Differentiation of trophoblast stem cells into giant cells is triggered by p57/Kip2 inhibition of CDK1 activity

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Genome endoreduplication during mammalian development is a rare event for which the mechanism is unknown. It first appears when fibroblast growth factor 4 (FGF4) deprivation induces differentiation of trophoblast stem (TS) cells into the nonproliferating trophoblast giant (TG) cells required for embryo implantation. Here we show that RO3306 inhibition of cyclin-dependent protein kinase 1 (CDK1), the enzyme required to enter mitosis, induced differentiation of TS cells into TG cells. In contrast, RO3306 induced abortive endoreduplication and apoptosis in embryonic stem cells, revealing that inactivation of CDK1 triggers endoreduplication only in cells programmed to differentiate into polyploid cells. Similarly, FGF4 deprivation resulted in CDK1 inhibition by overexpressing two CDK-specific inhibitors, p57/KIP2 and p21/CIP1. TS cell mutants revealed that p57 was required to trigger endoreduplication by inhibiting CDK1, while p21 suppressed expression of the checkpoint protein kinase CHK1, thereby preventing induction of apoptosis. Furthermore, *Cdk2*^{-/-} TS cells revealed that CDK2 is required for endoreduplication when CDK1 is inhibited. Expression of p57 in TG cells was restricted to G-phase nuclei to allow CDK activation of S phase. Thus, endoreduplication in TS cells is triggered by p57 inhibition of CDK1 with concomitant suppression of the DNA damage response by p21.

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The concept that nuclear genomes are duplicated once and only once each time a cell divides has become an established principle of the eukaryotic mitotic cell cycle (DePamphilis et al. 2006; Schwob and Labib 2006). Genome duplication is regulated through multiple convergent mechanisms. When these mechanisms are subverted, DNA rereplication occurs during S phase to produce abnormal replication structures and broken DNA molecules. This triggers a DNA damage response that arrests cell proliferation and eventually induces apoptosis. Nevertheless, developmentally programmed endoreduplication or “endocycles” (multiple rounds of genome duplication in the absence of an intervening mitosis) occur at specific times in the life cycle of protozoa, flies, mammals, and plants to produce terminally differentiated cells that remain physiologically active but nonproliferating for long periods of time (Lilly and Duronio 2005; Calvi 2006; Inze and De Veylder 2006). Thus, en-

docycles provide a mechanism by which cells can increase the number of gene copies at the expense of further cell proliferation, but how they switch from mitotic cycles to endocycles remains a mystery.

Initiation of DNA replication during mitotic cell cycles requires assembly of prereplication complexes (pre-RCs) at replication origins during the M-to-G1-phase transition (DePamphilis et al. 2006; Sivaprasad et al. 2006). Pre-RC assembly in metazoan cells requires the absence of both cyclin-dependent protein kinase (CDK) activity and geminin, a metazoan-specific protein that prevents loading of the replicative DNA helicase onto DNA. DNA synthesis (S phase), however, requires high CDK activity to initiate the process, and both CDK activity and geminin to prevent rereplication by modifying pre-RC proteins and blocking their interactions. In this way, replication origins are activated only once during a single cell division cycle. When S phase is completed, cells activate CDK1, the enzyme required for initiation of mitosis, by nuclear localization of mitotic cyclins A and B (Pesin and Orr-Weaver 2008). Once the metaphase spindle forms, the anaphase-promoting com-

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plex (APC) degrades both the mitotic cyclins and geminin, thus allowing cells to exit mitosis and begin pre-RC assembly. Therefore, the transition from mitotic cell cycles to endocycles must somehow prevent cells from entering mitosis while simultaneously allowing them to assemble pre-RCs and initiate DNA replication. In other words, endocycles represent a G-to-S-to-G-phase cycle with no cell division.

Developmentally programmed endoreduplication in mammals is a rare event. It occurs only twice; first when trophoblast stem (TS) cells differentiate into the trophoblast giant (TG) cells required for implantation and subsequent placentation (Cross 2005), and second when bone marrow megakaryoblasts differentiate into megakaryocytes (Ravid et al. 2002). Efforts to determine whether or not one or more CDK activities prevent endoreduplication in mammals have been inconclusive. First, none of the experiments have been carried out with cells that are programmed to endoreduplicate. Second, inhibition of both CDK1 and CDK2 activities in metaphase-arrested mammalian cells causes rapid reassembly of pre-RCs but not DNA synthesis, because CDK activity is required to initiate DNA replication (Ballabeni et al. 2004; Li et al. 2004). However, when CDK1 activity is inhibited selectively, endoreduplication is induced in some cell lines (Itzhaki et al. 1997; Vassilev et al. 2006; Hochegger et al. 2007), but not in others (Riabowol et al. 1989; Furukawa et al. 1990; Hamaguchi et al. 1992). Furthermore, when endoreduplication does occur, it occurs only after cells have activated CDK1 and entered mitosis (Vassilev et al. 2006; Hochegger et al. 2007).

Other evidence suggests that down-regulation of geminin triggers endoreduplication. When maternally inherited geminin is exhausted at the four- to eight-cell stage in mouse development, geminin nullizygous embryos undergo extensive DNA replication with concomitant formation of giant cells and expression of at least some genes characteristic of trophoblast (Gonzalez et al. 2006; Hara et al. 2006). However, these geminin-depleted embryos also contain damaged DNA and undergo apoptosis, suggesting that they have undergone DNA rereplication during S phase rather than endoreduplication of their genome. For example, siRNA depletion of geminin in either human cancer cells or *Drosophila* cells in culture induces random rereplication of DNA with formation of giant cells, thereby triggering the ATR, CHK1 DNA damage signaling pathway (Mihaylov et al. 2002; Melixetian et al. 2004; Zhu et al. 2004; Tachibana et al. 2005; Zhu and Dutta 2006). These cells arrest in G2 phase and soon die. In fact, geminin levels oscillate during endocycles in *Drosophila* (Zielke et al. 2008). Thus, the primary role of geminin appears to be suppression of rereplication during S phase.

To resolve these issues, we set out to determine whether or not CDK activity regulates the transition from mitotic cell cycles to endocycles when TS cells differentiate into TG cells. Mouse blastocysts consist of the trophoblast and the inner cell mass. TS cells arise from the trophoblast and differentiate exclusively into cells that comprise the placenta. Embryonic stem

(ES) cells are derived from the inner cell mass and differentiate into all of the cells that comprise the embryo. Here we show that selective inhibition of CDK1 activity, either by a chemical inhibitor called RO3306, or by developmentally programmed induction of the CDK-specific inhibitor p57 causes TS cells to endoreduplicate and differentiate into TG cells. Multiple rounds of genome duplication are then sustained by CDK2 and oscillating levels of p57. Induction of the CDK-specific inhibitor p21 serves to prevent apoptosis in cells undergoing multiple rounds of endoreduplication. These results not only identify the critical steps in programmed endoreduplication in mammalian cells, but account for the fact that loss of p57 results in placentomegaly.

Results

Inhibition of CDK1 activity induced endocycles in TS cells, but not in ES cells

TS cells in blastocysts are induced to differentiate into TG cells when deprived of fibroblast growth factor-4 (FGF4), a phenomenon that can be recapitulated in vitro (Simmons and Cross 2005). In the presence of FGF4 and medium conditioned by primary embryonic fibroblasts, TS cells proliferate in vitro to form tightly packed colonies (Fig. 1, 0 d). When FGF4 and conditioned medium are replaced with normal culture medium ("FGF4 deprivation"), TS cells spontaneously differentiate into TG cells, an event characterized by increased cell size, genome endoreduplication, and expression of specific genes. Therefore, to determine whether or not TG cell formation can be triggered by selective inhibition of CDK1 activity, TS cells were treated with RO3306, an

Figure 1. Selective inhibition of CDK1 activity induced differentiation of TS cells to TG cells. TS cells and ES cells were cultured in the presence of the CDK1 inhibitor RO3306 for the indicated times (days) and photographed at 10 $\times$ magnification.

ATP competitor that selectively inhibits CDK1 (Vassilev et al. 2006; Hochegger et al. 2007).

Within 24 h, RO3306-treated TS cells developed a giant cell morphology with an enlarged nucleus (Fig. 1, 1 d), reaching a maximum size after 3–6 d in culture (Fig. 1, 3 d). The morphological appearance of TS cells treated with RO3306 was indistinguishable from that of TS cells deprived of FGF4 for the same length of time (data not shown). Treatment of TS cells with RO3306 also induced transcription of genes characteristic of TG cells (Supplemental Fig. S1) that have been shown to be activated by FGF4-deprivation (Simmons and Cross 2005). Expression of genes characteristic of TS cells, however, was not suppressed by RO3306.

RO3306 treatment of ES cells also induced formation of large cells within 24 h, but in contrast to TS cells, RO3306 treated ES cells detached from the dish within 3 d (Fig. 1, 3 d) and contained <2N DNA (Fig. 2C), two characteristics of apoptosis. A third characteristic of apoptosis, the translocation of phosphatidylserine from the inner to the outer leaflet of the plasma membrane, can be detected by the calcium-dependent binding of annexin-V to living cells (Vermes et al. 1995). Annexin V staining detected apoptosis in ES cells treated with RO3306 as early as 24 h, but not in TS cells (data not shown). Primary mouse embryonic fibroblasts also ceased proliferation in the presence of RO3306, but they did not endoreduplicate their genome (data not shown).

To determine whether or not selective inhibition of CDK1 activity induced endoreduplication, TS cells were analyzed by fluorescence-activated cell sorting (FACS) following either FGF4 deprivation (Fig. 2A) or addition of RO3306 (Fig. 2B). Both procedures induced up to four endocycles, resulting in distinct cell populations with a DNA content of 8N, 16N, 32N, or 64N that amounted to the fourfold to 32-fold increase in nuclear DNA characteristic of primary TG cells in the placenta (Zybina and Zybina 2005). Endoreduplication was evident within 1 d of FGF4 deprivation, and no significant increase in the ploidy levels of TG cells was observed beyond 6 d. At this time, TG cells were at least 10 times the size of TS cells (Fig. 2E). Although the FACS profiles were ultimately indistinguishable, endoreduplication was induced more effectively with RO3306 than with FGF4 deprivation (Fig. 2D). Within 8 h of adding RO3306, TS cells accumulated in G2/M phase, as expected for selective inhibition of CDK1 activity. Endocycles appeared between 8 and 12 h, and by 3 d, ~70% of the TS cells had undergone up to four endocycles. The remaining cells underwent apoptosis, as evidenced by a decrease in DNA content and cell adhesion. Propagation of endocycles required continual suppression of CDK1 activity, since removal of RO3306 during the first 24 h permitted TS cells that had not yet entered the endocycle to continue with normal cell division (data not shown).

As with TS cells, treatment of ES cells for 8 h with RO3306-arrested cell division in G2 phase of the cell cycle (4N peaks in Fig. 2B,C). Endoreduplication (>4N peaks) appeared within 24 h, coincident with the appearance of large cells (Fig. 1). Within 48 h, however, most of

Figure 2. Selective inhibition of CDK1 activity mimicked FGF4 deprivation induced endoreduplication in TS cells, but not in ES cells. TS cells endoreduplicated their genome either upon FGF4 deprivation (A), or addition of RO3306 to the culture medium (B), as evidence by FACS analysis at the times (days) indicated. 2N DNA content indicates cells in G1 phase. 4N indicates cells in either G2 or M phase. Endocycles (>4N) were detected as peaks in the FACS profiles that represented cells with integral multiples of diploid (2N) DNA content. (C) Addition of RO3306 to ES cells at first induced endocycles (1 d), and then apoptosis (days 2 and 3), as evidenced by the appearance of cells with <2N DNA content in the FACS profile. Treatment of either TS or ES cells with DMSO (the solvent used to dissolve RO3306) had no effect (data not shown). The positions of diploid (2N) and tetraploid (4N) cells are indicated. (D) To quantify the data in A–C, the percentage of cells with >4N DNA content was plotted as a function of time either after FGF4 deprivation or treatment with RO3306. (E) Examples of TS cells and TG cells (6 d of FGF4 deprivation) stained with the TS-specific antibodies TROMA-1 (anti-cytokeratin EndoA: blue). Nuclei were stained with propidium iodide (red).

the ES cells were no longer attached to the dish, and contained <2 N DNA (Fig. 2C), characteristic of apoptotic cells. Thus, selective inhibition of CDK1 activity in cells programmed to endoreduplicate results in endocycles and the formation of stable nonproliferating giant cells, whereas the same treatment triggers abortive endoreduplication and apoptosis in cells that are not developmentally programmed to endoreduplicate (Fig. 2D).

Induction of endocycles in TS cells resulted from selective inhibition of CDK1

To determine whether or not RO3306 induction of endocycles was a consequence either of preventing mitosis or of inhibiting CDK1 activity, TS cells were incubated for 8 h with nocodazole, a specific inhibitor of microtubule assembly that arrest cells at metaphase. Mitotic cells were then collected and cultured in the presence of nocodazole and either in the presence or absence of RO3306 (Fig. 3A [+Noc]). Most of the nocodazole-arrested TS cells contained 4N DNA and about 80% exhibited metaphase morphology. In the absence of RO3306, metaphase-arrested cells did not endoreduplicate their genome but underwent apoptosis, as evidenced by the accumulation of cells with <2N DNA. In contrast, RO3306 induced endoreduplication in M-phase-arrested cells, as evidenced by accumulation of cells with either 8N or 16N DNA content (Fig. 3A [+Noc, +RO]). Therefore, endoreduplication in TS cells is not triggered by arresting TS cells in mitosis, but by selectively inhibiting CDK1 activity.

To confirm this conclusion, TS cells were treated with roscovitine, a CDK-specific inhibitor that acts on both CDK1 and CDK2 (Knockaert et al. 2002). Either CDK2 or CDK1 activity is required to initiate S phase in metazoan cells (Pacek et al. 2004; Aleem et al. 2005; Berthet and Kaldis 2006; Hochegger et al. 2007; Santamaria et al. 2007). Addition of roscovitine instead of RO3306 blocked both TS cell proliferation and endoreduplication

(Fig. 3B [+Ros]). Therefore, only selective inhibition of CDK1 activity triggers endoreduplication in TS cells. These results also demonstrate that, under the conditions used for TS cells, RO3306 inhibits CDK1 specifically. If RO3306 had inhibited both CDK1 and CDK2, then initiation of DNA replication would not have occurred; a conclusion subsequently confirmed by analysis of *Cdk2*^{-/-} TS cells (described below).

To determine whether or not the response of TS cells to RO3306 was cell cycle-sensitive, TS cells were synchronized near their G1/S interphase by suppressing DNA synthesis with excess thymidine, and then released in the presence of RO3306. Within 8 h, cells accumulated at their G2/M boundary, consistent with selective inhibition of CDK1, and then began to endoreduplicate their genome (data not shown), as observed with asynchronous cells (Fig. 2B). Thus, TS cells initiate endoreduplication by inhibiting CDK1 activity, not by arresting cells at a particular stage in their cell cycle.

In the absence of CDK1 activity, endoreduplication required CDK2

CDK2 is the CDK activity that normally drives S phase in metazoan cells. However, CDK1 can execute this function in mammals when the *Cdk2* gene is inactivated (Berthet et al. 2003; Ortega et al. 2003), particularly in preimplantation embryos (Santamaria et al. 2007). Therefore, to determine whether or not CDK2 is required to initiate multiple S phases when CDK1 activity is inhibited by RO3306, TS cells were derived from a *Cdk2*^{-/-} blastocyst. These TS cells expressed CDK1, but not CDK2 (Fig. 6C, below). As with wild-type TS cells, FGF4 deprivation induced *Cdk2*^{-/-} TS cells to differentiate into TG cells, although at a slower rate (Fig. 4A). This was consistent with hypothesis that CDK1 can substitute for CDK2. However, unlike wild-type TS cells, *Cdk2*^{-/-} TS cells did not endoreduplicate in response to RO3306 (Fig. 4B). Therefore, CDK2 is required for endoreduplication when CDK1 activity is suppressed continuously. These data confirm reports that only CDK2 and CDK1 can drive S phase in mammalian cells, and extend them to include endocycles. In addition, these results further confirm that RO3306 selectively inhibits CDK1 under the conditions employed in these experiments. Had RO3306 inhibited both CDK1 and CDK2, then endoreduplication could not have occurred.

Figure 3. Endoreduplication in TS cells was triggered by selective inhibition of CDK1, not by arresting cells in mitosis. (A) TS cell cultures were washed free of unattached cells and then cultured in the presence of 200 nM nocodazole (Sigma, M1404) for 8 h. Mitotic cells were released into the medium by sharp tapping of the dishes and recovered by centrifugation. One portion of the mitotic cells was again cultured in TS-cell medium supplemented with nocodazole while the other portion was cultured in the presence of both nocodazole and RO3306. Total cells were harvested on the indicated day, stained with propidium iodide, and analyzed by FACS to determine the ploidy levels. (B) TS cells were treated with 20 μ M roscovitine (Sigma, R7772) for 3 d before FACS analysis.

FGF4 deprivation triggered inhibition of CDK1 activity and induction of CDK-specific inhibitors p57 and p21

The results described above demonstrate that selective inhibition of CDK1 activity in TS cells can trigger endoreduplication with concomitant differentiation into TG cells. To determine whether or not this mechanism applies to natural differentiation of TS cells into TG cells, CDK1 protein was immunoprecipitated from lysates of FGF4-deprived TS cells and assayed for protein kinase

Figure 4. Endoreduplication required CDK2. (A) Wild-type and *Cdk2*^{-/-} TS cells were subjected to FGF4 deprivation for up to 6 d, during which time both cell lines differentiated into TG cells. The percentage of cells with >4N DNA content were determined by FACS analysis, and the percentage of cells with >4N DNA content was plotted as a function of time. (B) *Cdk2*^{-/-} and wild-type (wt) TS cells were cultured in TS medium containing FGF4 and RO3306 for 3 d before subjecting them to FACS analysis (shaded profiles). As a control, cells were cultured in TS medium containing FGF4 and DMSO for 3 d (solid line, open space).

activity. The results revealed a rapid decrease in CDK1 activity during the first 12 h of FGF4 deprivation (Fig. 5A). To determine what proteins may have accounted for this rapid loss of CDK1 activity upon FGF4 deprivation, cell lysates also were subjected to Western immunoblotting to detect changes in the levels of proteins known to regulate CDK activities (Fig. 5B). CDK1 protein levels fluctuated slightly within the first 48 h and then diminished continuously until the protein was barely detectable by 4 d of FGF4 deprivation (Fig. 5B; data not shown), suggesting that *Cdk1* gene activity is eventually silenced. The level of CDK2, on the other hand, showed a modest increase during the first 24 h that was maintained for several days, consistent with a requirement for CDK2 activity in endoreduplication. Surprisingly, the level of cyclin B1 (CCNB1), a CDK1 activator required for driving cells into mitosis, remained unchanged. In contrast, the levels of cyclin A2 (CCNA2) and cyclin E1 (CCNE1) decreased markedly after 2 d. The level of geminin, a specific inhibitor of pre-RC assembly, remained essentially unchanged during the first 3 d of FGF4 deprivation, and therefore did not appear to be involved in the mitotic-to-endocycle transition.

Two proteins that did appear to be involved in triggering endoreduplication in TS cells were the CDK-specific inhibitors CDKN1A/p21/CIP1 (p21) and CDKN1C/p57/

KIP2 (p57). The levels of these two proteins increased as much as 30-fold during the first 24 h of FGF4 deprivation when endoreduplication began (Fig. 5B). This was consistent with the report that p57 RNA is expressed specifically in the TG cells of mouse placenta, but not in their precursors in the ectoplacental cone (Hattori et al. 2000). In contrast, the level of the CDK-specific inhibitor CDKN1B/p27/KIP1 (p27), a regulator of the G1-to-S-phase transition in metazoan mitotic cell cycles, remained relatively constant. Immunoprecipitation of either p21 or p57 from cell lysates confirmed the absence of p21-CDK1 and p57-CDK1 complexes in TS cells and their presence in TG cells (Fig. 5C). Furthermore, p27-CDK1 complexes were not detected under these conditions. Thus, FGF4 deprivation resulted in rapid expres-

Figure 5. FGF4 deprivation rapidly suppressed CDK1 activity in TS cells with concomitant expression of CDK-specific inhibitors p21 and p57. (A) TS cells were differentiated into TG cells by FGF4 deprivation for the indicated times. Cell lysates were prepared, CDK1 protein was immunoprecipitated (IP), and the immunoprecipitate was assayed for its ability to phosphorylate histone H1. Coomassie-stained histone H1 protein used for the kinase assay is shown at the bottom. (B) TS cells were differentiated into TG cells by FGF4 deprivation (-FGF4), and total cell lysates were subjected to Western immunoblotting analysis for the indicated protein. "+FGF4, +RO" indicates TS cells that were cultured for 3 d in the presence of RO3306. (C) p21 p27 and p57 were independently immunoprecipitated from lysates of TS cells and of TG cells 24 h after removal of FGF4. CDK1 was detected by Western immunoblotting analysis. (D) Results in these and other experiments were quantified plotted as a function of time that TS cells were deprived of FGF4. (●) Relative change in CDK1 activity; (□) relative change in p57 protein level; (○) relative change in p21 protein level; (◆) fraction of cells with 4N DNA content or greater.

sion of CDK-specific inhibitors p21 and p57 with concomitant appearance of p21-CDK1 and p57-CDK1 protein complexes, inhibition of CDK1 protein kinase activity, and arrest of cells in G2/M phase (4N DNA content) (Fig. 5D). These events occurred in parallel with the onset of endocycles as TS cells differentiated into TG cells (Fig. 2D).

To identify changes in protein levels that may have resulted from CDK1 inhibition, lysates from RO3306 treated TS cells also were subjected to Western immunoblotting. RO3306 did not alter the level of CDK1, CDK2, cyclins A2, B1, and E1, or the CDK inhibitors p27 and p57, or geminin when compared with their level in untreated TS cells (Fig. 5B, cf. +FGF4 +RO and -FGF4, 0 d). However, RO3306 did induce expression of p21. Taken together, the results presented in Figure 5 suggest that induction of p57 is responsible for the rapid inhibition of CDK1 activity, and that induction of p21 gene expression is a consequence of CDK1 inhibition. No correlation was detected between the level of geminin protein and the transition from mitotic to endocycles.

p57 was required for endoreduplication

Western immunoblotting (Fig. 5B) and immunostaining (Fig. 8A,B, below; data not shown) revealed dramatic increases in the levels of both p57 and p21 proteins when TS cells were deprived of FGF4, suggesting that one or both of these CDK-specific inhibitors is responsible for triggering endoreduplication. To investigate this issue, *p57*^{-/-} and *p21*^{-/-} TS cell lines were derived from blastocysts nullizygous for the indicated gene and then examined for their response to FGF4 deprivation. FGF4-deprived *p57*^{-/-} TS cells did not endoreduplicate their genome. By 6 d of FGF4 deprivation, the fraction of *p57*^{-/-} cells with a DNA content >4N had increased slightly, but distinct populations of cells with ≥4N DNA were not evident (Fig. 6A,B). Nevertheless, *p57*^{-/-} TS cells were capable of endoreduplication, as evidenced by their response to RO3306 (Fig. 6D). Therefore, endoreduplication in response to FGF4 deprivation requires expression of p57.

FGF4 deprived *p57*^{-/-} TS cells not only failed to endoreduplicate their genome, but they continued to proliferate for several generations, whereas wild-type TS cells did not (Fig. 7A). FGF4 deprived *p57*^{-/-} TS cells continued to proliferate until they began to produce multinucleated giant cells around day 3 that failed to undergo cytokinesis (Fig. 7B,D). *p57*^{-/-} TG cells expressed the same genetic markers detected in wild-type TG cells (Supplemental Fig. S1), revealing that these events did not require p57. In fact, after they stopped proliferating, the fraction of multinuclear *p57*^{-/-} TS cells increased dramatically relative to wild-type cells (Fig. 7B). Most of these cells appeared as giant cells containing a tightly packed cluster of two to four nuclei (Fig. 7D) that appeared in FACS profiles as a peak of cells with 4N to 8N DNA content (Fig. 7C). Giant cells containing a tightly packed cluster of five to as many as 35 nuclei were also present after 8–10 d of culture (Fig. 7E,F). These results

Figure 6. p57 was required for endoreduplication during TS cell differentiation. (A) *p21*^{-/-} and *p57*^{-/-} TS cells were differentiated into TG cells by FGF4 deprivation for the indicated time (days), and subjected to FACS analysis. (B) The fraction of cells with >4N DNA content was determined from the data in A and Figure 2A (wt) as a function of time. (C) *Cdk2*^{-/-}, *p21*^{-/-}, and *p57*^{-/-} TS cells were differentiated into TG cells by FGF4 deprivation for the time indicated (days). Total cell lysates were prepared and analyzed for the proteins indicated by Western immunoblotting analysis. (D) *p21*^{-/-}, *p57*^{-/-}, and wild-type TS cells were cultured for 3 d in the presence of RO3306 and then analyzed by FACS (shaded profiles). The same cells were also cultured in parallel with DMSO instead of RO3306 (solid line, open profile).

confirm that expression of p57 prevents TG cells from undergoing mitosis, consistent with the role of p57 as an inhibitor of CDK1 activity.

p57 protein levels oscillated in TG cells

The simple fact that endoreduplication could not occur in the absence of CDK2 when CDK1 activity was inhibited continuously by RO3306 revealed that p57 activity must oscillate in *Cdk2*^{-/-} TS cells deprived of FGF4 so that CDK1 can drive S phase. Presumably, the same is also true in wild-type cells, because p57 is a potent inhibitor of both CDK1 and CDK2. Therefore, to determine whether or not p57 levels oscillated in TG cells, both TS and TG cells were stained with anti-p57 antibody (Fig. 8A,B). Consistent with the results of Western immunoblotting (Fig. 5B), p57 was not detected in TS cells but was detected in the nuclei of TG cells. However, p57 was present at high concentration in only 25% of the TG cells (Fig. 8A). Moreover, p57 was not present

Figure 7. p57 blocked mitosis in TG cells. (A) Equal numbers of wild-type (wt) and *p57*^{-/-} TS cells were differentiated into TG cells by FGF4 deprivation for the times indicated. Cells were then harvested and counted in triplicate. (B) Fraction of wild-type, *p21*^{-/-}, and *p57*^{-/-} cells with two or more nuclei present at the times indicated. Several fields of 100 cells were scored for each time point. (C) FACS analyses of *p57*^{-/-} TS cells before (solid line, open space) and at 10 d after FGF4 deprivation (shaded profile). Phase contrast images of *p57*^{-/-} TS cells at 3 d (D) and 10 d (E,F) of FGF4 deprivation.

in S-phase TG cells (those whose nuclei incorporated BrdU), but was present in TG cells that were not undergoing DNA replication (Fig. 8B). Similar results were obtained with both wild-type and *Cdk2*^{-/-} TS cells. Therefore, p57 protein levels in TG cells do, in fact, oscillate between high levels during Gap phase and low levels during S phase. This conclusion is consistent with a previous report that p57 protein accumulates at the end of S phase and then disappears before the onset of the next S phase in synchronized Rcho-1 cells, a rat choriocarcinoma cell line that exhibits characteristics of TS cells (Hattori et al. 2000). Cell cycle-dependent changes in p57 protein activity occur at the level of either RNA translation or protein stability, because p57 mRNA levels remained constant in either TS or TG cells (data not shown) as well as in Rcho-1 cells (Hattori et al. 2000).

p21 was not required for endoreduplication

As with wild-type TS cells, the level of p21 increased dramatically in *p57*^{-/-} cells while the levels of CDK2, cyclins A2, B1, and E1, as well as p27, remained constant (Fig. 6C). Therefore, p21 alone was not sufficient to induce endoreduplication. Moreover, FGF4-deprived *p21*^{-/-} TS cells also formed giant cells and expressed the same

genes detected in wild-type TG cells (Supplemental Fig. S1), revealing that these events do not require p21. As with *p57*^{-/-} and wild-type TS cells, RO3306 induced endoreduplication in *p21*^{-/-} TS cells was readily apparent within 3 d of treatment (Fig. 6D). Therefore, *p21*^{-/-} TS cells had not lost their ability to endoreduplicate in response to inhibition of CDK1 activity.

The delayed appearance of endocycles in *p21*^{-/-} TS cells corresponded to delayed expression of p57. Endoreduplication in *p21*^{-/-} TS cells was delayed at least 3 d after removal of FGF4 (Fig. 6A,B), and commenced upon the appearance of p57 (Fig. 6C). Therefore, the eventual induction of endoreduplication in *p21*^{-/-} TS cells results from expression of p57. This conclusion was confirmed by the observation that siRNA suppression of p57 in *p21*^{-/-} TG cells reduced by half the number of *p21*^{-/-} cells with >4 N DNA content, as well as the amount of p57 protein produced (data not shown). A similar correlation between induction of p57 and the onset of endocycles occurred with *Cdk2*^{-/-} TS cells. The onset of endoreduplication in *Cdk2*^{-/-} TG cells (Fig. 4A) was coincident with the delayed appearance of p57 protein and not with the earlier appearance of p21 (Fig. 6C).

The differences observed between *p21*^{-/-}, *p57*^{-/-}, and wild-type TS cells were not due to differences between

Figure 8. p57 protein levels oscillated during endoreduplication in TG cells. (A) TG cells after 3 d of FGF4 deprivation were stained with DAPI to identify nuclei and with anti-p57 antibodies to identify p57 protein. (B) TG cells after 3 d of FGF4 deprivation were cultured for 1 h in the presence of BrdU and then stained with both anti-BrdU antibodies (in situ cell proliferation kit, Roche) and anti-p57 antibodies.

mouse strains, because TS cells derived from wild-type embryos collected from the same female endoreduplicated to the same extent in response to either RO3306 or FGF4 deprivation.

p21 suppressed the DNA damage response in TG cells

Either DNA damage or stalled replication forks activate the ATR–CHK1 DNA damage response pathway that prevents entry into mitosis by inhibiting activation of CDK1. ATR activates the CHK1 protein kinase, which in turn, inactivates the CDC25 protein phosphatase that is required to convert the inactive form of CDK1 [CDK1-P(Y15, T14)] into the active form. Analysis of TS and ES cells revealed a strong correlation between expression of p21, suppression of CHK1, and reduced sensitivity to genotoxic stress.

Wild-type TS cells expressed CHK1 protein (Fig. 9A) but not p21 protein (Fig. 5B). FGF4 deprivation induced p21 expression (Fig. 5B) with concomitant suppression of both CHK1 and ATR levels (Fig. 9A). CHK2 levels were

Figure 9. Differentiation of TS cells into TG cells suppressed the DNA damage response. (A) Total cell extracts were prepared from wild-type TS cells before (0 h) and up to 48 h after FGF4 deprivation, and then subjected to Western immunoblotting analysis for the proteins indicated. TS cells also were cultured in the presence RO3306 for 48 h and analyzed for CDK1 protein (+FGF4 +RO). (B) ES cells were treated with RO3306 for the indicated times and then analyzed as in panel A. (C) TS cells were subjected to FGF4 deprivation for 3 d in order to produce TG cells. Then TS cells and TG cells were cultured for 3 d either in the presence (shaded bars) or absence (open bars) of 10 mM caffeine (Sigma) to inhibit ATR, or 200 nM UCN-01 (Sigma) to inhibit CHK1. Total cells were harvested and the fraction of cells with <2N DNA content was determined by FACS.

not affected. Selective inhibition of CDK1 activity with RO3306 also induced p21 expression in TS cells (Fig. 5B) with concomitant suppression of CHK1 levels (Fig. 9A), suggesting that CDK1 activity prevents expression of p21, which in turn, prevents a reduction in the level of CHK1 protein. CHK1 protein was not detected in TG cells using three different antibodies: one monoclonal, one polyclonal, and one that is specific for the phosphorylated form of CHK1. These results are consistent with a previous study showing that p21 can suppress expression of CHK1 in human colorectal cancer cells (Gottifredi et al. 2001). CHK1 levels were dependent on expression of p21, because FGF4 deprived *p21*^{-/-} TS cells continued to express CHK1 until the appearance of p57 (Fig. 6C). The inverse was also true. *p57*^{-/-} TS cells continued to express CHK1 during FGF4 deprivation until the appearance of p21 (Fig. 6C). Thus, either p21 or p57 appear capable of suppressing expression of CHK1.

To determine whether or not TG cells evade ATR, CHK1-dependent apoptosis, cells were treated either with caffeine to inhibit ATR or with UCN-01 to inhibit CHK1. Either treatment induced apoptosis in TS cells, but neither treatment had a comparable effect in TG cells (Fig. 9C). Thus, suppression of the ATR, CHK1-dependent checkpoint facilitates survival of cells undergoing programmed endoreduplication. In contrast with TS cells, RO3306 treatment of ES cells neither induced p21 nor suppressed CHK1 (Fig. 9B), which would account for the fact that it induced cell death rather than stable endoreduplication and giant cells.

These results suggest that the primary role of p21 up-regulation in TS cells following FGF4-deprivation is to prevent apoptosis by suppressing the DNA damage response pathway. Changes in CHK1 reflected changes in the level of protein, rather than its modification. The antibody used to detect CHK1 recognized both phosphorylated and unphosphorylated forms, and antibodies specific for the phosphorylated form of CHK1 did not detect CHK1 protein in cells (data not shown). Moreover, changes in the level of CHK1 were reflected in changes in the level of the inactive form of CDK1; both CHK1 and CDK1-P(T14) disappeared concurrently as TS cells differentiated into TG cells (Fig. 8A), as expected by up-regulation of Cdc25 activity in the absence of CHK1.

Discussion

TS cells derived from mouse blastocysts provide an opportunity to investigate the transition from mitotic to endocycles during mammalian development. These cells are programmed to differentiate into nonproliferating TG cells, during which time they undergo several rounds of endoreduplication. Here we show that selective inhibition of CDK1 activity by RO3306 triggers endoreduplication in TS cells along with acquisition of TG cell morphology and gene expression. ES cells also are derived from blastocysts, but they are not programmed for endoreduplication. ES cells responded to RO3306 by undergoing apoptosis.

In nature, TS cells differentiate into TG cells when

deprived of FGF4. In culture, FGF4 deprivation of TS cells induced the rapid appearance of two CDK-specific inhibitors, p57 and p21, with concomitant loss of CDK1 activity. However, of the two, only p57 is required to trigger endoreduplication, because only *p57*^{-/-} TG cells failed to endoreduplicate when deprived of FGF4. Since these cells were capable of endoreduplication when treated with RO3306, the role of p57 is to inhibit CDK1 activity. This conclusion was confirmed by the fact that FGF4 deprived *p57*^{-/-} TS cells continued to proliferate until confluent, at which time multinucleated TG cells accumulated. Thus, in the absence of p57, FGF4 deprivation induced repeated nuclear divisions even in the absence of cytokinesis. These results suggest that p57 plays a unique role in animal development by triggering endoreduplication during terminal differentiation of cells in order to amplify their genome.

The ability of p21 to prevent synthesis of CHK1 protects TG cells from undergoing apoptosis in response to incomplete DNA synthesis and DNA damage that may occur during endoreduplication. CHK1 is an essential protein kinase that is required for mammalian cell proliferation, and for preventing cells from entering mitosis with DNA damage or stalled replication forks (Liu et al. 2000; Takai et al. 2000). Either FGF4 deprivation or treatment with RO3306 selectively inhibited CDK1 activity in TS cells with concomitant induction of p21 and suppression of CHK1, a phenomenon also detected during the p53 response to DNA damage (Gottifredi et al. 2001). This would account for the fact that TS and ES cells, which expressed CHK1, were sensitive to genotoxic stress, whereas TG cells that did not express CHK1 were not. This would also account for the fact that loss of *p21* exacerbates the placental defects observed in *p57* mutant mice (Zhang et al. 1999). In *p21*^{-/-} TS cells, delayed expression of p57 in response to FGF4 deprivation occurred concurrently with suppression of CHK1 synthesis, suggesting that p57 can substitute for p21 in this role. In contrast, RO3306 induced aborted endoreduplication and apoptosis in ES cells where neither CDK inhibitor was expressed and CHK1 was present. These results suggest that p21 plays a unique role in animal development by suppressing the CHK1-dependent DNA damage response during terminal cell differentiation in order to prevent apoptosis. In addition, p21 may also help regulate initiation of S phase by inhibiting CDK2–CCNE activity during the Gap phase (Besson et al. 2008).

The results reported here also reveal that CDK2 is required for endoreduplication when CDK1 is inactive. *Cdk2*^{-/-} TS cells failed to endoreduplicate when cultured in the presence of RO3306, but they did endoreduplicate and differentiate into TG cells when deprived of FGF4. Therefore, CDK1 substituted for CDK2 in regulating S phase. The required oscillation of CDK1 activity under these conditions was accounted for by the fact that p57 was absent from S-phase cells but present in Gap-phase cells.

Finally, when together with published work from this and other labs, the results presented here suggest that

flies and mammals employ the same pathways to sustain endocycles, but initiate them by different means.

Triggering the endocycle

Among metazoa, the most well-characterized paradigm for developmentally programmed endoreduplication is the fruit fly, *Drosophila melanogaster*. Both mitotic cell cycles and endocycles in *Drosophila* are driven by oscillations in cyclin E-dependent CDK activity (Lilly and Duronio 2005). Low cyclin E–Cdk2 activity permits pre-RC assembly, whereas high cyclin E–Cdk2 activity initiates S phase. Thus, in order to transit from mitotic cell cycles to endocycles, other events must occur that prevent the onset of mitosis while permitting repeated G-to-S-phase transitions. Inactivation of the *Cdk1* gene in *Drosophila* can induce endoreduplication in cells that normally would have restricted genome duplication to once per cell division (Hayashi 1996; Weigmann et al. 1997). However, the mechanism by which mitosis is blocked during *Drosophila* development differs from that in mammals.

During oogenesis in *Drosophila*, the mitotic-to-endocycle transition in follicle cells is regulated by the Notch signaling pathway (Shcherbata et al. 2004). Notch down-regulates transcription of *String/Cdc25*, the protein phosphatase that is required to activate CDK1, and up-regulates Fizzy-related (Fzr), an activator of the APC that degrades geminin as well as mitotic cyclins A and B (Narbonne-Reveau et al. 2008; Zielke et al. 2008). These two events should prevent the onset of mitosis by suppressing CDK1 activity while at the same time promoting assembly of pre-RCs. *String*-null mutants arrest cells in G2 phase but they do not endoreduplicate their genome, revealing either that suppression of CDK1 activity alone is insufficient to trigger endoreduplication, or that inactivation of *String* does not sufficiently inactivate CDK1. Notch also down-regulates *Dacapo*, the only CDK inhibitor encoded by *Drosophila*. This assures that cells will enter S phase as soon as cyclin E is produced. *Dacapo* promotes replication licensing during *Drosophila* endocycles by reinforcing low CDK activity during the endocycle Gap phase, a role that it also appears to play during mitotic cell cycles (Hong et al. 2007). Thus, the role of *Dacapo* in mitotic cell cycles is equivalent to the role of *Sic1* in yeast, *Xic1* in frogs, and *p27* in mammals (Besson et al. 2008). The transition from mitotic to endocycles is accompanied by transcriptional down-regulation of the genes for CDK1 and cyclins A and B, all of which promote mitosis (Lilly and Duronio 2005).

Of these events, premature expression of Fzr is most likely responsible for triggering the mitotic-to-endocycle transition, as well as for maintaining subsequent endocycles. Fzr is dispensable for mitosis but essential for endocycles, and Fzr is expressed at the mitotic-to-endocycle transition in a Notch-dependent manner (Schaeffer et al. 2004). APC–Fzr activity, like that of geminin and cyclin E, oscillates during endocycles, and these oscillations are essential to sustaining endocycles (Narbonne-Reveau et al. 2008; Zielke et al. 2008). Since cyclin

E-Cdk2 can inactivate Fzr, these results suggest that the cyclic activity of APC-Fzr is driven by periodic inhibition of Fzr by cyclin E-Cdk2. Low cyclin E-CDK2 allows high APC-Fzr, which in turn, degrades mitotic cyclins and geminin. High cyclin E-CDK2 inactivates APC-Fzr, which allows the onset of S phase in the presence of geminin. Premature expression of APC-Fzr activity could trigger endoreduplication by preventing mitosis while simultaneously creating conditions that allow pre-RC assembly. APC-Fzr mediates ubiquitin-dependent degradation of mitotic cyclins that are required for CDK1 activity, degradation of Skp2 that is required for ubiquitin-dependent, SCF-Skp2-mediated degradation of CDK-specific inhibitors during G1 phase, and degradation of geminin that prevents assembly of pre-RCs.

All of these pathways are operative in mammals, but the transition from mitotic cycles to endocycles is different (Fig. 10). In flies, cyclin E-CDK2 is required to drive S phases in both mitotic cycles and endocycles, whereas in mammals cyclin E is required only for endocycles (Geng et al. 2003; Parisi et al. 2003), and CDK2 is dispensable for both mitotic and endocycles, because it can be replaced by CDK1. In flies, endocycles occur in the absence of mitotic cyclins A and B, whereas endoreduplication in mice is triggered in their presence. The critical difference is the availability of two CDK-specific inhibitors, p57 and p21, that are encoded by mammals, but not by flies. p57 has the same effect on endoreduplication as RO3306; both bind to and inhibit CDK1, regardless of cyclin levels. This represents a fundamental difference in the way endocycles are triggered in flies and mammals. In flies, premature activation of APC-Fzr would mediate destruction of both cyclins and geminin, conditions that prevent mitosis while allowing pre-RC assembly. In mammals, expression of p57 inhibits CDK1 which prevents mitosis and allows pre-RC assembly to begin (Noguchi et al. 2006; Vassilev et al. 2006; Hochegger et al. 2007) without interfering with the normal appearance of APC-Cdh1, the mammalian homolog of APC-Fzr that marks the transition from M-to-G1 phase (Pesin and Orr-Weaver 2008). APC-Cdh1, like APC-Fzr, is required for endoreduplication in mice (Garcı-Higuera et al. 2008). In contrast, APC-Cdc20, the primary mechanism for targeting cyclins for destruction (Pesin and Orr-Weaver 2008), is not. During the mitotic cycle, binding of Cdc20 to the APC is dependent on the phosphorylation of APC by CDK1 (Rudner and Murray 2000, and references therein). Therefore, expression of p57 should inhibit degradation of CCNA and CCNB, as observed in TG cells.

Once mitosis has been suppressed under conditions that allow pre-RC assembly, cells are locked in repeated cycles of genome endoreduplication. All that is needed to sustain these cycles are oscillations in the key regulators cyclin E-CDK2 and APC-Fzr/Cdh1, and the CDK-specific inhibitors Decapo, p57, and, presumably, p21. Both p57 and p21 are degraded in a reaction mediated by the E3-ubiquitin protein ligase SCF^{Skp2} (Kossatz et al. 2004) that is dependent on cyclin E-CDK2 phosphorylation of both inhibitors (Kamura et al. 2003). Transient

Figure 10. Mammalian endocycles. In TS cells, the transition from mitotic cell cycles to endocycles occurs when FGF4 deprivation induces expression of p57, a protein that selectively inhibits CDK1, the enzyme responsible for initiating mitosis. This allows cells to transit from G2 phase to G1 phase without passing through mitosis (M phase). Thus, mammalian endocycles consist of a Gap phase that is defined by the presence of p57 and an S phase that is defined by its absence. FGF4 deprivation also induces expression of p21, but the role of p21 is to suppress CHK1 and thereby prevent apoptosis in response to incomplete DNA replication. In addition, p21 may also suppress CDK2-CCNE activity during the Gap phase. CDK2-CCNE is required to initiate DNA replication during endocycles. Inhibition of CDK1 activity has at least two additional consequences. Inhibition of CDK1 prevents assembly of APC-Cdc20, the enzyme that targets cyclins A and B for degradation ($\emptyset$), and it prevents inactivation of the Orc1 subunit by phosphorylation when S phase is completed. Thus, p57 allows ORC assembly and binding to DNA replication origins under conditions that prevent these events during mitotic cell cycles. Furthermore, CDC6, which is phosphorylated by CDK2 during S phase, will become dephosphorylated in the presence of p57 and p21. These events allow initiation of pre-RC assembly. Geminin (Gem), a specific inhibitor of CDT1 during S phase, is targeted for degradation by the APC-Cdh1 ubiquitin ligase, an enzyme that is active during the late M and early G1 phases of mitotic cell cycles. CDT1 can then complete pre-RC assembly by loading MCM helicases. Since CDK1 activity is required to activate APC-Cdc20, this ubiquitin ligase will be inactive. CDK2-CCNE activity then concurrently promotes destruction of p57 and p21 by activating the SCF-Skp2 ubiquitin ligase, restores geminin expression by preventing assembly of APC-Cdh1, and initiates DNA replication.

expression of a stable mutant of p57 that cannot be phosphorylated inhibits DNA replication in Rcho-1 giant cells (Hattori et al. 2000). Thus, degradation of p27 and

p57 allow initiation of the first round of endoreduplication by cyclin E-CDK2. When S phase is completed, p57 and p27 are restored, CDK activity is inhibited, and pre-RC assembly begins again.

Role of p57 in placental development

Given the critical role of p57 in the development of TG cells, it is surprising that deletion of the p57 gene in mice does not arrest development. Nevertheless, *p57*^{-/-} embryos display placentomegaly, as well as hyperplasia involving both labyrinthine trophoblasts and spongiotrophoblasts (Zhang et al. 1998; Takahashi et al. 2000; Kanayama et al. 2002), suggesting that p57 expression serves to restrict TG cell proliferation as well as to trigger endoreduplication. In fact, *p57*^{-/-} TG cells continued to divide for 3 d in the absence of FGF4, and when cell division ceased, nuclear division continued resulting in accumulation of multinucleated giant cells. Moreover, *p57*^{-/-} TG cells expressed TG cell markers (Supplemental Fig. S1). Therefore, genome endoreduplication per se is not required for TG cell function, although it presumably facilitates it, since p57 is required for normal placental development. Placentomegaly may result both from an increase in the number of TG cells as well as from formation of multinucleated TG cells. Thus, the results presented here reveal a pivotal role for p57 in developmentally programmed endoreduplication, and provide insight into the relationship between p57 deficiency and placentomegaly, a condition associated premature birth among humans (Kanayama et al. 2002).

Materials and methods

Cell derivation and culture

p21^{+/-} mice (Brugarolas et al. 1995) and *CDK2*^{+/-} mice (Berthet et al. 2003) were provided by Philipp Kaldis (Institute of Molecular and Cell Biology, Singapore). *p57*^{+/-} mice (Matsuoka et al. 1995) were provided by Colin Stewart (Institute of Medical Biology, Singapore). TS and ES cells were derived from blastocysts produced by mating wild-type, *Cdk2* heterozygous mice, *Cdkn1a* (p21) heterozygous mice, or *Cdkn1c* (p57) heterozygous mice, as previously described (Yagi et al. 2007). Primary mouse embryonic fibroblasts either were isolated from wild-type embryos at E12.5, or were purchased from Chemicon and used at passage 4. Cells were seeded at 3.5×10^5 to 5×10^5 in T-75 flasks and cultured as described. RO3306 was synthesized at Hoffmann-LaRoche, prepared as a 10 mM solution in DMSO, and added to cells 24 h after seeding at a final concentration of 6 μ M (Vassilev et al. 2006).

Flow cytometry

Flow cytometry was performed using Cycletest kit (BD Biosciences) according to the manufacturer's instructions. Cells were analyzed by FACS Scan (Becton and Dickinson).

Protein kinase assay

Protein kinase assays were carried out using the kinase assay kit from Upstate Biotechnologies (catalog no. 17-137), except that

20 μ g of histone H1 was used as the protein kinase substrate and incubation with ³²P-ATP was for 5 min at 30°C.

Immunofluorescence

Cells were fixed with 3% paraformaldehyde for 10–20 min, rinsed briefly with PBS, and permeabilized for 5–10 min with methanol at –20°C. Primary antibodies (diluted 1:50 in PBS + 3% BSA) were applied for 1 h at room temperature. Cells were washed and secondary antibodies (diluted 1:300) were applied for 1 h at room temperature. Slides were fixed with Vectashield mounting medium and analyzed by fluorescent microscope (Nikon).

Immunoprecipitation and Western blotting

Cell extracts were prepared by sonication in HEMGT buffer (25 mM HEPES at pH 7.9, 12.5 mM MgCl₂, 150 mM NaCl, 10% glycerol, 0.1 mM EDTA, 0.5% Tween 20) containing protease inhibitor cocktail (Roche) and 25 μ M MG132 to inhibit the 26S proteasome. Extracts were centrifuged (10,000 rpm, 10 min, 4°C), and supernatants were used for immunoprecipitation (Ullah et al. 2007). Immunoprecipitation was done with 2 mg of total protein and 2–5 μ g of IgG overnight at 4°C. The extracts were diluted by adding four volumes of HEMGT buffer before adding protein G incubated for 30 min, centrifuged (1000 rpm, 5 min). Beads were collected, washed three times with HEMGT buffer for 10 min at room temperature with rotation. Beads were resuspended in 20 μ L of 1× LDS loading buffer (Invitrogen) and run on 4%–12% gradient polyacrylamide gel.

For Western blotting, proteins were transferred to PVDF membranes at room temperature, blocked with 3% nonfat milk for 1 h, incubated with primary antibodies for 1 h, washed three times with PBS containing 0.1% Tween 20, and incubated with secondary antibodies for 1 h. After three washes with PBS containing 0.1% Tween 20, chemiluminescence was detected using West Dura (Pierce).

Antibodies and oligonucleotides

Antibodies used were Cyclin A, (Santa Cruz Biotechnologies, SC596), Cyclin B1 Calbiochem (PC133), Cyclin E1 (SC481), CDK1/CDC2 (SC54, AB32384), Phospho T14 Cdc2 (Abcam, AB4823), Cdk2 (SC-6248), Chk1 (SC8408; AB63345), P-Chk1(ser345) (SC21866), Chk2 (SC9064, SC17747), p21 (SC397 and SC6246), p27 (SC776, SC1641), p57 (SC1037 and AB4058), Geminin (SC13015), Tubulin (Developmental Studies Hybridoma Bank, Iowa E7), and GAPDH (SC48166).

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