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14-3-3zeta cooperates with Phosphorylated Plk1 and is required for correct cytokinesis

Jian Du [1], Lijian Chen[2], Xin Luo[1], Yuxian Shen[3], Zhen Dou[4], Jilong Shen[5], Li Cheng[1], Ying Chen[3], Chengjin Li[3], Hua Wang[6], Xuebiao Yao[4]

[1]Department of Biochemistry and Molecular Biology, Anhui Medical University, Hefei 230032, China, [2]Department of Anaesthesiology, the First Affiliated Hospital of Anhui Medical University, Hefei 230032, China,[3]Key Laboratory of Gene Resource Utilization for Genetic Diseases of Educational Ministry and Anhui Province, Anhui Medical University,[4]Anhui Provincial Key Laboratory for Cellular Dynamics and Chemical Biology, Hefei 230027, China, [5]Department of Microbiology and Parasitology, Anhui Provincial Key Laboratory of Genomic Research, Anhui Medical University, Hefei 230032, China, [6] Department of Oncology, Anhui Provincial Hospital, Hefei 230001, China

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1. ABSTRACT

Proteins of the 14-3-3 family are functionally conserved in eukaryotic kingdom which participates in diversified and critical cellular processes. However, the exact roles of these proteins in mitotic regulation has remained elusive. Polo-like kinase 1 (Plk1) is a serine/threonine protein kinase that plays multiple critical functions such as centrosome maturation, mitotic chromosome segregation, cytokinesis, and the DNA damage response. Here we show that 14-3-3zeta interacts and cooperates with Plk1 in mitotic progress. 14-3-3zeta is associated with the spindle at metaphase and concentrated in the midbody during cytokinesis. Using yeast two hybrid assay, we found a functional connection between 14-3-3zeta and Plk1. We demonstrate that phosphorylation of Plk1 at S330 and S597 promotes its interaction with 14-3-3zeta. Importantly, 14-3-3zeta cooperates with Plk1 in ensuring successful cytokinesis. We conclude that mitotic phosphorylation of Plk1 promotes interaction with 14-3-3zeta and this interaction is required for faithful cytokinesis. Taken together with the results of previous studies, our results suggest 14-3-3 family emerges as a novel player in mitotic regulation: cooperation with Plk1 to ensure a faithful cytokinesis.

2. INTRODUCTION

Successful completion of the cell division cycle requires that the genome be duplictated accurately and apportioned equally to daughter cells. The process of cytokinesis divides the cytoplasm of a mitotic cell and finally yields two daughter cells. Defects in these processes cause genome instability and predispose to cancer(1). Many studies have focused on the early stage of cytokinesis, but the final stage of the process, termed abscission, where the thin strand of membrane and cytoplasm connecting the two cells is severed, remains elusive. During the final stage of cytokinesis, a structure called central spindle, becomes squeezed together as the cleavage furrow ingresses, eventually resulting a narrow tightly packed region of microtubules termed the midbody. Plk1, a mammalian M phase polo-like kinase, and Polo like kinase (Plks) in other organisms, have been implicated in several critical events in cell division(2,3). Plk1 localizes to centrosomes and kinetochores during early stages of mitosis and then to midbody during later stages(4,5). Such dynamic changes in subcellular localization of Plk1 are believed to be important for its function. Recently, Polo-like kinase1(Plk1) has emerged as a key regulator of cell divisions in eukaryotic cells. Several independent studies have now used chemical tools to specifically block the catalytic activity of Plk1

during anaphase. These studies have identified Plk1 as an essential early regulator of cytokinesis and anaphase spindle elongation in mammalian cells. While Plk1 activity was dispensable for the localization of both centralspindlin subunits Mklp1 and HsCyk-4 to the spindle midzone, recruitment of Ect2, the GEF protein critical for Rho activation, was abolished when anaphase cells were treated with Plk1 inhibitors(6,7,8,9) .Inactivation of Plk1 abrogated the formation of the contractile ring and prevents cleavage furrow ingression. The HsCyk-4/Ect2 complex might represent a crucial link between the mitotic spindle and the cell cortex. Inactivation of Plk1 prevented the interaction of Ect2 with its central spindle anchor protein HsCyk-4 and the localization of Ect2 to the spindle midzone.

The 14-3-3 proteins represent an evolutionarily conserved family that are involved in a wide range of biological processes(10,11). These small, acidic polypeptides of 28–32 kD are highly conserved, expressed in all eukaryotic organisms, and consist of seven different isoforms encoded by distinct genes in human with a variety of biological functions. Acting as either hetero- or homodimers, 14-3-3 proteins bind to specific phosphoserine and phosphothreonine sequence motifs and regulate target protein function by compartmental sequestration, altered enzymatic activity, or inhibition/promotion of protein-protein interactions(12,13,14). The 14-3-3 proteins have been implicated in G1/S and G2/M transition by binding to regulatory proteins and modulating their function. The CDC25C and CDC25B proteins are dual-specificity phosphatases that remove inhibitory phosphate groups from Thr14 and Tyr15, and thereby activate the cyclin-dependent kinase CDC2 — the main protein involved in driving cells through mitosis (15,16,17) During interphase, premature activation of CDC2 is prevented by the 14-3-3-mediated cytoplasmic sequestration. That is, 14-3-3 masks a nuclear localization sequence, preventing Cdc25 nuclear translocation and thereby inactivation of CDC25 proteins(18). Above all, 14-3-3 proteins function at several key points in G1/S- and G2/M-transition has been studied extensively; however, little is known about the exact roles of 14-3-3 proteins in regulation of mitotic progression (19,20,21).

Here, we demonstrate that *14-3-3* zeta is associated with the spindle in metaphase and concentrates in the midbody during cytokinesis. In addition, we found a functional connection between 14-3-3zeta and Plk1. Phosphorylation of Plk1 at S330/597 promotes its interaction with *14-3-3* zeta. *14-3-3* zeta and Plk1 may cooperate at the midbody to coordinate mitotic exit and cytokinesis. Our results have led us to predict that 14-3-3 family emerges as a novel player in mitotic regulation.

3. MATERIALS AND METHODS

3.1. Yeast two- hybrid screening

The Matchmaker GAL4 two-hybrid system 3 (BD Biosciences Clontech, Palo Alto, CA) was used to perform yeast two-hybrid screening. The Gal4-fused 14-3-3zeta was a generous gift from Dr. Haian, Fu (Emory University, USA) and then subcloned into pGBKT7vector (BD Clontech, Palo Alto, USA)and used as bait. 2 x 10^6 transformants from a human HeLa cDNA library (pGADT7 plasmid, Clontech) were screened in the yeast strain AH109 (Clontech) and 21 colonies were identified as positive clones. Among these 21 positive colonies, two were revealed to encode Plk1.

3.2. Reagents

The antibodies and reagents used in this study were as follows: mouse monoclonal anti-GFP antibody (BD Biosciences, San Diego, CA), mouse monoclonal anti-FLAG antibody (M2), mouse monoclonal anti-α-tubulin antibody , anti-FLAG M2 affinity gel, mouse monoclonal anti-His antibody , mouse monoclonal anti-Plk1 antibody (Invitrogen, Carlsbad, CA), rabbit polyclonal anti-14-3-3zeta antibody (Santa Cruz Biotechnology, INC.), FITC-conjugated goat anti-mouse IgG, Rhodamine-conjugated goat anti-mouse IgG, and Rhodamine-conjugated goat anti-rabbit IgG (Jackson ImmunoResearch, West Grove, PA), Lipofectamine 2000 and Oligofectamine reagent (Invitrogen, Carlsbad, CA), Glutathione-Sepharose 4B beads (Amersham Biosciences, Piscataway, NJ), Ni-NTA agarose beads (Qiagen, Valencia, CA).

3.3. DNA constructions

Human 14-3-3 isoforms (beta, gamma, epsilon, zeta, eta, sigma) cDNA were originally generous gifts from Dr. Haian Fu (Emory University, USA). Then, each cDNA was cloned into pEGFP-C1, p3XFLAG-myc-CMV-24 and pGEX-5X-3 vector respectively. The full-length cDNA encoding Plk1 (GenBank accession no. NM_005030) was then constructed into pGBKT7, pEGFP-N3, p3XFLAG-myc-CMV-24, and pET-28a(+) vectors. All plasmid constructs were sequenced for verification.

3.4. Recombinant protein production

GST-14-3-3zeta and His-Plk1 were expressed in *Escherichia coli* BL21 (DE3) and purified by affinity chromatography using Glutathione-Sepharose 4B beads (GST-PML3) or Ni-NTA agarose beads (His-Plk1).

3.5. GST Pull-down assay

GST-14-3-3zeta or GST was purified and conjugated to Glutathione-Sepharose 4B beads. Purified His-Plk1 was incubated with GST-Plk1-conjugated or GST-conjugated beads at 4˚C for 2 h. Resultant beads were washed three times with pre-cooled PBS plus 1% Triton X-100 and three times with PBS. Proteins bound by beads were fixed in Laemmli loading buffer, subjected to 10% SDS-PAGE, transferred to nitrocellulose membrane, probed with His monoclonal antibody, and finally developed with an ECL kit.

3.6. Cell culture, transfection and synchronization

HeLa cells, from American Type Culture Collection (Rockville, MD), were cultured as subconfluent monolayers in DMEM (Invitrogen, Carlsbad, CA) with 10% FBS (Hyclone, Logan, UT) and 100 U/ml penicillin plus 100 μg/ml streptomycin (Invitrogen, Carlsbad, CA). The transient transfection of plasmids and siRNA were

performed by Lipofectamine 2000 or Oligofectamine reagent respectively, according to the manufacturer's recommendations. For cell-cycle arrest in G1/S, HeLa cells were treated with 2 mM thymidine for 14 h, and then released for 10 h, after another round of thymidine treatment for 14 h, cells were arrested in G1/S transition. For cell-cycle arrest in G2/M, HeLa cells were treated with 100 ng/ml nocodazole for 24 h.

3.7. Co-immunoprecipitation assay

For co-immunoprecipitation experiment, Flag-14-3-3zeta was co-transfected with GFP-Plk1 wild type, GFP-Plk1$^{S330/597D}$, GFP-Plk1$^{S330/597A}$ or GFP vector into 293T cells by Lipofectamine 2000. Thirty-six hours after transfection, the cells were harvested and lysed in pre-cooled lysis buffer (50 mM HEPES, pH 7.4, 150 mM NaCl, 2 mM EGTA, 1% Triton X-100, 1mM phenylmethylsulfonyl fluoride, 10 g/ml leupeptin, and 10 g/ml pepstatin A) and /or with 1mM okadaic acid (Alexis) or 200 units/reaction λ-phosphatase (Invitrogen) for 30 minutes at 30□. After centrifugation, the supernatants were incubated with anti-FLAG M2 affinity gel at 4˚C for 4 h and then washed three times with lysis buffer and three times with PBS. Proteins were eluted with 50 µl Laemmli loading buffer, and resolved by 10% SDS-PAGE. Separated proteins were subsequently transferred to nitrocellulose membrane for immunoblotting with either GFP or FLAG monoclonal antibodies, respectively.

3.8. Small Interfering RNA (siRNA)

For the siRNA studies, the 23-mers of the siRNA duplex against Plk1(NO.NM_005030, target sequence, 5'-AAGGGCGGCTTTGCCAAGTGCTT-3') was designed as described previously(22) and synthesized by Dharmacon Research Inc. (Lafayette, CO). After trial experiments using a series of concentrations and time course assays, treatment at 150 nM for 48 h was finally selected as the most efficient conditions for repressing Plk1 protein .14-3-3zetasiRNA(NO. NM_001135702 , pooled sequences:#1, GCUUCCAUGUCUAAGCAAATT; #2, GGUACAUUGUGGCUUCAAATT; #3, CCAGUCACAGGUGUAGUAATT), 14-3-3zeta siRNA#3 treatment at 200nM for 48h was finally selected as the most efficient conditions for repressing 14-3-3zeta protein.

3.9. Immunofluorescence microscopy

Cells were grown on acid-treated glass coverslips. After transfection or drug treatment, cells were washed twice with PBS and one time with PHEM (60mM PIPES, 25mM HEPES, 10mM EGTA, 2mM $MgCl_2$, pH 6.9). Permeabilization was carried out for 1 min in PHEM buffer containing 0.1% Triton X-100 at room temperature. Pre-extracted cells were fixed in 3.7% formaldehyde in PHEM buffer for 10 min. After washing three times with PBS, cells were blocked with 1% BSA in PBS containing 0.05% Tween-20 for 30 min, and then incubated with primary antibodies for 1 h followed by secondary antibodies for 30 min. Slides were examined with a Zeiss Axiovert-200 fluorescence microscope, and images were collected and analyzed with Image-5 (Carl Zeiss, Germany). All images were processed with Adobe Photoshop 7.0 software.

4. RESULTS

4.1. Subcellular localization of 14-3-3 isoforms in cell cycle

Numerous genetic and biochemical studies have shown that 14-3-3 proteins play a key role in cell cycle regulation(11,13). It is conceivable that transient regulatory changes in expression and localization of 14-3-3 occur during the cell cycle. One possible regulatory mechanism that could account for isoform-specific cellular function is differential subcellular localization(23,24,25), presumably reflecting differences in biological functions. To address this possibility we investigated the localization of the various 14-3-3 isforms with isform specific antibodies (beta, epsilon, zeta, eta, sigma, gamma) throughout the cell cycle of the human *HeLa* cells. The beta isoform showed diffuse cytoplasmic staining (Figure1*A*, beta). The gamma isoform showed diffuse cytoplasmic staining and a significant Golgi presence (Figure1*A*, gamma, *yellow arrowhead*), indicating that this isoform may play a role in Golgi function. Immunofluorescence of HeLa cells showed that the cellular localization of 14-3-3epsilon is cytoplasmic, mostly of a diffuse nature but with some filament-like organization and no detectable nuclear presence (Figure1*A*, epsilon). The zeta isoform and eta isoform had diffuse cytoplasmic localization and weak nuclear staining (Figure1*A*, zeta and eta). The sigma isoform showed a diffuse cytoplasmic presence, and no detectable nuclear presence (Figure1*A*, sigma).

14-3-3 proteins are known to bind to several key cell cycle regulators, modulating their activity(11,26). Then, we investigated the subcellular localization of 14-3-3 isoforms with isform specific antibodies during the mitosis of *HeLa* cells. Cellular localization of the beta isoform was unvaryingly diffuse throughout mitosis(Figure1*B*, beta *yellow arrow*). We did not observe any significant localization of 14-3-3sigma to the spindle at least to detectable levels during mitosis (Figure1*B*, sigma). Just like the beta isoform, cellular localization of the gamma and eta isoforms were also unvaryingly diffuse throughout the mitosis with no particular localization to discrete mitotic structure (Figure1*B*, gamma and eta). epsilon isoform localized at the chromosome periphery when the nuclear membrane and nucleoli were disrupted at prometaphase (Figure1*B*, epsilon). Our studies, however, revealed the zeta isoform to be specifically associated with centrosomes and the spindle apparatus .We also found strong staining of the midbody within the area of constriction between the two daughter cells (Figure1*B*, *white arrowhead*) and suggested a possible novel role of this protein in mitotic exit. Immunofluorescence staining of the endogenous protein indicated that *14-3-3* zeta localized to the mitotic spindle regions at metaphase. At anaphase, *14-3-3* zeta localized at the midbody (Figure1C). Deconvolved image showed that 14-3-3 zeta exhibited two punctate stainings at the midbody in telophase (Figure1C, white arrowhead). All the localizations of 14-3-3 zeta observed here were specific because 14-3-3 zeta signals were abolished in 14-3-3 zeta siRNA-transfected cells (Figure5B). In addition, localization of other isforms was diffuse during cytokinesis is with no particular localization to discrete mitotic

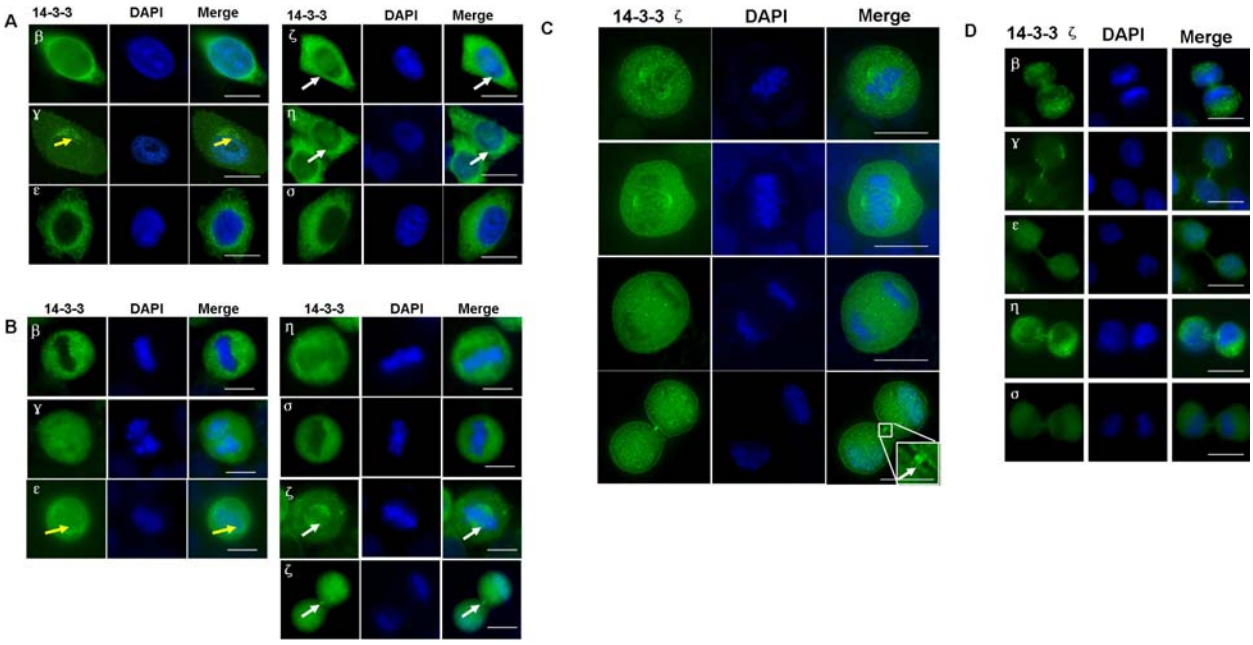

Figure 1. Subcellular distribution of 14-3-3 isoforms in HeLa cells. *A*, Cellular distribution of 14-3-3 isoforms beta, gamma, sigma, epsilon, zeta and eta in interphase cells. This set of montage represents optical images collected from HeLa cells stained for 14-3-3 isforms with specific antibodies (beta, gamma, sigma, epsilon, zeta and eta) (14-3-3 isforms, green), DAPI(DNA, blue) and their merged images. *Scale bar*, 10 μm. *B*, Localization of 14-3-3 isoforms during mitosis. HeLa cells were synchronized at the G1/S boundary by double thymide block, warmed with phosphate-buffered salie(PBS) and incubated with complete media for 8~9hr. synchronized HeLa cells were extracted, fixed. Optical images collected from one HeLa cell stained for 14-3-3 isforms with specific antibodies (beta, gamma, sigma, epsilon, zeta and eta) (14-3-3 isforms, green), , DAPI(DNA, blue) and their merged images. *Scale bar*, 10 μm. C, Deconvolved image of 14-3-3zeta during the cell cycle. 14-3-3 zeta is associated with the spindle at metaphase and concentrates in the midbody during cytokinesis. The *white arrowhead* indicates two punctate stainings at the midbody in telophase. Optical images collected from one HeLa cell double stained for anti-14-3-3zeta antibody(14-3-3zeta, green) and DAPI(DNA, blue)and their merged images. *Scale bar*, 10 μm. D, Localization of 14-3-3 isoforms during cytokinesis.HeLa cells were synchronized at the G1/S boundary by double thymide block, warmed with phosphate-buffered salie(PBS) and incubated with complete media for 9hr. synchronized HeLa cells were extracted, fixed. Optical images collected from one HeLa cell stained for 14-3-3 isforms with specific antibodies (beta, gamma, sigma, epsilon, zeta and eta) (14-3-3 isforms, green), , DAPI(DNA, blue) and their merged images. *Scale bar*, 10 μm.

structures (Figure1D). Together, these results show that *14-3-3* zeta is associated with the spindle at metaphase and concentrates in the midbody during cytokinesis.

4.2. Plk1 is a novel 14-3-3 binding protein

To better understand the role of *14-3-3*zeta in cellular dynamics, we performed a yeast two-hybrid to screen a human HeLa library using *14-3-3*zeta as the bait. Among the positive clones, one interesting candidate is Polo-like Kinase 1 (Plk1). To verify the interaction between Plk1 and *14-3-3*zeta, we co-transformed AH109 yeast competent cell with pGADT7- Plk1 and pGBKT7-*14-3-3*zeta. As shown in Figure2A, the co-transformation assay confirmed that Plk1 interacts with *14-3-3*zeta. To test whether Plk1 specifically interacts with *14-3-3*zeta, we co-transformed six BD-14-3-3 isoforms with AD-Plk1 in yeast AH109. Our results showed that 14-3-3 zeta and 14-3-3 eta could interact with Plk1 in yeast(Figure2B). If *14-3-3*zeta and 14-3-3eta are cognate binding partners of Plk1, they should co-distribute during the cell cycle. However, our previous study indicated that cellular localization of endogenous eta isoform was unvaryingly diffuse throughout the mitosis with no particular localization to discrete mitotic structure (Figure1B, eta and Figure1D , eta). Thus, we supposed that 14-3-3eta may not a bona fide binding partner of Plk1 during mitosis. To this end, we also examined the distribution of endogenous *14-3-3*zeta and Plk1 during mitosis in *HeLa* cells (Figure2C). *14-3-3*zeta signal at the spindle is strong in metaphase and weaker at anaphase and then *14-3-3*zeta localizes to the midbody during cytokinesis. Notably, *14-3-3*zeta colocalized with Plk1 at the midbody, demonstrating that these two proteins have similar spatiotemporal dynamics at the midbody during cytokinesis (Figure2 *C, white arrowhead*).

4.3. Evidence for the interaction between Plk1 and 14-3-3zeta *in vivo*

Our results presented above have confirmed the interaction specificity between Plk1 and *14-3-3* zeta by yeast two-hybird assay. To verify that the interaction between Plk1 and *14-3-3*zeta also occurs in mammalian cells, we carried out an exogenous immunoprecipitation assay. We used anti-FLAG antibodies to immunoprecipitate soluble Plk1 and its binding proteins

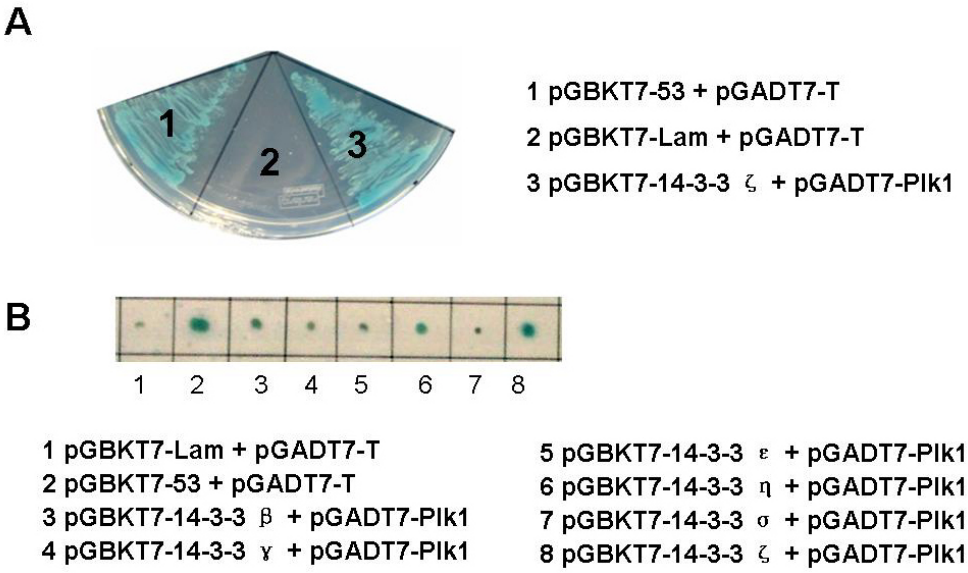

Figure 2. Identification of Plk1 and 14-3-3zeta interaction. A. Interaction of Plk1 and 14-3-3 zeta in the yeast. AH109 cells were co-transformed with indicated plasmids and then selected on supplemented minimal plates lacking tryptophan, leucine, histidine, and adenine. The interaction between Plk1 and 14-3-3zeta was revealed through staining for beta-galactosidase activity with X-α-Gal.1, Yeast cells were co-transformed with pGBKT7-53 and pGADT7(positive control); 2,Yeast cells were co-transformed with pGBKT7-lam and pGADT7(negative control). 3, Yeast cells were co-transformed with pGBKT7-14-3-3zeta and pGADT7-Plk1. B. Yeast cells were co-transformed with Plk1 bait construct and pGBKT7-lam (negative control), pGBKT7-53 (positive control) or 14-3-3 isoforms beta, gamma, sigma, epsilon, zeta and eta prey constructs as indicated. This experiment demonstrated that Plk1 interacts directly with the 14-3-3 zeta and eta isoforms. C. Plk1 co-distributes with 14-3-3 zeta to the midbody during cytokinesis. A merged image shows the co-localization of Plk1 and 14-3-3 zeta to the midbody mitotic cells. This set of optical images was collected from HeLa cells triple stained with mouse Plk1 antibody (red),DAPI (blue), anti-14-3-3 zeta antibody (green). *Scale bar*, 10 μm

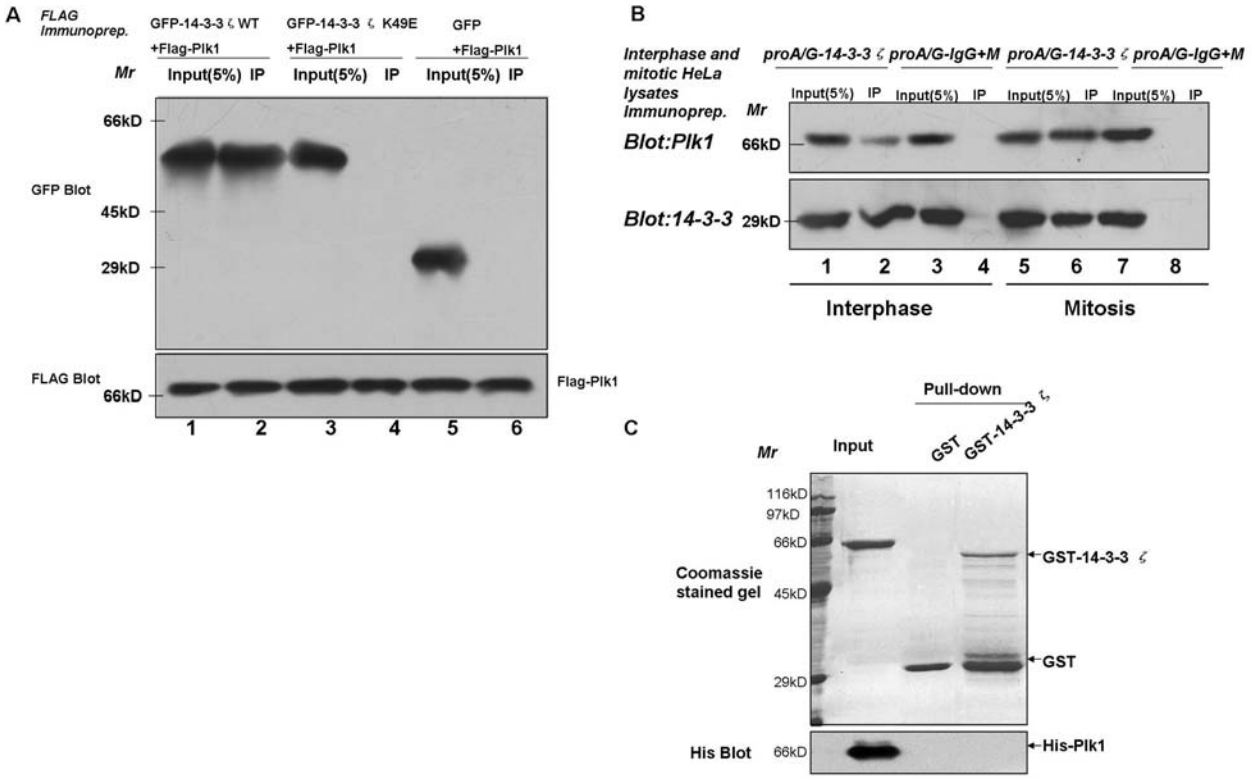

Figure 3. Evidence for the interaction between Plk1 and 14-3-3zeta. A, Exogenous Plk1 interacts with *14-3-3*zeta *in vivo*. HEK293T cells were transfected FLAG-tagged Plk1 with GFP tagged wild type 14-3-3zeta(WT) or GFPtagged 14-3-3zeta K49E or GFP. Thirty-six hours post-transfection, HEK293T cells were then extracted with 1% NP-40 plus 0.5% deoxycholate, and FLAG-tagged Plk1protein and its accessory were precipitated with 10 µl FLAG-M2-antibody-conjugated beads. Coprecipitated proteins were then immunoblotted for the presence of GFP- 14-3-3zeta WT, the binding null 14-3-3zetaK49E mutant proteins and GFP proteins (upper panel) or FLAG-Plk1(lower panel). B, Co-immunoprecipitation of endogenous Plk1 and *14-3-3*zeta from the interphase and mitotic cells. Extracts from the interphase or mitotic HeLa cells were extracted with 1% NP-40 plus 0.5% deoxycholate, and endogenous 14-3-3zeta protein and its accessory were precipitated with 10 µl 14-3-3zeta-antibody-conjugated proA/G beads. Coprecipitated proteins were then immunoblotted for the presence of Plk1 proteins (upper panel) or 14-3-3zeta (lower panel). C, *In vitro* Plk1 and *14-3-3*zeta interaction verified by pull-down assay. GST fused 14-3-3zeta recombinant protein was purified on glutathione-agarose beads and used as affinity matrix for absorbing histidine-tagged Plk1 as described in experimental procedure. Glutathione-agarose beads pre-bound GST protein was used as a negative control. The result suggests that high affinity 14-3-3zeta binding site might be buried in the over-expressed exogenous recombinant Plk1.

from lysates of mitotic 293 T cells transiently transfected FLAG-tagged Plk1 and GFP-tagged.*14-3-3*zeta , or the GFP-tagged binding-null 14-3-3zeta K49E mutant as a negative control.Western blot with GFP antibody validated that Plk1 is specially co-precipitated with *14-3-3*zeta as the binding-null 14-3-3zeta mutant K49E mutant can not bind to Plk1 (Figure3*A*). To examine whether endogenous 14-3-3zeta shows a similar interaction with Plk1 during mitosis, lysates from interphase and mitotic HeLa cells were incubated with an antibody against *14-3-3* zeta and the immunoprecipitates were examined for the presence of co-precipitating Plk1. Immunoprecipitates obtained with 14-3-3zeta antibodies were found to contain 14-3-3zeta (Figure3 *B*, *bottom panel*) as well as co-precipitating Plk1 during mitotic cell (Figure3 *B*, *upper panel*). Our result also showed the interaction between endogenous *14-3-3*zeta and Plk1 occurs specifically during mitosis because he interaction level between 14-3-3zeta and Plk1 during interphase cell is very low. Then, to test the direct binding between Plk1 and 14-3-3, we carried out a pull-down assay in which histidine-tagged recombinant Plk1 was purified on Ni-NTA agarose beads and used as an affinity matrix to absorb purified GST-*14-3-3*zeta in test tubes. However, GST-tagged 14-3-3 protein failed to pull down Plk1 recombinant protein (Figure3 *C*, *bottom panel*). We thought that this result may reflect that post-translational modifications of Plk1 might be required for its interaction with 14-3-3. We noticed that the differences in affinity that were evidenced between Plk1 and the *14-3-3* zeta in the two-hybrid assay were not reproduced *in vitro*.

4.4. Phosphorylation of Plk1 at S330/597 Enhances 14-3-3zeta Binding

Our results presented above have shown Plk1 is associated with *14-3-3* zeta in mitotic HeLa cells. We considered that post-translational modifications of Plk1might be required for the association with *14-3-3* zeta. Next, we addressed the mechanism underlying the Plk1/14-

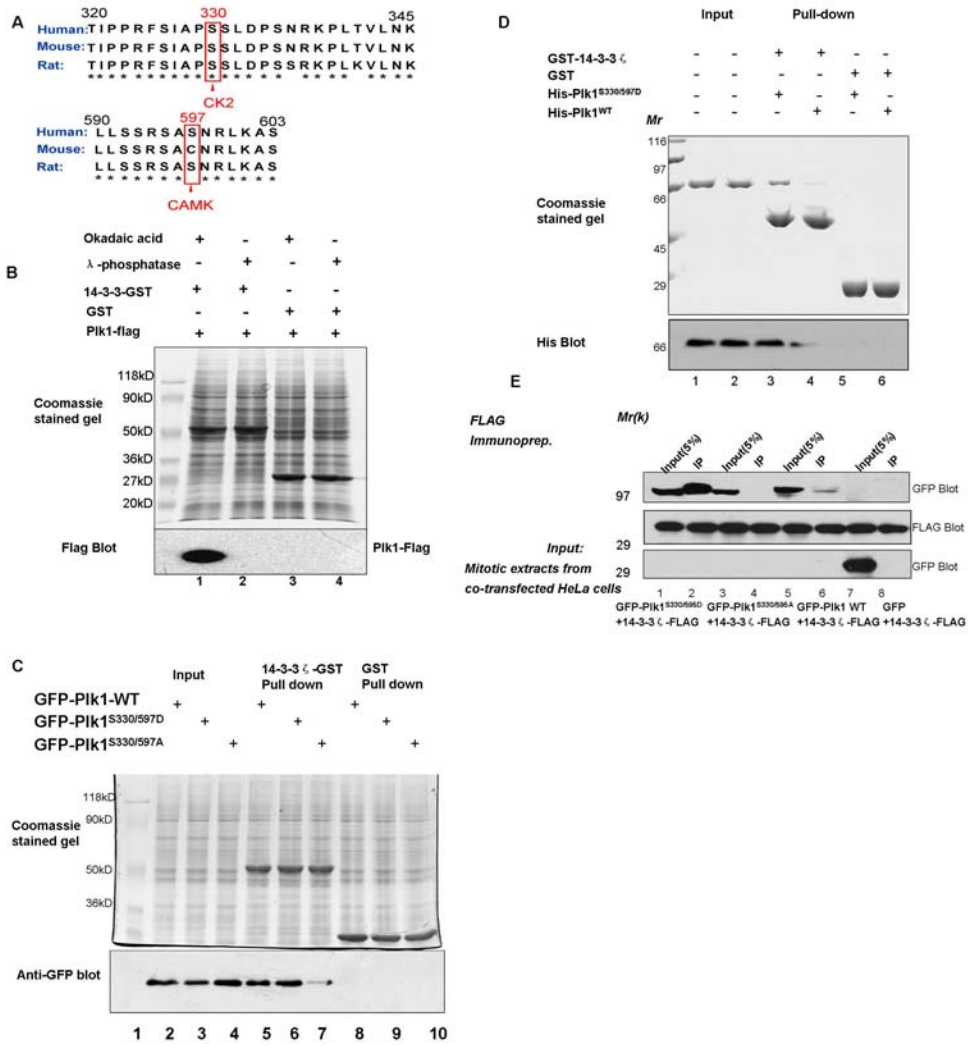

Figure 4. Phosphorylation of Plk1 at S330/597 Enhances 14-3-3zeta Binding. A, Comparison of the mode 14-3-3 binding consensus sequence bound by 14-3-3 with the sequence surrounding S330 and S597 indicates the presence of an evolutionarily conserved 14-3-3 binding site on human, mouse and rat. Conserved residues are indicated with asterisks and the two specific phosphorylation sites, S330 and S597, are underlined. B, an *in vitro* was used to validate whether phosphorylated Plk1 specifically interacts with 14-3-3zeta.Bacterial recombinant GST-14-3-3zetabound to glutathione beads was used as an affinity matrix to absorb Flag tagged Plk1. Extracts from 293T cells transfected with Flag tagged Plk1 were incubated with phosphatase inhibitor okadaic acid or λ -phosphagged followed by incubation with glutathione agarose tagged with GST or with the GST-fused 14-3-3zeta.Note that a significant amount of Plk1 protein was retained by the GST-14-3-3zeta while treatment with okadaic acid (lane 1). Upper panels, Coomassie blue stained . lower panels, immunoblots against Flag antibody. C, an *in vitro* pulldown assay was used to test whether phosphorylated Plk1 at S330/597 specifically interacts with *14-3-3* zeta, Bacterial recombinant GST-14-3-3 zeta bound to glutathione beads was used as an affinity matrix to absorb GFP tagged Plk1-WT, Plk1$^{S330/597D}$ and Plk1 $^{S330/597A}$. Note that a significant amount of Plk1$^{S330/597D}$ protein was retained by the GST-14-3-3zeta. *First lane*, molecular mass markers; *second* through *fourth lanes*, cell lysates of GFP- Plk1-WT, GFP-Plk1$^{S330/597D}$ and GFP Plk1 $^{S330/597A}$. *fifth* through *seventh lanes*, proteins retained on GST-*14-3-3* zeta affinity beads. D, *In vitro* Plk1-WT/Plk1 $^{S330/597D}$ and 14-3-3zeta interaction verified by pull-down assay. GST fused 14-3-3zeta recombinant protein was purified on glutathione agarose beads and used as affinity matrix for absorbing histidine-tagged Plk1 WT and Plk1 $^{S330/597D}$ as described in experimental procedure. Glutathione agarose beads pre-bound GST protein was used as a negative control (lane 5 and lane 6). Upper panel, Coomassie blue- stained . lower panels, western-blot result shows that ~66kDa his-tagged phospho-mimicking mutant Plk1 $^{S330/597D}$ recombinant protein was recognized by His tagged monocloned antibody (lane 3) while the association of wild type Plk1 with 14-3-3zeta is very low (lane 4). E, Co-immunoprecipitation of exogenously expressed GFP- Plk1-WT, Plk1$^{S330/597D}$ and Plk1 $^{S330/597A}$ with FLAG-*14-3-3* zeta from mitotic HeLa cells. Extracts from mitotic HeLa cells transiently transfected to co-express FLAG-14-3-3zeta and GFP- Plk1$^{S330/597D}$ (lane 1) , FLAG-14-3-3zeta and GFP- Plk1 $^{S330/597A}$ (lane3), FLAG-14-3-3zeta and GFP-WT (lane 5) or FLAG-14-3-3zetaand GFP (lane7) were incubated with an anti-FLAG mouse antibody and (lanes 2, 4, 6) immunoprecipitates were resolved by SDS-PAGE followed by western blotting analyses. Upper panel and lower panel, GFP blot; middle panel. FLAG blot.

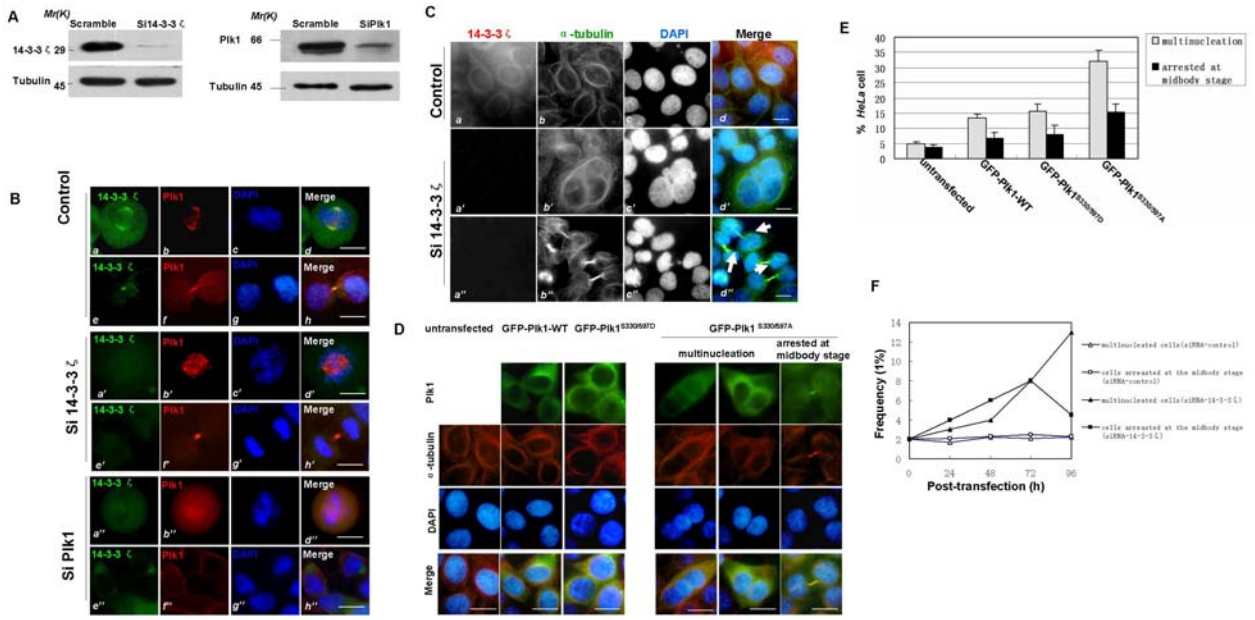

Figure 5. Ectopic Expression of the Plk1Phosphorylation Mutants S330/597A causes Cytokinesis Failure. A, Efficiency of siRNA treatments in HeLa cells. Aliquots of HeLa cells were transfected with 150nM siRNA oligonucleotide duplexes for 14-3-3zetaand Plk1 and their controls (scrambled oligonucleotide) for 48 h and subjected to SDS-PAGE and immunoblotting. *Upper panels*, immunoblots against targeted proteins; *lower panels*, immunoblots against tubulin. B, localization of 14-3-3zeta to the spindle and midbody depends on Plk1. Aliquots of HeLa cells were transfected with oligonucleotides (control and siRNA for 14-3-3zeta and Plk1) for 48 h, followed by fixation and immunocytochemical staining as described under "Materials and Methods." Optical images were collected from HeLa cells transfected with control siRNA (*Control panels*), 14-3-3 siRNA (*si14-3-3 panels*), and Plk1 siRNA (*siPlk1 panels*). *Scale bars* =10μm. C, Downregulation of 14-3-3zetaby siRNA results in cytokinesis defects. The multinucleated cells(middle panels) and the cells arrested at the midbody stage(bottom panels)are indicated with arrowheads and arrows, respectively. D, At 72 hr posttransfection, HeLa cells ectopically expressing wild-type GFP-Plk1 or the GFP-Plk1 phosphorylation mutants Plk1$^{S330/597D}$ and Plk1 $^{S330/597A}$ were stained with anti-α-tubulin antibodies. Transfected cells were scored for the indicated cytokinesis defects. *Scale bars* =10μm. E, The graph shows the mean percentage of cells with these cytokinesis defects in HeLa cells expressing wild-type Plk1, Plk1$^{S330/597D}$ and Plk1 $^{S330/597A}$, respectively. Error bars represent s.e.; n=3 preparations (~300 *HeLa* cells). *P ▪ 0.001. F, Time course analysis of percentage of cells arrested at the midbody stage or multinucleated cells. Dates are the mean of three independent experiments, and more than 250 cells were counted in each experiment.

3-3 interaction. The sequence surrounding the two phosphorylated serines of Plk1 proteins, RFSIAPS^{330} and RSAS^{597}, corresponds to the canonical Rx1-2Sx2-3S and RSxpS recognition motifs of 14-3-3 proteins(10,27) . Computational analysis suggest that Ser^{330} is potential CK2 phosphorylation site and Ser^{597} is potential site of CAMK/CAMK1 (Figure4*A*). To determine whether the interaction between 14-3-3zeta and Plk1 is phosphorylate dependent, we transiently transfected Flag-Plk1 into HEK293T cells, then treated cell lysate with the serine/ threonine phosphatase inhibitor okadaic acid or λ-phosphatase. Cell lysates were then subjected to incubation with GST or GST-fused 14-3-3zeta. Western blot analysis of both control and treated samples revealed that a high amount of Plk1 was retained by the GST-14-3-3zetawhile treatment with okadaic acid (Figure4B).

To test whether phosphorylated Plk1 at S330/597 specifically interacts with *14-3-3* zeta, constructs consisting of wild type Plk1 (Plk1-WT), phospho-mimicking mutant Plk1 (Plk1$^{S330/597D}$) and non-phosphorylatable mutants Plk1 (Plk1 $^{S330/597A}$) were fused to GFP. We transiently transfected the above GFP-tagged constructs into HEK293T. Cell lysates were then subjected to incubation with glutathione agarose tagged with GST or with the GST-fused *14-3-3* zeta. (Figure4C). As shown in Figure 4C, the results of Western analysis indicate that a significant amount of Plk1$^{S330/597D}$ protein was retained by the GST-14-3-3zeta (Figure4C, *bottom panel*).To evaluate the phosphor-regulation of Plk1and 14-3-3zetainteraction, we used a GST pull down assay *in vitro.* GST fused 14-3-3zeta recombinant protein was purified on glutathione-agarose beads and used as affinity matrix for absorbing histidine-tagged wild type Plk1 and histidine-tagged phosphor-mimicking mutant Plk1$^{S330/597D}$ as described in experimental procedure. Glutathione-agarose beads pre-bound GST protein was used as a negative control. The results of coomassie blue staining and western-blot were show in Figure4D. The western-blot probed with His mAb validates phosphor-mimicking mutant Plk1 $^{S330/597D}$ binds to 14-3-3zetawhile the interaction level between wild type Plk1 and 14-3-3zetais very low (Figure4C, bottom panel arrow).

To verify the interaction between phosphorylated Plk1 at S330/597 and *14-3-3* zeta occurs in mitotic HeLa cells, we used anti-FLAG antibodies to immunoprecipitate soluble *14-3-3* zeta and its binding proteins from lysates of mitotic arrested HeLa cells transiently transfected to express GFP-Plk1-WT, GFP- Plk1$^{S330/597D}$ and GFP-Plk1 $^{S330/597A}$ respectively. Western blot with GFP antibody validated that a significant amount of Plk1$^{S330/597D}$ protein is co-precipitated with *14-3-3* zeta (Figure4E, *upper panel*). However, the interaction level between wild type Plk1 and *14-3-3* zeta was very low. No GFP-Plk1 $^{S330/597A}$ was recovered in the 14-3-3zeta immunoprecipitates, suggesting that the interaction between Plk1 and 14-3-3zeta is dependent of the phosphorylation of Plk1 at S330/597.

4.5. Ectopic Expression of the Plk1 Phosphorylation Mutants, S330/597A Induces Cytokinesis Failure

The biochemical interaction between Plk1 and 14-3-3zeta and their co-distribution to the midbody propelled us to examine whether 14-3-3zeta is required for Plk1 localization to the midbody or vice versa. RNAi treatments successfully suppressed 14-3-3zeta protein accumulation without alteration of tubulin protein level (Figure5A). Other siRNA oligonucleotides (siRNA#1 and siRNA#2) targeted to a different sequence of 14-3-3zeta gave a similar suppression profile for 14-3-3zeta protein accumulation (date not shown). No significant effects on Plk1 localization were observed in response to 14-3-3zeta depletion (Figure5B *b' and f'*). However, the spindle and midbody localization of 14-3-3zeta was diminished when Plk1 protein expression was repressed (Figure5B *a'' and e''*), suggesting that Plk1 is essential for the mitotic localization of 14-3-3zeta. Several lines of evidence have implicated Plk1 in the regulation of cytokinesis(6,22). The midbody is a complex structure that forms between dividing cells during cytokinesis and is the site of cell cleavage. 14-3-3 proteins are known to bind to several key cell cycle regulators, such as CDC25C and WEE1, modulating their activity(20). To address the function of 14-3-3zeta during mitosis ,we investigated the role of 14-3-3zetain mitosis by depletion of endogenous 14-3-3zeta with siRNA in HeLa cells. We found that downregulation of endogenous 14-3-3zeta resulted in a population of cells arrested at midbody stage(Figure5C, *bottom panels*) and polyploidy(Figure5C, *middle panel*). Control-siRNA treated cells showed no significant cytokinesis defects (Figure5C, *upper panels*). Examination of 14-3-3zetasiRNA treated cells at various times showed that the percentage of multinucleated cells continued to increase after 72hr, whereas the percentage of cells at midbody stage peaked at 72hr post-transfection and then decreased at 96hr (Figure5F). Since phosphorylated Plk1 associates with 14-3-3zeta at the midbody during mitosis, we tested whether these proteins cooperate to regulate this final stage of cell division. In support of this idea, we demonstrated that endogenous 14-3-3zeta and Plk1 colocalize at the midbody during cytokinesis (Figure2C, *white arrowhead*). We next aimed to understand the function of Plk1 and14-3-3zeta association. In contrast to untransfected control cells (4.98±0.53%), two or more nuclei were observed in ~13% of HeLa cells expressing ectopic GFP-Plk1and in 32.14±3.55% of HeLa cells ectopically expressing the GFP-Plk1 phosphorylation mutants S330/597A (Figures 5D and E). Furthermore, a significant proportion of cells expressing GFP-Plk1$^{S330/597A}$ were arrested at the midbody stage compared to control cells. Immunofluorescence microscopy revealed that mutagenesis of these phosphorylation sites does not affect targeting of Plk1 to this intracellular domain (Figure5D), ruling out the possibility that these cytokinesis defects are due to an indirect effect caused by mislocalization. Thus, we demonstrate that Plk1 phosphorylation at S330 and S597 is required for its function during the final stages of cell division to complete cytokinesis successfully. These results suggest that phosphorylated Plk1 at S330/597 is essential for its association with 14-3-3zeta and reveal that 14-3-3zeta cooperates with Plk1 to complete correct cytokinesis.

5. DISCUSSION

In addition to its roles in cell division and proliferation, cytokinesis plays a key role in preventing genomic instability and aneuploidy that may then go on to form malignant tumors(28,29). Proteins required for cytokinesis are either upregulated in tumors or are encoded by genes that lie in regions of chromosomes found deleted or amplified in tumors and tumor-derived cell lines (30,31) It is therefore important to understand how these proteins normally function to promote cytokinesis, and how misregulation of this pathway can lead to division failure.

In this manuscript, we have provided evidence that *14-3-3* zeta is associated with Plk1 in yeast two hybrid assay. Immunofluorescence staining of the endogenous protein indicated Plk1 and 14-3-3zeta colocalize at the midbody. In our study, we have further explored the interaction between 14-3-3zeta and Plk1 using coimmunoprecipitation and pull-down studies. Indeed, we show that Plk1 is associated with *14-3-3* zeta at a low level in mitotic cells. Most interestingly, we found that phosphorylation of Plk1 at S330/597 promotes interaction with *14-3-3* zeta. These findings indicate that 14-3-3zeta and Plk1 may cooperate at the midbody to coordinate mitotic exit and cytokinesis. Thus, we propose that unphosphorylatable Plk1 at S330/597 may fail to bind or generate the required signal to downstream components of the mitotic exit pathway. Some researches show small-molecule inhibitors of Plk1 abrogate the formation of the contractile ring and prevent cleavage furrow ingression. Acute inactivation of Plk1 prevented the interaction of Ect2 with its central spindle anchor protein HsCyk-4 and the localization of Ect2 to the spindle midzone. These results established Plk1 as an essential early regulator of cytokinesis in mammalian cells and suggested that Plk1 controls cleavage furrow formation at the level of the HsCyk-4/Ect2 complex formation (7,8,9,32). Several Plk1 substrates at midbody during anaphase B have been identified. Plk1 phosphorylates NudC (Nuclear distribution gene C) at midbody and is required for cytokinesis(22). Other researches show that Erk2/cdk1-dependent phosphorylation at S425 and S428 of Cep55 (centrosome protein 55 kDa) is required for interaction with Plk1, enabling phosphorylation of Cep55 at S436(33). Some convincing studies demonstrated that the C-terminal region

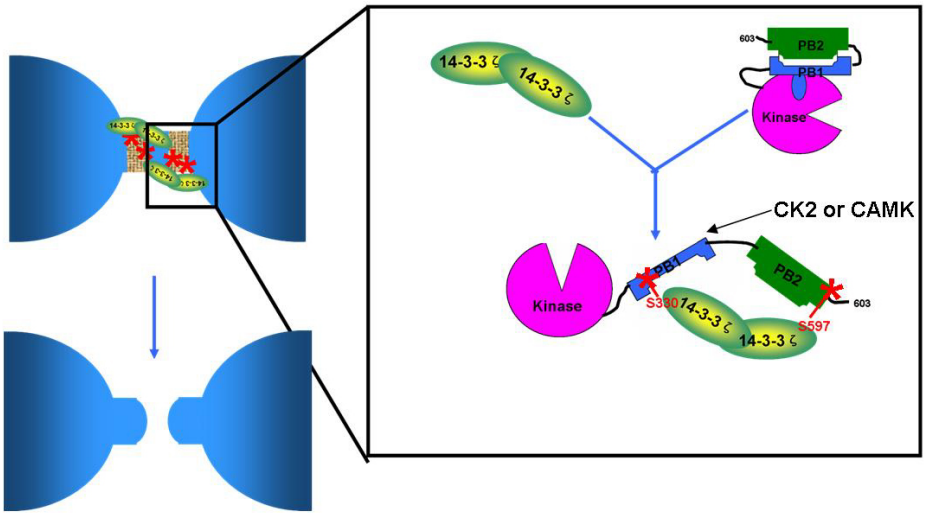

Figure 6. Model for phosphorylation of Plk1 is essential for its interaction with 14-3-3zeta and required for correct cytokinesis. Plk1 is phosphorylated at S330 and S597 by CK2 and CAMK or other kinase (red asterisks), and phosphorylated plk1 is required for the association with 14-3-3zeta. Phosphorylation of Plk1 at S330/597 by CK2 and CAMK or other kinase is required for the association of these two proteins. 14-3-3zeta translocated to midbody by its association with Plk1. Both Plk1and 14-3-3zeta accumulate on midbody during cytokinesis. The interaction of 14-3-3zeta with the PBD domain might therefore induce an open conformation of Plk1. This in turn may promote Plk1 enzymatic activity and permit interaction with its substrates. For example, Plk1 is thought to phosphorylate cep55 or NudC at the midbody to drive cells through cytokinesis.

of Plk1, as a phospho-dependent module, negatively regulates the catalytic activity of this kinase. In the absence of a binding partner, the PBD folds back to the kinase domain through an unknown mechanism, thereby impeding substrate binding and kinase activation. The interaction of an appropriate binding protein with the PBD might therefore induce a 'molecular switch' that supports an open conformation. This could promote enzymatic activity in conjunction with the phosphorylation of Thr-210 within the T loop of the PBD(32,34).

Based on our findings, we speculate a new model to explain the mechanism of Plk1 function during cytokinesis (Figure 6). Phosphorylation of Plk1 at S330/597 by CK2 and CAMK or other kinase is required for the association of 14-3-3zeta and Plk1. 14-3-3zeta translocated to midbody by its association with Plk1. Both Plk1and 14-3-3zeta accumulate on midbody during cytokinesis. The interaction of 14-3-3zeta with the PBD domain might therefore induce an open conformation of Plk1. This in turn may promote Plk1 enzymatic activity and permit interaction with its substrates. For example, Plk1 is thought to phosphorylate cep55 or NudC at the midbody to drive cells through cytokinesis(22,33). Whether the complex formed by phosphor-Plk1 and 14-3-3zeta interacts with other components is currently unknown.

Previous studies have shown that 14-3-3 proteins have emerged as key regulators in G1/S- and G2/M-transition by binding to regulatory proteins and modulating their function(18,35,36,37). In mammalian cells, an obligate step for entry into mitosis is the activation of the CDC2 protein kinase(38). The phosphatase CDC25C dephosphorylates CDC2 at Thr-14 and Thr-15, which results in CDC2 activation and initiates entry into mitosis(17,18). During unperturbed S-phase progression, CDC25C proteins are kept inactive by cytoplasmic sequestration mediated by 14-3-3 association. Thereby, premature activation of CDC2 is prevented(17). 14-3-3 proteins are involved in the regulation of G1/S-phase transition by several mechanisms. CDC25A is a central regulator of S-phase entry which dephosphorylates CDK2 on its inhibitory phosphates Thr-14 and Tyr-15 and is inactivated by cytoplasmic sequestration by 14-3-3 proteins. However, details of the molecular mechanisms of 14-3-3 proteins during cytokinesis are largely unknown. Recent research shows that phosphorylation at S708 of the kinesin-6 component MKLP1 is required for MKLP1 to stably localize to the central spindle. 14-3-3 protein binds centralspindiln when MKLP1 is phosphorylated at S710, and 14-3-3 binding of MKLP1 is a global inhibitor of centralspindle that allows Auora B to locally activate clustering and the stable accumulation of centralspindlin between segregating chromosomes(39). Here, we report a new mechanism of 14-3-3 protein involved in cytokinesis. Phosphorylation of Plk1 at S330/597 is required for the association of 14-3-3zeta and Plk1. 14-3-3zeta translocated to midbody by its association with Plk1. 14-3-3zetacooperates with Plk1 to complete correct cytokinesis. Taken together with the results of previous studies, our results suggest 14-3-3 family emerges as a novel player in mitotic regulation.

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Send correspondence to: Jian Du, Department of Biochemistry and Molecular Biology, Anhui Medical University, 81 Meishan Rd, Anhui, P.O. Box 71, Hefei 230032, China, Tel: 86-551-5161131, Fax: 86-551-5113575, E-mail: dudu@mail.ustc.edu.cn

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