



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

Received for publication, December 19, 2016, and in revised form, April 26, 2017. Published, Papers in Press, April 26, 2017, DOI 10.1074/jbc.M116.773226

Huabo Chen^{‡1}, Xiaowei Xu^{‡1}, Guopeng Wang^{‡1}, Boyan Zhang[‡], Gang Wang[‡], Guangwei Xin[‡], Junjun Liu[§], Qing Jiang^{‡2}, Hongyin Zhang[¶], and Chuanmao Zhang^{‡3}

From the [‡]Ministry of Education Key Laboratory of Cell Proliferation and Differentiation and the State Key Laboratory of Membrane Biology, College of Life Sciences, Peking University, Beijing 100871, China, the [¶]Cancer Research Center, Peking University Hospital, Peking University, Beijing 100871, China, and the [§]Department of Biological Sciences, California State Polytechnic University, Pomona, California 91768

Edited by Xiao-Fan Wang

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 CDK4 to initiate a new cell cycle in HeLa cells.

CDK4 regulates G₁/S phase transition in mammalian cells mainly by phosphorylating retinoblastoma family proteins (RBs)⁴ to release RB-bound E2F family transcription factors (1–3). D-type cyclins, including D1, D2, and D3, of which cyclin D1 has been extensively studied, are essential for the activation of CDK4 (1, 4). Dysfunction of CDK4 causes cells to be arrested at the G₁ phase in ovarian cancer cells, whereas elevation of

CDK4 activity results in overcoming of the G₁ phase arrest and promotes proliferation of the cells (5–7). CDK4 protein is nuclear, whereas cyclin D1 may be cytoplasmic and relocated to the nucleus for CDK4 kinase activation (8–13). In G₀ phase cells arrested by serum starvation, the protein level of cyclin D1 is low but elevates in response to growth factor stimulation (14). Degradation or cytoplasmic retardation of cyclin D1 reduces CDK4 activity (11, 14–16). Nuclear accumulation of cyclin D1 also regulates cell growth, migration, differentiation, and development in addition to cell cycle control (17–19). Overexpression of cyclin D1 is also linked with tumorigenesis (12, 20–22).

Although tremendous progress has been made in investigating the function of CDK4 during the G₁/S phase transition, many of those results were obtained from studies based on G₀ cells. In contrast, less attention has been given to the regulatory mechanisms controlling the fluctuation of CDK4 and cyclin D1 proteins and the activation of CDK4 in cycling cells (23), although deciphering these mechanisms is fundamentally important. In this work, we studied the mechanism regulating CDK4 activation in HeLa cells.

Results

CDK4 protein is degraded by APC/C during metaphase-anaphase (M-A) transition and reaccumulates in subsequent G₁ phase

To investigate the protein levels of CDK4 and cyclin D1 during the M-G₁ phase transition in cycling cells, we first performed immunofluorescence microscopy (IFM) in HeLa cells using specific antibodies. Surprisingly, we found that the protein level of CDK4 was decreased during the M-A transition, whereas that of cyclin D1 was unchanged (Fig. 1A). This result was confirmed in WI-38 and 3T3 cells (supplemental Fig. S1A). Then we reseeded mitotic HeLa cells collected by shake-off (Fig. 1B) and analyzed the protein levels of both cyclin D1 and CDK4 by Western blotting. We also observed that cyclin D1 protein level was stable during the G₂/M/G₁ phases (Fig. 1C), as reported previously (24). In contrast, CDK4 protein level was decreased, and the kinase activity of CDK4 was also impaired, as indicated by unphosphorylation of RB Ser-780 (*pS780-RB*), a preferential phosphorylation site for CDK4 (Fig. 1C) (25). We further analyzed synchronized HeLa cells at the G₂/M border

This work was supported by National Natural Science Foundation of China (NSFC) Grants 31371365, 31520103906, and 31430051 and Ministry of Science and Technology of China Grants 2016YFA0100501 and 2016YFA0500201. The authors declare that they have no conflicts of interest with the contents of this article.

This article contains supplemental Table 1, Figs. S1–S3, and Movies 1–7.

¹ These authors contributed equally to this work.

² To whom correspondence may be addressed. Tel.: 86-10-62757173; E-mail: jiangqing@pku.edu.cn.

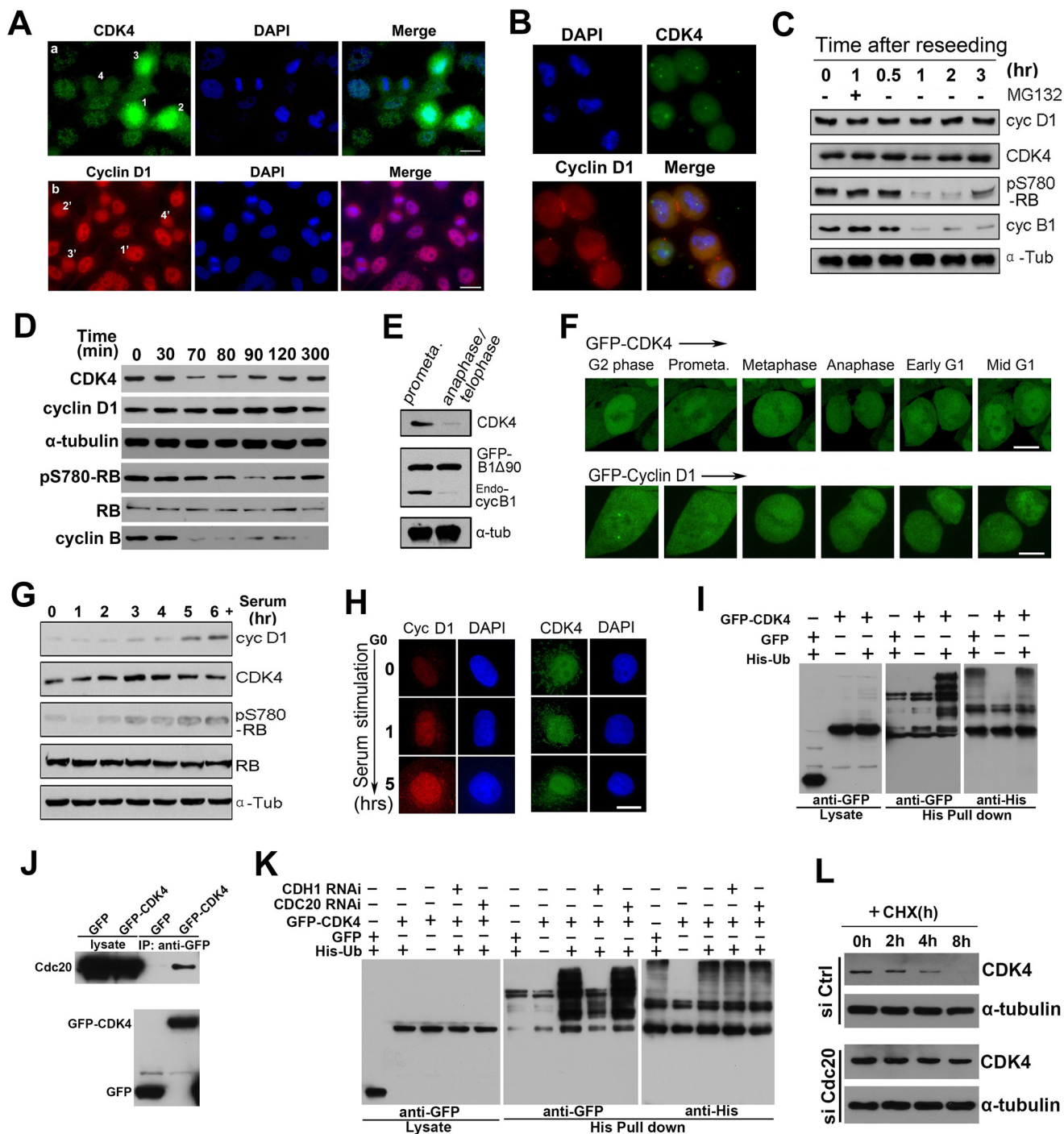
³ To whom correspondence may be addressed. Tel.: 86-10-62757173; E-mail: zhangcm@pku.edu.cn.

⁴ The abbreviations used are: RB, retinoblastoma protein; 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.

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-



sition, recovered, and was rapidly imported into the nucleus during the G₁ phase, whereas cyclin D1 was stable in the same period in all of the filmed cells (Fig. 1F and supplemental Movies 1 and 2). In contrast, the protein levels of CDK4 and cyclin D1 and the kinase activity of CDK4 in quiescent-like cells were low but quickly increased upon serum stimulation (Fig. 1 (G and H) and supplemental Fig. S1D). Moreover, in an *in vivo* ubiquitination assay, by co-expressing His-ubiquitin and GFP-CDK4 in HeLa cells followed by synchronization of the cells at the M-A transition, we also detected multiple ladder-like bands above the main band of GFP-CDK4 (Fig. 1I), indicating that CDK4 was ubiquitinated during the M-A transition. To investigate which E3 ubiquitin ligase was responsible for the ubiquitination of CDK4, we found that CDK4 interacted with Cdc20, one of the APC/C activators (Fig. 1J). By knocking down Cdc20 and Cdh1 separately, two activators for APC/C activity (26), we found that Cdc20-activated APC/C was responsible for the ubiquitination of CDK4 (Fig. 1K and supplemental Fig. S1E). To confirm this result, by treating Cdc20-knockdown cells with cycloheximide (CHX), we measured CDK4 degradation kinetics. The results showed that, whereas the protein level of CDK4 was decreased and eventually disappeared from 4 to 8 h with the treatment of CHX in control cells, the protein level of CDK4 in Cdc20-knockdown cells treated with CHX was stable (Fig. 1L), indicating that Cdc20-activated APC/C did mediate the ubiquitination and degradation of CDK4. Taken together, these data demonstrate that CDK4 protein is degraded by APC/C during the M-A transition and reaccumulates in the following G₁ phase, whereas cyclin D1 protein is stable during the M-G₁ transition in HeLa cells.

Slow nuclear import and fast export retain cyclin D1 in the cytoplasm in the absence of CDK4

Next, we investigated the subcellular localizations of CDK4 and cyclin D1 in HeLa cells. Through IFM using specific antibodies, we observed that cyclin D1 was extensively localized in the cytoplasm of the paired early G₁ cells, whereas CDK4, although at a very low level, was located in the nucleus (Fig. 2A). Along with the progress of the cell cycle, CDK4 protein was accumulated gradually and imported into the nucleus. Coincidentally, cyclin D1 protein was imported into the nucleus and co-localized with CDK4 (Fig. 2A). Through transient expres-

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 D1^{T286A} 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 D1^{T286A}-GFP (NLS-D1^{T286A}-GFP), we found that all of the NLS-D1^{T286A}-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 local-

Figure 1. CDK4 is degraded by APC/C-Cdc20 during M-A transition and reaccumulates from early to mid-G₁ phase. A, HeLa cells were immunostained with anti CDK4 and cyclin D1 antibodies. The early prometaphase, late prometaphase, metaphase, and anaphase/telophase cells are labeled 1, 2, 3, and 4, respectively, in a, whereas the G₂, metaphase, anaphase, and telophase cells are labeled with 1', 2', 3', and 4' in b. B, HeLa cells were synchronized at the G₁/S transition and were then released into fresh culture medium for 10 h. Mitotic cells that were in prometaphase were shaken off, reseeded, and stained with DAPI. C, the cell lysate of the reseeded mitotic and released cells was subjected to Western blotting. D, HeLa cells were arrested at the G₂/M border using RO3306 (9 μM) and released. The lysates from these cells were analyzed by Western blotting. E, HeLa cells transfected with GFP-cyclin B1Δ90 were arrested at prometaphase and anaphase/telophase and analyzed by Western blotting. F, HeLa cells expressing GFP-CDK4 and GFP-cyclin D1 were processed for time-lapse microscopy. More than 20 cells were filmed in each of three repeated experiments, and representative movie images are shown. G, HeLa cells arrested at the quiescent-like phase were released into G₁ phase by serum at the indicated time points. The cell lysates were analyzed by Western blotting. H, quiescent-like phase to G₁ phase cells were fixed and immunostained. DNA was stained by DAPI. 200 cells were randomly counted at each time point of the three repeated experiments. Representative images are shown. I, HeLa cells were co-transfected with His-ubiquitin and GFP or GFP-CDK4 or were transfected with GFP-CDK4 alone. Transfected cells were synchronized at the G₂/M border using RO3306 and then released to M-A transition. Lysate of cells was subjected to a pull-down assay using nickel-Sepharose beads. The isolated proteins were analyzed by Western blotting. J, HeLa cells were transfected with GFP-CDK4, and the total cell extract was used for immunoprecipitation with a GFP antibody and probed with Cdc20 and GFP antibodies. K, HeLa cells were separately transfected with negative control (NC), Cdc20, or Cdh1 RNAi double-stranded RNA followed by co-transfection with His-ubiquitin and GFP-CDK4. As negative controls, HeLa cells transfected with NC RNAi double-stranded RNA were transfected with GFP-CDK4 or co-transfected with His-ubiquitin and GFP. Transfected cells were synchronized at the G₂/M border using RO3306 and then released to M-A transition. Lysates of cells were subjected to a pull-down assay using nickel-Sepharose beads followed by Western blotting using anti-His and -GFP antibodies. L, HeLa cells were separately transfected with negative control or Cdc20 RNAi double-stranded RNA for 72 h. The cells were then treated with or without 30 μg/ml CHX for the indicated number of hours, lysed, and probed with CDK4 and α-tubulin antibodies. Scale bars, 10 μm.

CDK4 accumulation initiates the new cell cycle

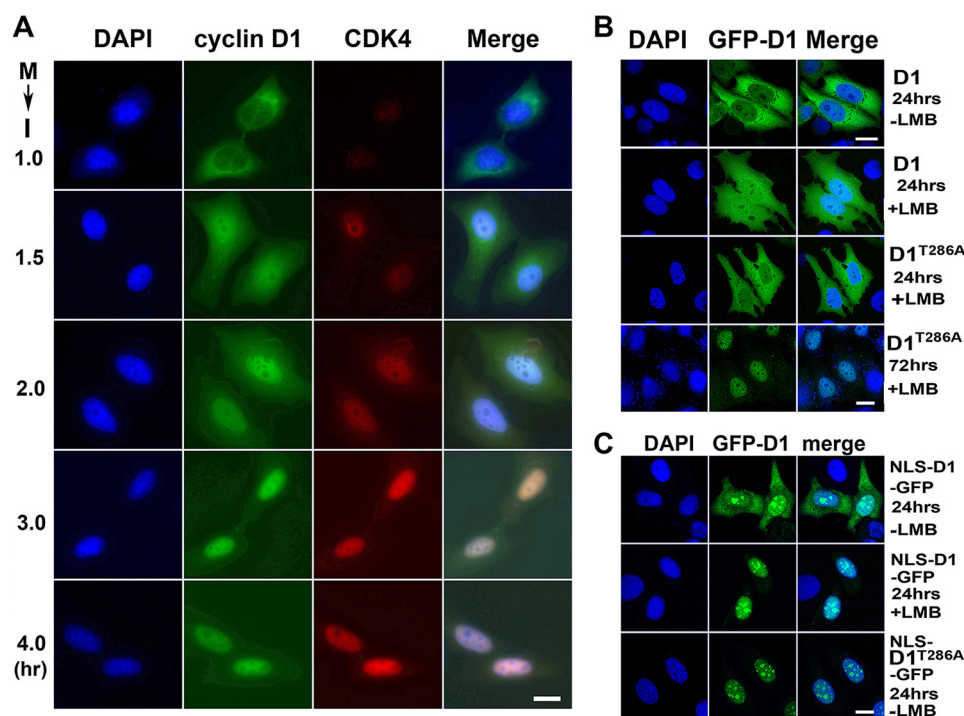


Figure 2. Slow nuclear import and fast export retain cyclin D1 in the cytoplasm in the absence of CDK4. *A*, mitosis (*M*)-arrested HeLa cells were released to interphase (*I*) at the indicated time points. Cyclin D1 and CDK4 were immunostained. The DNA was stained with DAPI. 200 pairs of the cells were randomly counted at each time point of three repeated experiments. Representative images are shown. *B*, HeLa cells were transfected with the wild-type GFP-cyclin D1 (*GFP-D1*) or mutant GFP-cyclin D1^{T286A} (*GFP-D1*^{T286A}) for 24 or 72 h. The cells were then treated with or without 10 ng/ml LMB for 4 h. *C*, HeLa cells transfected with the wild-type NLS-cyclin D1-GFP (*NLS-D1-GFP*) or mutant (*NLS-D1*^{T286A}-*GFP*) were treated with or without 10 ng/ml LMB for 4 h. More than 200 cells each of three experiments were randomly counted. Representative images are shown. Scale bars, 10 μ m.

ization of cyclin D1 was significantly decreased (Fig. 3, *B–D*). 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. 4*A*), 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. 4*B*). We also constructed another two mutants, CDK4^{K22A} and CDK4^{3A} (K22A/

R24A/D25A), that bind to cyclin D1 weakly (Fig. 4*C*) (33) and co-expressed each of them with cyclin D1. We observed that CDK4^{K22A}, but not CDK4^{3A}, was able to partially target cyclin D1 to the nucleus (Fig. 4*D*).

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 D1^{K112E/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^{K112E/K114E} did not bind to CDK4, whereas cyclin D1^{K114E} retained about 30% of binding capability with CDK4 compared with the wild type (Fig. 4*E*). Through IFM, we observed that only GFP-cyclin D1^{K114E} was co-localized with CDK4 in the nucleus (Fig. 4*F*). 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^{K112E/K114E} were retained in cytoplasm without co-localization with NLS-NoLS-CDK4-Myc (Fig. 4*G*). 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

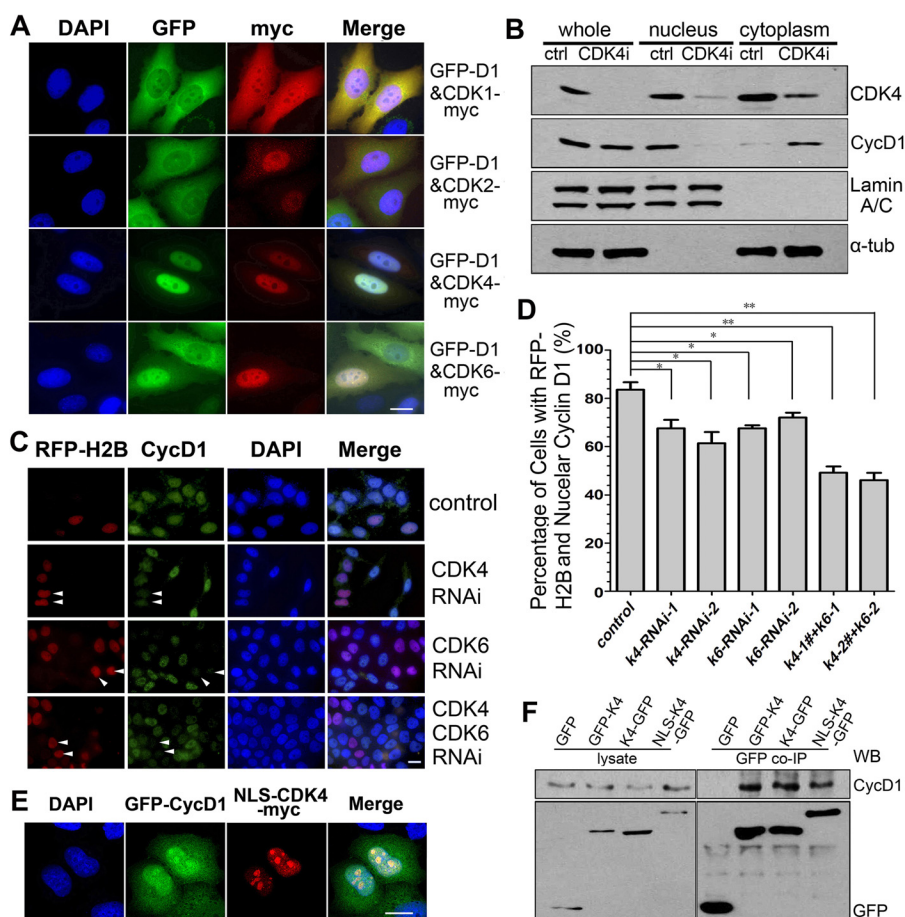


Figure 3. CDK4 shuttles between the nucleus and the cytoplasm and promotes the nuclear accumulation of cyclin D1. *A*, HeLa cells were co-transfected with GFP- or Myc-tagged cyclin D1 and CDK4 for 24 h. Myc-tagged protein was immunostained. 200 cells were randomly counted in each of three repeated experiments. 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 μ m) or a co-immunoprecipitation assay (*F*).

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. S2*F*), 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

P16^{INK4} disrupts the interaction by competing with cyclin D1 for binding with CDK4 and p21^{CIP1}/p27^{KIP1} directly binds and stabilizes cyclin D1-CDK4 complex (37, 38), we investigated the influence of P16^{INK4} and p21^{CIP1}/p27^{KIP1} 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-p16^{INK4}. In contrast, the stability of the cyclin D1-CDK4 complex was strengthened in the cells expressing GFP-p21^{CIP1} or -p27^{KIP1} (Fig. 5*A*). IFM showed that cyclin D1 was mostly localized in the cytoplasm in GFP-p16^{INK4}-expressing cells and in the nucleus without GFP-p16^{INK4} expression (Fig. 5*B*). On the contrary, cyclin D1 was localized exclusively in the nucleus in GFP-p21^{CIP1}- or GFP-p27^{KIP1}-expressing cells (Fig. 5*B*). Western blot analysis also confirmed that cyclin D1 was mainly cytoplasmic in p16^{INK4}-expressing cells (Fig. 5*C*). 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

CDK4 accumulation initiates the new cell cycle

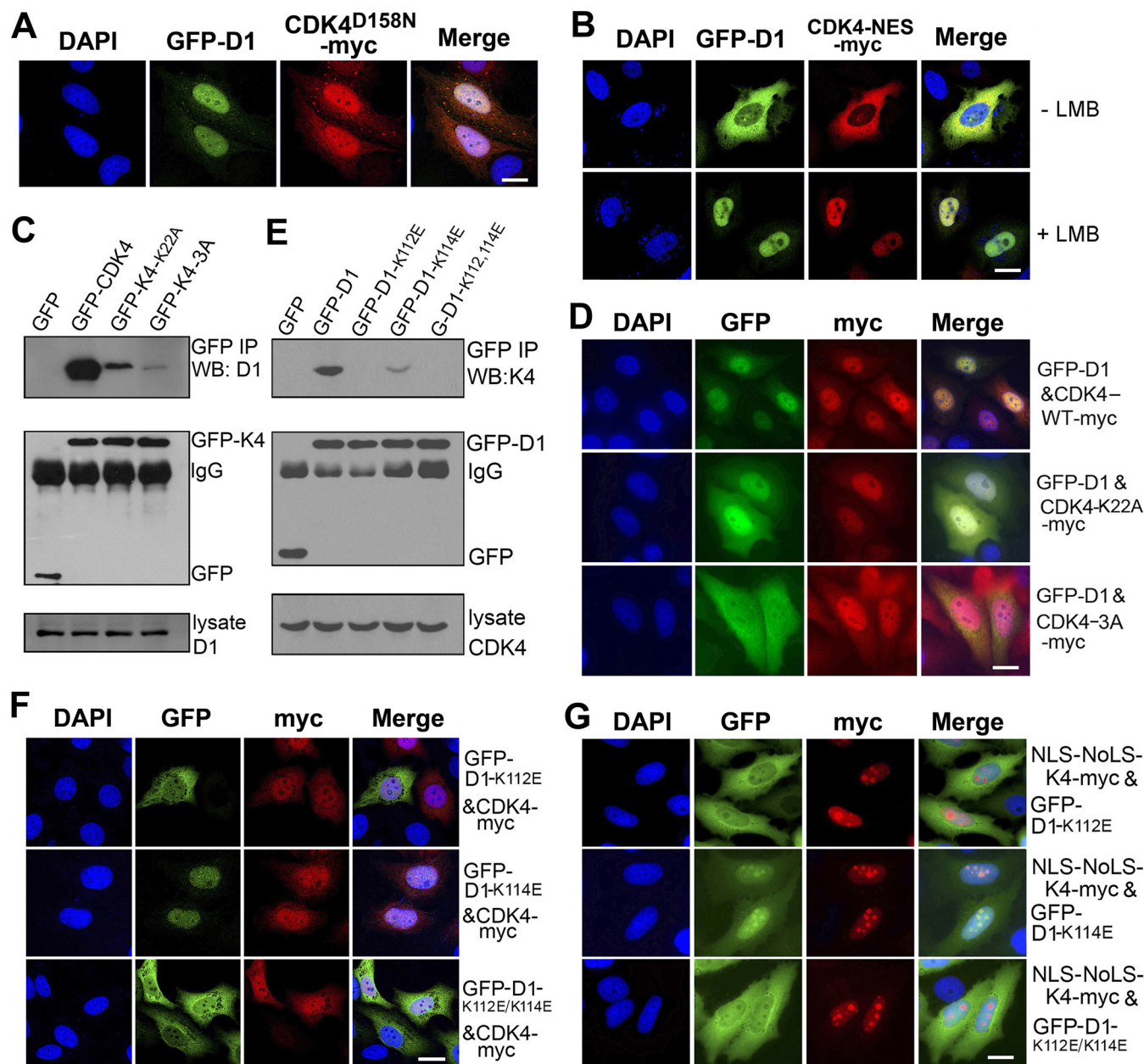


Figure 4. Direct binding is required for CDK4-mediated nuclear import of cyclin D1. *A*, HeLa cells co-transfected with GFP-cyclin D1 (GFP-D1) and CDK4^{K158N}-Myc plasmids for 24 h were visualized by IFM. *B*, asynchronous HeLa cells co-transfected with wild-type GFP-cyclin D1 (GFP-D1) and CDK4-NES-Myc plasmids for 24 h and treated without or with 10 ng/ml LMB for 4 h were visualized by IFM. *C*, HeLa cells were transfected with GFP-CDK4, and mutant plasmids for 22 h. The cell lysates were immunoprecipitated with a GFP antibody, and the co-immunoprecipitated cyclin D1 was detected. *D*, asynchronous HeLa cells co-transfected with GFP-cyclin D1 and CDK4-Myc mutant for 24 h were visualized by IFM. *E*, HeLa cells transfected with the wild-type GFP-cyclin D1 or mutant plasmid for 24 h followed by a co-immunoprecipitation assay using an anti-GFP antibody. The co-immunoprecipitated CDK4 with cyclin D1 or mutants was detected. *F*, HeLa cells co-transfected with CDK4-Myc and wild-type GFP-cyclin D1 or mutant cyclin D1 for 24 h were visualized by IFM. *G*, HeLa cells transfected with NLS-NoLS-CDK4-Myc and GFP-cyclin D1 mutant for 24 h were visualized by IFM. The DNA in all microscopy images was stained with DAPI. More than 200 cells in each of three repeated experiments were randomly counted. Representative images are shown. Scale bars, 10 μ m.

(supplemental Fig. S3A). 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 CDK4/6 knockdown with tet-on expression of wild-type GFP-cyclin D1 or mutant GFP-cyclin D1^{K112E/K114E}. Through time-

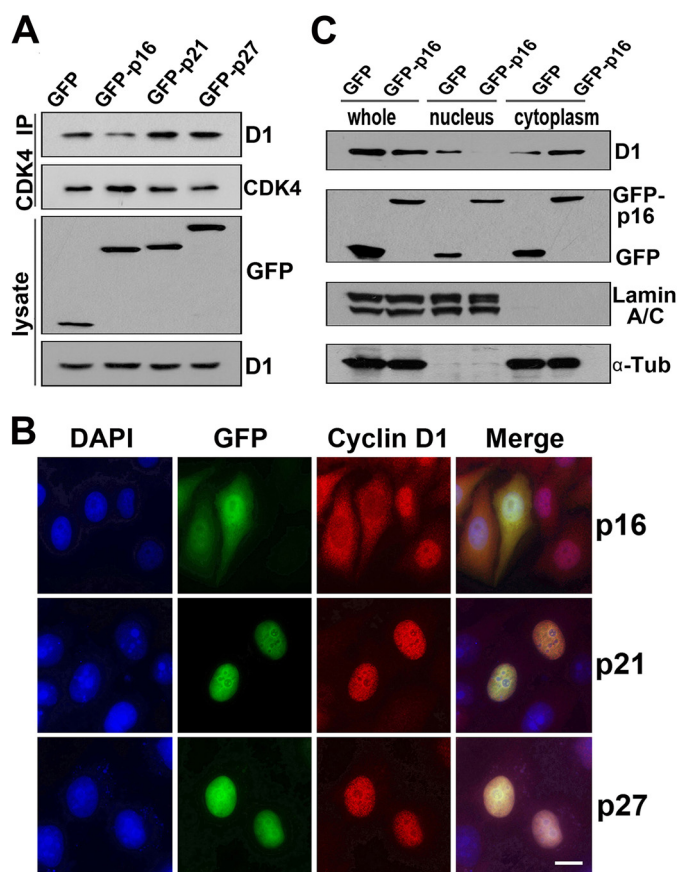


Figure 5. Stable binding of D-type cyclins with CDK4 enhances its nuclear localization, and CDK4/CDK6 knockdown results in cell-cycle arrest at the G₁ phase. A, HeLa cells were transfected with GFP, GFP-p16, GFP-p21, or GFP-p27 for 24 h. The cell lysates were immunoprecipitated with anti-CDK4 antibody. The co-immunoprecipitated cyclin D1 was detected by Western blotting. B, HeLa cells transfected with GFP-tagged p16, p21, or p27 for 24 h were visualized by IFM. The DNA was stained with DAPI. More than 200 cells in each of three repeated experiments were randomly counted. Representative images are shown. C, HeLa cells were transfected with GFP or GFP-tagged p16 for 24 h. The nuclear and cytoplasmic extracts were analyzed by Western blotting. Scale bars, 10 μ m.

lapse microscopy, we confirmed that only a small portion of wild-type cyclin D1 was able to enter the nucleus, whereas the total proteins of mutant cyclin D1^{K112E/K114E} were restrained in the cytoplasm in CDK4/CDK6-knockdown cells (Fig. 6C and supplemental Movies 6 and 7). We also examined the phosphorylation status of RB Ser-780 and found that CDK4 knockdown resulted in the accumulation of unphosphorylated RB at Ser-780 (supplemental Fig. S3B), indicating that the cells were arrested in the G₁ phase (39). Taken together, these results demonstrate that, to initiate a new cell cycle, the post-mitotic HeLa cells need to accumulate CDK4 to gain the CDK4 kinase activity.

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/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 μ g/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 primer is complementary to the 3'-end of the cyclin D1 sequence containing a Sall site but having a deleted cyclin D1 termination codon. The PCR product was gel-puri-

CDK4 accumulation initiates the new cell cycle

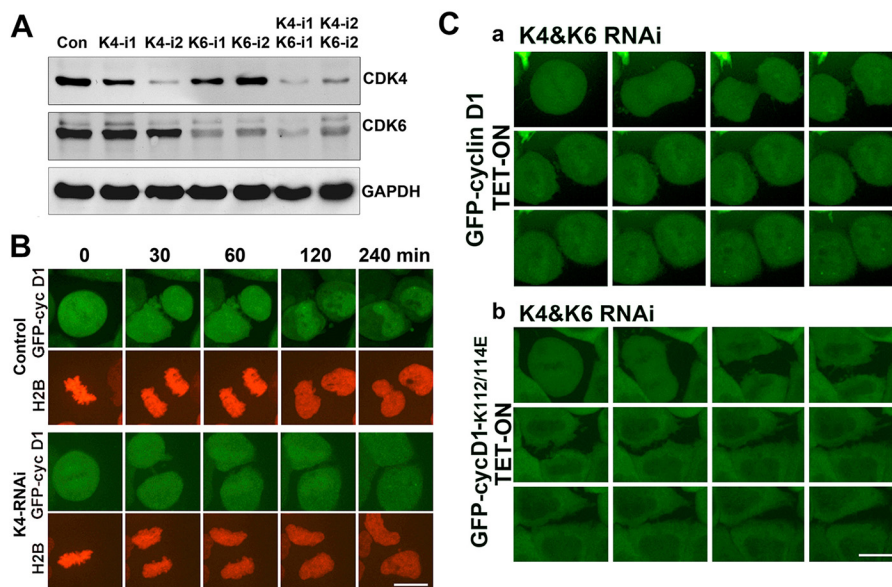


Figure 6. CDK4/CDK6 knockdown results in cell-cycle arrest at G₁ phase. A, HeLa cells were transfected with specific siRNA for 72 h to knock down CDK4 and CDK6 individually or simultaneously. Cell lysates were analyzed by Western blotting. B, GFP-cyclin D1 tet-on cells were transfected with specific double-stranded CDK4-knockdown siRNA and RFP-H2B for 64 h, treated with tetracycline for 8 h to induce GFP-cyclin D1 expression, and viewed by time-lapse microscopy. C, GFP-cyclin D1 and GFP-cyclin D1^{K112E/K114E} tet-on cell lines were transfected with specific CDK4/CDK6 knockdown siRNA for 64 h, treated with tetracycline for 8 h to induce GFP-cyclin D1 (a) and GFP-cyclin D1^{K112E/K114E} (b) expression, and viewed by time-lapse microscopy. More than 20 cells were filmed in each of three repeated experiments (a and b). Representative movie images are shown. Scale bars, 10 μ m.

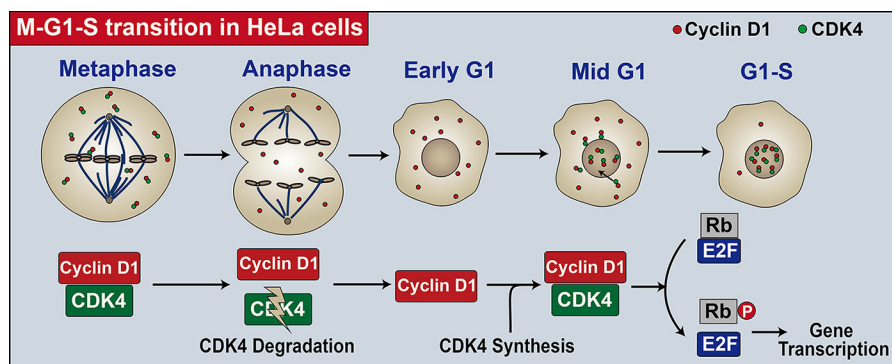


Figure 7. A CDK4 reaccumulation-dependent model elucidating the regulation of a new cell-cycle initiation in HeLa cells. In HeLa cells, CDK4 is degraded during the M-A transition and reaccumulates in the following early G₁ phase. The newly synthesized CDK4 shuttles between the nucleus and the cytoplasm and facilitates the nuclear transport of cyclin D1 to activate the kinase activity of CDK4. The activated nuclear CDK4 kinase then phosphorylates Rb to release RB-bound E2F family transcription factors for the new cell-cycle initiation.

fied and digested with BglII and Sall restriction endonucleases before ligation to pcDNA3.1myc vector that had been digested with BamHI and XhoI restriction endonucleases and pEGFP-C1 vector that had been digested with BglII and Sall 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 μ g/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; 1:50) and anti-c-Myc (M4439, Sigma; 1:200) and rabbit anti-CDK4 (sc-260, Santa Cruz Biotechnology; 1:50) antibodies were used. Anti-lamin A/C antibody used for Western blotting (1:1,000) was generated by immunizing a rabbit with bacterially expressed recombinant lamin A/C. For West-

ern blotting, mouse anti-GFP (M048-3, MBL; 1:3,000), 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- α -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 μ g/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'-GCAGCACUCUUAUCUACAU-3' and 5'-GCAGAGAU-GUUUCGUCGAA-3'. Two oligomers of CDK6-specific siRNA were synthesized with the following sequences: 5'-GCAGAAAUGUUUCGUAGAA-3' and 5'-GCAAAGACC-UACUUCUGAA-3'. 72 h 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 3T3 cells were achieved 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 PMSE, 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 blastidicin for 2 weeks. Positive clones were selected for further transfection of pcDNA4/TO-GFP-tagged wild-type cyclin D1 and mutant cyclin D1^{K112E/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 D1^{K112E/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.

Author contributions—C. Z., Q. J., H. C., and X. X. conceptualized the study; H. C., X. X., G. W., B. Y., G. W., and G. X. performed the experiments; J. J. L., Q. J., and H. Y. Z. discussed the project and analyzed the data. C. Z., Q. J., H. C., and X. X. wrote the manuscript; all authors approved the final version of the manuscript.

Acknowledgments—We thank the other members of the Zhang laboratory for helpful comments and critical discussion on this work.

References

- Murray, A. W. (2004) Recycling the cell cycle: cyclins revisited. *Cell* **116**, 221–234
- Nevins, J. R. (2001) The Rb/E2F pathway and cancer. *Hum. Mol. Genet.* **10**, 699–703
- Dyson, N. (1998) The regulation of E2F by pRB-family proteins. *Genes Dev.* **12**, 2245–2262
- Massagué, J. (2004) G₁ cell-cycle control and cancer. *Nature* **432**, 298–306
- Masamha, C. P., and Benbrook, D. M. (2009) Cyclin D1 degradation is sufficient to induce G₁ cell cycle arrest despite constitutive expression of cyclin E2 in ovarian cancer cells. *Cancer Res.* **69**, 6565–6572
- Weng, L. P., Brown, J. L., and Eng, C. (2001) PTEN coordinates G₁ arrest by down-regulating cyclin D1 via its protein phosphatase activity and up-regulating p27 via its lipid phosphatase activity in a breast cancer model. *Hum. Mol. Genet.* **10**, 599–604
- Retzer-Lidl, M., Schmid, R. M., and Schneider, G. (2007) Inhibition of CDK4 impairs proliferation of pancreatic cancer cells and sensitizes towards TRAIL-induced apoptosis via downregulation of survivin. *Int. J. Cancer* **121**, 66–75
- Alt, J. R., Gladden, A. B., and Diehl, J. A. (2002) p21(Cip1) Promotes cyclin D1 nuclear accumulation via direct inhibition of nuclear export. *J. Biol. Chem.* **277**, 8517–8523
- Musgrove, E. A., Caldon, C. E., Barraclough, J., Stone, A., and Sutherland, R. L. (2011) Cyclin D as a therapeutic target in cancer. *Nat. Rev. Cancer* **11**, 558–572
- Diehl, J. A., and Sherr, C. J. (1997) A dominant-negative cyclin D1 mutant prevents nuclear import of cyclin-dependent kinase 4 (CDK4) and its phosphorylation by CDK-activating kinase. *Mol. Cell. Biol.* **17**, 7362–7374
- Alt, J. R., Cleveland, J. L., Hannink, M., and Diehl, J. A. (2000) Phosphorylation-dependent regulation of cyclin D1 nuclear export and cyclin D1-dependent cellular transformation. *Genes Dev.* **14**, 3102–3114
- Dhar, K. K., Branigan, K., Parkes, J., Howells, R. E., Hand, P., Musgrove, C., Strange, R. C., Fryer, A. A., Redman, C. W., and Hoban, P. R. (1999) Expression and subcellular localization of cyclin D1 protein in epithelial ovarian tumour cells. *Br. J. Cancer* **81**, 1174–1181
- Holland, T. A., Elder, J., McCloud, J. M., Hall, C., Deakin, M., Fryer, A. A., Elder, J. B., and Hoban, P. R. (2001) Subcellular localisation of cyclin D1 protein in colorectal tumours is associated with p21(WAF1/CIP1) expression and correlates with patient survival. *Int. J. Cancer* **95**, 302–306
- Aggarwal, P., Vaites, L. P., Kim, J. K., Mellert, H., Gurung, B., Nakagawa, H., Herlyn, M., Hua, X., Rustgi, A. K., McMahon, S. B., and Diehl, J. A. (2010) Nuclear cyclin D1/CDK4 kinase regulates CUL4 expression and triggers neoplastic growth via activation of the PRMT5 methyltransferase. *Cancer Cell* **18**, 329–340
- Aggarwal, P., Lessie, M. D., Lin, D. I., Pontano, L., Gladden, A. B., Nuskey, B., Goradia, A., Wasik, M. A., Klein-Szanto, A. J., Rustgi, A. K., Bassing, C. H., and Diehl, J. A. (2007) Nuclear accumulation of cyclin D1 during S phase inhibits Cul4-dependent Cdt1 proteolysis and triggers p53-dependent DNA rereplication. *Genes Dev.* **21**, 2908–2922
- Barbash, O., Egan, E., Pontano, L. L., Kosak, J., and Diehl, J. A. (2009) Lysine 269 is essential for cyclin D1 ubiquitylation by the SCF(Fbx4/ α B-crystallin) ligase and subsequent proteasome-dependent degradation. *Oncogene* **28**, 4317–4325
- Pestell, R. G., Albanese, C., Reutens, A. T., Segall, J. E., Lee, R. J., and Arnold, A. (1999) The cyclins and cyclin-dependent kinase inhibitors in hormonal regulation of proliferation and differentiation. *Endocr. Rev.* **20**, 501–534
- Fantl, V., Stamp, G., Andrews, A., Rosewell, I., and Dickson, C. (1995) Mice lacking cyclin D1 are small and show defects in eye and mammary gland development. *Genes Dev.* **9**, 2364–2372
- Sicinski, P., Donaher, J. L., Parker, S. B., Li, T., Fazeli, A., Gardner, H., Haslam, S. Z., Bronson, R. T., Elledge, S. J., and Weinberg, R. A. (1995) Cyclin D1 provides a link between development and oncogenesis in the retina and breast. *Cell* **82**, 621–630
- Alao, J. P. (2007) The regulation of cyclin D1 degradation: roles in cancer development and the potential for therapeutic invention. *Mol. Cancer* **6**, 24
- Yu, Q., Geng, Y., and Sicinski, P. (2001) Specific protection against breast cancers by cyclin D1 ablation. *Nature* **411**, 1017–1021
- Lu, F., Gladden, A. B., and Diehl, J. A. (2003) An alternatively spliced cyclin D1 isoform, cyclin D1b, is a nuclear oncogene. *Cancer Res.* **63**, 7056–7061
- Stacey, D. W. (2010) Three observations that have changed our understanding of cyclin D1 and p27Kip1 in cell cycle control. *Genes Cancer* **1**, 1189–1199
- Hitomi, M., and Stacey, D. (1999) Cyclin D1 production in cycling cells depends on ras in a cell-cycle-specific manner. *Curr. Biol.* **9**, 1075–1084
- Day, P. J., Cleasby, A., Tickle, I. J., O'Reilly, M., Coyle, J. E., Holding, F. P., McMenamin, R. L., Yon, J., Chopra, R., Lengauer, C., and Jhoti, H. (2009) Crystal structure of human CDK4 in complex with a D-type cyclin. *Proc. Natl. Acad. Sci. U.S.A.* **106**, 4166–4170
- Teixeira, L. K., and Reed, S. I. (2013) Ubiquitin ligases and cell cycle control. *Annu. Rev. Biochem.* **82**, 387–414
- Glutzer, M., Murray, A. W., and Kirschner, M. W. (1991) Cyclin is degraded by the ubiquitin pathway. *Nature* **349**, 132–138
- Murray, A. W., and Kirschner, M. W. (1989) Cyclin synthesis drives the early embryonic cell cycle. *Nature* **339**, 275–280
- Zur, A., and Brandeis, M. (2001) Securin degradation is mediated by fzy and fzr, and is required for complete chromatid separation but not for cytokinesis. *EMBO J.* **20**, 792–801
- Nishi, K., Yoshida, M., Fujiwara, D., Nishikawa, M., Horinouchi, S., and Beppu, T. (1994) Leptomycin B targets a regulatory cascade of crm1, a fission yeast nuclear protein, involved in control of higher order chromosome structure and gene expression. *J. Biol. Chem.* **269**, 6320–6324
- Diehl, J. A., Zindy, F., and Sherr, C. J. (1997) Inhibition of cyclin D1 phosphorylation on threonine-286 prevents its rapid degradation via the ubiquitin-proteasome pathway. *Genes Dev.* **11**, 957–972
- Liu, Q., Yu, J., Zhuo, X., Jiang, Q., and Zhang, C. (2010) Pericentrin contains five NESs and an NLS essential for its nucleocytoplasmic trafficking during the cell cycle. *Cell Res.* **20**, 948–962
- Coleman, K. G., Wautlet, B. S., Morrissey, D., Mulheron, J., Sedman, S. A., Brinkley, P., Price, S., and Webster, K. R. (1997) Identification of CDK4 sequences involved in cyclin D1 and p16 binding. *J. Biol. Chem.* **272**, 18869–18874
- Baker, G. L., Landis, M. W., and Hinds, P. W. (2005) Multiple functions of D-type cyclins can antagonize pRb-mediated suppression of proliferation. *Cell Cycle* **4**, 330–338
- Li, Z., Jiao, X., Wang, C., Ju, X., Lu, Y., Yuan, L., Lisanti, M. P., Katiyar, S., and Pestell, R. G. (2006) Cyclin D1 induction of cellular migration requires p27(KIP1). *Cancer Res.* **66**, 9986–9994
- Fu, J., Bian, M., Liu, J., Jiang, Q., and Zhang, C. (2009) A single amino acid change converts Aurora-A into Aurora-B-like kinase in terms of partner specificity and cellular function. *Proc. Natl. Acad. Sci. U.S.A.* **106**, 6939–6944
- LaBaer, J., Garrett, M. D., Stevenson, L. F., Slingerland, J. M., Sandhu, C., Chou, H. S., Fattaey, A., and Harlow, E. (1997) New functional

- activities for the p21 family of CDK inhibitors. *Genes Dev.* **11**, 847–862
38. Bagui, T. K., Mohapatra, S., Haura, E., and Pledger, W. J. (2003) P27Kip1 and p21Cip1 are not required for the formation of active D cyclin-cdk4 complexes. *Mol. Cell. Biol.* **23**, 7285–7290
39. Fry, D. W., Bedford, D. C., Harvey, P. H., Fritsch, A., Keller, P. R., Wu, Z., Dobrusin, E., Leopold, W. R., Fattaey, A., and Garrett, M. D. (2001) Cell cycle and biochemical effects of PD 0183812. A potent inhibitor of the cyclin D-dependent kinases CDK4 and CDK6. *J. Biol. Chem.* **276**, 16617–16623
40. Baldin, V., Lukas, J., Marcote, M. J., Pagano, M., and Draetta, G. (1993) Cyclin D1 is a nuclear protein required for cell cycle progression in G1. *Genes Dev.* **7**, 812–821
41. Matsushime, H., Quelle, D. E., Shurtleff, S. A., Shibuya, M., Sherr, C. J., and Kato, J. Y. (1994) D-type cyclin-dependent kinase activity in mammalian cells. *Mol. Cell. Biol.* **14**, 2066–2076