CDK4 protein is degraded by anaphase-promoting complex/cyclosome in mitosis and reaccumulates in early G1 phase to initiate a new cell cycle in HeLa cells

CDK4 regulates G1/S phase transition in the mammalian cell cycle by phosphorylating retinoblastoma family proteins. However, the mechanism underlying the regulation of CDK4 activity is not fully understood. Here, we show that CDK4 protein is degraded by anaphase-promoting complex/cyclosome (APC/C) during metaphase-anaphase transition in HeLa cells, whereas its main regulator, cyclin D1, remains intact but is sequestered in cytoplasm. CDK4 protein reaccumulates in the following G1 phase and shuttles between the nucleus and the cytoplasm to facilitate the nuclear import of cyclin D1. Without CDK4, cyclin D1 cannot enter the nucleus. Point mutations that disrupt CDK4 and cyclin D1 interaction impair the nuclear import of cyclin D1 and the activity of CDK4. RNAi knockdown of CDK4 also induces cytoplasmic retention of cyclin D1 and G0/G1 phase arrest of the cells. Collectively, our data demonstrate that CDK4 protein is degraded in late mitosis and reaccumulates in the following G1 phase to facilitate the nuclear import of cyclin D1 for activation of CKD4 to initiate a new cell cycle in HeLa cells.

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4 The abbreviations used are: RB, retinoblastoma; APC/C, anaphase-promoting complex/cyclosome; M-A, metaphase-anaphase; IFM, immunofluorescence microscopy; CHX, cycloheximide; LMB, leptomycin B; NLS, nuclear localization sequence; NoLS, nucleolus localization signal; NES, nuclear export sequence; TRITC, tetramethylrhodamine isothiocyanate.

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CDK4 accumulation initiates the new cell cycle and the indicated time points after G2/M release. Strikingly, we found that CDK4 protein was degraded during the M-A transition, and the phosphorylation of RB Ser-780 was decreased accordingly (Fig. 1D and supplemental Fig. S1B). Interestingly, from middle to late G1 phase, the protein level of CDK4 was recovered gradually, and the phosphorylation of RB Ser-780 was increased (Fig. 1D and supplemental Fig. S1B). These results were also confirmed in 3T3 cells (supplemental Fig. S1C). When the mitotic cells were treated with proteasome inhibitor MG132, CDK4 protein was kept at a steady high level (supplemental Fig. S1B), suggesting that CDK4 protein was degraded in mitosis via a ubiquitin-proteasomal pathway. To define the timing of ubiquitination and degradation of CDK4 proteins, we transfected HeLa cells with non-degradable GFP-cyclin B1Δ90 (27, 28), which sustains CDK1 activity, and arrested cells at the anaphase-telophase transition (29), treated the cells with nocodazole, and then released them into fresh medium. We observed that the protein levels of endogenous CDK4 and cyclin B1 were high in prometaphase and low in anaphase-telophase, whereas exogenous GFP-cyclin B1Δ90 was kept stable (Fig. 1E). As analyzed by microscopy, we also found that CDK4 protein was decreased during the M-A tran-
sion of GFP-cyclin D1 and treatment of leptomycin B (LMB), an effective inhibitor for nuclear exportin CRM1 (30), we also found that GFP-cyclin D1 was mainly localized in the cytoplasm of the paired G₁ phase cells, even in the presence of LMB (Fig. 2B). Because it was reported that mutation at Thr-286 prevents cyclin D1 from binding to CRM1 and displays low nuclear export efficiency (11, 31), we generated GFP-cyclin D1T286A mutant and expressed it in HeLa cells. Interestingly, we found that this mutant also became localized in the cytoplasm within 24 h, although eventually it was imported to the nucleus in the presence of LMB by 72 h (Fig. 2B), suggesting that nuclear import of this mutant was also insufficient in early G₁ phase. By linking a nuclear localization signal (NLS) to cyclin D1 (NLS-D1-GFP) (32), we also observed that only a fraction of these fusion proteins were in the nucleus and that, when treated with LMB, all of the NLS-D1-GFPs were localized to the nucleus (Fig. 2C). Furthermore, when linking an NLS to cyclin D1T286A-GFP (NLS-D1T286A-GFP), we found that all of the NLS-D1T286A-GFPs were localized in the nucleus despite the LMB treatment (Fig. 2C). Taken together, these results indicate that slow nuclear import and fast nuclear export retain cyclin D1 in the cytoplasm of the early G₁ phase cells in the absence of CDK4.

**CDK4 shuttles between the nucleus and cytoplasm and promotes the nuclear import of cyclin D1**

Through separate expression or co-expression of CDK4 and cyclin D1 in HeLa cells, we investigated how these proteins are transported to the nucleus. We observed that, when expressed separately, CDK4 entered the nucleus efficiently, whereas cyclin D1 was retained in the cytoplasm. When co-expressed, both CDK4 and cyclin D1 were imported and co-localized in the nucleus (Fig. 3A). In comparison, when cyclin D1 was co-expressed with CDK1 or CDK2, cyclin D1 was retained in the cytoplasm, although both CDK1 and CDK2 entered the nucleus efficiently (Fig. 3A). These results indicate that CDK4 promotes the nuclear import of cyclin D1. Because CDK6 also binds to cyclin D1 directly, we co-expressed CDK6 with cyclin D1 and found that both cyclin D1 and CDK6 were imported into the nucleus (Fig. 3A and supplemental Fig. S2 (A and B)). These results were also confirmed by experiments done in HEK 293T and MCF-7 cells (supplemental Fig. S2C). When CDK4 was knocked down by RNA interference (RNAi), the nuclear-localized CDK4 accumulation initiates the new cell cycle
CDK4 accumulation initiates the new cell cycle

To understand whether the nuclear accumulation of cyclin D1 was due to CDK4-promoted nuclear import or CDK4-mediated nuclear retention, we linked an NLS to CDK4-Myc (NLS-CDK4-Myc) and expressed this fusion protein in HeLa cells. The results showed that the forced nuclear import of NLS-CDK4-Myc, which does not weaken the binding of CDK4 with cyclin D1, impaired the nuclear localization of cyclin D1 (Fig. 3, A, E, and F), indicating that disrupting nucleocytoplasmic shuttling of CDK4 reduced the nuclear import of cyclin D1. Taken together, these data demonstrate that CDK4 shuttles between nucleus and cytoplasm, and this shuttling facilitates the nuclear import of cyclin D1.

Direct binding of CDK4 with cyclin D1 is required for CDK4-mediated nuclear import of cyclin D1

Next, we tested how CDK4 mediates the nuclear translocation of cyclin D1. First, by co-expressing a CDK4 kinase-dead mutant CDK4\(^{D158N}\) with cyclin D1 in HeLa cells, we observed that both CDK4\(^{D158N}\) and cyclin D1 were imported into the nucleus (Fig. 4A), indicating that the kinase activity of CDK4 was not required for their nuclear import. In contrast, by co-expressing GFP-cyclin D1 with CDK4 fused with an NES sequence of PKI-α and a Myc tag (CDK4-NES-Myc), we observed that GFP-cyclin D1 was co-localized with this NES-containing CDK4 in cytoplasm. Interestingly, when the cells were treated with LMB, both GFP-cyclin D1 and CDK4-NES-Myc were co-localized in the nucleus (Fig. 4B). We also constructed another two mutants, CDK4\(^{K22A}\) and CDK4\(^{K22A/R24A/D25A}\), that bind to cyclin D1 weakly (Fig. 4C) (33) and co-expressed each of them with cyclin D1. We observed that CDK4\(^{K22A}\), but not CDK4\(^{K22A/R24A/D25A}\), retained about 30% of binding capability with CDK4 compared with the wild type (Fig. 4D). It has been reported that the residues Lys-112 and Lys-114 within the cyclin box of cyclin D1 are important for its interaction with CDK4, although it is ambiguous which one is pivotal (34, 35). In this work, we generated the mutants cyclin D1\(^{K112E}\), cyclin D1\(^{K114E}\), and cyclin D1\(^{K22A/K114E}\) and co-expressed each of them with CDK4 in HeLa cells. Through co-immunoprecipitation assays, we found that cyclin D1\(^{K112E}\) and cyclin D1\(^{K22A/K114E}\) did not bind to CDK4, whereas cyclin D1\(^{K114E}\) retained 30% of binding capability with CDK4 compared with the wild type (Fig. 4E). Through IFM, we observed that only GFP-cyclin D1\(^{K114E}\) was co-localized with CDK4 in the nucleus (Fig. 4F). We also generated a CDK4 construct containing an NLS and a nucleolus localization signal (NoLS) (NLS-NoLS-CDK4-Myc) and co-expressed it with each of the cyclin D1 mutants. We observed that only cyclin D1\(^{K114E}\) was co-localized with NLS-NoLS-CDK4-Myc in the nucleus and the nucleoli, whereas most of cyclin D1\(^{K112E}\) and cyclin D1\(^{K22A/K114E}\) were retained in cytoplasm without co-localization with NLS-NoLS-CDK4-Myc (Fig. 4G). By establishing a tet-on system (36) to induce the expression of GFP-cyclin D1\(^{K112E/K114E}\) by tetracycline and time-lapse microscopy, we also observed that GFP-cyclin D1\(^{K112E/K114E}\) was persistently cytoplasmic throughout the cell cycle (supplemental Movie 3). Collectively, these results demonstrate that only Lys-112 on cyclin D1, but not Lys-114, is
essential for direct binding with CDK4 and also that this direct binding is required for the nuclear import of cyclin D1. We further tested whether another part of the cyclin D1 molecule is involved in its nuclear import. By co-expressing the GFP- or Myc-tagged cyclin box, C and N terminus deletion mutants of cyclin D1 with CDK4, we found that, no matter whether the mutant without cyclin box was expressed alone or co-expressed with CDK4, this mutant remained in the cytoplasm (supplemental Fig. S2, D and E). In contrast, when the mutants with either C or N terminus deletion were expressed alone, they were retained in cytoplasm, but, once co-expressed with CDK4, they entered the nucleus (supplemental Fig. S2F), indicating that neither the N nor C terminus of cyclin D1 contributes to its nuclear import. Taken together, we conclude that the Lys-112 residue of cyclin D1 is required for the interaction of cyclin D1 with CDK4 and the nuclear import of cyclin D1 in HeLa cells.

Stable binding of D-type cyclins with CDK4 enhances their nuclear localization

Because physical interaction between CDK4 and cyclin D1 promotes the nuclear import of cyclin D1, we decided to test whether the stability of CDK4-cyclin D1 complex influences the subcellular localization of cyclin D1 in HeLa cells. Because P16INK4 disrupts the interaction by competing with cyclin D1 for binding with CDK4 and p21CIP1/p27KIP1 directly binds and stabilizes cyclin D1-CDK4 complex (37, 38), we investigated the influence of P16INK4 and p21CIP1/p27KIP1 on the complex stability of CDK4-cyclin D1 and the subcellular localization of cyclin D1. Through a co-immunoprecipitation assay, we found that the stability of cyclin D1-CDK4 complex was compromised in the cells expressing GFP-p16INK4. In contrast, the stability of the cyclin D1-CDK4 complex was strengthened in the cells expressing GFP-p21CIP1 or -p27KIP1 (Fig. 5A). IFM showed that cyclin D1 was mostly localized in the cytoplasm in GFP-p16INK4-expressing cells and in the nucleus without GFP-p16INK4 expression (Fig. 5B). On the contrary, cyclin D1 was localized exclusively in the nucleus in GFP-p21CIP1-, or GFP-p27KIP1-expressing cells (Fig. 5B). Western blot analysis also confirmed that cyclin D1 was mainly cytoplasmic in p16INK4-expressing cells (Fig. 5C). Because the other two D-type cyclins, D2 and D3, can also bind to CDK4 directly, by co-expressing these proteins in HeLa cells, we tested whether D2 and D3 cyclins are also imported into the nucleus along with CDK4. We found that, as a control, cyclin E was persistently in the nucleus, whereas cyclin D2 and D3 were cytoplasmic when expressed alone and entered the nucleus when co-expressed with CDK4.

Figure 3. CDK4 shuttles between the nucleus and the cytoplasm and promotes the nuclear accumulation of cyclin D1. A, HeLa cells were co-transfected with GFP- or Myc-tagged cyclin D1 and CDK4 for 24 h. Myc-tagged protein was immunostained. 200 cells were randomly counted in each of three repeated experiments. Representative images are shown. B, HeLa cells were transfected with CDK4 siRNA for 72 h and fractionated into nuclear and cytoplasmic fractions, followed by Western blotting. C and D, asynchronous HeLa cells co-transfected with control, CDK4 or CDK6 siRNA, and RFP-H2B for 72 h were stained using an anti-cyclin D1 antibody and DAPI (C), and the cells with cyclin D1 in the nucleus were counted (D). *, p < 0.05; **, p < 0.01 (Student’s t test). E and F, HeLa cells co-transfected with GFP-cyclin D1 (GFP-D1) and NLS-CDK4-Myc for 24 h were visualized by IFM (E; scale bars, 10 μm) or a co-immunoprecipitation assay (F).
CDK4 accumulation initiates the new cell cycle

These results indicate that cyclins D1, D2, and D3 share a very similar mechanism for their nuclear import and retention. Taken together, we conclude that a stable binding between D-type cyclins and CDK4 enhances their nuclear localization in HeLa cells.

CDK4/CDK6 knockdown results in cell-cycle arrest at the G1 phase

Knowing that accumulation of CDK4 in cells facilitates nuclear import of cyclin D1 and activation of CDK4 kinase in the nucleus to promote the M/G1/S transition, we wanted to verify whether knocking down CDK4 would arrest post-mitotic cells in G1 phase due to cytoplasmic retention of cyclin D1 and low CDK4 kinase activity. To do this, we knocked down CDK4 with siRNA and simultaneously expressed GFP-cyclin D1 in HeLa cells. The results showed that cyclin D1 could not be imported to the nucleus in CDK4-knockdown cells (Fig. 6 (A and B) and supplemental Movies 4 and 5). We also combined CDK4/CDK6 knockdown with tet-on expression of wild-type GFP-cyclin D1 or mutant GFP-cyclin D1K112E/K114E. Through time-
CDK4 accumulation initiates the new cell cycle

Discussion

Collectively, in this work, we show that periodic degradation of CDK4 is directly related to the activation of CDK4 in HeLa cells. The CDK4 protein level declines dramatically during metaphase-anaphase transition, and this decline results in a dramatic loss of CDK4 kinase activity in late M and early G1 phases. Importantly, the protein level of CDK4 increases during the following early and middle G1 phase, and accordingly, the kinase activity of CDK4 is recovered to enable the phosphorylation of RB again from middle to late G1 phase. Although we currently do not know why CDK4 is degraded in late mitosis, we suspect that this degradation may be crucial for a cell to decide to either continue cycling or arrest at G0 phase.

Cyclin D1 is regarded as a nuclear protein (40), although it is localized in the cytoplasm at certain stages of the cell cycle. Because cyclin D1 does not possess a recognized NLS, it may be co-imported into the nucleus with other factors. In this work, we have identified that CDK4 protein shuttles between the nucleus and cytoplasm and promotes the nuclear import of cyclin D1. CDK4 may enter the nucleus without cyclin D1, but cyclin D1 alone cannot enter the nucleus. Due to the degradation of CDK4 in mitosis, cyclin D1 will be retained in the cytoplasm in early G1 phase, although its protein level remains unchanged. Therefore, CDK4 reaccumulation after mitosis is also required for nuclear translocation of cyclin D1.

Based on our results and previously reported data, we propose a working model for the role of CDK4 in initiating a new cell cycle in HeLa cells (Fig. 7). In this model, CDK4 protein is degraded during the metaphase-anaphase transition in a proteasome-dependent manner, whereas cyclin D1 protein remains stable and is retained in the cytoplasm. CDK4 protein accumulates from the next early to mid-G1 phase and shuttles between the nucleus and the cytoplasm to facilitate the nuclear import of cyclin D1 and the M/G1/S transition (Fig. 7). When the divided cells lack nutrients, such as during serum starvation, they do not synthesize CDK4 and remain in the G0 phase. Accordingly, cyclin D1 is degraded to a basal level in these G0 cells. Once supplied with nutrients, these G0 cells begin to synthesize CDK4 and cyclin D1, as well as other essential factors, and initiate a new cell cycle starting from the G0 phase (G0/G1/S transition) (Fig. 7). In conclusion, this work reveals a CDK4 degradation/reaccumulation-dependent mechanism for initiating a new cell cycle in HeLa cells. Our findings may have important implications, potentially helping us to understand cell cycle control and tumorigenesis.

Materials and methods

Cell culture and transfection

HeLa, HEK 293T, MCF-7, WI-38, or NIH3T3 cell lines were maintained at 37°C in Dulbecco’s modified Eagle’s medium containing 10% fetal bovine serum (FBS), 100 units/ml penicillin, and 100 μg/ml streptomycin in the presence of 5% CO2. WI38 cells were cultured in Eagle’s minimum essential medium with Earle’s balanced salts containing 10% FBS and 1% non-essential amino acids in the presence of 5% CO2. To express exogenous proteins, the cells were transiently transfected via the calcium phosphate or liposome techniques according to the manufacturer’s instructions.

Plasmids and antibodies

Cyclin D1 cDNA was cloned from MCF-7 cells by PCR with relevant oligonucleotides. Upstream sense primer contains a BgLII site and the cyclin D1 initiation codon, whereas the downstream antisense prime is complementary to the 3’-end of the cyclin D1 sequence containing a SalI site but having a deleted BglII site and the cyclin D1 initiation codon, whereas the down...
fied and digested with BglII and SalI restriction endonucleases before ligation to pcDNA3.1myc vector that had been digested with BamHI and XhoI restriction endonucleases. Other genes were cloned by the same method with corresponding oligonucleotides. A point mutation was constructed by a PCR-based technique with relevant oligonucleotides. All constructs were verified by DNA sequence analysis (supplemental Table 1).

Anti-GFP antibody used for immunoprecipitation (4 μg/sample) was generated by immunizing rabbits with bacterially expressed recombinant GFP tagged with His. For immunofluorescence, mouse anti-cyclin D1 (sc-20044, Santa Cruz Biotechnology; 1:500), anti-CDK4 (sc-23896, Santa Cruz Biotechnology; 1:500), anti-CDK6 (sc-7961, Santa Cruz Biotechnology; 1:500), anti-CDK6-tubulin (T6199, Sigma; 1:2,000), anti-Cdh1 (sc-56312; Santa Cruz Biotechnology, 1:500), and anti-GAPDH (60004-1-Ig, Proteintech; 1:3,000) and rabbit anti-p286-cyclin D1 (ab62151, Abcam; 1:1,000), anti-Rb (sc-50, Santa Cruz Biotechnology; 1:500), anti-p780-Rb (555S, MBL; 1:1,000), anti-cyclin B1 (sc-752, Santa Cruz Biotechnology; 1:1,000), and anti-Cdc20 (A1231, ABclonal; 1:1,000) antibodies were used. All animal experiments were performed according to approved guidelines.

**Western blotting and immunoprecipitation assay**

HeLa or 3T3 cells were washed twice with calcium/magnesium-free PBS and lysed in lysis buffer (0.5% Nonidet P-40, 50 mM HEPES, 150 mM NaCl, 1 mM EDTA, 1 mM DTT, 1 mM NaF, 0.1 mM NaVO₄, 0.2 mM PMSF, and 10 μg/ml protein inhibitors).
GFP-tagged proteins were immunoprecipitated with an anti-GFP antibody. The protein samples were denatured and separated by 10% SDS-polyacrylamide gel electrophoresis and transferred onto nitrocellulose membranes. The membranes were probed with primary antibodies for 2 h at 37 °C, and the positive bands were detected by using the specific secondary antibody conjugated with horseradish peroxidase followed by a chemiluminescence detection system.

**His6 tag pull-down assays**

HeLa cells were seeded on 10-cm tissue culture plates and transfected with relevant siRNA and vectors. 40 h post-transfection, cells were treated with 10 μM MG132 for an additional 8 h. Treated cells were washed by PBS and lysed with 6 ml of lysis buffer (6 M guanidinium-HCl, 0.1 M Na2HPO4/NaH2PO4, 0.01 M Tris/HCl, pH 8.0, 5 mM imidazole, and 10 mM β-mercaptoethanol), rotating 30 min at room temperature. 75 μl of Ni2+-nitrilotriacetic acid-agarose beads (Qiagen) were then added, and lysates were rotated at room temperature for 4–5 h. Lysates were placed on the rack, kept standing for 5 min, and centrifuged at 2,000 rpm for 5 min at room temperature. The beads were roughly washed and rotated for 5 min at room temperature with 750 μl of wash buffer 1 (6 M guanidinium-HCl, 0.1 M Na2HPO4/NaH2PO4, 0.01 M Tris/HCl, pH 8.0, plus 10 mM β-mercaptoethanol); then the samples were kept standing for 5 min and centrifuged at 2,000 rpm for 5 min at room temperature. The pelleted beads were sequentially washed with wash buffer 2 (8 M urea, 0.1 M Na2HPO4/NaH2PO4, 0.01 M Tris/HCl, pH 8.0, 10 mM β-mercaptoethanol), wash buffer 3 (8 M urea, 0.1 M Na2HPO4/NaH2PO4, 0.01 M Tris/HCl, pH 6.3, 10 mM β-mercaptoethanol plus 0.2% Triton X-100), and wash buffer 4 (8 M urea, 0.1 M Na2HPO4/NaH2PO4, 0.01 M Tris/HCl, pH 6.3, 10 mM β-mercaptoethanol plus 0.1% Triton X-100). Finally, proteins were eluted by incubating the beads in 40 μl of elution buffer (500 mM imidazole, 0.15 M Tris/HCl, pH 6.7, 30% glycerol, 0.72 M β-mercaptoethanol, 5% SDS) for 20 min at room temperature. 35 μl of eluates were mixed with 2× loading buffer in a 1:1 ratio and analyzed by Western blot analysis.

**RNAi**

HeLa cells were transfected with siRNA especially designed to silence the CDK4 or CDK6 gene. Two oligomers of CDK4-specific siRNA were synthesized with the following sequences: 5’-GCAGCAUCUGUUAUCAUGAUCACAGUUACUCGAAUCAAG-3’ and 5’-GCAGGAGAUCUCUGGACCGGAAACGUGUC-3’. Two oligomers of CDK6-specific siRNA were synthesized with the following sequences: 5’- GCACGAGAGAUCUCUGGACCGGAAACGUGUC-3’ and 5’-GCAGGAGAUCUCUGGACCGGAAACGUGUC-3’. The transfection efficiency was monitored by using GFP indicator vectors. Twenty-four hours after transfection, the cells were subjected to immunofluorescence or nuclear and cytoplasmic fractionation.

**Cell-cycle synchronization**

HeLa cells were synchronized at the G1/S phase transition by double thymidine blocks. Briefly, cells were treated with 2.5 mM thymidine for 16 h, released for 10 h, and incubated again for an additional 14 h in thymidine-containing medium. Cells blocked in the G1/S boundary were released for 8 h in complete medium and subsequently treated with 0.25 μg/ml nocodazole for an additional 6 h to obtain prometaphase cells. Prometaphase cells were then released and treated with 5 mM MG132 for 2 h to obtain metaphase cells. Quiescent G0 to G1 cells were harvested by serum starvation for 72 h and release as described previously (41).

**IFM**

HeLa, HEK 293T, MCF-7, or WI-38 cells grown on coverslips in Petri dishes were fixed with 4% paraformaldehyde for 15 min at room temperature and permeabilized with 0.2% Triton X-100 in PBS, pH 7.4, on ice for 2 min. The cells were sealed with Mowiol containing 1 μg/ml DAPI. For indirect immunofluorescence, cells were incubated with the respective primary antibodies for 1 h at 37 °C. After washing, the cells were successively incubated with the secondary antibodies conjugated with TRITC or FITC for 1 h at room temperature, and the cells were sealed with Mowiol containing 1 μg/ml DAPI.

**Nuclear and cytoplasmic cell fractionation**

HeLa cells were washed twice with cold low-penetration buffer (20 mM HEPES (pH 7.8), 5 mM potassium acetate, 0.5 mM MgCl2, 0.5 mM DTT, 1 mM PMSF, 20 units/ml aprotinin, 5 mg/ml leupeptin, and 0.4 mM NaF). After incubation on ice for 15 min, cells were collected with a scraper. 50 μl of cell suspension mix was incubated with 50 μl of 2× loading buffer (12.5 mM Tris, pH 6.8, 2% SDS, 2 mM DTT, 20% glycerine, 5% β-mercaptoethanol, and 0.2% bromphenol blue) to obtain whole-cell sample. The rest of the cell suspension was added to a Dounce homogenizer. Nuclei were pelleted at 8,000 rpm for 5 min, and the supernatant (cytoplasmic extracts) was centrifuged at 13,200 rpm for 5 min. Nuclei were washed three times with 1 ml of PBS and suspended in 50 μl of sample buffer to obtain nuclear sample.

**Cell line generation and cell culture**

Tet-on cell lines were generated according to the supplier’s instructions (plasmids and drugs from Invitrogen) as we described previously (36). HeLa cells were transfected with pcDNA6/TR, and cultured in the presence of 10 μg/ml blasticidin for 2 weeks. Positive clones were selected for further transfection of pcDNA4/TO-GFP-tagged wild-type cyclin D1 and mutant cyclin D1K112E/K114E and cultured in the presence of 200 μg/ml zeocin for an additional 2 weeks. Again, positive clones were selected and cultured for use. GFP-tagged wild-type cyclin D1 and mutant cyclin D1K112E/K114E were expressed when 1 μg/ml tetracycline was added.

**Living cell imaging and microscopy**

HeLa cells were cultured on dishes with glass bottoms. The dynamics of the targeting proteins during the cell cycle were recorded by spinning disc microscopy with a heated culture chamber (37 °C, 5% CO2) and a Nikon ×60 (numeric aperture 1.43) Plan-apo oil objective.

**Statistical analyses**

HeLa cells transfected with double-stranded siRNA and RFP-H2B for 72 h were immunostained with antibody against
CDK4 accumulation initiates the new cell cycle

cyclin D1. The cells with obviously less cyclin D1 in the nucleus were counted. Each experiment was repeated three times, and error bars in Fig. 3 represent S.D.

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