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CDK11^{p58} Phosphorylation of PAK1 Ser174 Promotes DLC2 Binding and Roles on Cell Cycle Progression

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CDK11^{p58}, a CDK11 family Ser/Thr kinase, is a G2/M specific protein and contributed to regulation of cell cycle, transcription and apoptotic signal transduction. Recently, CDK11^{p58} has been reported to exert important functions in mitotic process, such as the regulation of bipolar spindle formation and sister chromatid cohesion. Here, we identified p21 activated kinase 1 (PAK1) as a new CDK11^{p58} substrate and we mapped a new phosphorylation site of Ser174 on PAK1. By mutagenesis, we created PAK1^{174A} and PAK1^{174E}, which mimic the dephosphorylated and phosphorylated form of PAK1; further analysis showed PAK1^{174E} could be recruited to myosin V motor complex through binding to dynein light chain 2 (DLC2). PAK1^{174E} could accelerate the mitosis progression in a nocodazole blocked cell model, while PAK1^{174A} exhibited an opposite role. Our results indicated PAK1 may serve as a downstream effector of CDK11^{p58} during mitosis progression.

Key words: CDK11p58, cell cycle, dynein light chain 2, mitosis, myosin V, PAK1, phosphorylation.

Abbreviations: DLC1, dynein light chain 1; DLC2, dynein light chain 2; GIT, G-protein-coupled receptor (GPCR)-kinase interacting protein; IRES, internal ribosome entry site; PAK1, p21 activated kinase 1; MTOCs, microtubule organizing centers; PDK1, 3-phosphoinositide-dependent kinase-1; PIX, PAK1-interacting exchange factor; PKA, protein kinase A.

P21 activated kinase 1 (PAK1) is a PAK/STE family Ser/Thr protein kinase. Upon binding to the activated form (GTP binding) of Rho GTPase family members Rac or cdc42 with its amino-terminal PBD domain, PAK1 undergoes a conformational change, which leads to autophosphorylation at several sites, including Thr423, and activation of kinase activity (*1*). Other than Rac and cdc42, many protein kinases or small molecules have also been reported to regulate PAK1 activity via different mechanisms, including PDK1, AKT, PIX, Nck, PKA, p110c and sphingosine (*2–8*). As a protein kinase, PAK1 plays an important role in diverse cellular processes, including regulation of apoptosis signal (*9*), cytoskeleton dynamics and cell motility (*10–13*), transcriptional machinery (*14*). Besides its functions depending on kinase activity, PAK1 also participate in other signal transducing pathways independent of its kinase activity, such as the inhibition of human scavenger receptor class B, type I promoter or cell cycle (*15*, *16*).

PAK1 is phosphorylated on T212 by cdc2 in cells undergoing mitosis (*17*, *18*). Moreover, activated PAK1 can accumulate at chromosomes at prophase and move to the contract ring during cytokinesis. Further analysis made the finding that PAK1 can phosphorylate histone H3 in breast cancer cells during mitosis, thus it is conceivable that PAK1 may play a role in mitotic events (*19*).

CDK11^{p58} is a Ser/Thr kinase and belongs to CDK11 family which has three yet identified isoforms CDK11^{p110}, CDK11^{p58} and CDK11^{p46} (*20*). In human CDK11 family proteins are encoded by two highly similar genes called cdc2L1 and cdc2L2 (*21*). CDK11^{p110} is a 110 kD protein that is ubiquitously expressed in all cell cycles and functions in transcription and RNA processing (*22*). CDK11^{p46} (CDK11^{p50}) is a smaller 46–50 kD peptide containing C-terminal kinase domain of larger CDK11 member proteins whose function is linked with apoptotic signalling (*23*, *24*). CDK11^{p58} is a G2/M specific protein kinase and is translated from the same mRNA as CDK11^{p110} by a CAP-independent translation mechanism through targeting an internal ribosome entry site (IRES) (*25*). Thus, CDK11^{p58} kinase activity achieves its peak during the onset of mitosis. The CDK11^{p58} protein or kinase activity is hardly detected in interphase cells (unpublished data). Like other CDKs, CDK11^{p58} is involved in cell cycle control, apoptosis signal as well as transcriptional repression (*26–29*). CDK11^{p58} also seems to be essential to the maintenance of normal cell cycle. Early embryonic lethality is found with CDK11 knockout mice due to apoptosis of the blastocyst cells between 3.5 and 4 days post-coitus (*30*). Recent studies revealed that CDK11^{p58} promotes centrosome maturation and bipolar spindle formation (*31*). CDK11^{p58} is also essential for the maintenance of sister chromatid cohesion (*32*).

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CDK11 is identified as a RanGTP-dependent microtubule stabilization factor that can regulate spindle assemble rate in Xenopus (*33*). Although CDK11^{p58} plays such important role in mitotic events, little is known about the exact underlying mechanisms.

In our previous report, CDK11^{p46} was characterized as a binding partner and negative regulator of PAK1 in NIH3T3 cells undergoing anoikis, and in our efforts to testify whether PAK1 was a CDK11^{p46} substrate, we found that PAK1 could also be phosphorylated by CDK11^{p58} *in vitro*. In this report, we identified PAK1 as a new substrate of CDK11^{p58} kinase. We also managed to identify a new phosphorylation site of Ser174 on PAK1, as judged by both *in vitro* kinase assay and *in vivo* analysis. Further analysis showed constitutively phosphorylated form of PAK1 on Ser174 could be recruited to myosin V motor complex via binding to dynein light chain 2 (DLC2). Moreover, A375 cells expressing the dephosphorylated form of Ser174 (174A) exhibited delayed M-phase progression after released from nocodazole block. Our studies support the model that PAK1 Ser174 phosphorylation by CDK11^{p58} created a new docking site for PAK1 itself during the progression of mitosis. This is also the first report with regard to potential substrates of CDK11^{p58} in mitosis and provides clues to understanding the mechanism of mitosis regulation.

MATERIALS AND METHODS

Materials—Hela cells, COS-7 cells, 293T cells and the human melanoma cancer cells A375 were obtained from the Institute of Cell Biology Academic Sinica. Dulbecco's modified Eagle's medium (DMEM), Hoechst 33258 were purchased from Sigma. Lipofectamine^{TM} 2000 Reagent, G418 and rabbit polyclonal anti-myc antibody were purchased from Invitrogen. Monoclonal anti-HA antibody, PVDF membrane, leupeptin, aprotinin and phenylmethylsulfonyl fluoride were purchased from Roche. Polyclonal anti-PAK1, anti-PITSLRE anti-GAPDH antibodies, HRP-conjugated goat anti-mouse, goat anti-rabbit secondary antibody were from Santa Cruz Biotechnology. Rabbit monoclonal anti-Fas ligand antibody and anti-phosphoserine antibody (4A4) were from upstate. Phosphor-PAK1 (Thr423) antibody was from Cell Signaling. The enhanced chemiluminescence (ECL) assay kit and [γ-^{32}P] ATP (>3,000 Ci/mM) were purchased from Amersham Biosciences.

Plasmids Construction—The expression plasmid Myr-AKT encoding constitutively active AKT was kindly provided by Dr Jin Q. Cheng (Department of Pathology and Cell Biology and Cell Biology, H. Lee Moffitt Cancer Center and Research Institute). pcDNA3.0-HA-CDK11^{p46}, pcDNA3.0-HA-D149N, PAK1 fragments including 1–394 aa, 1–332 aa, 240–332 aa, 210–332 aa, 148–332 aa cloned into pcDNA3.0-HA and PGEX-4T1 vectors and PAK1-PET-22b were constructed as described previously(*7*, *34*). All of the PAK1 site mutant fragments were generated using site-mutagenesis with the primers as follows (the mutation sites are underlined): S165A (5′-AATGTGAAGGCTGTGGCTGAG ACTCCTGCAGTG-3′ and 5′-GCAGGAGTCTCAGCCACA GCCTTCAC-3′), S174A (5′-GCACTGCCACCAGTTGCAG AAGATGAGGATGAT-3′ and 5′-CATCCTCATCTTCTGC AACTGGTGGC-3′), T185A (5′-GATGATGATGATGCT GCCCCACCACCAGTGATTGCT-3′ and 5′-CACTGGTGG TGGGGCAGCATCATCATC-3′), T197A (5′-CCACGCCCA GAGCACGCAAAATCTGTATACACA-3′ and 5′-GTATAC AGATTTTGCGTGCTCTGGGCGTGG-3′), and were further amplified by PCR then subcloned into pcDNA3-HA and PGEX-4T1 vectors with the primers 5′-GATGAATTC TCAGCTGAGGATTAC-3′ (EcoRI site underlined) and 5′-GATCTCGAGTTAGTCCAAGTAATTCAC-3′ (XhoI site underlined). PAK1 full-length site mutants S174A, S174E and S174AK299R were generated using the same mutagenesis primers as PAK1 site mutant fragments and were then further cloned into pEGFP-N3 and pcDNA3. 1A-myc vectors by PCR amplification with the primers 5′-GATGAATTCTCAGCTGAGGATTA C-3′ (EcoRI site underlined) and 5′-ATAGGATCCGAGA TTGTTCTTTGTTGCCTC-3′ (BamHI site underlined), PCR templates PAK1-PCMV and K299R-PCMV were kind gifts from Dr Steen H. Hanson. PAK1-K299RS174A-PGEX4T1 were generated with the primers 5′-GATGAATTCTCAGCTGAGGATTAC-3′ (EcoRI site underlined) and 5′-GATCTCGAGATGTCAAATAACGGC CTAGAC-3′ (XhoI site underlined) using K299RS174A-pEGFPN3 as template. All of the plasmids constructed above were verified by sequencing.

Cell Culture, Transfection, and Stable Lines Selection—All the cells were cultured in DMEM medium supplemented with 10% (v/v) fetal bovine serum, 100 U/ml penicillin, 50 μg/ml streptomycin and maintained under 5% CO_2 in humidified air. For transient transfection assays, cells were seeded on cell culture dishes 24 h before transfection. The following day, when the cells became 70–80% confluent, totally 8 μg (for 100 mm dishes) of plasmids were transfected with Lipofectamine^{TM} 2000 reagent following the manufacturer's recommendations. After 48 h, the cells were harvested for further analysis. Stable clone selection was conducted by culturing with G418 (400 μg/ml for A375 and HeLa cells) 24 h after transfection. Single clones were isolated after 2–3 weeks of culturing. Stable transfectants with high expression of the target genes were identified by western blot analysis using corresponding antibodies.

Immunoprecipitation and Immunoblot Assays—Exactly 48 h after transfection, cells were washed three times with ice-cold PBS and solubilized with 0.5 ml of lysis buffer [50 mM Tris–HCl (pH 7.5), 150 mM NaCl, 0.1% NP-40, 5 mM EDTA, 5 mM EGTA, 15 mM $MgCl_2$, 60 mM β-glycerophosphate, 0.1 mM sodium orthovanadate, 0.1 mM NaF, 0.1 mM benzamide, 10 μg/ml aprotinin, 10 μg/ml leupeptin and 1 mM PMSF]. Detergent-insoluble materials were removed by centrifugation at 12,000 r.p.m. for 15 min at 4°C. Totally, 200 μg of the whole-cell lysates were incubated with normal mouse IgG (Santa Cruz Biotechnology) or relevant antibody at 4°C for 1 h. Pre-equilibrated protein G-agarose beads (Roche Applied Science) were then added and collected by centrifugation after overnight incubation at 4°C and then gently washed three times with the lysis buffer. The beads were used for *in vitro* kinase assays or

for immunoblot by boiling off the binding proteins in SDS sample buffer.

In Vitro *Kinase Assay*—The CDK11 kinase assay was performed as described previously (*35*), with minor modifications. In brief, HA-tagged CDK11^{p58} or CDK11^{D224N} were transfected in COS-7 cells. Exactly 48 h post-transfection, cells were solubilized in IP lysis buffer, and totally 200 μg of whole-cell extracts were precipitated using HA antibody. Protein kinase activity of the immunoprecipitates was assayed in 30 μl reactions containing kinase buffer {50 mM Tris–HCl (pH 7.5), 15 mM $MgCl_2$, 1 mM dithiothreitol, 50 μM ATP, 1 μCi of [γ-^{32}P]ATP} and 1 μg of purified PAK1 fragments or GST peptide alone as substrates. PAK1 kinase assay was performed in 30 μl reactions containing kinase buffer [100 mM NaCl, 50 mM Tris (pH 7.5), 10 mM $MgCl_2$, 1mM $MnCl_2$, 50 mM ATP], 1 μCi of [γ-^{32}P]ATP, 1 μg of myelin basic protein (MBP) as substrate. After incubation at 30°C for 30 min, the samples were boiled with 4× SDS loading buffer and separated by 10% SDS–PAGE. The gel was then fixed, dried and analysed by FLA-5100 phosphoimaging system (Fujifilm).

In Vitro *Binding Assay*—Briefly, His-tagged PAK1 protein was purified from *Escherichia coli* using Ni Sepharose high performance (GE Healthcare Life Sciences). GST control, GST-DLC1, GST-DLC2 were expressed in *E. coli*, and then purified, and pre-immobilized onto glutathione-Sepharose 4B beads, and incubated with 10 μg of His-PAK1 pre-phosphorylated by CDK11^{p58}. After gentle rotation for 12 h at 4°C, the reactions were washed with lysis buffer [20 mM Tris (pH 7.5), 50 mM NaCl, 10% glycerol, 10 mM NaF, 1% Nonidet P-40, 1 mM $NaVO_4$, 10 μg/ml aprotinin, 10 μg/ml leupeptin, 1 mM PMSF] three times. Bound proteins were eluted by boiling with SDS sample buffer and then subjected to western blot analysis.

RESULTS

PAK1 could be Phosphorylated by CDK11^{p46} and CDK11^{p58} In Vitro—Our group previously found that CDK11^{p46} could form complexes with PAK1 in NIH3T3 cells undergoing anoikis. Moreover, the kinase activity of PAK1 was inhibited by CDK11^{p46}, depending on the kinase activity of CDK11^{p46} itself (*7*). But whether the inhibition is direct or indirect was unknown. In order to testify if CDK11^{p46} could phosphorylate PAK1 directly, *in vitro* kinase assay was performed using purified GST-K299R (a kinase dead form of PAK1, to preclude the possibility of autophosphorylation) or GST peptide expressed in *E. coli* as substrate. As shown in Fig. 1A, while CDK11^{p46} kinase could phosphorylate K299R, no signal was found in GST control lane. CDK11^{D149N} lacking kinase activity was unable to phosphorylate K299R. Since CDK11^{p46} and CDK11^{p58} share the same kinase domain, the possibility whether CDK11^{p58} could also phosphorylate PAK1 was tested. The results of *in vitro* kinase assay are shown in Fig. 1B, intensity of radioactive signal elevated with CDK11^{p58}, while CDK11^{D224N}, a kinase-dead mutant of CDK11^{p58}, failed to phosphorylate PAK1. This result suggested PAK1 could serve as substrate for both CDK11^{p58} and CDK11^{p46} *in vitro*.

CDK11p^{58} Phosphorylates PAK1 on Serine 174 both In Vitro *and In Vivo*—To map the exact phosphorylation site of PAK1 by CDK11^{p58}, we constructed and expressed a series of GST-tagged PAK1 fragments including 1–394 aa, 1–332 aa, 240–332 aa, 210–332 aa and 148–332 aa in *E. coli*. These GST fusion proteins were purified and subjected to *in vitro* kinase assays as potential CDK11^{p58} substrates. As seen in Fig. 1C and D, PAK1 1–394 aa, 1–332 aa and 148–332 aa could be phosphorylated by CDK11^{p58} kinase, although the signal intensity may varied. But CDK11^{p58} failed to phosphorylate 210–332 aa as well as 240–332 aa. The expression level of full-length PAK1 was relatively low due to the expression efficiency of longer fragments in *E. coli* expression system. In summary, the phosphorylation sites of PAK1 may lie between 148 and 210 aa.

The classic CDKs phosphorylate the consensus sequence S/T-P-X-K/R, but they may also have other substrate consensus and specificities. For example, the regulatory myosin-II light chain is phosphorylated *in vitro* and *in vivo* by cdc2 on serines that lack an adjacent proline (*36*). Similarly, some target sites lack adjacent basic residues (*37*). Thus, the absence of this consensus site should not necessarily rule out a protein as a potential CDK11^{p58} target. So we explored all of the possible Ser/Thr phosphorylation sites on PAK1 using NetPhos 2.0 (*38*), the predicted phosphorylation sites were shown in Fig.1E and Fig.1F. We finally selected four candidate sites between 148–210 aa with relatively high prediction scores, including Ser165, Ser174, Thr185 and Thr197. By using site mutagenesis, we were able to change these sites to alanine on the previously constructed 148–332 aa fragment, respectively. Then all of the four site-mutated 148–332 aa mutants were expressed in the form of GST fusion peptides by *E. coli*. *In vitro* kinase assay was performed as illustrated in Fig. 1G. Interestingly, single mutating Ser174 to alanine completely abolished the radioactive signal in gel, while the other three single site mutations had no difference in radioactive signal with wide-type 149–332 aa peptide. Likewise, CDK11^{p58} failed to phosphorylate full-length PAK1-K299RS174A compared to PAK1-K299R (Fig. 1H). These results suggested that Ser174 of PAK1 might be a major phosphorylation site by CDK11^{p58} *in vitro*.

To test whether serine 174 is also phosphorylated by CDK11^{p58} *in vivo*, COS-7 cells were transfected with HA-tagged CDK11^{p58}, CDK11^{D224N}, empty vector and GFP-tagged K299R or K299R^{S174A} as indicated in Fig. 2A. Cells were harvested after 48 h and the GFP-tagged K299R or K299R^{S174A} were immunoprecipitated using monoclonal anti GFP antibody (Roche). Then the binding proteins were subjected to western blot analysis using an antibody that recognizes phosphoserine (Upstate) to detect the serine phosphorylation level of the PAK1 peptides. As shown in Fig. 2A, compared with empty vector control, expression of CDK11^{p58} rather than CDK11^{D224N} promoted PAK1's serine phosphorylation (compare lane 1 and 4, 5, 8). CIP treatment completely abolished the corresponding band, indicating that the antibody was specific to recognize phosphorylated serine. MyrAKT was used here as a positive

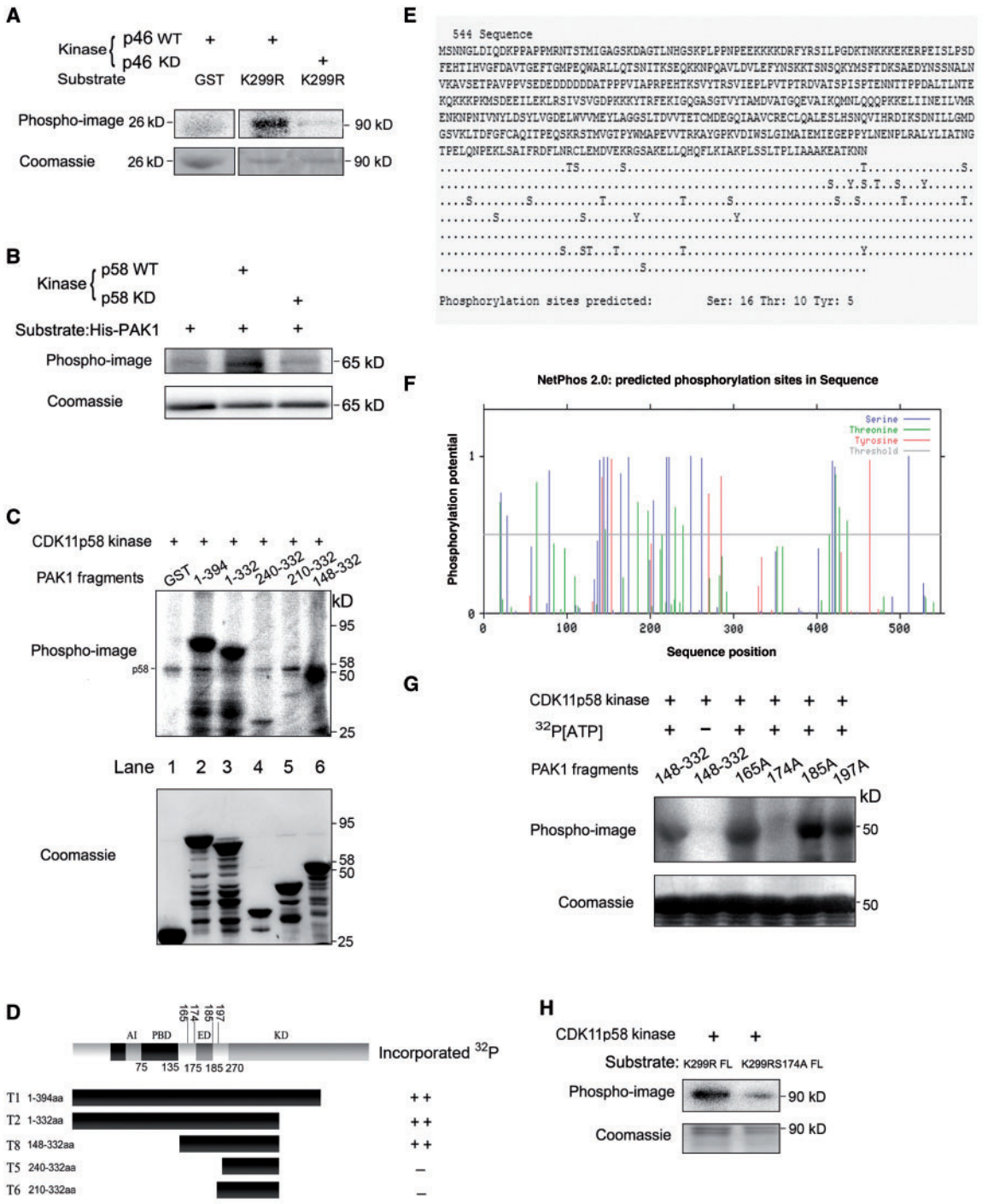

Fig. 1. **CDK11^{p58} phosphorylates PAK1 at Ser174 *in vitro*.** (A) CDK11^{p46} phosphorylates PAK1 *in vitro*. 1 μg of K299R or GST peptide control was incubated with wild-type CDK11^{p46} or kinase dead form (KD) and [γ-^{32}P] ATP in an *in vitro* kinase assay. The reaction mix was separated with 10% SDS–PAGE and incorporated ^{32}P was detected by phosphoimaging. (B) CDK11^{p58} phosphorylates His-PAK1 *in vitro*. One microgram of His-PAK1 expressed by *E. coli* and purified with Ni Sepharose High performance (Amersham) was used as substrate in each reaction. Lower panel shows the Coomassie stain of purified substrates. (C) The same *in vitro* kinase assay of CDK11^{p58} using *E. coli* expressed PAK1 K299R or PAK1 fragments 1–394 aa, 1–332 aa, 240–332 aa, 210–332 aa and 148–332 aa as substrates (upper panel). Lower panel: Coomassie stain of purified substrates used in the *in vitro* kinase assay. (D) Schematic display of the ^{32}P incorporation of PAK1 fragments. (E) Predicted PAK1 potential phosphorylation sites by Netphos 2.0. (F) Schematic display of Netphos 2.0 predicted phosphorylation sites of PAK1 in sequence. Different amino acid residues are displayed with different colours. Threshold score is 0.5. (G) *In vitro* kinase assay of PAK1 site mutants. Indicated PAK1 fragments from *E. coli* was purified and then subjected to *in vitro* kinase assay. Phosphoimaging signals are showed in upper panel. Lower panel: Coomassie staining of PAK1 site mutants after purification with Sepharose 4B beads. (H) *In vitro* kinase assay of CDK11^{p58} using GST tagged full-length PAK1-K299R and PAK1-K299RS174A as substrates.

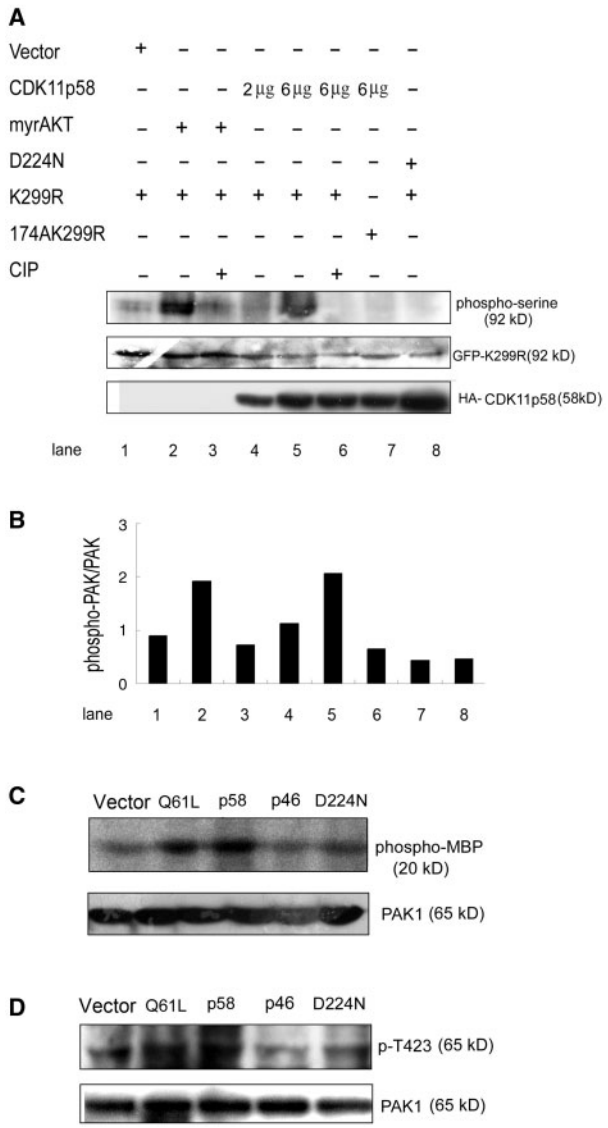

Fig. 2. **CDK11^{p58} phosphorylates and activates PAK1 Ser174 *in vivo*.** (A) COS-7 cells were transfected with 4 μg of GFP-K299R or GFP-K299R174A and 4 μg (or with the indicated amount) CDK11^{p58} or CDK11^{D224N} or myrAKT (as positive control). At 48 h after transfection, cells were immunoprecipitated with 1μg of anti-GFP polyclonal antibody (Roche). The immunoprecipitated proteins were pre-dephosphorylated by CIP (only for + indicated lanes 3 and 6) or directly subjected to western blot and probed with anti-phosphoserine antibody (Upstate). (B) Relative Serine phosphorylation of PAK1 from (A) determined by compares serine phosphorylation level with total K299R protein level of each lane using Total Lab software. Control lane is arbitrarily set at 1. (C) A375 cells transfected with 2 μg of vector control, Q61L, CDK11^{p58}, CDK11^{D224N} and CDK11^{p50}, respectively were immunoprecipitated with PAK1 antibody (Santa Cruz). The immunoprecipitants were subjected to *in vitro* kinase assay to detect endogenous PAK1 activity using MBP as substrates. Upper panel shows the phosphoimaging signal. Fifteen percent of cell lysates were analysed by western blot to detect endogenous PAK1 levels (lower panel). (D) Western blot detection of the duplicate immunoprecipitants from (C) with anti-P-T422 antibody. The bottom panel shows endogenous PAK1 levels of each lane from 15% of the cell lysates.

control (*3*). Interestingly, K299R^{174A} had no serine phosphorylation when co-transfected with wild-type CDK11^{p58}, suggesting that Ser174 is a major phosphorylation site by CDK11^{p58} *in vivo*.

PAK1 is Activated upon CDK11^{p58} Phosphorylation—PAK1 protein could be phosphorylated at multiple serine/threonine sites by a series of protein kinases including PKA, PI3K, AKT, as well as PAK1 itself (autophosphorylation), thus regulating its kinase activity towards various substrates. To explore whether CDK11^{p58} phosphorylation also has an influence on PAK1 kinase activity, we transfected A375 cells with plasmids encoding full-length cdc42-Q61L, CDK11^{p58}, CDK11^{D224N}, CDK11^{p46} or vector control, endogenous PAK1 kinase was then immunoprecipitated to perform *in vitro* kinase assay using MBP as substrates. As depicted in Fig. 2C, overexpression of cdc42-Q61L (activated form of cdc42) caused a drastically increased kinase activity of endogenous PAK1 while CDK11^{p46} inhibited PAK1 activity, consistent with our previous reports (*7*). Unexpectedly, CDK11^{p58} stimulated PAK1 kinase activity to the same extent as Q61L did. This effect seems to be closely related to phosphorylation process because CDK11^{D224N}, a kinase defect form of CDK11^{p58} failed to activate PAK1. We were also able to detect PAK1 activity by the implication of pT423 antibody, which could recognize a single threonine 423 phosphorylation site in the activation loop of PAK1 protein. As seen in Fig. 2D, CDK11^{p58} also stimulated the phosphorylation of T423 as Q61L did, while D224N showed no ability to facilitate this process. Taken together, these results support the idea that CDK11^{p58} can activate PAK1, depending on its kinase ability.

Serine 174 Phosphorylation of PAK1 Promotes PAK1-DLC2 as well as PAK1–myosin V Association—We then examined PIX-PAK1 interaction after CDK11^{p58} phosphorylation since PIX could associate with PAK1 at a proline-rich SH3 site in the region near serine 174 (*39*). We created plasmids encoding both the phospho-mimic and dephosphorylated form of PAK1 on Ser174 by mutant serine 174 to glutamine or alanine (called 174E and 174A, respectively). But PAK1 Ser174 phosphorylation alone did not alter PAK1 binding to PIX or GIT1 (data not shown). We previously reported that DLC2, a 8 kD dynein light chain, is a PAK1 interaction partner upon EGF stimulation but its exact physiological consequence is still under investigation (*40*). Interestingly, we found that DLC2 binding was significantly increased upon Ser174 phosphorylation in co-immunoprecipitation assay, as shown in Fig. 3A (compare lane 2 and 3). As a control, dynein light chain 1, also reported by other groups as a PAK1 binding partner (*41*), associating with 174A and 174E to the same extent (Fig. 3A, lane 4 and 5).

Although DLC1 and DLC2 share >93% amino acid sequence similarity, they have different binding partners and subcellular localization. DLC1 is a component of dynein motor complex, while DLC2 forms complex with myosin V, an actin-based mechanoenzyme (*42*, *43*). Both DLC1 and DLC2 can connect their cargos with motor protein complexes. The fact that DLC2 prefers to bind to 174E suggests PAK1 may be recruited to myosin V

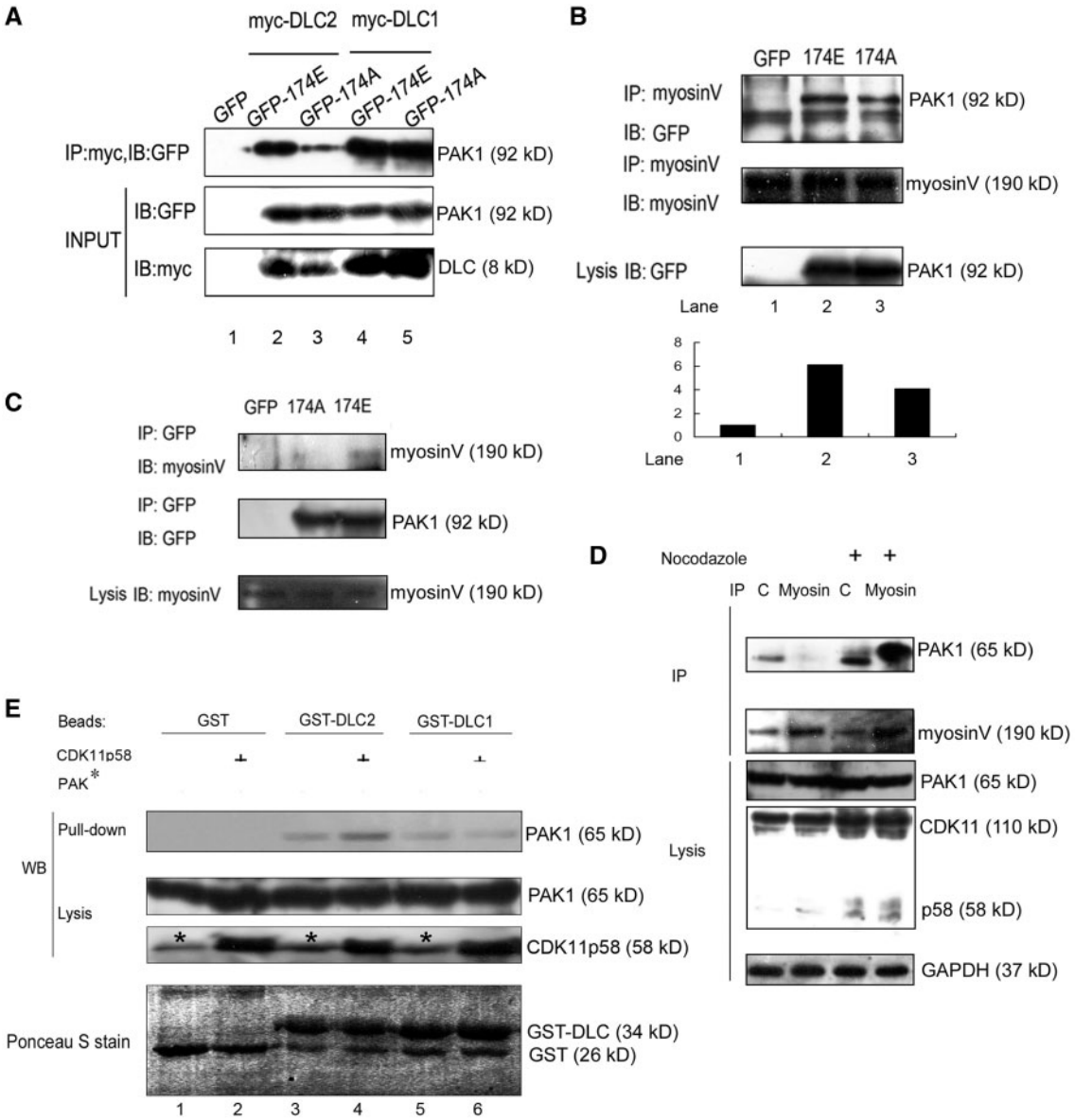

Fig. 3. **Ser174 phosphorylation of PAK1 facilitates PAK1-DLC2 binding.** (A) 293T cells were transfected with the indicated combinations of GFP-174A or GFP-174E and myc-DLC1 or myc-DLC2 expression plasmids. Forty-eight hours after transfection, cells were lysed and performed immunoprecipitation experiments with myc antibody. The bound PAK1 was detected by performing western blot using GFP antibody. Bottom panel shows the expression levels of PAK1 mutants and DLC2 in each lane. (B) Co-immunoprecipitation of transfected GFP-PAK1^{174A} or PAK1^{174E} with endogenous myosin V motor complexes in A375 cells. A 2 μg of anti-myosin VA antibody was used for each immunoprecipitation reaction. The cell lysates were also blotted with GFP-antibody (bottom panel) to show the equal expression levels of PAK1^{174A} or PAK1^{174E}. Lower panel: quantitative data of the Co-IP results using using Total Lab software. The control lane's signal density was set to 1. Similar Co-IP experiments were performed in (C), except that antibody for immunoprecipitation is replaced with anti-GFP. (D) Co-immunoprecipitation of endogenous PAK1 and myosin VA in unsynchronized or nocodazole-blocked A375 cells. A375 cells was treated with 10 ng/ml nocodazole or ethanol (control) for 16 h, then cells were harvested and performed co-IP experiments using anti-myosin VA antibody or normal goat serum as control. PAK1-myosin V complex binding was determined followed by blotting with PAK1 antibody. Lower panels show the expression levels of several proteins indicated. Note that CDK11^{p58} expression was up-regulated by nocodazole treatment. (E) *In vitro* binding of DLCs with His-PAK1 treated with or without CDK11^{p58} kinase. GST control, GST-DLC1, GST-DLC2 was expressed in *E. coli*, purified, and pre-immobilized onto glutathione-Sepharose 4B beads, and incubated with 10 μg of His-PAK1 pretreated with immunoprecipitated CDK11^{p58} or vector control. After gentle rotation for 12 h, bound proteins were eluted by boiling with SDS sample buffer and then subjected to western blot analysis. Bottom panel: Ponceau staining shows the Sepharose 4B beads bound DLCs or GST peptides on the same PVDF membrane. *, unspecific bands.

motor complex upon Ser174 phosphorylation. To investigate this possibility, *in vivo* binding experiments were performed in A375 melanoma cells which express high levels of endogenous myosin V (data not shown). Both 174E and 174A could be co-immunoprecipitated with endogenous myosin V using an anti-myosin VA antibody, but 174A binding to less myosin V compared to 174E. The myosin VA antibody did not pull down GFP in control lane (Fig. 3B). Myosin V could also be pulled down by GFP-tagged 174E using GFP antibody while 174A or GFP alone could not (Fig. 3C). To further prove this possibility in a physiological condition, A375 cells were synchronized in G2/M phase with nocodazole, high levels of CDK11^{p58} was detected, while the protein level of 110 kD CDK11 kinase remained unchanged (Fig. 3D, lower panel), in accordance with previous report that CDK11^{p58} is a G_2/M specific protein. Endogenous PAK1 was co-immunoprecipitated with myosin V but not with immunoprecipitants using normal goat IgG (Fig. 3D, upper panel). PAK1 band could not be found in unsynchronized A375 immunoprecipitants. This indicates that upon CDK11^{p58} phosphorylation at Ser174, PAK1 translocated to myosin V motor complex through binding to DLC2.

To test the direct binding between phosphorylated PAK1 and DLCs, *in vitro* binding assay was also performed. His-PAK1, DLC2 and DLC1 were expressed in *E. coli* as GST fusion proteins. GST pull-down assays demonstrated that CDK11^{p58}-phosphorylated PAK1 had a significant binding to DLC2 compared with untreated PAK1 (Fig. 3E, lanes 3–4). In fact, CDK11^{p58} treatment had no influence on PAK1 binding to DLC1 (Fig. 3E, lanes 5–6). This provides evidence for the direct interaction of DLC2 and PAK1 upon CDK11^{p58} phosphorylation.

174A could Delay Mitotic Progression—To further explore the functional consequences after PAK1 Ser174 phosphorylation, we generated 174A and 174E stable A375 cell lines. Western blot was performed to confirm the recombinant protein expression in selected clones (Fig. 4A). Lines A2 and E1 were chosen for further analysis because of the relative equal recombinant protein expression level compared to endogenous PAK1. Then the two stable lines were analysed using flow cytometry and found no significant difference in cell cycle

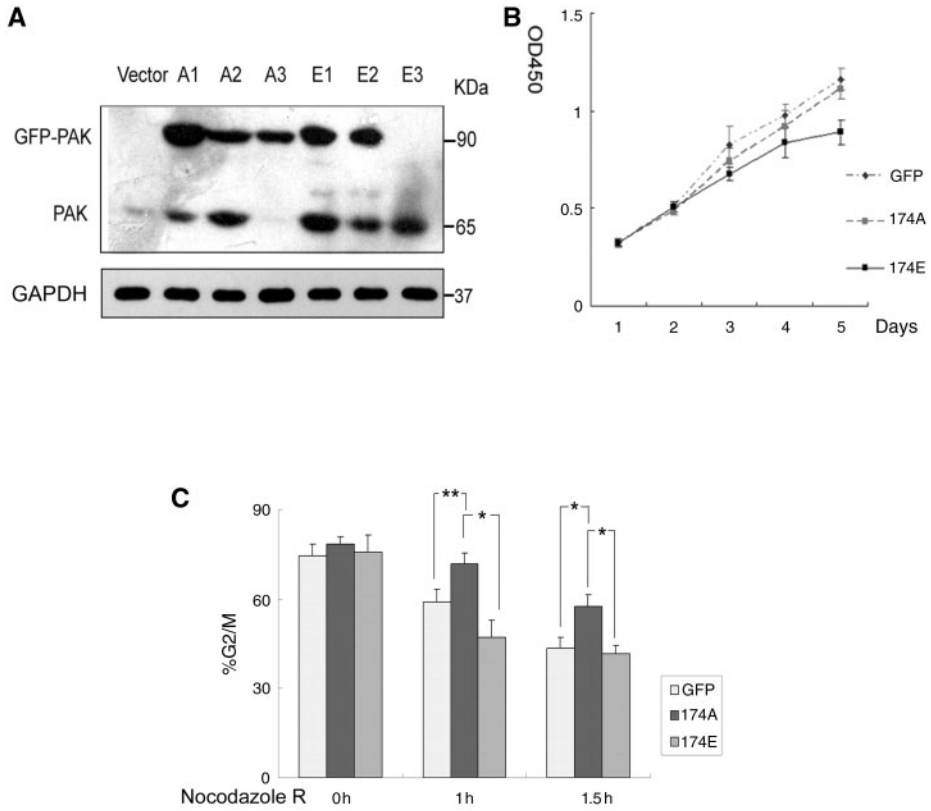

Fig. 4. **Functional analysis of PAK1^{174A} or PAK1^{174E} stable lines.** (A) Identification of A375 stable lines expressing PAK1 site mutants. Western blot analysis of seven selected clones after selection with G418 using PAK1 antibody. GAPDH implicated as a sample loading control. Vector, GFP control; A1, A2, A3, GFP-PAK1^{174A}; E1, E2, E3, GFP-PAK1^{174E}. (B) Equal number of A375 stable cells expressing GFP control, PAK1^{174A} or PAK1^{174E} was cultured. MTT assay was performed at the indicated intervals. Each clone was plated in six wells, the experiments were repeated independently for three times for the same results. Data shown here are the means and standard errors for one representative experiment. (C) A375 stable lines treated with 10 ng/ml nocodazole for 16 h were allowed for re-entering cell cycle by washing with complete growth medium to remove residual nocodazole. At the indicated time points, cells were trypsinized and analysed the percentages of cells in G2/M phase by flow cytometry. $^{*}P < 0.01$; $^{**}P < 0.05$. Data shown are the means and standard errors for three independent experiments.

distribution (data not shown). Next, nocodazole was used to block cells at the beginning of G_2/M phase. After 12 h of treatment with nocodazole, as shown by FACS analysis, $74.54 \pm 3.77\%$ of the vector control cells, $78.45 \pm 2.36\%$ of 174A and $75.84 \pm 5.70\%$ of 174E were blocked at G_2/M phase. Then cells were allowed to re-enter cell cycle by changing the medium containing nocodazole with fresh complete medium. At 1 h after release, the percentages of cells in G_2/M were $59.09 \pm 4.15\%$ of control, $71.89 \pm 3.58\%$ of 174A and $47.04 \pm 5.72\%$ of 174E, respectively. At 1.5 h after release, the percentages were $43.60 \pm 3.56\%$ of control, $57.60 \pm 3.80\%$ of 174A and $41.58 \pm 2.87\%$ of 174E (also displayed in Fig. 4C), indicating that 174A might delay the mitotic progression and 174E could accelerate it, compared with the control.

Cell cycle progression is closely related with normal cell growth kinetics. So we then detect the growth curves of the three stable lines using an improved MTT assay. As shown in Fig. 4B, to our surprise, 174E had a delayed growth curve compared to the vector control and 174A, while 174A and vector control have a similar growth curve. Taken together, these results support the notion that the tight control of Ser174 phosphorylation by CDK11^{p58} is critical to the normal mitosis progression.

DISCUSSION

Many signal pathways have been reported to contribute to mitotic control. Among them, CDK1/cyclinB signaling has been studied extensively. In interphase cells, CDK1 is phosphorylated on Thr14 and Tyr15 by Wee1 and Myt1 (*44*, *45*), sequestering it in inactive state and preventing the immature entry into mitosis. At the onset of G2-M transition, these sites are dephosphorylated by cdc25, and then CDK1 becomes active and triggers the onset and progression of mitosis though phosphorylating a series of substrates. PAK1 is also reported to be phosphorylated by CDK1/cylinB on Thr212. PAK1T212(PO_4) was enriched at MTOCs and spindle to regulates microtubule dynamics (*17*, *18*). Recently, CDK11 family protein CDK11^{p58} is also linked with some mitosis events (*30*). In this report, we found PAK1 was phosphorylated by CDK11^{p58} at a new site of Ser174, and PAK1^{174A} exhibited reduced ability to bind with DLC2-myosin V complex compared with PAK1^{174E}. Since CDK11^{p58} kinase has influences on mitotic spindle dynamics as well as sister chromatid cohesion through some not yet identified mechanisms, identification of new substrates of CDK11^{p58} kinase can help to gain insight to how CDK11^{p58} could link with cytoskeleton dynamics and chromosome–microtubule interplay during mitosis. We show that PAK1 is a substrate of CDK11^{p58} and can be strongly activated upon phosphorylation. Our data provide preliminary evidence for the notion that PAK1 could mediate the CDK11^{p58} regulation towards cytoskeleton dynamics in cells undergoing mitosis. This is also the first report with regard to new substrates of CDK11^{p58} in mitosis and provides new insight into the mechanism underlying the eukaryotic mitosis regulation of CDK11^{p58}.

PAK1 is recently reported to be involved in the regulation of mitotic events. Some aspects of the regulation need PAK1 kinase activity, like histone H3 phosphorylation and PAK1 kinase-dependent cytoskeleton rearrangement. Although cdc2 phosphorylates T212, it does not activate PAK1, so single T212 phosphorylation may not provide full upstream activation signals for the regulation of PAK1 downstream targets. Our experiments showed that CDK11^{p58} could strongly activate PAK1. Depending on the kinase activity of CDK11^{p58}, this activation may help PAK1 to change the cytoskeleton dynamics through kinase-dependent mechanism or phosphorylating its mitotic-specific targets, causing the morphological changes in mitosis.

Although the 8 kD Dynein light chain family (LC8, DLC8) members DLC1 and DLC2 share 93% sequence identity they have unique functions. *In vivo* DLC2 mainly forms complex with myosin V motor, while DLC1 is an integral component of dynein motor complex. DLC1 binding to a BH3 only proapoptotic protein Bim regulates its proapoptotic activity (*46*). DLC2 prefers to form complexes with Bmf (*42*). PAK1 could be a binding partner of both DLC1 and DLC2, but they bind to different regions of PAK1. DLC1 interacts with PAK1 near the region of aa 150–270, while DLC2 binds to aa 210–332 (*40*, *41*). This may explain why CDK11^{p58} treated PAK1 has binding preference only toward DLC2. Unlike other DLC-binding cargo proteins, our sequence analysis revealed that PAK1 does not contain the DLC binding consensus (K/R)XTQT (*47*), indicating that PAK1 is not merely a non-selective cargo of DLCs.

Myosin V was reported to concentrate at mitotic spindles as well as mid-body of the cell (*48*). Myosin V was also detected at MTOCs throughout the whole-cell cycle (*49*). The detailed mechanism is not known yet, but at least, myosin V–microtubule compartments interaction can partly account for this localization pattern. Following Ser174 phosphorylation, PAK1 is activated and recruited to myosin V motors through binding with DLC2, might changing its localization with myosin V complex, then execute its functions on cytoskeleton regulation or other functions via kinase-dependent or independent mechanisms. This is not the first report for the linkage between PAKs and myosin V motors for other group has found that p21-activated kinases Cla4 and Ste20 regulate vacuole inheritance in *Saccharomyces cerevisiae (*50*)*. Further analysis needed to be carried out in order to examine the colocalization of PAK1 and myosin V complex during the cell cycle progression.

Notably, CDK11^{p46} also phosphorylates PAK1 in an *in vitro* kinase assay. It was revealed that Ser174 was a preferred phosphorylation site by CDK11^{p46}, but none *in vivo* phosphorylation signal of PAK1 was detected when CDK11^{p46} was transiently expressed in COS-7 or 293T cells (data not shown). This is not inconceivable because in many cases substrates *in vitro* were not bona fide substrates physiologically. Besides since CDK11^{p46} is cleaved from large CDK11 proteins only when cells undergo apoptosis, the physiological relevance between CDK11^{p46} and PAK1 in mitotic cells is weak. Although the function of CDK11^{p46} as a kinase suppressor of PAK1 depends on its kinase activity, this suppression seems to be indirect rather than direct because PAK1 is not a substrate of CDK11^{p46} *in vivo*.

Unlike CDK11^{p46}, CDK11^{p58} did not form a complex with PAK1 in some *in vivo* binding assays, indeed we failed to detect CDK11^{p58}–wtPAK1 interaction in unsynchronized or M-phase cells. But we did detect CDK11^{p58}–174A interaction in stable A375 cells (Supplementary Fig. 1). CDK11^{p58} was found to associate with 174A, indicating that CDK11^{p58} kinase may form a complex with PAK1 transiently *in vivo*, phosphorylate PAK1 at Ser174, and then disassociate quickly.

The PAK1 Ser174 dephosphorylated form 174A could impair the M-phase progression in a nocodazole release experiment, underlining the importance of correct localization of PAK1 in cell cycle progression. Although release from nocodazole block depends on many parameters, though primarily how fast microtubules re-polymerize and the spindle re-assembles. Since CDK11 could affect microtubule dynamics, it is possible to speculate that PAK1 also plays a role in the process downstream of CDK11^{p58}. It seems controversial that 174A stable lines have unaffected growth kinetics. There are two possible explanations for this. One is in 174A cell lines CDK11^{p58} signal remained intact and could phosphorylate endogenous PAK1, which then acts as CDK11^{p58} downstream effectors in promoting morphological changes of mitotic cells. The other explanation is although 174A expression displayed impaired M-phase progression in G_2/M blocked cell model, it is not enough to cause the abnormalities in the whole cell cycle. This notion is confirmed by the fact that the cell cycle distribution remains unaltered in 174A stable lines. The slowed growth kinetic exhibited by the 174E stable line could be partly explained by the fact that 174E caused an immature exit to mitosis and thus impaired the cell cycle control machinery. As we failed to generate antibody specific to recognize Ser174 phosphorylation, we could not provide direct evidence for the hypothesis that PAK1 could act as a CDK11^{p58} effecter as well as whether CDK11^{p58} is the unique Ser174 kinase of PAK1, but it is obvious that the tight control of PAK1 Ser174 phosphorylation status is critical to normal cell cycle progression as well as cell growth. To further explore the detailed roles of PAK1 downstream of CDK11^{p58} in mitosis, the scrutiny of changes including bipolar spindle dynamics, chromosomes condensation or separation needs to be pre-formed in future studies. CDK11 RNAi caused severe impairments in cell cycle, proliferation and apoptosis, the consequences of 174A overexpression seems not as severe as CDK11 RNAi, so the identification of other CDK11^{p58} downstream targets will also be helpful to the further study of CDK11^{p58} functions in mitosis.

SUPPLEMENTARY DATA

Supplementary data are available at *JB* online.

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CONFLICT OF INTEREST

None declared.

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