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Human Cdc14A Phosphatase Modulates the G2/M Transition through Cdc25A and Cdc25B*[S]

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The Cdc14 family of serine-threonine phosphatases antagonizes CDK activity by reversing CDK-dependent phosphorylation events. It is well established that the yeast members of this family bring about the M/G1 transition. Budding yeast Cdc14 is essential for CDK inactivation at the end of mitosis and fission yeast Cdc14 homologue Flp1/Clp1 down-regulates Cdc25 to ensure the inactivation of mitotic CDK complexes to trigger cell division. However, the functions of human Cdc14 homologues remain poorly understood. Here we have tested the hypothesis that Cdc14A might regulate Cdc25 mitotic inducers in human cells. We found that increasing levels of Cdc14A delay entry into mitosis by inhibiting Cdk1-cyclin B1 activity. By contrast, lowering the levels of Cdc14A accelerates mitotic entry. Biochemical analyses revealed that Cdc14A acts through key Cdk1-cyclin B1 regulators. We observed that Cdc14A directly bound to and dephosphorylated Cdc25B, inhibiting its catalytic activity. Cdc14A also regulated the activity of Cdc25A at the G2/M transition. Our results indicate that Cdc14A phosphatase prevents premature activation of Cdk1 regulating Cdc25A and Cdc25B at the entry into mitosis.

In eukaryotes, periodic fluctuations in the activity of cyclin-dependent kinases (CDKs)[4] govern the cell division cycle by controlling the precise timing and spatial distribution of phosphorylation events, essential for cell duplication and the maintenance of genomic stability (1–3). Different Cdk-cyclin complexes form at specific cell-cycle stages and initiate the events of the S and M phases. Thus, Cdk1-cyclin B complexes are key regulators of mitosis in mammalian cells. Cdk1 kinase activity drives cells into mitosis and governs early mitotic events such as chromosome condensation, nuclear envelope breakdown, and the assembly of the mitotic spindle (1, 4–6). At metaphase, Cdk1-cyclin B complexes have completed their main functions and are inactivated for cells to exit from mitosis.

Like all CDKs, Cdk1 is subject to complex regulation (7). As the Cdk1-Cyclin B1 complexes accumulate in S and G2 phases of the cell cycle, Myt1 and Wee1 kinases keep Cdk1 inactive by phosphorylating two neighboring residues in its ATP-binding site: Thr-14 and Tyr-15 (8–10). At the G2/M transition, the activation of Cdk1 is achieved by Cdc25 phosphatases, which dephosphorylate those two residues (11). The exact trigger that activates the mitotic Cdk1-cyclin B1 complexes remains unknown, but it is clear that the balance between the inhibiting Wee1/Myt1 kinases and the activating Cdc25 phosphatases determines Cdk1 activity and hence mitotic entry.

In mammals, all three Cdc25 family members, Cdc25A, Cdc25B, and Cdc25C, identified have been implicated in the control of the G2/M transition through the activation of Cdk1-cyclin B1 complexes (12–15). The initial stimulation of Cdk1 has been attributed to Cdc25B (12), which acts on the centrosomes (13, 16, 17). Cdc25A and Cdc25C complete the activation of Cdk1-cyclin B1 as part of the autoamplification loop, in which phosphorylation by Cdk1 activates Cdc25C and stabilizes Cdc25A, thereby further increasing the phosphatase pool needed to reach the threshold of Cdk1 activity required to enter mitosis (14, 18–20).

The highly conserved Cdc14 family of dual-specificity phosphatases reverses CDK-dependent phosphorylation events (21–23). In the budding yeast *Saccharomyces cerevisiae*, Cdc14 is essential for CDK inactivation at the exit of mitosis (23, 24). Cdc14 is kept inactive in the nucleolus for most of the cell cycle (24, 25), but at the onset of anaphase it is released from its nucleolar confinement by the sequential action of the FEAR (fourteen early anaphase release) (26, 27) and MEN (mitotic exit network) (28) regulatory networks. Active Cdc14 promotes CDK inactivation by both promoting mitotic cyclin destruction and CDK inhibitor Sic1 accumulation (23, 24). In the fission yeast *Schizosaccharomyces pombe*, the Cdc14 homologue Flp1/Clp1 is released from the nucleolus earlier in mitosis than *S. cerevisiae* Cdc14 and it is not essential for mitotic exit, although it does play roles in Cdk1/Cdc2 inhibition and in the regulation of septum formation and cytokinesis (29–31). Flp1/Clp1 inhibits mitotic CDK activity by promoting the phosphorylated state of Cdk1 at Tyr-15 through, at least in part, the direct down-regulation of Cdc25 protein (29–31).

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[4] The abbreviations used are: CDK, cyclin-dependent kinase; PD, phosphatase-dead.

Cdc14 homologues have also been found in metazoans. In the nematode *Caenorhabditis elegans*, genetic analysis have suggested that Cdc14 probably promotes the stabilization of the CDK inhibitor CKI-1, contributing to the G1 arrest of specific precursor cells during larval development (32). In humans, there are two Cdc14 homologues, termed Cdc14A and Cdc14B (33), whose specific functions and substrates are still poorly understood. During interphase, Cdc14A localizes to the centrosome and Cdc14B to the nucleolus, but both are dispersed at the onset of mitosis (22, 34). Previous studies have shown that human Cdc14s are able to rescue *cdc14* yeast mutant phenotypes, suggesting some functional conservation among these phosphatases (33, 35). Cdc14A has been identified as a regulator of centrosome duplication and has been suggested to participate in the control of mitotic entry (34). In addition, Cdc14A dephosphorylates human Cdh1, promoting APC activation *in vitro* (36), suggesting that Cdc14A may also regulate Cdk1 inactivation in human cells. Moreover, human Cdc14A is a functional homologue of fission yeast Flp1/Clp1, being able to dephosphorylate *S. pombe* Cdc25p both *in vitro* and *in vivo*, like Flp1/Clp1 does (35). Here we investigated the role of Cdc14A in cell cycle control, focusing on the regulation of Cdc25s in human cells. We found that deregulated human Cdc14A phosphatase impairs the timing of mitotic entry by the effect on Cdk1-cyclin B1 activity. Furthermore, Cdc14A inhibited both Cdc25A and Cdc25B catalytic activities at the G2/M transition. Thus, our results support a novel role for human Cdc14A phosphatase as a regulator of Cdk1 at the entry into mitosis.

EXPERIMENTAL PROCEDURES

Cell Culture—Derivatives of the U-2-OS cell line (51) conditionally expressing Cdc14A alleles were a generous gift from Jiri Lukas (Institute of Cancer Biology, Danish Cancer Society). Cells were maintained and induced to express the transgenes as described previously (34). Wild type U-2-OS cells were cultured in Dulbecco's modified Eagle's medium (DMEM) plus 2 mM glutamine, 100 units ml^{-1} penicillin G, 0.1 mg ml^{-1} streptomycin, and 10% fetal bovine serum at 37 °C/5% CO_2 and used for transient transfection experiments. To synchronize cells in early S phase, thymidine was added at 2.5 mM to subconfluent cells; 24 h later the cells were washed with fresh culture medium, followed by a second addition of thymidine 12–15 h later. After 24 h of the second thymidine treatment, cells were released by extensive washing.

Flow Cytometry—For cell cycle analysis, cells were fixed in ice-cold 70% ethanol for 1 h and stained with 8 μg/ml-propidium iodide, 20 μg/ml-RNase A solution for 1 h at 37 °C. For phosphohistone H3 positivity, cells were harvested and fixed in ice-cold 70% ethanol. Cells were washed with PBS-0.15% Triton X-100 and subsequently stained with anti-phosphohistone H3 and FITC-conjugated anti-rabbit. Then, they were incubated with a 8 μg/ml propidium iodide and 20 μg/ml RNase A solution for 1 h at 37 °C. Stained cells were analyzed using a FACSCalibur device (Becton Dickinson).

Plasmids, siRNA and Virus Production—A plasmid encoding GST-Cdc14A was a gift from Peter Jackson. GST-Cdc14A was purified from bacteria (22). Cdc14A(PD) is a mutant C278S, which causes a complete loss of phosphatase activity. Full-length, N-terminal (amino acids 1–373) and C-terminal (amino acids 374–566) Cdc25B fragments were amplified by PCR and fused to an N-terminal HA-tag in pcDNA3 (Invitrogen). siRNAs (Dharmacon) used were: Cdc25B (5′-AAUCCUCCCUGUCGUCUGAAU-3′), GL2 (control) (5′-AACGUACGCGGAAUACUUCGA-3′), Cdc14A siRNA 2 (5′-GCACAGTAAATACCCACTATT-3′), Luc (control) (5′-ACGUACGCGGAAUACUUCGAAUU-3′). Cdc14A siRNA 1 (5′-GGGACATTGATAGCCTGTTATGTAA-3′) and its corresponding siRNA control (low GC) were from Invitrogen. Cells were transfected with Lipofectamine 2000 or Lipofectamine RNAiMAX reagents (Invitrogen) according to the manufacturer's instructions. The final concentration of the siRNA duplex in the culture medium was 60–100 nM. Four extra silent mutations were introduced to Cdc14A cDNA in the area corresponding to siRNA duplex 1 by polymerase chain reaction (PCR) mutagenesis. The PCR oligos used are: 5′-AGAACAGGGACCCTGATCGCCTGCTATGTAATG-3′ and 5′-CATTACATAGCAGGCGATCAGGGTCCCTGTTCT-3′. The resulting Cdc14A mutant (Cdc14A (R)) was subcloned into pBABE-puro retroviral expression vector. Retroviruses were produced in 293T cells and used to infect U-2-OS cells. After 24 h of infection, cells were grown in medium containing 0.5 μg/ml of puromicine for 48 h and then transfected with the corresponding siRNAs. pBABE-puro-GFP plasmid was used to control the infection efficiency.

mRNA Analyses—RNA was extracted using the RNeasy kit (Qiagen). cDNA synthesis was performed using SuperScript First-Strand kit (Invitrogen). Cdc14A primers were 5′-GTTCCTGAACATCTGTGA-3′ and 5′-GCATGTGTAAACCTGTAG-3′. Cdc14B primers were 5′-GTGCCATTGCAGTACATT-3′ and 5′-AGCAGGCTATCAGAGTG-3′. 18 S rRNA primers, used as internal control, were 5′-CGCCGCTAGAGGTGAAATTC-3′ and 5′-CTTTCGCTCTGGTCCGTCTT-3′. Quantitative PCR analysis was performed according to standard procedures using an iQ5 Multicolor Real-Time PCR Detection System (Bio-Rad).

Immunochemical Techniques—The antibodies used in this study included mouse monoclonal to Cdc14A (DCS-291, NeoMarkers), Cdc25A (sc-7389, Santa Cruz Biotechnology), Cdc25B (DCS-164, (52), Myc tag (sc-40, Santa Cruz Biotechnology), Cdk1 (sc-54, Santa Cruz Biotechnology), β-actin (AC-15, Sigma), rabbit polyclonals to Cdc25A (sc-7157), Cdc25B (sc-5619), Cdc25C (sc-327), cyclin A (sc-751), cyclin B1 (sc-752), GFP (FL), and HA (Y-11), all from Santa Cruz Biotechnology, and phospho-Cdk1-Tyr15 (9111, Cell Signaling) and phosphohistone H3-Ser10 (Upstate). Immunoprecipitation, immunoblotting, and Cdc25 phosphatase assays were performed as described (52).

Kinase and Phosphatase Assays—Immunoprecipitated N- and C-terminal halves of Cdc25B were phosphorylated by Cdk1-cyclin B1 (New England Biolabs) in the presence of 0.15 μCi of [γ-^{32}P]ATP. Samples were then incubated for 30 min at 30 °C with GST-Cdc14A or GST-Cdc14A(PD) (100 ng) in phosphatase buffer (20 mM Tris, pH 8.3; 150 mM NaCl, 2 mM EDTA, 0.1% Triton X-100; 5 mM DTT), or with λ phospha-

tase (New England Biolabs) according to the manufacturer's instructions. To measure Cdk1 activity, Cdk1-cyclin B1 complexes were immunoprecipitated from cellular extracts using anti-cyclin B1 antibodies. Then, they were incubated for 15 min at 30 °C with histone H1 in kinase buffer (50 mM Hepes pH 7.5, 10 mM $MgCl_2$, 1 mM DTT) in the presence of [γ-^{32}P]ATP. Reactions were stopped by the addition of loading buffer and boiled for 5 min at 95 °C. Proteins were resolved by SDS-PAGE and visualized by immunoblotting or autoradiography. Cdc25 phosphatase activities were measured as activation of Cdk1-cyclin B1 complexes as described previously (52). Briefly, inactive Cdk1-cyclin B1 complexes were immunoprecipitated from cells treated with doxorubicin using anti-cyclin B1 antibodies. Cdc25 proteins were immunoprecipitated with specific antibodies and incubated for 45 min at 30 °C with Cdk1-cyclin B1 immunoprecipitates in phosphatase buffer. Reaction was stopped by addition of kinase assay buffer. Then, to assay the activity of Cdk1, kinase reactions were carried out in the presence of [γ-^{32}P]ATP for 5 min using histone H1 as substrate.

Immunofluorescence Microscopy—Cells were grown on coverslips, induced to express Myc-Cdc14A for 48 h and fixed for 20 min in 100% methanol at −20 °C. Myc-tagged Cdc14A was detected with rabbit polyclonal antibody against Myc tag (clone 9E10; Sigma-Aldrich). Centrosomes were detected by co-staining with mouse monoclonal antibody against γ-tubulin (clone GTU-88; Sigma-Aldrich). Secondary antibodies were Alexa Fluor 488 goat anti-mouse (Invitrogen) and FITC-conjugated goat anti-rabbit (Jackson ImmunoResearch Laboratories). DNA was counterstained by DAPI. Images were acquired using a ×40 or ×60 objectives under a Leica DM6000B microscope and a digital camera (Hamamatsu ORCA-ER) and processed with Metamorph program.

Live Cell Imaging—Human BJ cells were grown in six well plates. Cells were synchronized in G1/S by double thymidine treatment and transfected with Cdc14A, or control siRNAs after the first thymidine arrest. Cells were then released into fresh medium containing the kinesin Eg5 inhibitor, Dimethylenastron (10 μM), to prevent exit from mitosis, and transferred to the microscope. Recording was started right after release from the second thymidine treatment. For rescue of Cdc14A knockdown experiments, U-2-OS cells were first infected with the corresponding retroviruses and then treated as above. Nocodazole (50 nM) was used instead of dimethylenastron. Time-lapse frames were acquired using a ×20 EC Plan-Neofluar objective on an Eclipse TE2000-E microscope (Nikon) controlled by Metamorph software and equipped with a Control Unit of temperature, air, and CO_2 (Okolab), and a Hamamatsu ORCA-ER camera. Bright-field frames were recorded every 5 min. Time-lapse data were processed using Metamorph and ImageJ 1.42q software.

RESULTS

Ectopic Expression of Cdc14A Delays the Onset of Mitosis—Using U-2-OS clones expressing Myc-tagged Cdc14A or its phosphatase-dead (PD) mutant form in a tetracycline-repressible manner (34), we observed that induction of active Cdc14A, but not the inactive Cdc14A(PD), delayed cells in G2/M (Fig. 1, *A* and *B*). This effect was correlated with an inhibitory effect on mitotic Cdk1-cyclin B1 complexes (Fig. 1*C*). In these cells, we also checked the cell cycle-regulated localization of Myc-Cdc14A, which was, as it has been described (22), centrosomal during interphase and cytosolic since early prophase to the end of mitosis (supplemental Fig. S1). Previously reported effects of Cdc14A overexpression, such as lagging and missegregated chromosomes, misshapen nuclei, progressive cell death, and centrosome splitting out of mitosis (34) were also observed (supplemental Fig. S2).

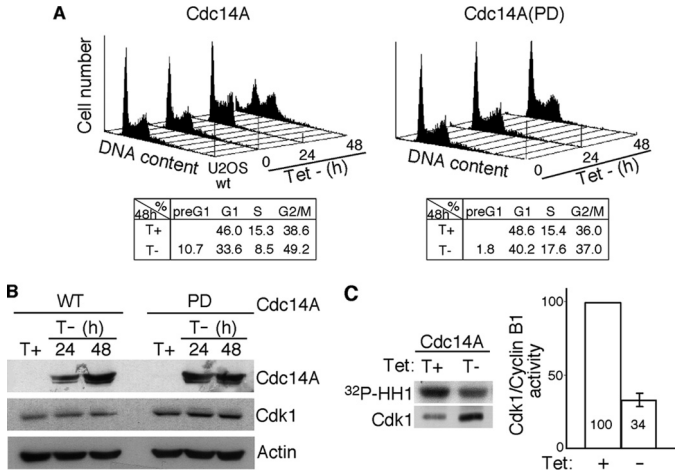

FIGURE 1. **Ectopic expression of Cdc14A results in G2/M delay.** *A*, U-2-OS cell lines conditionally expressing active Cdc14A or its phosphatase-dead form, Cdc14A(PD), following removal of tetracycline from the medium (*T*−) were analyzed by flow cytometry at the indicated time points. Untreated parental U-2-OS cells were also monitored as control. *B*, Cdc14A U-2-OS cell lines induced as in *A* were subjected to immunoblot analysis with the indicated antibodies. *C*, Cdk1-cyclin B1 complexes were immunoprecipitated with anti-cyclin B1 antibody from cells induced to express Cdc14A for 48 h. The ability of these complexes to phosphorylate histone H1 was assessed in the presence of [γ-^{32}P]ATP and normalized by the amount of immunoprecipitated Cdk1. Cdk1-cyclin B1 activity in non-induced cells (*T*+, control conditions) was considered 100%. *Left panels* correspond to a representative experiment. Bars in the graph show means from three independent experiments.

To define the cell cycle delay caused by Cdc14A overexpression more precisely, cells were synchronized in G1/S by double thymidine treatment, and then released and monitored as they progressed through G2/M. Cdc14A expression was induced as indicated in the experimental setup depicted in Fig. 2*A*. As shown in Fig. 2*B*, the ectopic expression of active Cdc14A, led to a higher number of cells with a 4N DNA at all the time points analyzed as compared with non-induced cells or cells overproducing the inactive phosphatase form, indicating that Cdc14A up-regulation slows cells in G2/M. The same effect was observed when Cdc14A expression was induced after the second thymidine treatment (supplemental Fig. S3). Because cyclin A starts to be degraded in prometaphase (37, 38) and cyclin B1 in metaphase (39), we used these two proteins as markers for the characterization of the G2/M delay. As shown in Fig. 2*C*, while in control cells cyclin A declined 14 h after release, in cells overexpressing Cdc14A the level of cyclin A remained constant, indicating a delay

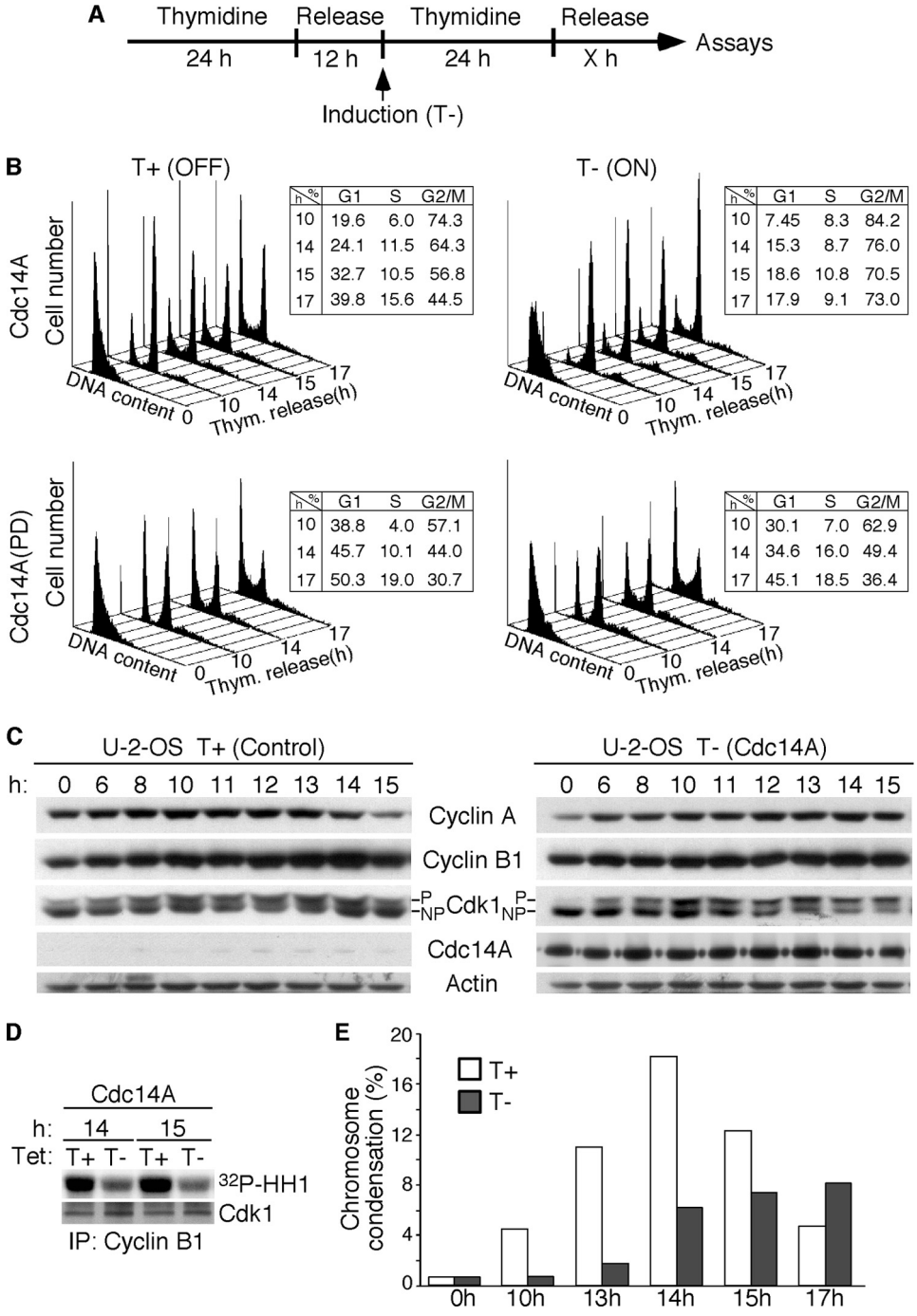

FIGURE 2. **Cells overexpressing active Cdc14A accumulate before prophase.** *A*, scheme of the experimental setup used to induce Cdc14A or Cdc14A(PD) expression in cells synchronized at G1/S. Before the second thymidine treatment, half of the cells were induced to express the transgenes removing tetracycline from the medium (*T*−) and the other half were kept non-induced (*T*+). Samples were taken for flow cytometry (*B*), immunoblot (*C*), kinase assays (*D*), and chromosome condensation (*E*) analyses at the indicated times after release. *B*, FACS profiles of synchronized cells. Samples of induced and non-induced cells were taken for FACS analysis at 0, 10, 14, 15, and 17 h after release from the double thymidine treatment. *C*, cellular extracts from the U-2-OS cell line conditionally expressing active Cdc14A, synchronized, and induced to express the transgene or kept non-induced as indicated in *A*, were prepared at the indicated times and immunoblotted with the indicated antibodies. *P*: phosphorylated; *NP*: non phosphorylated. *D*, Cdk1-cyclin B1 complexes were immunoprecipitated from cellular extracts corresponding to 14- and 15-h samples of *C* using anti-cyclin B1 antibodies. Then, their activity was determined by their capacity to phosphorylate histone H1 in the presence of [γ-^{32}P]ATP. The *upper gel* shows ^{32}P incorporation, and the *lower panel* the amount of immunoprecipitated Cdk1 detected by immunoblot. *E*, U-2-OS cells conditionally expressing active Cdc14A were grown on glass coverslips and treated as indicated in *A*. Cells were taken at the indicated times after release, fixed, stained with 4,6-diamidino-2-phenylindole, and analyzed by microscopy. More than 1000 cells were analyzed per time point.

before prometaphase. Consistently, cyclin B1 levels also remained high throughout the experiment in cells overproducing Cdc14A. In parallel, we monitored the phosphorylation shift of Cdk1 by immunoblot. While in control cells the non-phosphorylated form of Cdk1 was significantly increased 14 h after release, indicating Cdk1 activation, in cells overexpressing Cdc14A phosphorylated and inactive Cdk1 persisted up to the last time point (Fig. 2*C*). This observation was confirmed

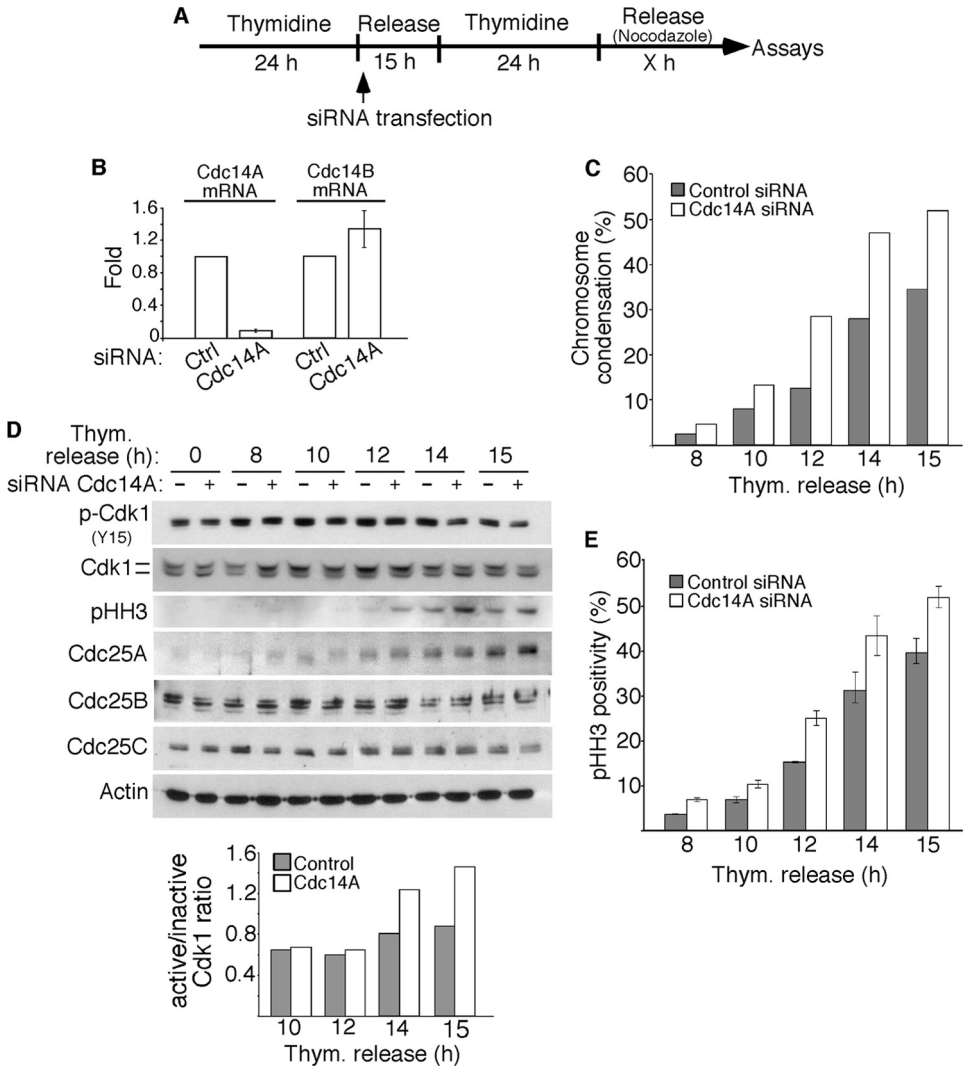

FIGURE 3. **Down-regulation of Cdc14A accelerates mitotic entry.** *A*, outline of the experimental setup used to transfect cells with siRNAs. U-2-OS cells released from a first thymidine treatment were transfected with Cdc14A or control siRNAs and grown for 15 h, after which they were subjected to a second thymidine treatment for 24 h. Next, the cells, synchronized at early S phase, were released into the cell cycle by the addition of fresh medium containing nocodazole to prevent mitotic exit. Cells were then collected at the indicated times for chromosome condensation (*C*), immunoblotting (*D*), and phosphohistone H3 positivity (*E*) analyses. *B*, Cdc14A and Cdc14B mRNA levels were analyzed at 10 h after release using real-time PCR in triplicate measurements (±S.D.). The value given for Cdc14A and Cdc14B mRNAs in control cells was set as 1. *C*, cells were fixed and stained with 4,6-diamidino-2-phenylindole and analyzed by fluorescence microscopy. The percentage of cells with condensed chromosomes was obtained by counting a minimum of 800 nuclei for each sample. *D*, cells were collected at the indicated times and then cell lysates were immunoblotted with antibodies to the indicated proteins. The graph shows the quantification of dephosphorylated/phosphorylated Cdk1 forms shown in *D* at the indicated time points. The results of *C* and *D* correspond to the same experiment and are representative of three independent ones. *E*, phosphohistone H3 positivity was determined by flow cytometry, using anti-phosphohistone H3/FICT antibodies. The graph shows the quantification of three independent experiments.

by *in vitro* Cdk1 kinase assays (Fig. 2*D*). These results show that Cdc14A inhibits Cdk1-cyclin B1 complexes activity at the G2/M transition.

Because chromosome condensation is an early event in mitosis and depends on Cdk1 activity (5), we wondered whether the kinetics of chromosome condensation might be affected by ectopic expression of Cdc14A. As shown in Fig. 2*E*, in cultures in which Cdc14A was up-regulated chromosome condensation was also consistently delayed.

Expression of the Cdc14A(PD) form did not cause, however, any delay at the G2/M transition, as observed by measuring cyclin B1 degradation, Cdk1 activation and chromosome condensation (supplemental Fig. S4). Taken together, these data indicate that the excess of active Cdc14A delays the G2/M transition, suggesting that Cdc14A regulates mitotic entry.

Depletion of Cdc14A Accelerates Entry into Mitosis—To confirm the possible role of Cdc14A in mitotic entry, we down-regulated Cdc14A expression using small interfering RNA (siRNA) in U-2-OS cells (Fig. 3*A*). Cells were synchronized in G1/S by double thymidine treatment and transfected with Cdc14A or control siRNAs after the first thymidine arrest. Cells were then released into fresh medium containing nocodazole, to prevent exit from mitosis, and were monitored as they progressed into mitosis. Because the available Cdc14A-specific antibodies did not recognize the endogenous protein, siRNA oligos were validated by RT-PCR, checking the two Cdc14 isoforms, Cdc14A and Cdc14B. As shown in

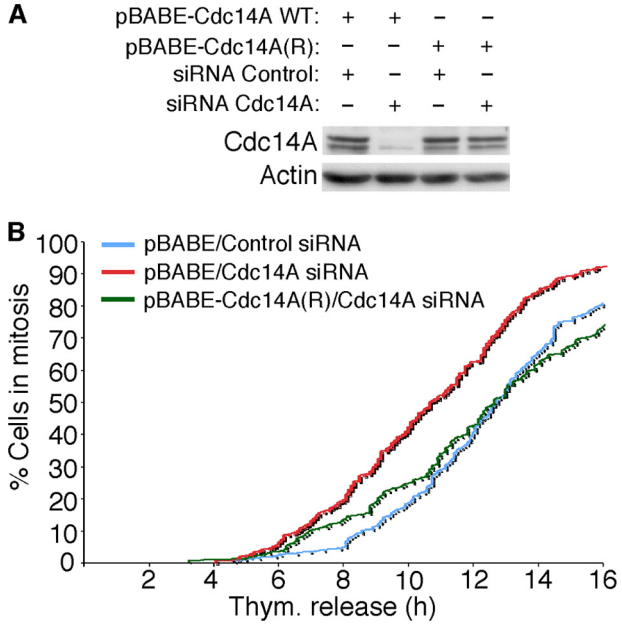

FIGURE 4. **Expression of siRNA-resistant Cdc14A in Cdc14A siRNA-treated cells restores the timing of mitotic entry.** *A*, U-2-OS cells infected with retroviruses expressing Cdc14A wild type (pBABE-Cdc14A WT) or Cdc14A cDNA with four silent mutations in the sequence corresponding to siRNA sequence (pBABE-Cdc14A(R)) were transfected with control or Cdc14A siRNA. After 48 h of transfection, cell lysates were immunoblotted for Cdc14A or actin. *B*, U-2-OS cells infected with empty vector (pBABE) or retroviruses expressing Cdc14A(R) were synchronized in G1/S by double thymidine treatment and transfected with Cdc14A or control siRNAs after the first thymidine arrest as described in the legend to Fig. 3*A*. Cells were then released into fresh medium containing nocodazole and monitored as they progressed into mitosis by time-lapse microscopy. Recording was started right after release. Accumulative plots of cells that enter in mitosis, judged by cell rounded-up, are shown in percentage of the total number of cell in each case ($n = 201$, $n = 227$, and $n = 161$ for pBABE/control siRNA, pBABE/Cdc14A siRNA, and pBABE-Cdc14A(R)/Cdc14A siRNA, respectively). The results are representative of three independent experiments.

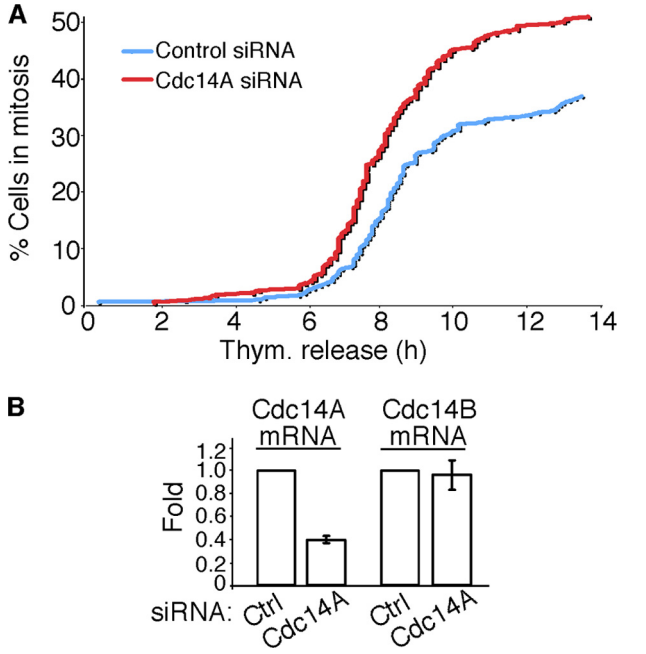

FIGURE 5. **Down-regulation of Cdc14A accelerates mitotic entry in BJ human fibroblasts.** Cells were synchronized in G1/S by double thymidine (5 mM) treatment and transfected with Cdc14A (siRNA 1) or control siRNAs after the first thymidine arrest as described in the legend to Fig. 3*A*. Cells were then released into fresh medium containing the kinesin Eg5 inhibitor, Dimethylenastron (10 μM), to prevent exit from mitosis, and were monitored as they progressed into mitosis by time-lapse analysis. Recording was started right after release. The results are representative of three independent experiments. *A*, quantification of time-lapse series. Accumulative plots of cells that enter in mitosis, judged by cell rounded-up, are shown in percentage of the total number of cell in each case ($n = 239$ and $n = 240$ for control and Cdc14A siRNAs, respectively). *B*, Cdc14A and Cdc14B mRNA levels were analyzed when cells were released from the second thymidine treatment using real-time PCR in triplicate measurements (±S.D.). The value given for Cdc14A and Cdc14B mRNAs in control cells was set as 1.

Fig. 3*B*, Cdc14A siRNAs (siRNA 1) were efficient and specific against this transcript. We found that the kinetics of chromosome condensation was accelerated when Cdc14A was down-regulated as compared with control cultures (Fig. 3*C*). Consistently, depletion of Cdc14A also accelerated Cdk1 activation and histone H3 phosphorylation (Fig. 3, *D* and *E*), indicating that entry into mitosis was faster in Cdc14A siRNA-transfected cells.

Because specificity is a major concern for siRNA duplexes, to strengthen the conclusion that Cdc14A slows the entry into mitosis, two types of experiments were performed. First, we down-regulated Cdc14A by an independent siRNA duplex (siRNA 2). Under similar experimental conditions, siRNA 2 also accelerated chromosome condensation (supplemental Fig. S5, *A* and *B*). Moreover, the effect obtained with siRNA 2 was less marked than with the first siRNA duplex, consistent with a lower Cdc14A depletion (Fig. 3*B* and supplemental Fig. S5, *A* and *C*). These results suggest that Cdc14A is a rate-limiting factor for the entry into mitosis. Second, we performed a siRNA-resistant transgene rescue experiment for siRNA 1, using a Cdc14A cDNA with several silent mutations in the sequence corresponding to siRNA 1 as the rescue construct (Cdc14A(R)) (Fig. 4*A*). U-2-OS cells were first infected with retroviruses expressing pBABE empty vector or pBABE-Cdc14A(R) and then transfected with siRNA 1 or siRNA control as indicated in Fig. 3*A*. Entry into mitosis was monitored by time-lapse microscopy. As shown in Fig. 4*B*, cells infected with empty vector and transfected with siRNA 1 accelerated entry into mitosis as judged by the faster accumulation of mitotic cells. However, in cells infected with retroviruses expressing Cdc14A(R), and then transfected with siRNA 1 the kinetics of mitotic cells accumulation was similar to the observed in the control siRNA-treated cells (Fig. 4*B*). We conclude that the faster entry into mitosis caused by siRNA 1 is specific to knockdown of Cdc14A.

To know whether or not Cdc14A also regulates the mitotic entry in normal, nontransformed human cells, we analyzed whether Cdc14A depletion in human BJ-TERT fibroblasts would also accelerate the entry into mitosis. Upon transfection with Cdc14A or control siRNAs as described in Fig. 3*A*, we found that reduction of Cdc14A in these cells also resulted in a faster mitotic entry (Fig. 5, *A* and *B*). All together, these data indicate that, in a normal cell cycle, Cdc14A negatively regulates entry into mitosis.

Cdc14A Dephosphorylates Cdc25B and Inhibits Its Catalytic Activity—We wished next to investigate the molecular mechanism by which Cdc14A negatively regulates entry into mitosis. Because we found no direct effect on Cdk1-Cyclin B1 complexes (data not shown), we speculated that Cdc14A

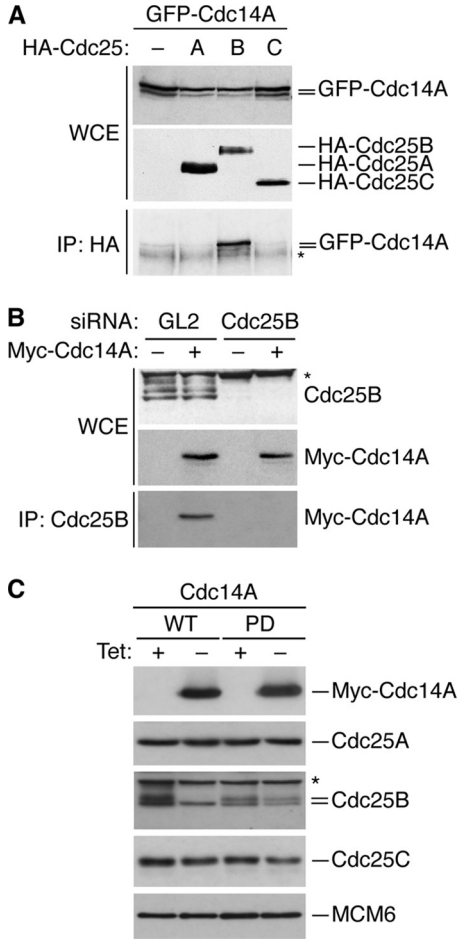

FIGURE 6. **Cdc14A interacts with Cdc25B.** *A*, U-2-OS cells were cotransfected with GFP-tagged Cdc14A and each of the HA-tagged Cdc25 proteins as indicated. Cdc14A-Cdc25 interactions were assessed by immunoblotting of HA-immunocomplexes with anti-GFP antibody. *WCE*, whole cell extracts. *B*, cells were transfected with GL2 (control) or Cdc25B siRNAs for 48 h and transfected with Myc-Cdc14A plasmid for an additional 24 h. Cell lysates were subjected to immunoprecipitation with Cdc25B antibody followed by immunoblotting with the indicated antibodies. *C*, U-2-OS-Cdc14A cell lines were left untreated or induced to express the transgenes for 48 h. Cell lysates were processed for immunoblotting with the indicated antibodies. *Asterisks* show cross-reacting bands.

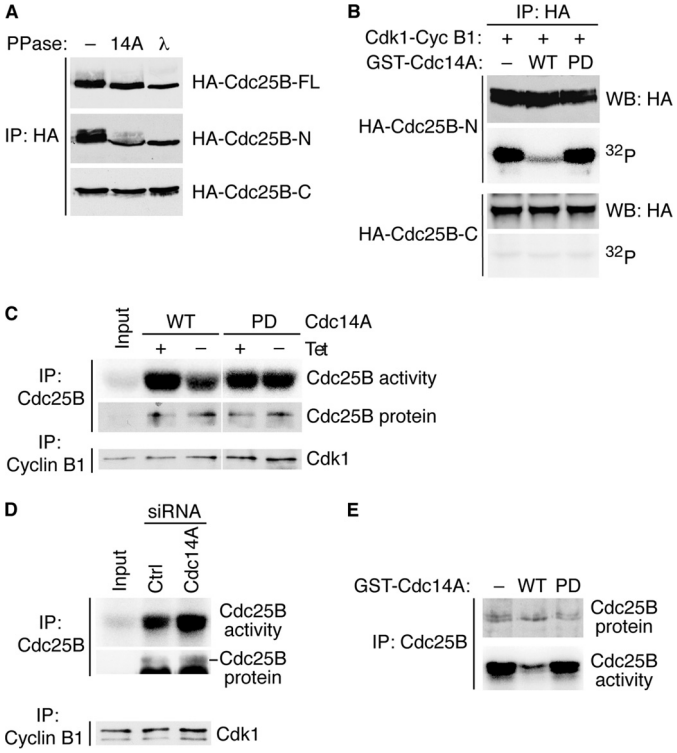

FIGURE 7. **Cdc14A dephosphorylates and inhibits Cdc25B.** *A*, full-length, N-, or C-terminal Cdc25B forms were expressed in U-2-OS cells for 24 h, immunoprecipitated with anti-HA antibody, and incubated with GST-Cdc14A or λ phosphatase. Dephosphorylation of Cdc25B was monitored by immunoblotting. *B*, Cdc25B-N or -C immunocomplexes, prepared as in *A*, were incubated with recombinant Cdk1-cyclin B1 and [γ-^{32}P]ATP, and then with GST-Cdc14A or GST-Cdc14A(PD) forms. Phosphorylated proteins were visualized by autoradiography. *C*, U-2-OS-Cdc14A cell lines were left untreated or induced to express the transgenes for 48 h. Cells were then processed for the measurement of Cdc25B activity on Cdk1-cyclin B1 complexes, whose activation was then measured by kinase assays on histone H1. The *input lane* shows the activity of Cdk1-cyclin B1 not incubated with Cdc25B. *D*, U-2-OS cells were synchronized and transfected as described in the legend to Fig. 3*A*. Cells were collected at 12 h after release and processed for Cdc25B activity analysis. The results correspond to the 12-h release samples of Fig. 3 and are representative of two independent experiments. *E*, Cdc25B was immunoprecipitated from U-2-OS cells and incubated with GST-Cdc14A forms. The samples were then divided and processed for the immunoblotting of Cdc25B or the measurement of Cdc25B activity.

might regulate Cdc25 mitotic activators. The fact that Cdc14A is able to dephosphorylate *in vitro* and to inhibit *in vivo* Cdc25 protein in *S. pombe* cells (35) supported this hypothesis. To test this idea, we first checked Cdc14A binding to each of the Cdc25s. Cdc14A was co-expressed with Cdc25A, -B, or -C in U-2-OS cells and analyzed by co-immunoprecipitation. Cdc14A co-immunoprecipitated with Cdc25B, but not with Cdc25A or Cdc25C (Fig. 6*A*). Furthermore, Cdc14A also bound to endogenous Cdc25B (Fig. 6*B*), suggesting that it may be a substrate of Cdc14A. Indeed, only the faster migrated, non-modified form of Cdc25B was observed in U-2-OS cells conditionally expressing high levels of wild-type Cdc14A, whereas cells expressing the inactive Cdc14A(PD) mutant exhibit both the modified and non-modified forms (Fig. 6*C*).

To confirm Cdc25B dephosphorylation by Cdc14A, full-length Cdc25B or its N- or C-terminal halves were subjected to *in vitro* phosphatase assays using GST-Cdc14A or λ phosphatase. The Cdc25B N-terminal half contains the known CDK phosphorylation sites. As shown in Fig. 7*A*, both full-length and N-terminal Cdc25B, but not the C-terminal domain, were equally modified by both the Cdc14A and λ phosphatase treatments, suggesting that Cdc14A dephosphorylates the Cdc25B N-terminal domain. This is consistent with the notion of Cdc14A phosphatase removing CDK-dependent phosphorylation events. Next, the two N- and C-terminal halves of Cdc25B, immunoprecipitated as HA-tagged protein constructs from U-2-OS-transfected cells, were first phosphorylated *in vitro* by Cdk1-cyclin B1, and then incubated with the GST-Cdc14A or its inactive GST-Cdc14A(PD) form. As shown in Fig. 7*B*, only the N-terminal half of Cdc25B was phosphorylated by Cdk1-cyclin B1 and was efficiently dephosphorylated by Cdc14A. Together, these data indicate that Cdc14A directly binds to and dephosphorylates Cdk1-dependent phosphorylation sites of Cdc25B.

Because Cdc25B activity peaks at G2/M, coinciding with its phosphorylated state, we surmised that Cdc14A might regu-

late it. To address this issue, we first analyzed the activity of Cdc25B in cells overexpressing Cdc14A. Thus, Cdc25B was immunoprecipitated from U-2-OS cells conditionally expressing Cdc14A or the inactive Cdc14A(PD) form, and its phosphatase activity was assessed by their ability to dephosphorylate inactive Cdk1-cyclin B1 complexes, whose activation was then measured by kinase assays on histone H1. We found that Cdc25B was inhibited in cells overexpressing active Cdc14A but not the Cdc14A(PD) mutant form (Fig. 7*C*), indicating that Cdc14A negatively regulates Cdc25B activity. Next, we determined whether the down-regulation of Cdc14A expression affects Cdc25B activity at the G2/M transition. Indeed, when the Cdc25B activity was tested in G2/M synchronized cells treated with Cdc14A siRNA (Fig. 3*B*) we found that it was significantly increased (Fig. 7*D*). Finally, in order to check that Cdc25B inhibition was in fact a direct effect of Cdc14A activity, immunoprecipitated Cdc25B from untreated U-2-OS cells was subjected to *in vitro* phosphatase assays with both GST-Cdc14A or the inactive GST-Cdc14A(PD) form. Cdc25B activity was then assessed on inactive Cdk1-cyclin B1 complexes. As shown in Fig. 7*E*, the *in vitro* Cdc14A-mediated dephosphorylation of Cdc25B led to a dramatic inhibition of its activity. Together, these data indicate that Cdc14A dephosphorylates Cdc25B, inhibiting its catalytic activity.

Cdc14A Regulates the Activity of Cdc25A—As mentioned, the three Cdc25 isoforms are involved in the activation of Cdk1 at the G2/M transition. We therefore also analyzed the ability of Cdc14A to regulate Cdc25A and Cdc25C at mitotic entry. Neither protein levels nor the subcellular locations of Cdc25A and Cdc25C were modified when cells misexpressed Cdc14A (Figs. 3*D* and 5*C*, and data not shown). Cdc25A and Cdc25C activities were also analyzed in cells in which Cdc14A was deregulated. Thus, both Cdc25A and Cdc25C proteins were immunoprecipitated with specific antibodies from asynchronous U-2-OS cells conditionally expressing Cdc14A, and their activity was assessed on inactive Cdk1-cyclin B1 complexes. Surprisingly, while Cdc25C was not affected (data not shown), the activity of Cdc25A was significantly decreased in cells overexpressing Cdc14A (Fig. 8*A*). Because the induction of the catalytic dead form of Cdc14A did not have any effect (Fig. 8*B*), we surmised that the inhibition of Cdc25A would depend on Cdc14A activity. Moreover, we determined whether the Cdc14A-mediated inhibition of Cdc25A occurred at the G2/M boundary. We found that in G2/M-synchronized cells Cdc25A activity was inhibited when Cdc14A was overexpressed (Fig. 8*C*), indicating that Cdc14A inhibits Cdc25A activity at the G2/M transition. Again, Cdc25C activity was not significantly affected under these experimental conditions (Fig. 8*D*). Finally, if the inhibition of Cdc25A activity is mediated by Cdc14A, the opposite effect should be reflected in cells where Cdc14A is down-regulated. Indeed, when the Cdc25A activity was tested in G2/M-synchronized cells treated with Cdc14A siRNA (Fig. 3*B*), we found that it was significantly increased (Fig. 8*E*). We have previously reported that Cdc14A reverses the Cdk1-cyclin B1 phosphorylation of Cdc25A on residues involved in its stability (40). Indeed, *in vitro* dephosphorylation of Cdc25A by Cdc14A does not inhibit its catalytic activity (data not shown). Taken together, our results suggest that at the G2/M transition Cdc14A acts on an unknown protein, which in turn inhibits Cdc25A phosphatase activity.

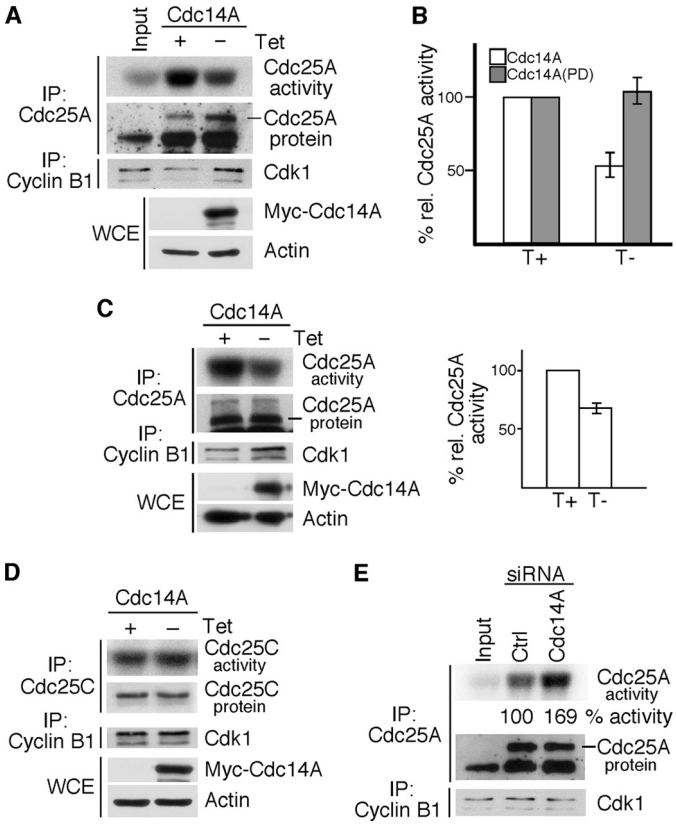

FIGURE 8. **Cdc14A regulates Cdc25A activity.** *A* and *B*, U-2-OS-Cdc14A clones were left untreated or induced to express Cdc14A or Cdc14A(PD) for 48 h. Cells were then processed for measurement of Cdc25A activity. Phosphorylated bands were quantified and normalized with respect to immunoprecipitated Cdc25A. The Cdc25A activity from untreated cells (*T*+) was considered 100%. The graph represents the means from three independent experiments. *C* and *D*, U-2-OS-Cdc14A cell line was synchronized and treated as in the legend to Fig. 2*A*. Cdc25A and Cdc25C activities were analyzed from 14 h samples. *C*, result shown in the *left panel* corresponds to the 14 h samples of Fig. 2. The graph shows the quantification of three independent experiments. The value given for the Cdc25A activity in the control samples was set as 100%. *D*, Cdc25C activity was analyzed from the same samples as in *C*. *E*, U-2-OS cells were synchronized and transfected with Cdc14A siRNA as described in the legend to Fig. 3*A*. Cdc25A activity was analyzed from 12 h samples. The result is representative of two independent experiments and corresponds to the 12 h samples of Fig. 3.

DISCUSSION

The results presented here demonstrate that misregulation of Cdc14A phosphatase in the cell affects the onset of mitosis, indicating that Cdc14A modulates the G2/M transition. In this sense, we found that when both asynchronous and G2-phase synchronized cells had increased levels of active Cdc14A phosphatase they accumulated at the G2/M boundary and slowly progressed through the early stages of mitosis. Moreover, cells where Cdc14A expression was down-regulated by siRNAs accelerated entry into mitosis and this effect can be rescued by an RNAi-resistant transgene. These data indicate that Cdc14A is involved in the timing of the G2/M transition in human cells. It has been reported that the overexpression of the other human

Regulation of G2/M Transition by Cdc14A

Cdc14 family member, Cdc14B, does not have a major effect on the distribution of cells in different parts of the cell cycle (34). Thus, the observed effects seem to be specific for Cdc14A phosphatase.

The key switch for the onset of mitosis is the activation of cyclin-dependent kinase Cdk1 (41). Because yeast Cdc14 homologues have been shown to negatively regulate Cdk1 activity (23, 24, 29, 31, 42), we reasoned that the effect produced by Cdc14A misregulation at the G2/M transition could be due to its role in Cdk1 regulation. In fact, high levels of Cdc14A in the cell inhibited the Cdk1-cyclin B1 complexes activity (Fig. 1*C*). We investigated the mechanism by which Cdc14A regulates Cdk1 activity. Because we did not find evidence for a direct effect of Cdc14A on Cdk1-cyclin B1 complexes, we analyzed whether Cdc14A phosphatase might act through their regulators, the Cdc25 phosphatase family. In mammalian cells, all three Cdc25 isoforms contribute to achieving and maintaining the high activity of Cdk1-cyclin B1 complexes at mitosis (12–15, 43) (see Ref. 44 for a review). It is accepted that Cdc25B starts the activation of Cdk1-cyclin B1 at centrosomes at the G2/M transition (13, 16, 17). Later on, Cdc25A and Cdc25C complete Cdk1-cyclin B1 complexes activation as a part of the autoamplification loop, in which phosphorylation by Cdk1 activates Cdc25C and stabilizes Cdc25A, which in turn cooperate to reach the threshold of mitotic Cdk1 activity (14, 18–20). We therefore speculated that Cdc14A could control Cdk1 activation by acting on these Cdk1 regulatory proteins.

First, we demonstrate that Cdc14A interacts with and dephosphorylates Cdc25B at Cdk1-cyclin B1 phosphorylation sites contained within its N-terminal domain, inhibiting its catalytic activity. Phosphorylation of Cdc25B has been reported to coincide with an increase in its phosphatase activity at the G2 and M phases (12, 45). Several kinases seem to be involved in this Cdc25B activation among which are Aurora A and CK2 (46, 47). We show that Cdc14A inhibits Cdc25B activity by reversing Cdk1-cyclin B1-mediated phosphorylation. Our data indicate that Cdk1 phosphorylates and activates Cdc25B at mitosis. Thus, we have identified a new substrate of Cdc14A, the Cdc25B protein, whose inhibitory dephosphorylation by Cdc14A leads to a decrease in Cdk1 activity at the onset of mitosis. Because both Cdc14A and Cdc25B localize to centrosomes at some point during the cell cycle, it will be of interest to understand whether the regulation of Cdc25B activity by Cdc14A occurs in centrosomes or whether is performed by the centrosome-released pool of Cdc14A at early mitosis.

Moreover, we show that misregulation of Cdc14A produces a change in the catalytic activity of Cdc25A. Thus, in G2/M-synchronized cells, high levels of Cdc14A induced a significant reduction in Cdc25A catalytic activity, whereas the down-regulation of endogenous Cdc14A produced the opposite effect, *i.e.* an increase in its phosphatase activity. This was indeed the most intriguing finding of our study. To date, the regulation of Cdc25A at the G2 and M phases seems to be tightly associated with its protein turnover or stability (48). Accordingly, Cdc25A protein levels change in a cell cycle-dependent manner, being predominantly expressed in G1 (49)

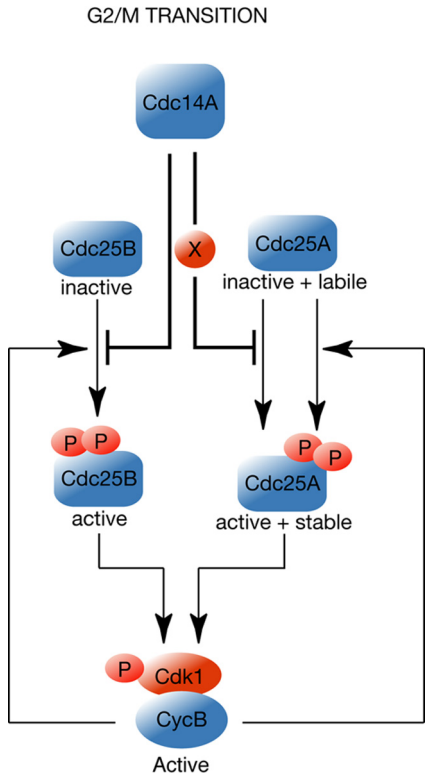

FIGURE 9. **Model: function of Cdc14A at the G2/M transition.** In late G2 Cdc14A is proposed to negatively regulate Cdk1 activation to control the timing of mitosis by inhibiting Cdc25A and Cdc25B activities. Cdc14A inhibits Cdc25A activity through an unknown mechanism. Cdc14A directly binds to and dephosphorylates Cdc25B.

and stabilized in mitosis through Cdk1-cyclin B1-mediated phosphorylation (14). In contrast, destruction of Cdc25A prevents premature mitosis of cells with an incompletely replicated and/or damaged genome (for a review, see Ref. 50). Our results demonstrate that Cdc14A affects the activity of Cdc25A and not its stability at the G2/M transition. These data suggest a new mechanism of Cdc25A regulation at G2/M boundary, based on changes in its catalytic activity mediated by Cdc14A phosphatase. We have previously reported that Cdc14A reverses the Cdk1-cyclin B1 phosphorylation of Cdc25A on residues involved in its stability (40). Indeed, *in vitro* dephosphorylation of Cdk1-cyclin B1-phosphorylated Cdc25A by Cdc14A did not inhibit its catalytic activity (data not shown). Our results suggest that at the G2/M transition exists an inhibitory effect of Cdc14A on Cdc25A, which might likely involve an unknown protein modulating the activity of Cdc25A phosphatase.

It has been shown that Cdc25A and Cdc25B are required for mitotic entry (13, 14). Cdc25A is involved in the initiation of chromosome condensation and Cdc25B initiates the activation of Cdk1-cyclin B1 at centrosomes (13). In summary, we show that depletion of Cdc14A accelerates the timing of mitotic entry through deregulation of Cdc25A and Cdc25B at the G2/M transition. Cdc14A inhibits Cdc25A and Cdc25B activity, the latter through direct binding and dephosphorylation (Fig. 9). Our findings indicate that in human cells Cdc14A plays a role in preventing premature entry into mitosis.

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