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Phosphorylation of Right Open Reading Frame 2 (Rio2) Protein Kinase by Polo-like Kinase 1 Regulates Mitotic Progression*

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Background: Rio2 is a protein kinase and involved in ribosomal subunit maturation.
Results: Rio2 is a novel substrate of Plk1. Overexpression of Rio2 causes a prolonged mitotic exit whereas knockdown of Rio2 accelerates mitotic progression.
Conclusion: Plk1-dependent phosphorylation of Rio2 regulates the timing of the metaphase-anaphase transition.
Significance: We unveiled the novel role of Rio2 and its phosphorylation by Plk1 in regulating mitotic progression.

Polo-like kinase 1 (Plk1) plays essential roles during multiple stages of mitosis by phosphorylating a number of substrates. Here, we report that the atypical protein kinase Rio2 is a novel substrate of Plk1 and can be phosphorylated by Plk1 at Ser-335, Ser-380, and Ser-548. Overexpression of Rio2 causes a prolonged mitotic exit whereas knockdown of Rio2 accelerates mitotic progression, suggesting that Rio2 is required for the proper mitotic progression. Overexpression of phospho-mimicking mutant Rio2 S3D but not the nonphosphorylatable mutant Rio2 S3A displays a profile similar to that of wild-type Rio2. These results indicate that the phosphorylation status of Rio2 correlates with its function in mitosis. Furthermore, time-lapse imaging data show that overexpression of Rio2 but not Rio2 S3A results in a slowed metaphase-anaphase transition. Collectively, these findings strongly indicate that the Plk1-mediated phosphorylation of Rio2 regulates metaphase-anaphase transition during mitotic progression.

Polo-like kinase 1 (Plk1)[2] is a highly conserved Ser/Thr kinase in eukaryotes and plays a critical role in various aspects of mitotic events, such as G_2/M transition, spindle formation, chromosome congression and segregation, as well as cytokinesis (1–4). Plk1 exerts its multiple functions by phosphorylating a number of substrates. For example, Plk1 regulates G_2/M transition through direct phosphorylation of cyclin B1, Cdc25, and Wee1, which contributes to the activation of cyclin-dependent kinase 1 (5, 6). During spindle formation, Plk1 maintains the integrity of the spindle poles by phosphorylating Kizuna (7, 8). Plk1 phosphorylates Emi1, an inhibitor of APC/C, causing Emi1 degradation and allowing progression beyond prometaphase (9, 10). Phospho-regulation of Cdc14A by Plk1 is essential for faithful chromosome segregation (11). Phosphorylation of Mklp2 by Plk1 is necessary for Plk1 localization to the central spindle during anaphase and telophase, and the interaction of these two proteins is required for cytokinesis (12). Numerous substrates of Plk1 have been identified and studied extensively these years. These findings were helpful to a better understanding of the multiple roles of Plk1 in cell cycle regulation.

Rio2 was first identified as a member of RIO (right open reading frame) family which belongs to atypical protein kinases in yeast. Rio proteins, Rio1 and Rio2, are conserved from archaea to human, whereas Rio3 subfamily presents only in multicellular eukaryotes (13, 14). Both yeast Rio1 and Rio2 are necessary for processing of 20 S pre-rRNA to the 18 S rRNA in 40 S ribosomal subunit synthesis (15, 16). Human Rio2 (hRio2) was also found to be involved in late 40 S ribosomal subunit maturation (17, 18). As known, many ribosome biogenesis-associated proteins also play very important roles in cell cycle progression (19). Previous studies in yeast indicated that depletion of Rio1 arrested cell cycle progression at S phase and mitosis (19–21), which suggested that Rio1 may also be involved in cell cycle regulation. Rrp14p is required for both ribosomal subunit synthesis and correct positioning and elongation of the mitotic spindle (22). Casein kinase I isoform Hrr25 not only regulates the maturation of 40 S ribosomal subunits (23), but also participates in the transcriptional response to DNA damage (24). It has been reported that Rio2 interacted with many proteins involved in ribosome biogenesis in yeast as well as proteins involved in cell proliferation (25–29), indicating that Rio2 may have dual functions in ribosome biogenesis and cell proliferation. However, the role of Rio2 in cell proliferation has not been addressed. Rio2 was identified to be phosphorylated at mitosis by mass spectrometry recently, and two of the phospho-

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[2] The abbreviations used are: Plk1, polo like kinase 1; APC/C, anaphase-promoting complex or cyclosome; KD, kinase-dead; Rio, right open reading frame; tet, tetracycline.

rylated sites were proposed to be Plk1 consensus phosphorylation sites (9, 30). Based on these findings, we assume that Rio2 may function in cell cycle progression. In this study, we demonstrate that Rio2 is a novel substrate of Plk1 and its phosphorylation by Plk1 plays an important role in the regulation of proper metaphase-anaphase transition.

EXPERIMENTAL PROCEDURES

Plasmids and Antibodies—Myc-Plk1 and FLAG-Plk1 were made by subcloning Plk1 cDNA into the pCMV-myc and pFLAG-CMV-2 vector, respectively. FLAG-Rio2 was made by cloning the full length of Rio2 cDNA into pFLAG-CMV-2 at BglII-KpnI sites. The full length of Plk1 and Rio2 was cloned into pET30a and pET41b (Novagen), respectively. The deletion and point mutants of Rio2 were cloned into pET41b.

The rabbit anti-Rio2 polyclonal antibody was generated by immunizing rabbits with glutathione S-transferase (GST)-Rio2 (280–450 amino acids). The antibody was affinity-purified with antigen-conjugated agarose. FLAG antibody (M2) was purchased from Sigma. Myc (9E10), Plk1 (F-8), cyclin B1 (GNS1), β-actin (1–19), and Ser(P)-10-H3 (sc-8656-R) antibodies were purchased from Santa Cruz Biotechnology. Anti-phosphoserine antibody (AB1603) was purchased from Millipore.

Co-immunoprecipitation and GST Pulldown Assays—293T cells were lysed in lysis buffer (50 mM Tris, pH 8.0, 150 mM NaCl, 0.5% Triton X-100) with protease inhibitor (Roche Applied Science) at 4 °C for 15 min. Then the cell lysates were incubated with antibodies at 4 °C for 2 h followed by addition of protein A-agarose beads. After 2 h of incubation at 4 °C, the beads were washed with the lysis buffer. The immunoprecipitates were separated by SDS-PAGE and immunoblotted with the indicated antibodies. For the GST pulldown assays, GST-Rio2 or GST immobilized on Sepharose 4B-glutathione beads (Pharmacia) were incubated with purified His-Plk1 at 4 °C for 2 h followed by washing with lysis buffer. The bound proteins were dissolved in SDS loading buffer, separated by 10% SDS-PAGE, and immunoblotted with Plk1 antibody.

In Vitro Kinase Assay—His-tagged constitutively active Plk1 T210D (Plk1-TD) and kinase deficient Plk1 (Plk1-KD) (31) were purified from *Escherichia coli* with nickel-nitrilotriacetic acid beads (Qiagen). Purified GST-Rio2, GST-Rio2 mutants were incubated with Plk1-TD or Plk1-KD in kinase buffer (50 mM Tris, pH 7.5, 10 mM $MgCl_2$, 1 mM DTT) with 1 mM cold ATP, 1 μCi of [γ-^{32}P]ATP at 30 °C for 30 min. The samples were resolved by 10% SDS-PAGE and subjected to autoradiography.

Flow Cytometry—Cells were harvested, fixed with ice-cold 70% ethanol at −20 °C, and stained with PBS/1% BSA containing 20 μg/ml propidium iodide and 100 μg/ml RNase A. Stained cells were analyzed on a FACScan instrument (BD FACSCalibur).

Mass Spectrometry—GST-Rio2 was incubated with His-Plk1-TD or His-Plk1-KD and cold ATP for the *in vitro* kinase assay. Subsequently, GST-Rio2 was isolated using SDS-PAGE, digested with trypsin, and then subjected to HPLC-MALTI-TOF mass spectrometry analysis to identify the phosphopeptides.

RNA Interference—Double-strand RNA oligonucleotides were purchased from Ribobio (Guangzhou, China). Rio2 siRNAs were synthesized corresponding to the following cDNA sequences: siRio2-1, 5′-ACAUGGUGGCUGUAAUAAA-dTdT3′; siRio2-2, 5′-GGAUCUUGGAUAUGUUUAAdTdT3′. HeLa cells were transfected with siRNA duplexes using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions.

Generation of Tet-on Stable Cell Lines—FLAG-tagged Rio2, Rio2 S3A, and Rio2 S3D were cloned into the KpnI-XhoI sites of pcDNATM/TO vector (Invitrogen) and transfected into T-REx™-HeLa cells (Invitrogen). 48 h after transfection, cells were selected with 100 μg/ml Zeocin and 5 μg/ml blasticidin for 3 weeks. Clones were picked, and expression of exogenous Rio2 was tested by immunoblotting in the presence of tetracycline.

Cell Culture and Synchronization—293T and HeLa cells were cultured in DMEM supplemented with 10% FBS. T-REx™-HeLa cell lines were maintained in DMEM containing 10% FBS plus 5 μg/ml blasticidin. Cells were synchronized at G_1/S transition by a double thymidine block as described in a previous report (32). In brief, HeLa cells were incubated for 16 h in complete medium with 2 mM thymidine released in fresh medium for 8 h and then incubated with 2 mM thymidine for another 16 h. For S phase synchronization, cells were released from the double thymidine treatment for 3 h. After 8 h, most of the cells were in G_2 phase. To obtain cells synchronized in mitosis, HeLa cells were incubated for 16 h in the presence of 2 mM thymidine and released for 6 h followed by treatment with 100 ng/ml nocodazole for 6 h. Mitotic cells were collected by shake-off. Cells in G_1 phase were obtained from this pool of cells by resuspending them in fresh medium and allowing them to exit from mitosis for 4 h, and discarding the unattached cells by shake-off.

Time-lapse Imaging—To monitor the cells during mitosis, HeLa cells were treated with thymidine for 16 h and released for 5 h, then were placed in microscope *Delta Vision.* Experiments were performed in a temperature-controlled chamber, which was maintained at 37 °C with a humidified atmosphere of 5% CO_2 in air. Cells were observed, and images were taken every 6 min. SoftWoRx was used for acquisition and analysis.

RESULTS

Rio2 Interacts with Plk1 in Vivo and in Vitro—To investigate the interaction between Plk1 and Rio2, pCMV-myc-Plk1 and pFLAG-CMV-2-Rio2 were co-transfected into 293T cells. The cell lysates were immunoprecipitated with FLAG antibody and subjected to immunoblotting with myc antibody. As shown in Fig. 1*A*, Rio2 associated with Plk1 *in vivo*. Next, we examined whether the kinase activity of Plk1 was involved in the interaction. FLAG-tagged Plk1 and a kinase-defective mutant FLAG-tagged Plk1-KD (K82R) were expressed, respectively, in 293T cells and then immunoprecipitated with FLAG antibody followed by immunoblotting with Rio2 antibody. The result indicated that the interaction between Plk1 and Rio2 was independent of Plk1 kinase activity (Fig. 1*B*). To confirm further the interaction between endogenous Rio2 and Plk1, the cell lysates from 293T cells were immunoprecipitated with Plk1 or Rio2 antibodies and immunoblotted with Rio2 or Plk1 antibodies.

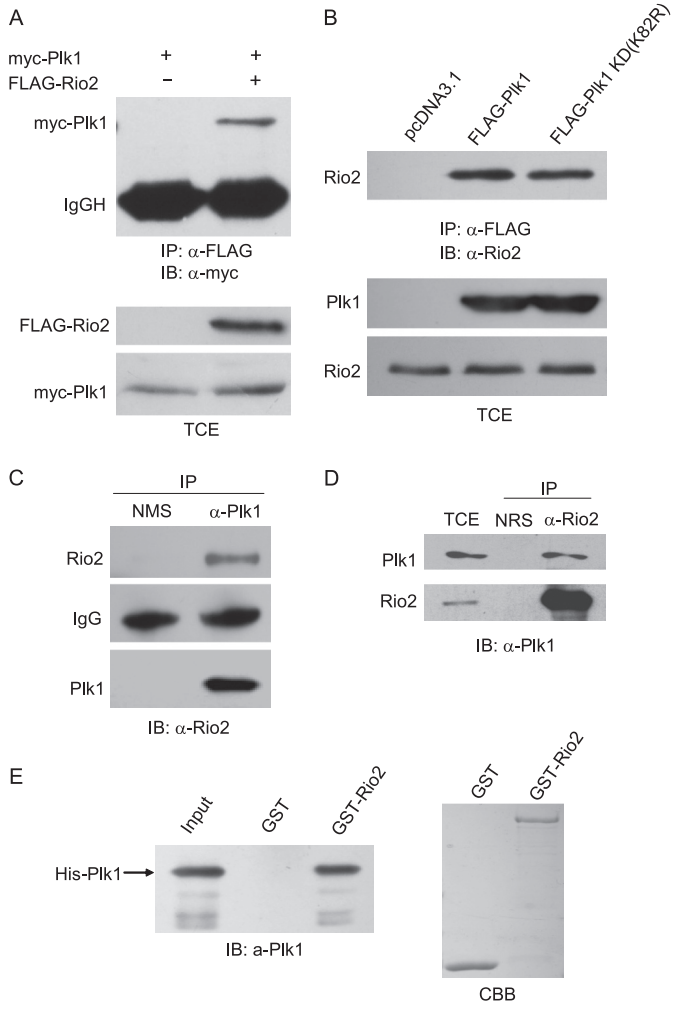

FIGURE 1. **Rio2 interacts with Plk1.** *A*, 293T cells co-transfected with FLAG-tagged Rio2 and myc-Plk1. The total cell extracts (*TCE*) were immunoprecipitated (*IP*) with FLAG antibody and immunoblotted (*IB*) with myc antibody. *B*, 293T cells transfected with pcDNA3.1, FLAG-tagged Plk1, and Plk1-KD (K82R). The cell lysates were immunoprecipitated with FLAG antibody and immunoblotted with Rio2 antibody. *C*, lysates from 293T cells immunoprecipitated with normal mouse serum or Plk1 antibody, respectively, followed by immunoblotting with Rio2 antibody. *D*, lysates from 293T cells immunoprecipitated with normal rabbit serum or Rio2 antibody and subjected to immunoblotting with Plk1 antibody. *E*, GST pulldown assay. Purified His-Plk1 was incubated with immobilized GST or GST-Rio2, respectively. The bound protein was detected by immunoblotting with Plk1 antibody. *CBB*, Coomassie Brilliant Blue.

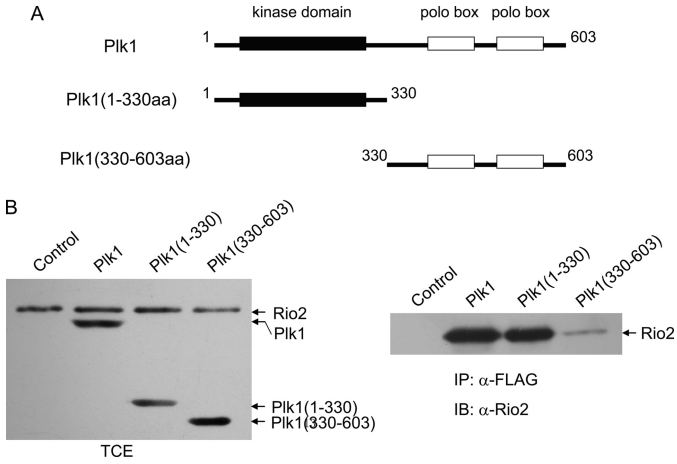

FIGURE 2. **Rio2 interacts with the N-terminal domain of Plk1.** *A*, schematic of Plk1 deletion mutants. *B*, 293T cells transfected with FLAG-tagged full-length of Plk1 or the indicated Plk1 deletion mutants. The total cell extracts (*TCE*) were immunoprecipitated (*IP*) with FLAG antibody and immunoblotted (*IB*) with Rio2 antibody. The total cell lysates were immunoblotted with mixed Rio2 and FLAG antibodies.

These data showed that endogenous Rio2 and Plk1 can associate with each other (Fig. 1, *C* and *D*). To verify the direct interaction between Plk1 and Rio2, we did the GST pulldown assay with purified GST-Rio2 and His-Plk1. As shown in Fig. 1*E*, Plk1 can interact with Rio2 *in vitro*.

Plk1 contains the kinase domain at its N terminus and two polo-box domains at its C terminus. To address which region of Plk1 is required for the interaction with Rio2, FLAG-tagged Plk1 (1–330 amino acids) and FLAG-tagged Plk1 (330–603 amino acids) were generated (Fig. 2*A*) and transfected into 293T cells. The cell lysates were immunoprecipitated with FLAG antibody and subjected to immunoblotting with Rio2 antibody. Results shown in Fig. 2*B* demonstrated that the N-terminal region of Plk1 is responsible for its binding with Rio2.

Plk1 Phosphorylates Rio2 at Ser-335, Ser-380, and Ser-548—To examine whether Rio2 is a substrate for Plk1, we performed the *in vitro* kinase assay. Purified GST or GST-Rio2 was incubated with His-Plk1-TD (constitutively active form of Plk1). As shown in Fig. 3*A*, GST-Rio2 was phosphorylated. To confirm this result and exclude the autophosphorylation of Rio2, GST-Rio2 was incubated with His-Plk1-TD and His-Plk1–KD in kinase buffer. Plk1-TD could phosphorylate GST-Rio2 whereas Plk1-KD could not (Fig. 3*B*). These data indicated that Rio2 can be phosphorylated by Plk1.

Next, we sought to define the phosphorylation sites on Rio2. GST-Rio2 was incubated with His-Plk1-TD or His-Plk1-KD for the *in vitro* kinase assay and subjected to a mass spectrometry analysis. Three phosphorylation sites in Rio2 (Ser-335, Ser-380, and Ser-548) were identified, and two of them, Ser-335 and Ser-380, were Plk1 consensus phosphorylation sites, E/D-*X*-S/T (Fig. 3*C*).

To confirm the phosphorylation sites of Rio2 by Plk1, a series of deletion forms of Rio2 and their corresponding mutants with serine to alanine point mutation were prepared for the *in vitro* kinase assay (Fig. 3*D*). These data demonstrated that the Ser-335, Ser-380, and Ser-548 of Rio2 were phosphorylated by Plk1 (Fig. 3, *D* and *E*).

The *in vitro* kinase assay with full-length Rio2 and its mutants indicated that Rio2 was highly phosphorylated by Plk1, whereas the phosphorylation of Rio2 S335D/S380D/S548A triple mutant (Rio2 S3A) was greatly reduced. The phosphorylation signals of Rio2 double mutants were also decreased compared with that of wild-type Rio2 (Fig. 3*F*). These results confirmed that Ser-335, Ser-380, and Ser-548 of Rio2 were the major phosphorylation sites for Plk1 *in vitro*. To confirm further that the three sites of Rio2 can be phosphorylated *in vivo*. pCMV-myc-Rio2 wild type or Rio2 S3A mutant was co-transfected with pFLAG-CMV-2-Plk1-TD into 293T cells. The cell lysates were immunoprecipitated with myc antibody and immunoblotted with phosphoserine antibody. As shown in Fig. 3*G*, The phosphor-

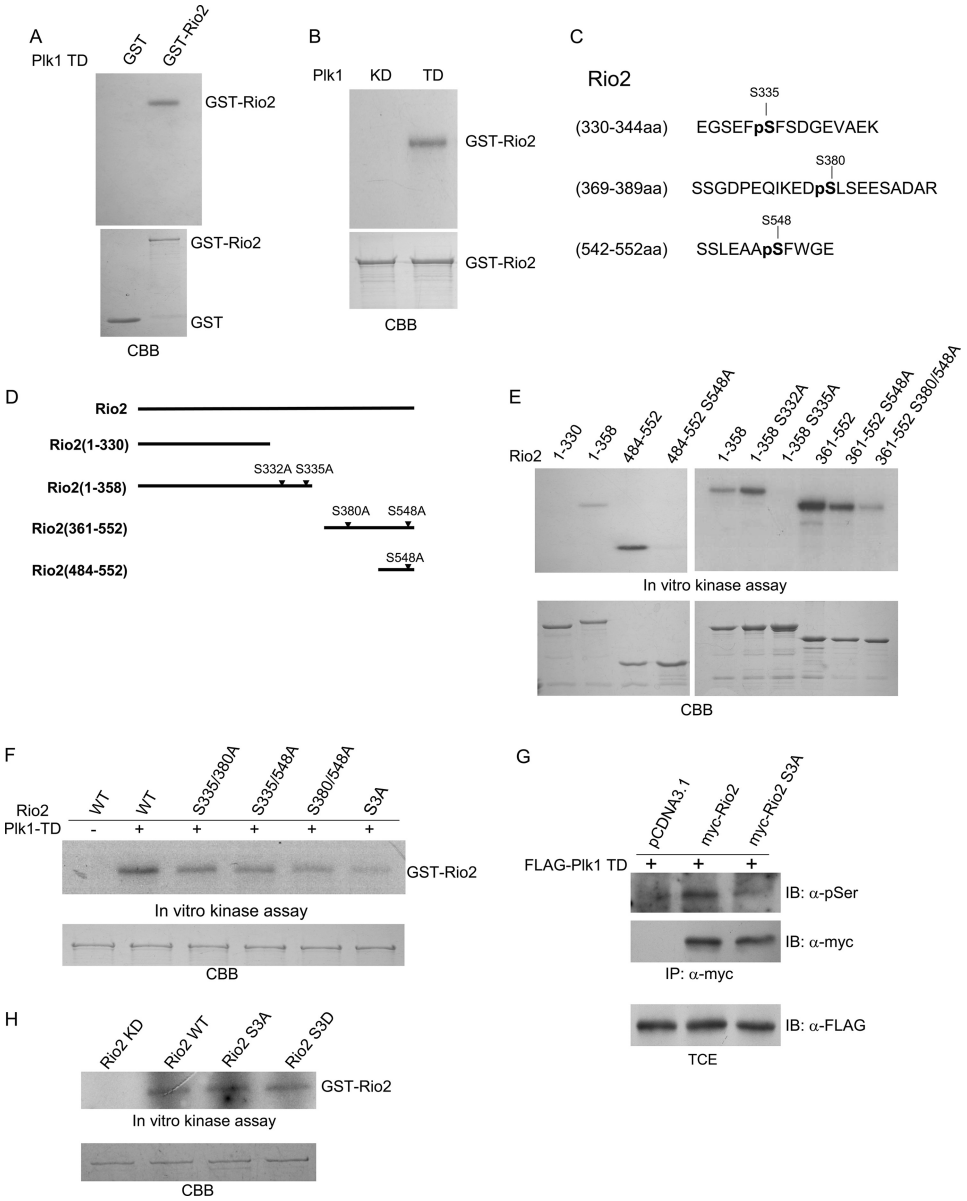

FIGURE 3. **Plk1 phosphorylates Rio2 at the sites of Ser-335, Ser-380, and Ser-548.** *A*, purified GST or GST-Rio2 incubated with His-Plk1-TD for the *in vitro* kinase assay. *CBB*, Coomassie Brilliant Blue. *B*, GST-Rio2 incubated with His-Plk1-KD or His-Plk1-TD for the *in vitro* kinase assay. *C*, phosphopeptides identified by mass spectrometry. GST-Rio2 was incubated with His-Plk1-KD or His-Plk1-TD for the *in vitro* kinase assay in the presence of cold ATP and then subjected to mass spectrometry analysis. *D*, schematic figure of Rio2 deletion and point mutation mutants. *E* and *F*, GST-Rio2 mutants (*E*) and GST-Rio2 full-length and its Ser/Ala mutants (*F*) incubated with His-Plk1-TD for the *in vitro* kinase assay. *G*, 293T cells co-transfected with FLAG-tagged Plk1-TD and myc-Rio2 or myc-Rio2 S3A. The total cell extracts (*TCE*) were immunoprecipitated (*IP*) with myc antibody and followed by immunoblotting (*IB*) with anti-phosphoserine (*pSer*) and myc antibodies, respectively. *H*, purified GST-Rio2 wild-type or mutant Rio2 proteins (KD, S3A, or S3D) subjected to the *in vitro* kinase assay.

ylation of Rio2 S3A was reduced significantly compared with that of wild-type Rio2, indicating that Rio2 can be phosphorylated *in vivo*.

Rio2 has been identified as a Ser/Thr kinase and is capable of autophosphorylation (13). However, the substrates of Rio2 still remain to be discovered. To determine whether Rio2 phosphorylation by Plk1 regulates the kinase activity of Rio2, we examined the autophosphorylation of Rio2. The KD mutant of Rio2, which lost the autophosphorylation activity, was taken as a negative control (18). Rio2 KD, Rio2 WT, Rio2 S3A, and Rio2 S3D were prepared for the *in vitro* kinase assay (Fig. 3*H*). The data showed that the autophosphorylation of Rio2 was not affected by Plk1 phosphorylation.

Rio2 Phosphorylation by Plk1 Influences Mitotic Exit—Plk1 is a pivotal mitotic kinase of which both protein level and kinase activity are peaked at mitosis (33). Rio2 interacted with Plk1, suggesting that it may function in mitosis. To investigate the functional relevance of Plk1-dependent Rio2 phosphorylation in mitosis, we generated the tet-on stable HeLa cell lines in which Rio2, nonphosphorylatable mutant Rio2 S335A/S380A/S548A (Rio2 S3A) or phospho-mimicking mutant S335D/S380D/S548D (Rio2 S3D) expressed in the presence of tetracycline (Fig. 4*A*). The cells were arrested in mitosis with nocodazole. Mitotic cells were shaken off and released into fresh medium, and their progression through mitosis was monitored by flow cytometry. The data showed

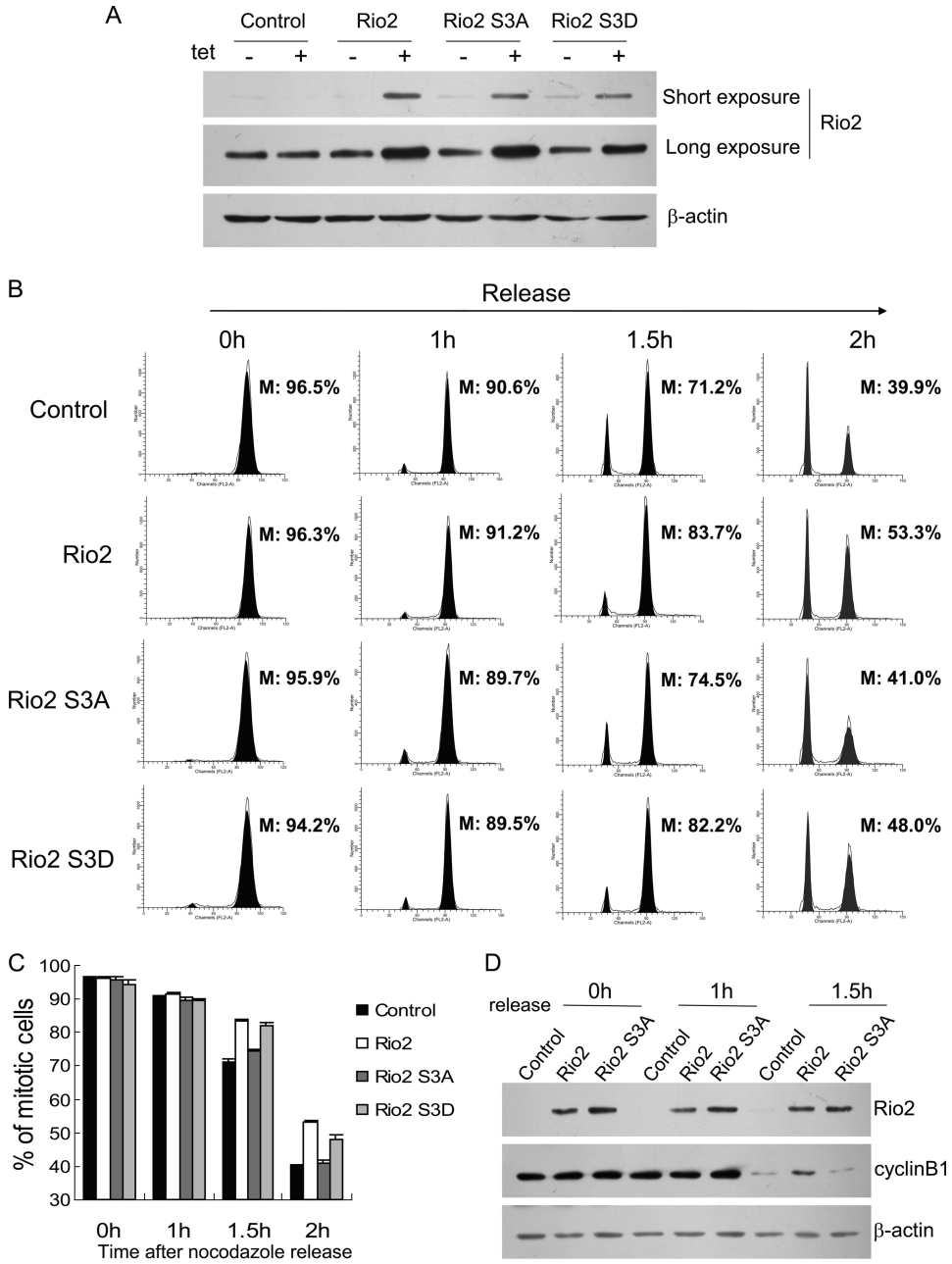

FIGURE 4. **Overexpression of Rio2 influences mitotic exit.** *A*, tet-on inducible HeLa cells (control, Rio2, Rio2 S3A, and Rio2 S3D) were harvested at 24 h in the presence or absence of tetracycline and subjected to immunoblotting with Rio2 antibody. *B* and *C*, tet-on inducible HeLa cells (control, Rio2, Rio2 S3A, and Rio2 S3D) were synchronized at M phase in the presence of tetracycline. Then, the mitotic cells were released and harvested at the indicated times and subjected to flow cytometry analysis (*B*); the percentage of mitotic cells was calculated (*C*). *D*, cell lysates from the above experiment were subjected to immunoblotting with the indicated antibodies.

that the percentage of cells at M phase in Rio2- and Rio2 S3D-overexpressed cells were 10–13% higher than that in the control and Rio2 S3A-overexpressed cells at 1.5 h and 2 h after release (Fig. 4, *B* and *C*). The data suggested that the phosphorylation of Rio2 was involved in controlling the proper timing of mitotic exit. The amount of cyclin B1 was higher in Rio2-overexpressed cells than in control and Rio2 S3A-overexpressed cells at 1.5 h after release from nocodazole block (Fig. 4*D*), which supported the phenotype observed in flow cytometry.

Rio2 Controls the Timing of Metaphase-Anaphase Transition—To investigate further which stage of mitosis was influenced by Rio2, we monitored living cells through mitosis with time-lapse imaging microscopy. As clearly shown in Fig. 5*A*, Rio2-overexpressed cells stayed at metaphase longer than the control and Rio2 S3A-overexpressed cells. However, there seemed to be only marginal differences in the duration of other mitotic phases. The data of statistic analysis indicated that Rio2-overexpressed cells exhibited a slower metaphase-anaphase transition (40.7 ± 2.1 min) than the control (19.6 ± 0.8 min) and Rio2 S3A-overexpressed cells (23.4 ± 1.4 min) (Fig. 5*B*). Taken together, we propose that Rio2 is an important regulator for controlling the proper timing of metaphase-anaphase transition, and its phosphorylation, most likely by Plk1, regulates its function.

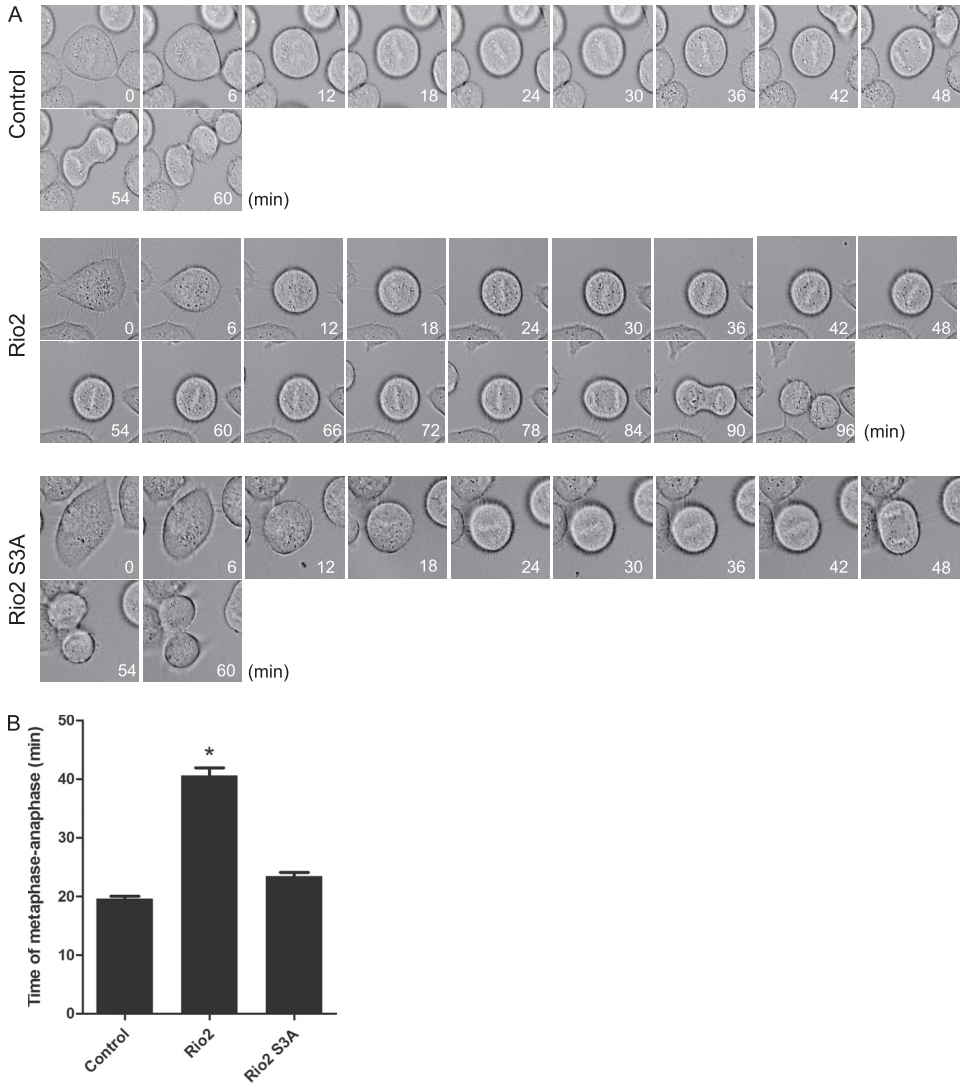

FIGURE 5. **Phosphorylation of Rio2 affects the metaphase-anaphase transition.** *A*, the tet-on inducible HeLa cells (control, Rio2, and Rio2 S3A) were monitored under a microscope. The time-lapse movies were taken, and representative images at the indicated times during mitosis are shown. *B*, the time of metaphase-anaphase transition for tet-on inducible HeLa cells (control, Rio2, and Rio2 S3A) was assessed by video time-lapse microscopy. Results are representative of the average ± S.E. (*error bars*) of 100 mitotic cells from each group monitored individually. *, $p < 0.01$.

Knockdown of Rio2 Accelerates Mitotic Progression—To see whether the expression pattern of Rio2 is cell cycle-regulated, we synchronized HeLa cells at different phases of the cell cycle and detected the protein level of Rio2. The data showed that Rio2 is expressed in different phases of cell cycle at a similar level whereas Plk1 peaked at M phase (Fig. 6*A*). To investigate further the function of Rio2 in cell cycle progression, we took the RNA interference approach to determine whether knockdown of Rio2 affects mitotic progression. As shown in Fig. 6*B*, both sets of siRNA targeting to different regions of the Rio2 gene (siRio2-1, siRio2-2) (17, 18) could knock down Rio2 efficiently. We treated HeLa cells with either control or Rio2 siRNA and subsequently arrested them in mitosis with nocodazole. Mitotic cells were shaken off and released into fresh medium. The flow cytometry analysis data showed that the Rio2-depleted cells exited from mitosis faster than control cells (Fig. 6, *C* and *D*). Immunoblotting analysis showed that knockdown of Rio2 resulted in an accelerated kinetics for the degradation of cyclin B1 (Fig. 6*E*). These data indicated that depletion of Rio2 accelerates mitosis progression. To investigate whether the faster mitosis exit is due to inactive spindle checkpoint, we arrested HeLa cells with thymidine-nocodazole or thymidine-paclitaxel treatment. The mitotic index was determined by FACS using Ser(P)-10-H3 antibody (a well established marker of mitosis). As shown in Fig. 6, *F* and *G*, Rio2-depleted cells arrested at mitosis similar to control cells in the presence of nocodazole or paclitaxel. These results suggested that knockdown of Rio2 did not affect the spindle checkpoint.

DISCUSSION

In this study, we identified Rio2 as a novel substrate of Plk1, and it can be phosphorylated by Plk1 at Ser-335, Ser-380, and Ser-548 *in vitro*. It has been reported that Ser-335 and Ser-380 of Rio2 were phosphorylated at mitosis *in vivo* (30), but the kinase responsible for the phosphorylation was unclear. Our results here suggest that Plk1 is likely to be the kinase that phosphorylates Rio2 at Ser-335 and Ser-380 at mitosis.

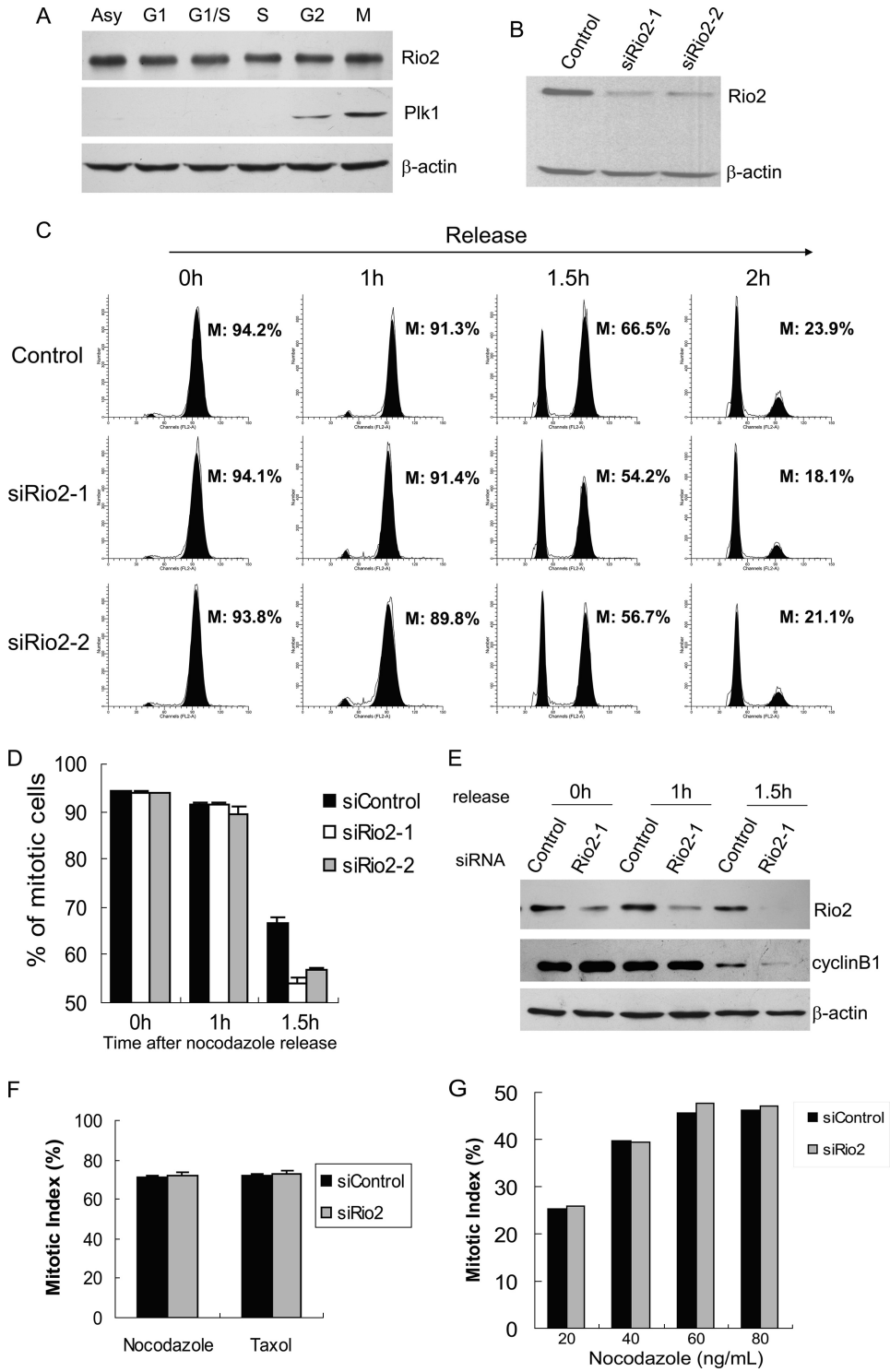

FIGURE 6. **Knockdown of Rio2 causes the accelerated mitotic progression.** *A*, HeLa cells were synchronized at different stages of the cell cycle. The amounts of Rio2, Plk1, and β-actin (as a loading control) were analyzed by immunoblotting. *Asy*, asynchronize cells. *B*, HeLa cells were transfected with control siRNA or siRNA against Rio2 (siRio2-1 or siRio2-2) for 72 h. The cell lysates were harvested and immunoblotted with Rio2 and β-actin antibodies, respectively. *C* and *D*, HeLa cells were transfected with control siRNA, siRio2-1, and siRio2-2 and synchronized to M phase. Mitotic cells were released and collected at the indicated times for flow cytometry analysis (*C*), and the percentage of mitotic cells was calculated (*D*). *E*, cell lysates from the above experiment were subjected to immunoblotting with Rio2, cyclin B1, and β-actin antibodies. *F*, HeLa cells were transfected with control siRNA and siRio2-1 followed by thymidine-nocodazole or thymidine-paclitaxel (Taxol) arrest. Mitotic index was determined by FACS using Ser(P)-10-H3 antibody. *G*, HeLa cells were transfected with control siRNA and siRio2-1 and arrested by treatment with the indicated concentrations of nocodazole. Mitotic index was determined by FACS using Ser(P)-10-H3 antibody.

Rio2 has been found as a Ser/Thr kinase and is capable of autophosphorylation (13). However, the substrates of Rio2 still remain to be discovered, which presents a hurdle to our understanding of the biological function of Rio2 as a kinase. Thus, the autophosphorylation of Rio2 was investigated to see whether Plk1 phosphorylation affected Rio2 kinase activity. Rio2 and

Rio2 S3A mutant showed similar autophosphorylation activity, which means that the kinase activity of Rio2 is probably not influenced by its phosphorylation by Plk1. The kinase domain of Rio2 is at its N-terminal region whereas its phosphorylation sites by Plk1 were all located at C-terminal domain which may explain why its phosphorylation by Plk1 does not influence its kinase activity. It is possible that the phosphorylation of Rio2 by Plk1 may regulate its interaction with other proteins.

Plk1 phosphorylating Rio2 leads us to speculate that Rio2 may play a role in cell cycle progression, especially in mitosis. Considering the result that overexpression of Rio2 does not affect mitotic entry (data not shown), we wonder whether the mitotic exit will be influenced. Interestingly, our results show that overexpression of wild-type Rio2 and phospho-mimicking mutant Rio2 S3D prolong mitotic progression whereas knockdown of Rio2 accelerates mitotic exit, suggesting that Plk1-mediated phosphorylation of Rio2 regulates mitotic progression. In addition, time-lapse imaging data shows that the Rio2-overexpressed cells take a longer time at metaphase than Rio2 S3A-overexpressed cells, although the cells could eventually exit from mitosis (Fig. 5*A*). These results demonstrate that Rio2 plays an important role in controlling the metaphase-anaphase transition. Based on these observations, we propose that the phosphorylation of Rio2 by Plk1 will allow cells to complete mitosis in a proper timing. The exact molecular mechanism by which Rio2 regulates mitotic progression needs to be studied further.

It has been reported that Plk1 activity is involved in a variety of pathways to ensure correct metaphase-anaphase transition. Most of these functions are linked to the regulation of APC/C, an E3 ubiquitin ligase that is responsible for the timely degradation of various mitotic proteins (securin, cyclin B1), thereby regulating chromosome segregation and initiating anaphase (34–36). We find that Rio2 interacts with Apc3 and Apc5 (data not shown), subunits of APC/C complex, suggesting that Plk1-mediated phosphorylation of Rio2 may control the timing of metaphase-anaphase transition by regulating the activity of APC/C. We analyzed whether overexpression of Rio2 influenced the function APC/C by checking the half-life of cyclin B1 in the presence of cyclohexmide. There was no significant difference observed on the half-life of endogenous cyclin B1 between control and Rio2-overexpressed cells (data not shown). It is possible that interaction of Rio2 and APCs may control Rio2 degradation, or Rio2 may play a role in APC/C complex targeting other proteins.

In summary, we have identified Rio2 as a novel substrate of Plk1 and Plk1-dependent phosphorylation of Rio2 regulates the normal timing of the metaphase-anaphase transition. Our studies unveil a novel role of Rio2 in regulating mitotic progression, in addition to its function in ribosome biogenesis. These findings are helpful to a better understanding of the various roles of Plk1 in controlling metaphase-anaphase transition.

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