probed by immunoblotting with the indicated antisera. Note the absence of Cdt1 and Mcm3 in the Roscovitine treated samples containing the indestructible Geminin mutant (lane 12).

Our results show that proteolysis has opposing effects on endoreplication during G2 and M phase. One of these differences could to be the stability of licensing factors such as Cdt1. We compared Cdt1 levels in G2 and M phase using two different Cdt1 antibodies. For this purpose, we synchronized HeLa cells by a double thymidine release in G2 phase and inhibited cell cycle progression into M phase by Roscovitine addition, or allowed the cells to proceed into M phase by adding nocodazole. Fig. 7 D shows that Cdt1 levels decreased after Cdk inhibition by Roscovitine, but were markedly increased in the Nocodazole treated cells. These results indicate that Cdt1 levels are kept low during G2 phase independently of Cdk activity, and are rapidly increased after mitotic Cdk1 activation and entry into M phase.

Discussion

A chemical genetics approach to study Cdk1 in vertebrate cells

This study describes the use of chemical genetics to study vertebrate Cdk1. We generated a chicken DT40 cell line, in which we compensated the deleted endogenous Cdk1 with a *cdk1as* mutant cDNA of the *Xenopus laevis* Cdk1 orthologue. The *as*-mutation of the active site has varying effects on different kinases. In the case of Cdk1, the mutation of F80G significantly reduces the activity of the kinase. To overcome this obstacle, we chose two different strategies. First, we used the *Xenopus* cDNA of Cdk1, because we found that this Cdk1 orthologue was relatively resistant to the introduction of the F80G mutation, retaining 20% of the WT kinase activity (see Fig. 1, A and B). Second, we aimed to compensate this fivefold reduction by overexpression of the mutant kinase, and selected a stably transfected clone that showed a fourfold increase of Cdk1 levels when compared to *Xenopus* WAK cells (unpublished data). However, using a PSTAIRE antibody that should cross-react to frog and chicken Cdk with the same affinity, we determined that the levels of the exogenously expressed Cdk1 were approximately the same as the endogenous chicken Cdk1 (Fig. S1 D). Our approach proved to be successful, and we were able to fully reconstitute the loss of the endogenous Cdk1 with the *Xenopus cdk1as* transgene.

Compared to conventional small molecule inhibitors, the advantage of this genetic approach lies primarily in its highly increased specificity, rapid action and reversibility (Shokat and Velleca, 2002). Accordingly, 1NM-PP1 had no discernable effect on *cdk1WT* cells. In *cdk1as* cells, on the other hand, the cellular Cdk1 activity was annulled within 10 minutes after inhibitor addition, as judged by dephosphorylation of Histone H3 and the Cdk1 substrate Cdc27 (Fig. 5, C and D). This study shows that DT40 cells are a useful tool for chemical genetic analysis of vertebrate Cdk1. This cell line is characterized by a very stable karyotype and high gene targeting frequencies, allowing the establishment of double and even triple mutants (Sonoda et al., 2001). The transformed character of the cell line and the use of *Xenopus* Cdk1 might be a potential drawback of

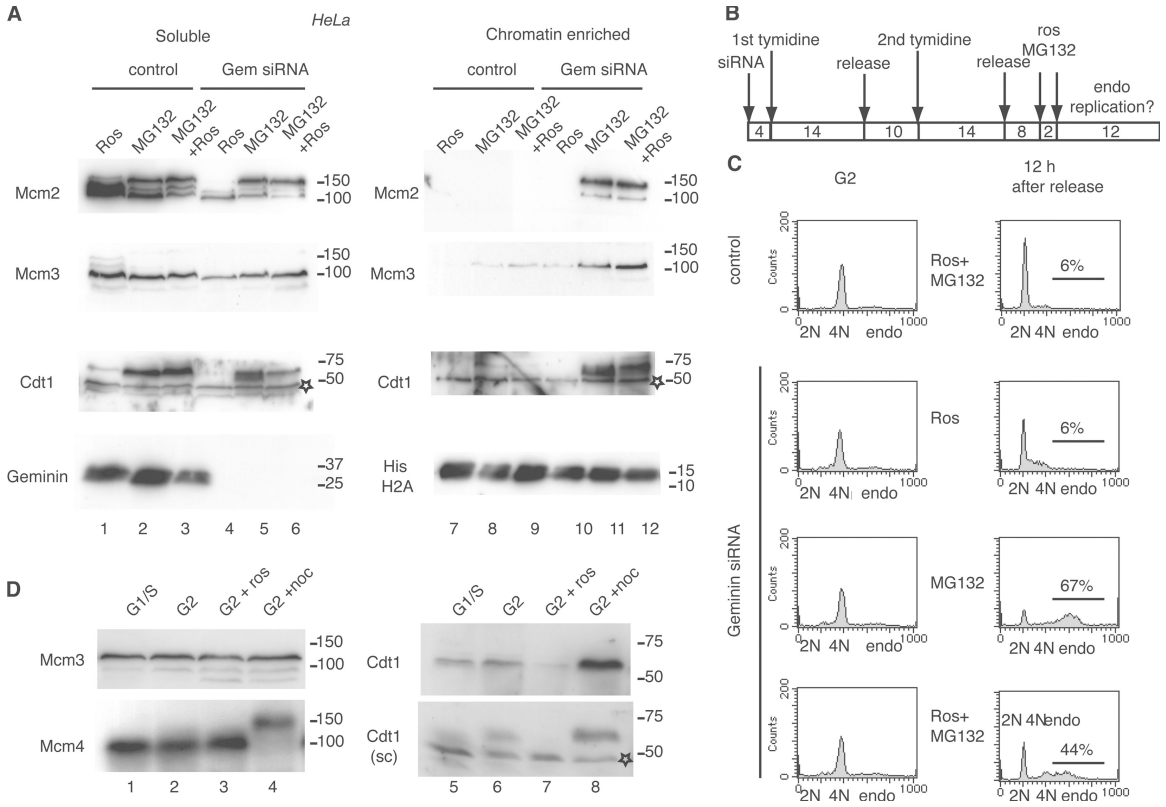

Figure 7. **Mechanisms of endoreplication control during G2 phase.** (A) HeLa cells were transfected with scrambled siRNA (lanes 1–3) or Geminin siRNA (lanes 4–6) and synchronized by a double thymidine block. 8 h after release we added 50 μM Roscovitine (lane 1 and 4), 10 μM MG132 (lane 2 and 5), or both (lane 3 and 6). After a further 2 h of incubation we prepared soluble (left) and chromatin-enriched (right) fractions and analyzed for the presence of the indicated proteins by immunoblotting. (B) Diagram of the synchronization protocol used to induce endoreplication in G2 cells. (C) Left panel: DNA content analysis of the same cells as described in A. Right panel: a part of these cells were washed in excess medium and cultured for a further 12 h, before DNA content analysis. The percentage of cells with DNA content greater then 4N is shown. (D) Cells were synchronized by a double thymidine block (lane 1 and 5), released for 8 h (lane 2 and 6), further incubated for 4 h in 50 μM roscovitine (lane 3 and 7), or Nocodazole (lane 4 and 8). Extracts of these cells were analyzed by immunoblotting with two different Cdt1 antibodies and antibodies raised against Mcm3 and 4.

this approach. However, Cdk functions in cell cycle control are highly conserved among different species and cell lines, and the S phase functions of Cdk1 described here are likely to be of general relevance.

Redundant and specific functions of Cdk/cyclin complexes in the cell cycle

Our phenotypic comparison of *cdk1as* and *cdk1as/cdk2*$^{-/-}$ cells reveals redundant as well as specific roles of vertebrate Cdk1 in the control of DNA replication, centrosome duplication, and mitosis (Fig. 7 A). We show that the deletion of Cdk2 renders both the initiation of DNA replication and centrosome duplication dependent on Cdk1 (Fig. 2 and Fig. 3). This suggests that Cdk1 and Cdk2 share an essential function in the control of S phase. Our findings also suggest that the centrosome cycle and the cell cycle can be uncoupled simply by blocking entry into mitosis, and that either Cdk1 or 2 activity is required to drive this cell cycle–independent centrosome amplification.

In principle, the experiment in Fig. 3 C shows that mid S phase cells do not need Cdk1/2 activity to continue replication. However, the dynamics of S phase completion appear to be changed after Cdk1 inhibition in *cdk1as/cdk2*$^{-/-}$ cells. A detailed analysis of late origin firing and the dynamics of replication elongation will be necessary to determine the roles of Cdk1/2 during ongoing replication.

The functional overlap of Cdk1 and Cdk2 does not include the mitotic functions of Cdk1, which cannot be compensated by Cdk2. We also found that neither Cdk1 nor Cdk2 appear to control the events of early G1 phase such as initiation of de novo cyclin synthesis (Fig. 3 D), which is likely to be triggered by Cdk4/6. Moreover, we cannot exclude at this point that either kinase carries out other specific functions that evade our phenotypic analysis.

It remains to be addressed, which of the different cyclins is primarily responsible for the S phase functions of Cdk1. A-type and B-type cyclins, the predominant binding partners of Cdk1as in the DT40 cells (Fig. S3 E) can both initiate DNA replication (Strausfeld et al., 1996; Moore et al., 2003). Cyclin A is the more likely candidate for this function, because cyclin B/Cdk1 is rapidly exported from the nucleus during S phase (Yang et al., 1998) and kept inactive by the Wee1 kinase (Chow et al., 2003). However, a recent study suggests that cyclin E is also capable of binding and activating Cdk1, especially after deletion of Cdk2 (Aleem et al., 2005). The precise functions of the individual cyclins during S phase need to be addressed in future studies.

Differential control of endoreplication during G2 and M phase

Our study points to fundamentally different mechanisms in the control of endoreplication before and after mitotic Cdk1 activation and entry into M phase. The three main players of licensing control in vertebrate appear to be Geminin and Cdk1 (and possibly Cdk2), as well as proteolysis pathways such as the degradation of Geminin and Cdt1. In the following section we will discuss our findings on the function of these players comparing G2 to M phase. A summary of this discussion is presented in Fig. 8 B.

Geminin inhibits origin licensing independently of Cdks

Our results in Fig. 6 and Fig. 7 show that removal of Geminin is a necessity for origin licensing both in G2 and M phase, and that Geminin can act independently of Cdk activity. This idea is not supported by a previous study (Ballabeni et al., 2004), where Roscovitine treatment does not cause APC/C activation and Geminin degradation in mitotic HeLa cells. In contrast, we observed in consistence with previous studies (Listovsky et al., 2000) that the APC/C was rapidly activated upon mitotic Cdk1 inactivation causing the degradation of Geminin as well as other APC/C substrates (Fig. 6 A). We also confirmed that a degradation resistant Geminin mutant inhibited origin licensing, even after Cdk1 inactivation (Fig. 6 C). The discrepancy between our and Ballabeni et al.'s (2004) results may be due to the twofold lower doses of Roscovitine used in the previous study, which may trigger Geminin degradation only partially.

Effects of Cdk1 inhibition on endoreplication in G2 and M phase

We found that specific inhibition of both Cdk1 and Cdk2 initiated neither origin licensing nor DNA re-replication during G2 phase in DT40 (Fig. 4 B). The same result was obtained from Roscovitine-treated HeLa cells (Fig. 4 D). Paradoxically, Cdk1 inhibition in prometaphase rapidly triggered origin licensing and endoreplication (Fig. 5, B and G, lane 2 and lane 5; and Fig. 6 A). Accordingly, Cdk1 inhibition during G2 phase does not lead to APC/C activation and Geminin destruction, whereas in M phase Cdk1 inactivation results in the rapid removal of Geminin and other APC/C substrates (Fig. 6, A and B). The presence of the APC/C inhibitor Emi1 during G2 but not during M phase (Reimann et al., 2001; Margottin-Goguet et al., 2003) is a likely explanation for this difference in APC/C activity between the two different cell cycle phases. This idea is supported by a recent report by Machida and Dutta (2007), which demonstrates that depletion of the APC/C inhibitor Emi1 is sufficient to induce endoreplication in human cells.

In contrast to our results, the APC/C appears to be activated during G2 phase in Cdk1 depleted HT2-19 human cells and *Drosophila* cells (Hayashi, 1996; Laronne et al., 2003). This could explain the observed endoreplication in these cells after Cdk1 inactivation. The premature APC/C activation in G2 might be an effect of incomplete Cdk1 inactivation, which could allow Emi1 degradation, while not being sufficient to trigger mitosis. Alternatively, Emi1 levels might differ among different cell lines.

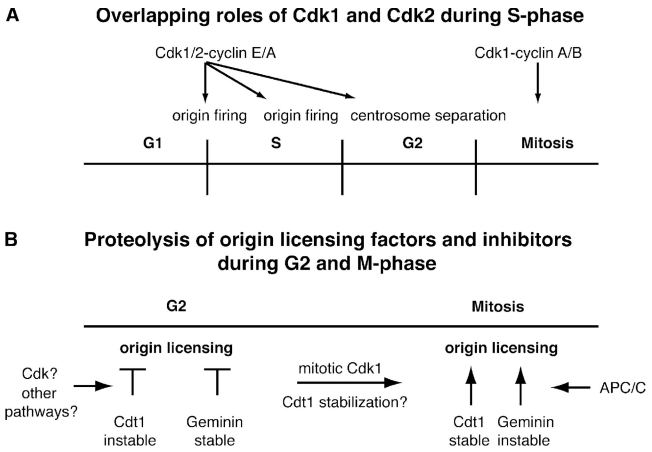

Figure 8. **Model of Cdk1 function in controlling DNA replication in vertebrate cells.**

Licensing control by proteolysis during the G2 phase

When we analyzed the redundant roles of Geminin and Cdks in the control of origin licensing in the G2 phase, we made the surprising observation that Cdk inhibition did not trigger Mcm loading onto chromatin, even after Geminin depletion. This suggests the presence of an additional control mechanism that suppresses endoreplication in the G2 phase. Accordingly, we found that the licensing factor Cdt1 did not accumulate during G2 phase even after Cdk1 inhibition (Fig. 7 A, lane 1; and Fig. 7 D). Conversely, Cdt1 appears to accumulate once cells enter M phase (Fig. 7 D).

Accordingly, we found that transient inhibition of proteolysis stabilized Cdt1, and was sufficient to induce a new round of DNA replication in Geminin-depleted cells. This MG132 induced endo-replication occurred regardless of the state of Cdk activity, suggesting that Geminin and proteolysis are the major control mechanisms that block endoreplication in G2 phase. The question remains what the essential targets of these proteolysis pathways are. Degradation of the licensing factor Cdt1 is an obvious candidate (Zhong et al., 2003), but other players such as orc1 (Mendez et al., 2002), and the degradation of Cdk inhibitors such as p21 and p27 might also be involved (Nakayama et al., 2004). Defining these essential proteolysis targets for licensing control in G2 phase will be an important challenge for future studies.

Collectively, a model emerges in which differential proteolysis of licensing factors and licensing inhibitors control origin licensing during G2 and M phase (Fig. 8 B). In G2 phase the licensing inhibitor Geminin is stable, while the licensing factor Cdt1 is degraded. Once cells enter mitosis Cdt1 is stabilized and Geminin degradation by the APC/C is initiated upon induction of anaphase. In this way multiple mechanisms ensure in human cells that chromosomes are not replicated before the sister chromatids are separated.

Materials and methods

Construction of targeting constructs and vectors

A schematic overview of the *CDC2* targeting construct is shown in Fig. S1 A. The upstream arm was amplified using 5′-AACGCGTAACTAGGACGGCTCCCGAGCAGG-3′ and 5′-GAAACGCACATAGCAAATACCAGTCTCAGG-3′

and cloned into pBS via SacI and BamHI. The downstream arm was amplified using the primers 5′-TCCTAAACTGCTTGTGAAGAAATAAGCAGG-3′ and 5′-CCTGCTTATTTCTTCACAAGCAGTTTAGGA-3′ cloned into pBS via EcoRI and SalI. BSR or Neomycin selection markers were cloned into the BamHI site of the construct. Gene targeting of DT40 cells was performed as described previously (Sonoda et al., 1998). The Neomycin resistance cassette was flanked by two loxP sequences, and was removed from the chicken genome by transient expression of the Cre-recombinase. Neomycin sensitive cells were isolated by subcloning. *Xenopus leavis* Cdk1myc was cloned into pIRES2-EGFP (CLONTECH Laboratories, Inc.) via BamHI–EcoRI. Mutagenesis was performed using a standard PCR protocol. Stable integration of this vector in the DT40 genome was achieved by electroporating 20 μg of the linearized Cdk1 expression vector into DT40 cells, and selection of Neomycin resistant colonies as described previously (Sonoda et al., 1998).

The chicken cDNA for Cdk2 was cloned by RT-PCR using the primers 5′-ATGGAGAACTTTCAAAAGGTGGAGA-3′ and 5′-GGCTGTCCCCCA-CCTGCGCCTGTGA-3′, and the sequence was submitted to the NCBI GenBank (accession number EF182713). A schematic overview of the *CDK2* gene disruption construct is shown in Fig. S3 A. There is no genomic information available for the chicken *CDK2* gene, and we were not able to amplify its genomic sequences by PCR. We screened a chicken genomic DNA pool (provided by RZPD Deutsches Ressourcenzentrum für Genomforschung GmbH) for a *CDK2* gene containing fragment using PCR with chicken *CDK2* cDNA primers 5′-GTCGTGTACAAGGCCCGGAACAAGG-TCACG-3′ and 5′-CGCTGCCTTGGCCGAGATGCGCTTGTTGGG-3′, and analyzed isolated clones by Southern blot analysis with a chicken Cdk2 cDNA probe. We generated the targeting constructs by restriction enzyme digestion of the isolated cosmid clone. A BglII fragment was isolated upstream of exon 2 for the 5′ arm, and an EcoRV NotI fragment between exon 5 and 6 was isolated for the 3′ arm. The fragments were cloned into pCR 2.1-TOPO vector (Invitrogen). Selection cassettes (either His or Puro flanked with two LoxP sequences) were inserted into the construct at EcoRI–EcoRV sites by blunt-end ligation.

The following primers were used to detect Cdk1 and 2 orthologues by RT-PCR: *Xenopus laevis* Cdk1 (5′-ATGAAGAAAATTCGATT-GGAAAACG-3′ and 5′-GATCTCCGAGGAGGACCTGAACTAA-3′), *Gallus* Cdk1 (5′-ATGGAGGATTACACGAAGATAGAGAAGATT-3′ and 5′-CTTCCTGCTAATCTGATTAAGAAATTCTAA-3′), and *Gallus* Cdk2 (5′-TACCTGTTCCAGCTGCTGCAAGGCC-3′ and 5′-GGCTCAAATGCTG-CACTACGATCCC-3′).

Human Geminin was cloned by RT-PCR and the destruction box (amino acid residues 23–30) was deleted as described by Benjamin et al., (2004). Mouse cyclin B1 destruction box mutant (R42A; L45A) in pEVT7 was a gift from Stephan Geley (Medical University Innsbruck, Austria).

Cell culture, transfection, and synchronization

DT40 cells were cultured, and transfected as described previously (Sonoda et al., 1998). Takata et al. (1998) described the synchronization of DT40 cell by elutriation. 1NM-PP1 was obtained from Cellular Genomics and used at the indicated concentrations (1–10 μM). We used a 50-μM concentration of Roscovitine (Calbiochem), and a 5-μM concentration of MG-132 (Calbiochem). Cell cycle analysis of BrdU-pulsed and PI-stained samples was performed on a FACScan (Becton Dickinson) using CellQuest software (Takata et al., (1998). HeLa cells were synchronized in mitosis by incubation in 100 ng/ml Nocodazole, and in S phase by a double thymidine block. In brief, cells were incubated for 14 h in 2 mM thymidine, washed in PBS, released for 10 h, and then blocked for another 14 h in 2 mM thymidine. Transient transfection of HeLa cells by Lipofectamine (Invitrogen) was performed following the second thymidine block as described in the manufacturer's protocol. To target Geminin we transfected a QIAGEN-validated siRNA SI02653805 at a final concentration of 100 nM by oligofectamine (Invitrogen), following the manufacturer's instructions.

Preparation of total cell extracts and chromatin fractionation

For total cell extraction, 10^6 DT40 cells or 10^5 HeLa cells were lysed in 10 μl ECB buffer (50 mM Tris, pH 7.5, 120 mM NaCl, 0.5% NP-40, and 1 mM EDTA) containing 1 mM DTT and a 1:100 dilution of a protease inhibitor cocktail (Nakalai Tesque 25955-11). The extracts were incubated for 20 min on ice, sonicated, and suspended in 2× Laemmli buffer. For chromatin fractionation, we separated soluble and insoluble fractions as described by Ballabeni et al. (2004). 10^6 DT40 or 10^5 HeLa cells were first lysed for 20 min in 10 μl CSK buffer containing DTT and protease inhibitors as above. Following centrifugation, the supernatant was mixed with 2× Laemmili buffer, and the pellet was washed and resuspended in 10 μl CSK buffer, sonicated and suspended in 2× Laemmli buffer.

Immunoblotting, Immunoprecipitation, immunofluorescence, and Histone H1 kinase assays

Immunoblotting and immunoprecipitation, and histone H1 kinase assays were done as described earlier (Sonoda et al., 1998; Chow et al., 2003). Immunofluorescence of cyclin B was performed with formaldehyde fixed samples that were spun on a glass slide by centrifugation in a Cytospin centrifuge. α- and γ-tubulin immunofluorescence was performed as described earlier (Yamaguchi-Iwai et al., 1999).

Image acquisition and manipulation

All images were taken with an Olympus BX61 microscope, equipped with a Photometrics CoolSnap HQ camera, and Olympus Uplan/APO 100× lens (NA 1.35). Samples were mounted in Vectashield mounting medium with DAPI (Vector Laboratories, Inc.) and analyzed at room temperature. Image acquisition was performed using MetaMorph software.

Antibodies

Antibodies used in this study comprised the anti Cdk1 mouse monoclonal A17 antibody (Gannon et al., 1998), rabbit polyclonal anti Cdk1 antibody from Upstate Biotechnologies, rabbit polyclonal anti Cdk2 antibody from Abcam (ab-7954-1), anti-PSTAIRE (Poon et al., 1997), and rabbit polyclonal anti-phosphotyrosine15 Cdk1 antibody from Cell Signaling (Nr. 9111S). (06-923). Anti-chicken cyclin A (Maridor et al., 1993) and anti-chicken cyclin B2 (Gallant and Nigg, 1992) antibodies were gifts from E. Nigg's laboratory (MPI of Biochemistry, Munich, Germany); monoclonal anti-α-tubulin FITC conjugate (No. F2168), and γ-tubulin polyclonal (No. T3559) antibodies were obtained from Sigma-Aldrich; Mcm2, 3, 4, and Histone H2A polyclonal antibodies were obtained from Abcam (ab 4461-50, 4460-50, 3728-50, 13923-100, respectively); and anti–human Cdt1 antibody was a gift from H. Nishitani (Kyushu University, Fukuoka, Japan; Nishitani et al., 2001). The Cdt1 antibody used in Fig. 7 was purchased from Santa Cruz Biotechnology, Inc. (sc-28262). Aurora kinase A rabbit polyclonal antibodies were purchased from Abcam (ab12875), and to detect Cdc20 we used a mixture of two monoclonal anti-cdc20 antibodies from Santa Cruz Biotechnology, Inc. (SC1907 and SC1906). Human cyclin B1 monoclonal V152 antibodies (ab-72) and rabbit polyclonal anti-Geminin antibodies (ab-12147-50) were obtained from Abcam. For FACS analysis, ethanol-fixed cells were stained with anti-BrdU monoclonal antibody (BD Biosciences; Nr. 555627) and anti-phospho Ser10 rabbit polyclonal Histone H3 antibody (Upstate Biotechnology; Nr. 06-570). Alexa-labeled secondary antibodies were purchased from Molecular Probes, and HRP-labeled secondary antibodies were from Santa Cruz Biotechnology. Cdc27 was detected by a monoclonal antibody from Abcam (ab10538).

Online supplemental material

Fig. S1 shows generation of *cdk1as* cells. Fig. S2 shows synchronous mitosis after release from G2 arrest. Fig. S3 shows gene targeting of CDK2 in *cdk1as* cells. Fig. S4 shows G1 arrest in *cdk1as/cdk2*$^{-/-}$ cells.

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