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ORIGINAL ARTICLE

IK-guided PP2A suppresses Aurora B activity in the interphase of tumor cells

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Abstract Aurora B activation is triggered at the mitotic entry and required for proper microtubule-kinetochore attachment at mitotic phase. Therefore, Aurora B should be in inactive form in interphase to prevent aberrant cell cycle progression. However, it is unclear how the inactivation of Aurora B is sustained during interphase. In this study, we find that IK depletion-induced mitotic arrest leads to G2 arrest by Aurora B inhibition, indicating that IK depletion enhances Aurora B activation before mitotic entry. IK binds to Aurora B, and colocalizes on the nuclear foci during interphase. Our data further show that IK inhibits Aurora B activation through recruiting PP2A into IK and Aurora B complex. It is thus believed that IK, as a scaffold protein, guides PP2A into Aurora B to suppress its activity in interphase until mitotic entry.

Keywords Aurora B · Cell cycle · IK · Interphase · Mitosis · PP2A · Protein RED

Abbreviations

IK	Inhibitor K562
PP2A	Protein phosphatase 2A

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Introduction

Inhibitor K562 (IK) inhibits interferon gamma-induced expression of major histocompatibility complex (MHC) class II antigen in the K562, a human erythroleukemic cell line, thus named as IK [1, 2]. Later on, several immune related functions of IK have been found. IK expressed in $CD34^+$ hematopoietic progenitor cells play a role in $CD34^+$ cell proliferation and differentiation, and IK also ameliorates autoimmune disease by reducing MHC class II expression [3, 4]. On the other hand, there are several reports showing that IK is associated with cell cycle regulation. IK is required for kinetochore localization of MAD1, mitotic progression, and activation of the SAC [5], and IK depletion causes cell cycle arrest in the mitosis, which in turn leads to cell death [6, 7]. IK appears as a ubiquitously expressed intracellular protein with clear nuclear foci, but IK does not colocalize with previously known nuclear foci [8]. At the entry of mitosis, IK nuclear foci are dismantled without showing any clear localization during whole mitosis period [7]. Until now, the identity and role of IK nuclear foci in the interphase completely remains unknown.

Aurora serine/threonine kinase is a family of proteins consisting of Aurora A, B, and C. Of these three aurora kinases, the functions of Aurora A and B in mitosis are well defined [9–13]. Aurora A localizes on centrosomes from the end of the S phase to G2 and then moves to the spindle poles during mitosis. Aurora A plays a critical role in the maturation of centrosome by recruiting other centrosomal proteins [14, 15]. Aurora A is activated by its binding partner TPX2, a microtubule associated protein. TPX2 binding alone can enhance the kinase activity of unphosphorylated Aurora A [16] or stimulate autophosphorylation and autoactivation [17, 18]. Aurora B, the catalytic subunit

of chromosome passenger complex (CPC), is located on chromosomes during prophase and moves to the centromeres during metaphase [19, 20]. During mitosis, Aurora B corrects microtubule attachment at the centromere. Aurora B then moves to the midbody during cytokinesis. Aurora B kinase activity peaks in mitosis during the cell cycle [21]. Aurora B is activated in a sequential manner. In the first step, Aurora B binds to the C-terminal IN box sequence of INCENP. This binding enables Aurora B to autophosphorylate Thr232 within its activation loop [22, 23]. The partially activated Aurora B then phosphorylates INCENP at two adjacent serine residues of the conserved TSS sequence [24]. Phosphorylation of the TSS motif and release of the C-terminal tail of Aurora B can generate the fully active kinase in *trans* [25]. Despite it is well known that Aurora B is associated with INCENP in the interphase, how the activation mechanism of Aurora B is suppressed during interphase remains largely elusive.

The function of Aurora B is opposed directly by phosphatases or indirectly by dephosphorylation of Aurora B substrates. Aurora kinase B directly forms kinase-phosphatase complexes with PP1 or PP2A. Both PP1 and PP2A binding can inactivate Aurora B through dephosphorylation of Aurora B kinase itself [26]. On the other hand, PP2A can indirectly oppose the Aurora B activity through dephosphorylation of Aurora B substrates. For example, distribution of PP2A from the centromere towards the kinetochore ensures a timely dephosphorylation of Aurora B and Plk1 substrates on amphitelic kinetochores for the stabilization of microtubule-kinetochore attachment [27]. When errors in kinetochore-microtubule attachment occur, Mps1 or Aurora B-mediated phosphorylation of Knl1 will result in the recruitment of spindle assembly checkpoint (SAC) proteins to the kinetochore. The result of SAC activation will block anaphase entry until all chromosomes are correctly attached to the mitotic spindle. Subsequently, when amphitelic attachment is achieved, BubR1-associated PP2A-B56 will dephosphorylate Knl1, leading to SAC silencing [28]. During anaphase, Aurora B is concentrated at the central spindle and then phosphorylates KIF4A. The accumulation of phosphorylated KIF4A will suppress microtubule dynamics and prevent the full extension of central spindle. PP2A-B56γ can simultaneously dephosphorylate Aurora B to regulate central spindle dynamics as a negative feedback loop [29]. Although the regulation mechanism of Aurora B activity is well studied in mitosis, it is not well understood during interphase. Only a few studies have shown that several phosphatases can influence Aurora B activity during interphase [30]. However, little is known about the precise mechanisms by which they modulate Aurora B activity.

In this study, we demonstrate that IK is a novel binding partner for Aurora B and PP2A. These three proteins form a complex in interphase. In the complex, IK-dependent PP2A recruitment is required for the dephosphorylation of Aurora B. These results imply that IK spatiotemporally regulates Aurora B activation during interphase.

Materials and methods

Plasmids

Full-length human IK cDNA was purchased from Origene (MD, USA) and cloned into the pcDNA 3.1 vector (Invitrogen, CA, USA), pCMV tag-2B vector (Stratagene, Amsterdam, Netherlands), and pEGFP-c2 vector (Clonetech, CA, USA). GST-Aurora B was obtained from Dr. Changwoo Lee at Sungkyunkwan University School of Medicine. For Bimolecular Fluorescence Complementation assay, IK and Aurora B were cloned into pBiFC-VN173 and pBiFC-VC155 provided by Dr. Keun Il Kim at Sookmyung Women's University. The plasmid transfection was performed using polyethylenimine (PEI) solution or X-tremeGENE HP DNA Transfection Reagent (Roche Diagnostic Systems, IN, USA) according to the manufacturer's instructions.

Antibodies

Primary antibodies for immunoblots, immunofluorescence, and flow cytometry analysis were as follows: rabbit polyclonal anti-IK (Santa Cruz, sc-1335485), mouse monoclonal anti-GST (Abcam, ab18183), mouse monoclonal anti-β-actin (Santa Cruz, sc-47778), mouse monoclonal anti-pHistone H3Ser10 (Millipore, #05-806), rabbit polyclonal anti-Aurora B (Abcam, ab2254), rabbit polyclonal anti-pAurora B Thr232 (Rockland, 600-401-677), rabbit monoclonal pAurora B Thr232 (Cell Signaling, #2914), rabbit polyclonal anti-INCENP (Cell Signaling, #2786), mouse monoclonal anti-Borealin (Santa Cruz, sc-376635), rabbit monoclonal anti-Survivin (Cell Signaling, #2808), mouse monoclonal anti-FLAG (Sigma, F1804), mouse monoclonal anti-GFP (Thermoscientific, MA5-15256), and mouse monoclonal anti-PP2Ac (Millipore, #05-421) antibodies. Secondary antibodies were purchased from Bethyl laboratories (TX, USA).

Cell cultures

HeLa and HEK 293T cells were maintained in DMEM supplemented with 10 % heat-inactivated fetal bovine

serum (HyClone Laboratories, UT, USA) at 37 °C in a humidified 5 % CO_2 incubator as described previously [6]. Cells were treated with inhibitors of Aurora A (MLN8237 at 2 μM) and Aurora B (ZM447439 at 2 μM). For double thymidine block assay, HeLa cells were plated onto 100 mm tissue culture dishes at 20 % confluence (1.2×10^6 cells) and then treated with thymidine at 2 mM for 18 h, released for 9 h, and treated with thymidine at 2 mM for 17 h as previously described [6]. For Cell Clock assay, cells were treated with redox dye, observed color changes 1 h after the redox dye treatment, and then photographed.

Transfection of siRNAs

For RNA interference assay, siRNA duplexes (Santa Cruz, CA, USA) were designed to repress IK (A, 5′-GAAGAAACCGAGCUUAUCA-3′; B, 5′-CUACCAAGGAGUUGAUCAA-3′; and C, 5′-GCAUUCCAGUAUGGUAUCA-3′). The transfection was performed with final concentration of 20 nM. The IK siRNA duplex in the 3′UTR was 5′-CAAAGGUUGCAAGAUGUUU-3′ and used in all experiments. Control siRNAs were synthesized by Dharmacon Research (CO, USA). The siRNAs were transfected into HeLa cells using RNAi-MAX (Invitrogen, CA, USA). For IK restoration assay, cells were transfected with human IK expressing plasmid followed by transfection with IK siRNA 18 h after IK plasmid transfection. Cell lysates were prepared 48 h after the IK siRNA transfection and level of pAurora B was measured.

Production of shRNA

A lentivirus co-expressing GFP and IK shRNA was produced according to the manufacturer's protocol (Open Biosystems, AL, USA) with the following modifications. HEK 293T cells were simultaneously transfected with 2.2 μg of the pGIPZ lentiviral IK shRNA, 4.4 μg of the pAX2 packaging plasmid, and 3.3 μg of the pMD2G envelope plasmid using PEI transfection method. Virus-containing supernatant was collected 72 h after the transfection, filtrated through a 0.45 μm filter, and used to infect cells for 96 h.

Immunofluorescence

HeLa cells on coverslips were fixed with −20 °C methanol for 5 min. Subsequently, cells were permeabilized and blocked with PBS-BT (1 × PBS, 3 % BSA, and 0.1 % Triton X-100) for 30 min at RT. The coverslips were then incubated with primary and secondary antibodies diluted in PBS-BT for 1 h. The cells were then stained with DAPI mounting medium (Sigma-Aldrich, MO, USA). Images were acquired as described previously [6].

Immunoprecipitation and immunoblot analysis

One microliter of anti-FLAG M2 or anti-Aurora B antibody was incubated with 1 ml of pre-cleared lysate from HEK 293T and HeLa cells transfected with the indicated plasmid or siRNA for overnight at 4 °C with rotation. Anti-IgG antibody was used as a nonspecific control. The antibody-coupled samples were mixed with 30 μl of Protein G-agarose (Roche, IN, USA) at RT for 2 h with rotation. The agarose beads were washed five times with 1 ml of cold buffer (50 mM Tris–HCl pH 7.5, 0.5 % Igepal CA-630, 0.5 mM EDTA, and 150 mM NaCl). Immunoprecipitated proteins were resolved by SDS-PAGE and transferred to nitrocellulose membranes (Amersham Pharmacia Biotech, IL, USA). Blocking and antibody incubations were performed in 5 % low-fat milk in TBS-T (150 mM NaCl, 20 mM Tris–HCl pH 8.0, 0.05 % Tween20). Blots were developed by ECL (Amersham Pharmacia Biotech, IL, USA).

G2/M population analysis

HeLa cells were transfected with control and IK siRNA. Cells were collected 48 h after the transfection. Cells were fixed with 70 % ethanol and washed with PBS followed by staining in PI buffer (50 μg/ml propidium iodide, 10 mM Tris–HCl pH 7.5, 5 mM $MgCl_2$, and 200 μg/ml RNase A) at 37 °C for 30 min. Stained cells were analyzed on a FACS Canto II cytometer using FACS Diva software (BD Bioscience, CA, USA) as described previously [6]. The percent of G2 and mitotic populations were calculated from a quadrant plot, in which the *x*-axis is the DNA content (PI) and the *y*-axis is FITC intensity (pH3S10). FITC-negative among G2/M population is G2 population and FITC-positive among G2/M population is M population.

Production and purification of recombinant IK

The pET28a-IK constructs were transformed into *E. coli* strain BL21 and recombinant protein was induced by 1 μM of isopropyl-beta-D-thiogalactopyranoside (IPTG) treatment for 3 h. The cells were harvested by centrifugation at 5000 rpm for 15 min at 4 °C, resuspended in 0.1 % Triton-X-100 in PBS, and sonicated with 10 cycles for 10 s. After centrifugation at 13,500 rpm for 15 min at 4 °C, the lysates were incubated Ni–NTA beads for 24 h at 4 °C, the His-IK-coupled Ni–NTA beads were collected by centrifugation at 11,000 rpm for 1 min, and washed three times with washing buffer (50, 150, 5 mM EDTA, and 1 % NP-40). The recombinant IK was eluted with 500 μl of 20 mM

imidazole and subsequently concentrated using a Centrifugal Filter column (Amicon, Millipore).

In vitro kinase assays

For the measurement of Aurora B kinase activity in vitro, 0.5 μg of recombinant Aurora B was incubated with 10 μl of purified His-IK (0.5 μg of recombinant PP2A was used for additional experiment) and 1 μg of MBP protein substrate for the kinase reaction in the presence of 100 μM ATP at RT for 30 min. The reaction was stopped by adding 10 μl of ADP-Glo reagent for 40 min at RT and luminescence was recorded after incubation with 20 μl of kinase detection reagent according to the ADP-Glo kinase assay kit (Promega, WI, USA). For the measurement of Aurora B kinase activity in vivo, Aurora B was immunoprecipitated from lysates and the kinase reaction was performed under the same conditions used for the in vitro assay described above.

Statistical analysis

Student's t test and Scheffe's multiple comparison tests (IBM SPSS statistics Version 21) were employed for statistical analysis. $p < 0.05$ was considered as a significant difference ($*p < 0.05$, $**p < 0.01$, $***p < 0.001$). Data were presented as means ± standard deviations (SD).

Results

IK depletion leads to mitotic arrest through aberrant spindle dynamics

From the previous siRNA library screening, IK was identified as a cell death-inducing gene [31]. In an attempt to clarifying the molecular basis of the cell death, HeLa cells were transfected with IK siRNA. The transfected cells showed severe defects in chromosome alignment, and the misaligned chromosomes increased in the IK-depleted cells as much as 15-fold compared to the control siRNA-treated cells, indicating that IK plays a role in proper chromosome alignment (Fig. 1a). To determine whether IK depletion causes the chromosome misalignment by aberrant spindle dynamics, IK-depleted cells were stained with anti-α-tubulin and anti-γ-tubulin antibodies to examine spindle formation and centrosomal integrity. The intensities of α-tubulin and γ-tubulin in IK-depleted cells were greatly reduced, which is indicative of spindle dynamics defects (Fig. 1b, c). In addition, when pole-to-pole distance was measured, IK-depleted cells showed an increase in distance (Fig. 1d). These findings collectively imply that IK depletion alters spindle dynamics and centrosome function.

IK depletion-induced mitotic arrest is prevented by Aurora B inactivation through G2 arrest

It is well known that Aurora B plays a pivotal role in proper chromosome attachment and spindle dynamics during mitosis. Whereas lack of Aurora B activity impairs ability to correct erroneous attachment of microtubule to kinetochore, hyperactivity of Aurora B causes defects in chromosome segregation by promoting continuous disruption of chromosome-microtubule attachments [32, 33]. Based on this fact, we tested whether Aurora B activity is implicated in aberrant spindle dynamics and centrosome function in IK-depleted cells. To this end, IK-depleted cells were treated with Aurora B inhibitor (ZM447439) and Aurora A inhibitor (MLN8237), stained with FITC-labeled anti-pH3S10 antibody and PI, and then analyzed using flow cytometry. The percent of G2 and mitotic populations were calculated from a quadrant plot (Fig. 2a). To our surprise, treatment of Aurora B inhibitor completely blocked the mitotic entry induced by IK depletion (Fig. 2b) through the G2 arrest (Fig. 2c). However, Aurora A inhibitor failed to do so, indicating that the molecular functions of IK is closely linked with Aurora B. To further confirm that the Aurora B inhibitor prevents mitotic arrest induced by IK depletion, Cell Clock assay was performed. In the Cell Clock assay, cell population in a different stage of cell cycles can be visualized by different colors; yellow is indicative of G1/S, dark green is G2 and blue is M. The number of colored cells was counted after combined treatment of IK siRNA and ZM447439, and graphed (Fig. 2d). As expected, Aurora B inhibition in IK-depleted cells enhanced G2 population and reduced mitotic population. Taken together, these findings suggest that IK together with Aurora B regulates mitotic entry.

IK depletion activates Aurora B kinase in interphase

Because Aurora B inhibition prevents G2/M transition in IK-depleted cells (Fig. 2), we hypothesized that Aurora B activity may be hyper-activated in the absence of IK. To examine Aurora B activity, four siIKs targeting different IK regions were transfected into HeLa and the activation of Aurora B was determined by measuring the phosphorylation on Thr232 of Aurora B. Interestingly, the phosphorylation of Aurora B was significantly enhanced in IK-depleted cells by all different siIKs (Fig. 3a). To examine whether IK-depletion enhances Aurora B phosphorylation at a single cell level, pGIPz-shIK lentivirus, which simultaneously expresses GFP and shIK, was used to select infected cells from non-infected cells. The phosphorylation level of Aurora B was examined in GFP-expressing cells. A significant increase in Aurora B phosphorylation was observed in GFP-expressing cells, but not

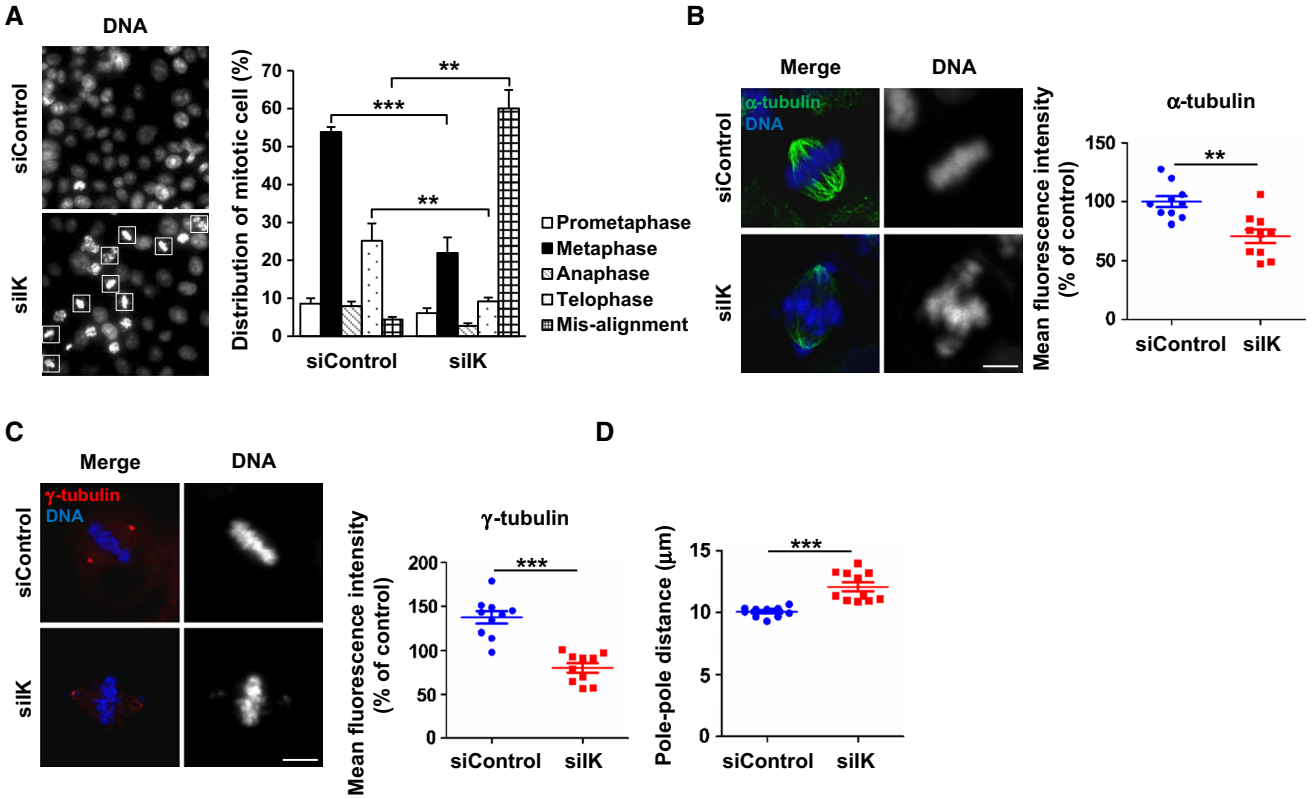

Fig. 1 IK depletion causes aberrant spindle dynamics. **a** HeLa cells were transfected with siControl or siIK for 48 h and stained with Hoechst 33258. The change in nuclear morphology was examined under fluorescence microscopy. *White squares outline* misaligned chromosomes and scattered chromosomes ($N > 600$). **b**, **c** HeLa cells were transfected with siControl or siIK for 48 h and then stained with α-tubulin or γ-tubulin after fixation. The intensities of α-tubulin and γ-tubulin were quantified by AutoDeblur and graphed ($N = 10$). **d** Distance between two stained γ-tubulins in images of (**c**) was measured by AutoDeblur and graphed ($N = 11$). *Scale bars* indicate 5 μm. $^{**}p < 0.01$, $^{***}p < 0.001$

in non-GFP-expressing cells and control shRNA infected-cells (Fig. 3b).

On the other hand, Aurora B is likely to be activated in interphase because nuclear envelope breakdown does not occur yet (Fig. 3b). For the detailed analysis of Aurora B kinase activity, cells were transfected with siIK and then synchronized using a double thymidine block (DTB), followed by a release. The cell lysates prepared in each time point were immunoprecipitated with anti-Aurora B antibody to measure its kinase activity directly. In consistent with the increase in Aurora B phosphorylation, Aurora B kinase activity in IK-depleted cells was increased in the interphase (Fig. 3c). To exclude the possibility that the increase in Aurora B activity is due to IK depletion-induced mitotic cells, single thymidine block assay after siIK transfection was performed to prevent mitotic entry of IK-depleted cells. Flow cytometry analysis showed that thymidine efficiently arrested the cell cycle at the interphase and blocked the mitotic entry of IK-depleted cells. In this experimental condition, the Aurora B activity was increased at IK-depleted interphase cells, implying that IK-depleted Aurora B activation occurs at the interphase, not mitotic phase cells (Fig. S1). To rule out the IK siRNA-induced off-targeting effects, restoration experiments were performed with siRNA targeting 3′-UTR of IK and IK expression vector. Aurora B activity and histone H3 phosphorylation was successfully rescued by IK expression, further supporting that Aurora B is abnormally activated in the absence of IK (Fig. 3d).

IK is a novel binding partner for Aurora B

To find out the underlying mechanism that IK depletion causes Aurora B activation, IK and Aurora B interaction was examined. HEK293T cells were co-transfected with FLAG-IK and GST-Aurora B expressing plasmids, and then cell lysates were immunoprecipitated with anti-FLAG antibody. GST-Aurora B strongly bound to FLAG-IK (Fig. 4a). To exclude the possibility that other cellular proteins would be associated with IK and Aurora B interaction, bacterially purified His-IK and GST-Aurora B were incubated, and then pull-down assay was performed. GST-

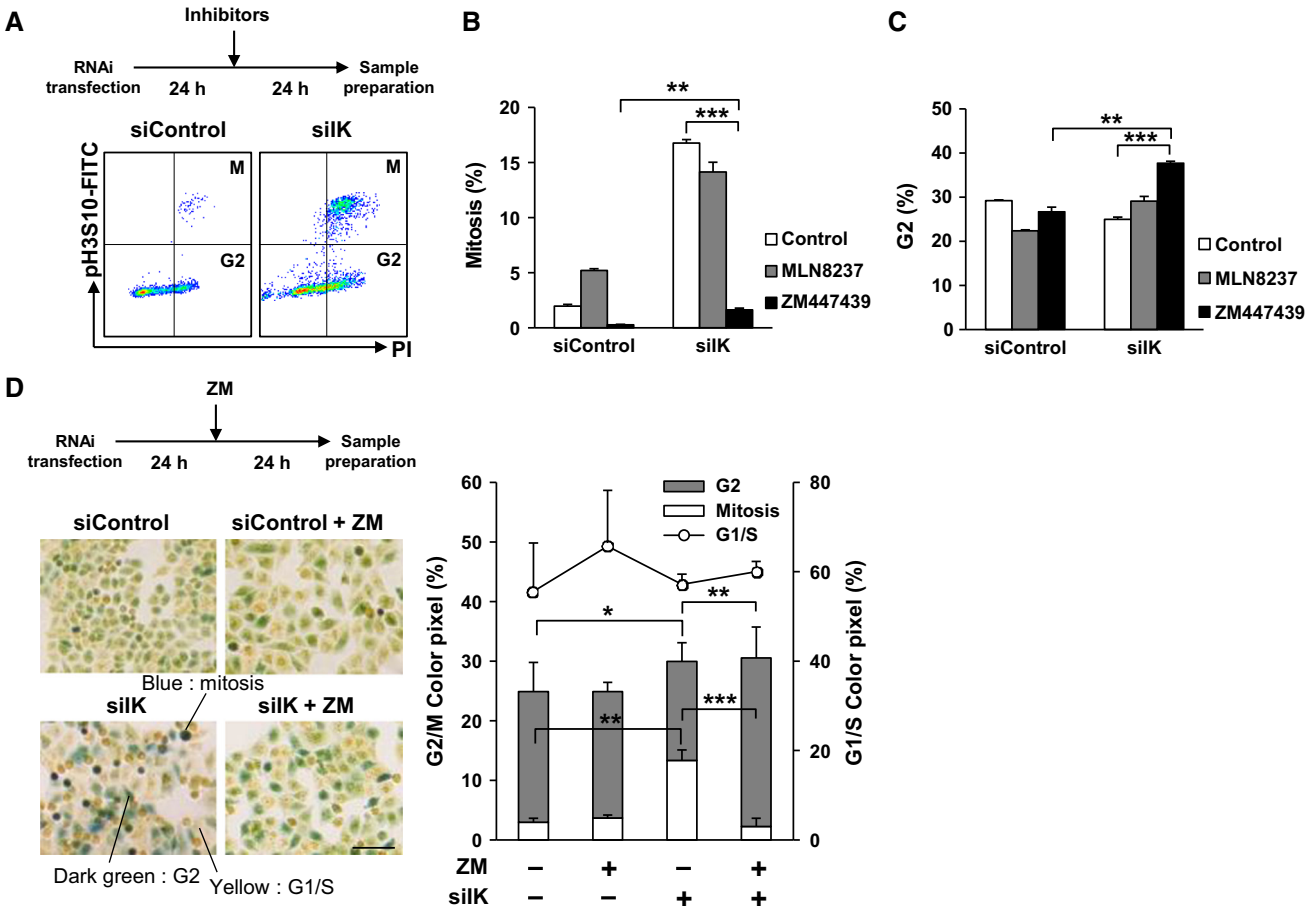

Fig. 2 Aurora B inhibition in IK-depleted cells arrests at G2 phase, not at mitosis. **a** The *top of the figure* indicates the scheme of the experiment. HeLa cells were transfected with siControl or siIK, and treated with Aurora B inhibitor (ZM447439) and Aurora A inhibitor (MLN8237) for additional 24 h. **b**, **c** The cells from (**a**) were analyzed using flow cytometry. The percent of G2 (quadrant IV) and mitotic populations (quadrant I) were calculated from a quadrant plot, in which the *x*-axis is the DNA content and the *y*-axis is FITC intensity. **d** The cells from (**b**, **c**) were also analyzed using Cell Clock assay. The populations of each phase were measured using image J and graphed. *Scale bar* indicates 5 μm. $*p < 0.05$, $**p < 0.01$, $***p < 0.001$

Aurora B was detected when His-IK was pulled down with Ni-beads (Fig. 4b), implying that IK is able to directly interact with Aurora B without other protein involvement.

Next, to test if Aurora B and IK can colocalize, location of both proteins was observed using immunofluorescence microscopy. IK showed a pattern of nucleus foci, which was clearly colocalized with Aurora B at the interphase (Fig. 4c). To further confirm the IK and Aurora B colocalization, bimolecular fluorescent complementation (BiFC) experiment was performed. Plasmids that *N*-terminal Venus fused to Aurora B and C-terminal Venus to IK were constructed and two constructs were transfected. Consistent with the immunofluorescence result, the direct intermolecular interaction between IK and Aurora B was observed at the interphase, but not at the mitotic phase (Fig. 4d). To determine at which phase that IK binds to Aurora B, the interaction was examined in synchronized and released cells. Unexpectedly, the interactions of IK and Aurora B were found in interphase and mitosis. Their interaction was also strongly detected in G2 phase (Fig. 4e). The discrepancy of IK and Aurora B interaction at mitotic phase was explained in "Discussion" section.

IK promotes Aurora B dephosphorylation through PP2A recruitment

It was previously reported that PP2A plays a critical role in Aurora B inactivation in the interphase [34], and we demonstrated above that IK may be another factor able to modulate Aurora B phosphorylation. Therefore, we asked if IK cooperatively regulates the Aurora B phosphorylation together with PP2A. To this end, we first analyzed if IK and PP2A form a complex with Aurora B. Aurora B pull down assay showed that PP2A and IK existed in a Aurora B-complex with chromosome passenger complex including INCENP, Borealin, and Survivin (Fig. 5a), and reverse pull

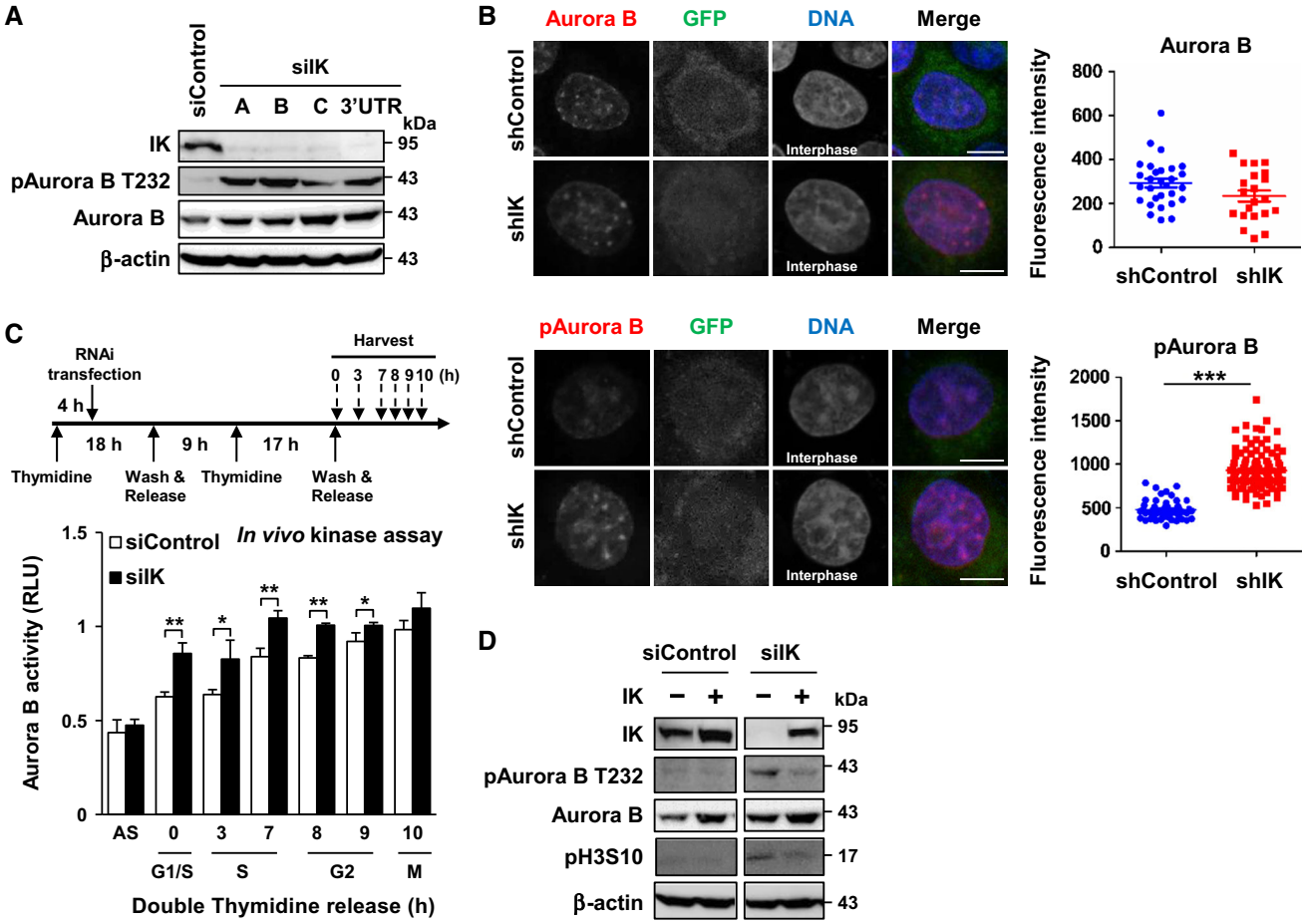

Fig. 3 IK depletion activates Aurora B in the interphase. **a** HeLa cells were transfected with four types of siRNA targeting different IK regions for 48 h and then levels of IK, pAurora B, and Aurora B were examined using immunoblot assay. **b** HeLa cells were infected with lentivirus co-expressing IK shRNA and GFP or GFP only for 96 h. Cells were stained with anti-pAurora B T232 and anti-Aurora B antibodies, and analyzed using confocal microscopy. Fluorescence intensities were measured and graphed ($N > 23$). **c** HeLa cells were synchronized by the scheme of the experiment. The lysates were immunoprecipitated using anti-Aurora B antibody. Aurora B kinase activities were then measured. **d** For the rescue experiment, HeLa cells were transfected with IK expression plasmid together with IK siRNA targeting 3′-UTR of IK. The lysates were obtained 48 h after the transfection and levels of IK, pAurora B T232, Aurora B, and pH3S10 were determined using immunoblot assay. *Scale bars* indicate 5 μm. $*p < 0.05$, $**p < 0.01$, $***p < 0.001$

down assay also showed that IK bound to Aurora B, PP2A, and Survivin (Fig. 5b). These results strongly suggest that IK, Aurora B, and PP2A form a complex. Next, to determine whether PP2A dependent dephosphorylation of Aurora B is mediated by IK, IK immunoprecipitates were treated with PP2A inhibitor, okadaic acid. The treatment of PP2A inhibitor significantly enhanced the level of phospho-T232 in Aurora B (Fig. 5c), indicating that IK-guided PP2A negatively regulates Aurora B activation. To exclude the possibility that the IK immunoprecipitates contain other protein phosphatases which can dephosphorylate Aurora B, bacterially purified His-IK and PP2A were incubated with recombinant active Aurora B, followed by the measurement of Aurora B activity in vitro. PP2A significantly reduced the Aurora B activity. Interestingly, co-incubation of IK and PP2A with Aurora B decreased the activity more efficiently (Fig. 5d) and recombinant IK did not affect PP2A activity (Fig. 5e), indicating that association of IK and PP2A efficiently prevents Aurora B activation.

IK depletion disrupts PP2A-Aurora B interaction

It was reported that Aurora B dephosphorylation in the interphase is achieved by direct interaction of Aurora B and PP2A [34]. Our findings described above propose a possibility that IK may guide the interaction between Aurora B and PP2A. To test this possibility, we tested if Aurora B and PP2A interaction perturbed in IK-depleted cells, since IK did not affect PP2A activity. Aurora B pulldown assay proved that Aurora B and PP2A interaction was significantly disrupted in IK-depleted cells, while INCENP interaction was strongly increased (Fig. 6a). To further

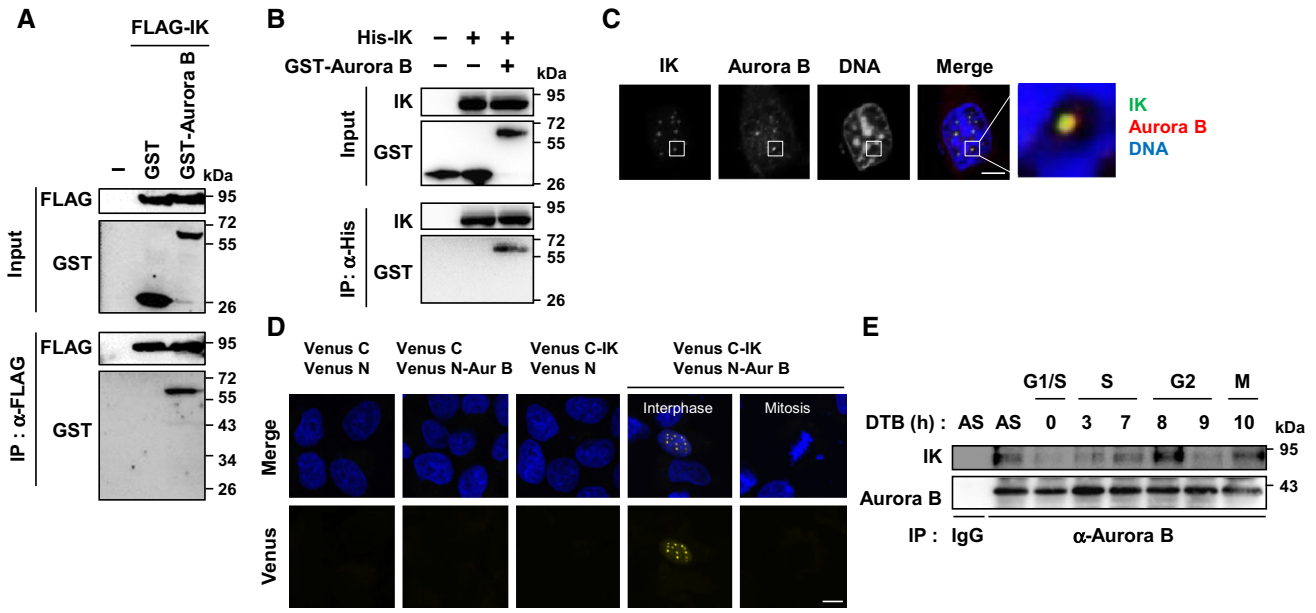

Fig. 4 IK binds to Aurora B. **a** HEK 293T cells were co-transfected with FLAG-IK and GST-Aurora B and then pulled down with anti-FLAG antibody. Anti-FLAG and anti-GST antibodies were used for immunoblot assay. **b** Mixture of His-IK and recombinant GST-Aurora B was incubated with Ni–NTA agarose beads and levels of IK and Aurora B were determined using immunoblot assay. The recombinant GST was used as a negative control. **c** GFP-IK-transfected HeLa cells were stained with anti-Aurora B antibody and analyzed using confocal microscopy. *White box* indicates region shown at higher magnification. *Scale bar* indicates 5 μm. **d** Plasmids containing the *N* terminus of Venus fused to Aurora B (Venus N-Aur B) and the *C* terminus of Venus fused to IK (Venus C-IK) were cotransfected in HeLa cells. The *yellow foci* expressing cells were examined by confocal microscopy. *Scale bar* indicates 10 μm. **e** HeLa cells were synchronized with the scheme of the experiment in Fig. 3c and immunoprecipitated with anti-Aurora B antibody. Levels of IK and Aurora B were determined using immunoblot assay

support this result, Aurora B and PP2A interaction was also examined in IK-depleted cells arrested at the interphase by the single thymidine block. The Aurora B and PP2A interaction was also dissociated (Fig. S1). To determine whether the disrupted interaction of Aurora B and PP2A is indeed due to the IK depletion, 3′-UTR IK siRNA and IK vector co-transfected into HeLa cells. The exogenous IK expression successfully restored the interaction Aurora B with PP2A (Fig. 6b).

Taken together, our findings suggest that IK, as a scaffold, contributes to PP2A and Aurora B interaction. The IK-guided PP2A dephosphorylates Aurora B, leading to the inactivation. In the absence of IK, Aurora B and PP2A interaction is disrupted, leading to Aurora B activation during the interphase. This model was schematically depicted in the Fig. 6c.

Discussion

IK was first identified as a 19 kDa immune-related secretary cytokine, but later studies have shown that IK is a ubiquitously expressed intracellular and nuclear protein, and the depletion of IK causes mitotic arrest [35]. It is thus believed that IK plays an important role in cell cycle control. This is also supported by our observation that the IK expression was detected in all tissues examined (Fig. S2).

Aurora B localizes in a cell cycle dependent manner [36]. In early mitosis, Aurora B is localized to the inner centromere on chromosomes. At the metaphase-anaphase transition Aurora B moves from centromere to the central spindle and then concentrates in midbody in telophase cells. However, the localization of Aurora B at interphase remained largely unknown. Through this study, we show that Aurora B interacts and colocalizes with IK at nuclear foci in the interphase, which was revealed by immunocytochemistry and BiFC analysis in transformed and tumor cells. On the other hand, IK and Aurora B interaction was also detected in mitotic phase when immunoblot assay was performed (Fig. 4e). However, the centromeric Aurora B at mitosis is unlikely to colocalize with IK, because nuclear foci containing IK dispersed into cytosol from the entry of mitosis, which was examined through time-lapse microscopy (Fig. S3). Therefore, their interaction at mitotic phase would be an artifact occurred by the breakdown of cells. Although if it is true, at this moment it is not clearly exclude the possibility that non-localized Aurora B into centromere binds to dispersed IK at mitotic phase in tumor cells.

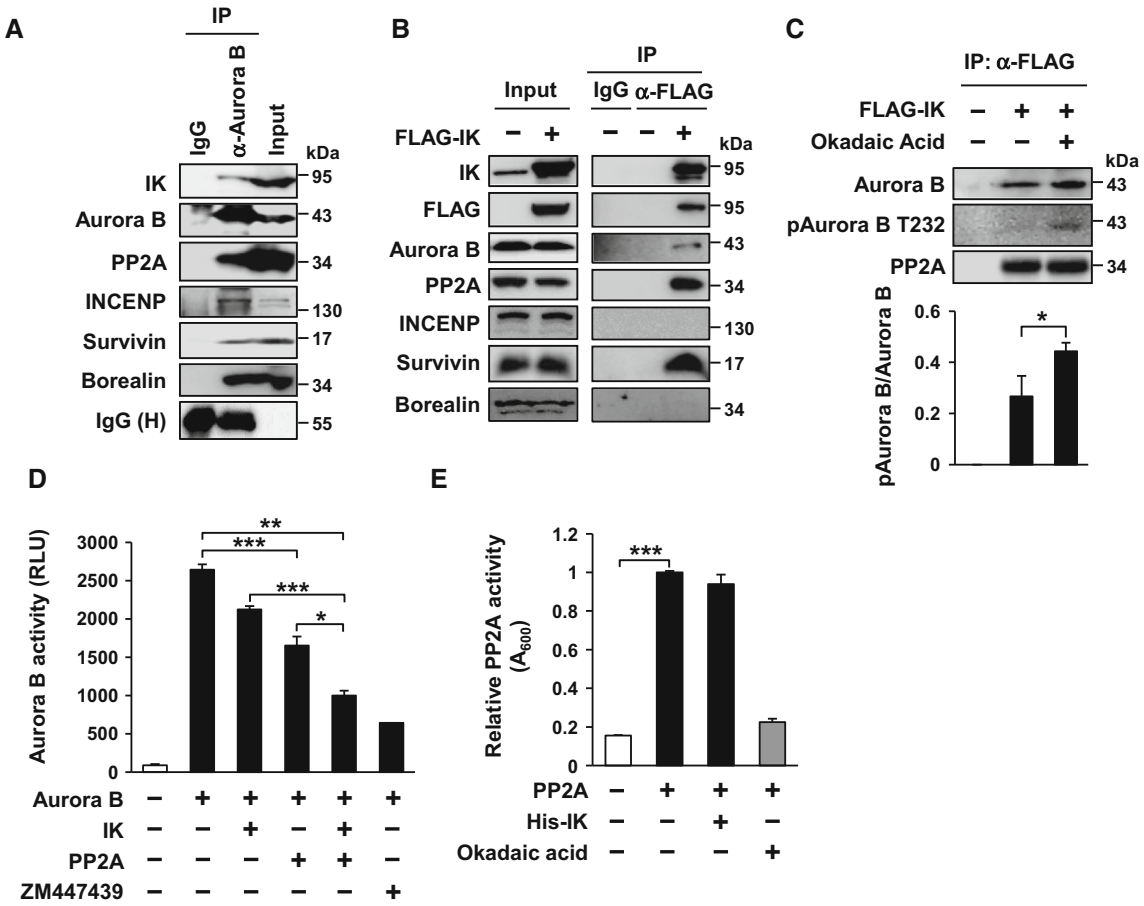

Fig. 5 IK-guided PP2A prevents Aurora B phosphorylation. **a** HeLa cells were lysed and immunoprecipitated with anti-Aurora B and IgG antibodies. Levels of IK, Aurora B, PP2A, INCENP, Survivin, and Borealin were examined using immunoblot assay. **b** HEK293T cells were transfected with 10 μg of FLAG-IK. Cell lysates were immunoprecipitated with anti-FLAG antibody. Levels of IK, FLAG, Aurora B, PP2A, INCENP, Survivin, and Borealin were detected using immunoblot assay. **c** Lysates from (**b**) were treated with okadaic acid at 1 nM at 30 °C for 30 min. Levels of Aurora B, pAurora B, and PP2A were examined. The relative intensities of pAurora B and Aurora B bands were calculated by imageJ software and graphed. **d** Recombinant GST-Aurora B were incubated with recombinant PP2A and purified IK, and Aurora B kinase activity was measured. Luminescence signal indicative of Aurora B activity was recorded. **e** Recombinant PP2A were incubated with recombinant Aurora B and purified IK. PP2A activity was measured by a Ser/Thr PPase assay and the relative phosphatase activity was determined by measuring at 600 nm. PP2A inhibitor, okadaic acid, was used at 10 nM concentration. $*p < 0.05$, $**p < 0.01$, $***p < 0.001$

In the present study, we show that IK depletion induces improper Aurora B activation in tumor and transformed cells, but IK-depleted normal cells may not show aberrant Aurora B activation in the interphase. Unlike tumor cells, Aurora B expression in the normal cells is very low in G1/early S phase, increased in late S phase, and peaked near the G2/M abundant [37, 38]. Therefore, the disruption of Aurora B and PP2A interaction in absence of IK could be marginal, because there would be a very low colocalization of Aurora B and IK in the nucleus during the interphase in normal cells. Further study is required to resolve effect of IK on the regulation of Aurora B in the interphase of normal cells. On the other hand, Aurora B is overexpressed in many types of cancer in association with tumor aneuploidy. Accordingly, the distribution and suppression of overexpressed Aurora B during the interphase in tumor cells are expected to be involved in a special pathological process of tumor progress. Since the suppression of IK in tumor cells induces aberrant Aurora B activation and leads to the eventual tumor cell death, the regulation of IK could be a potential strategy to develop anti-tumor therapeutic agents if IK depletion does not affect Aurora B activation in the normal cells.

Aurora B activation begins at mitotic entry through phosphorylation at threonine 232 [39]. At the time of mitotic entry, Aurora B is partially activated by binding with INCENP and then fully activated by sequential autophosphorylation. It suggests that the interaction of Aurora B and INCENP is required for proper Aurora B activation. However, although Aurora B is in a complex

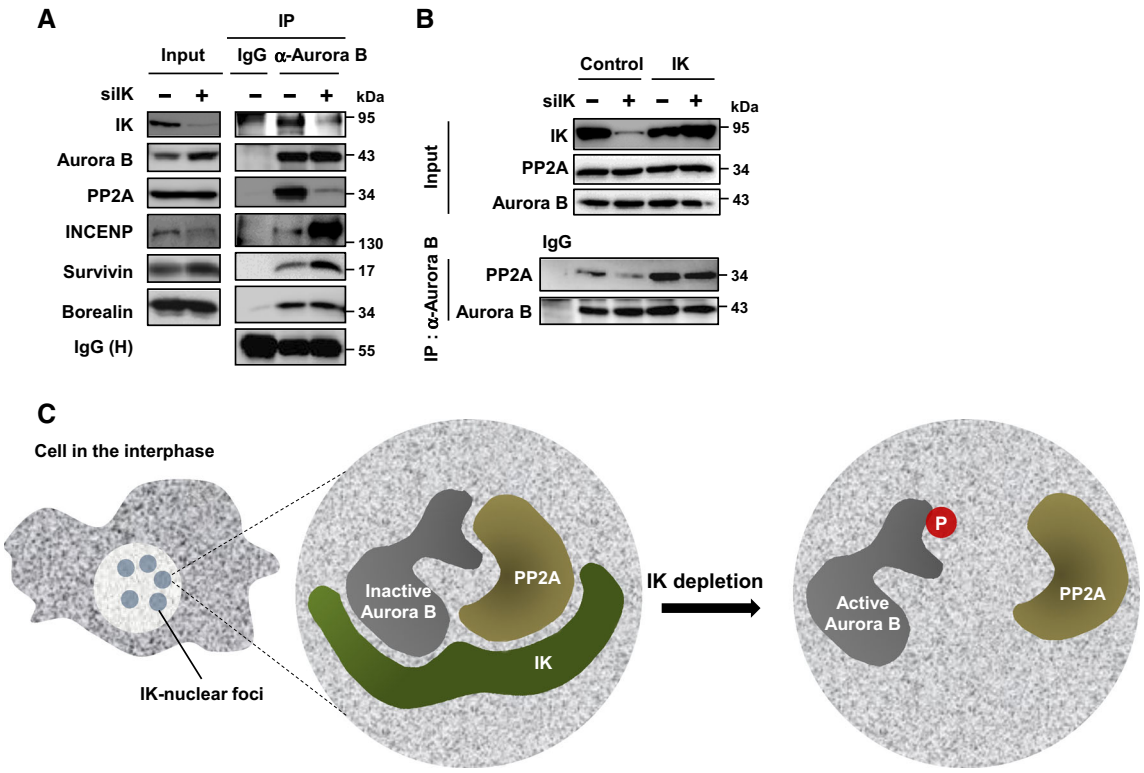

Fig. 6 IK depletion disrupts PP2A and Aurora B interaction. **a** HeLa cells were transfected with siIK for 48 h and the cell lysates were immunoprecipitated with anti-Aurora B antibody. Levels of IK, Aurora B, PP2A, INCENP, Survivin, and Borealin were detected by immunoblot assay. **b** For the rescue experiment, HeLa cells were co-transfected with IK expression plasmid and IK siRNA targeting 3′-UTR of IK. Cell lysates were immunoprecipitated with anti-Aurora B antibody 48 h after the transfection. Levels of IK, PP2A, and Aurora B were detected by immunoblot assay. **c** Schematic drawing of IK complex containing Aurora B and PP2A. In the absence of IK, interaction of PP2A and Aurora B is disrupted

with INCENP and Survivin in Xenopus embryo during both interphase and mitosis [21], how Aurora B exists an inactivated form in interphase was still uncertain. In this study we tested the possibility that IK negatively regulates Aurora B activity in the interphase, since IK depletion caused Aurora B activation and Aurora B inhibitor prevented mitotic arrest caused by IK depletion. When bacterially purified IK and PP2A incubated with recombinant Aurora B, IK-guided PP2A greatly inhibited Aurora B activity compared to PP2A incubation with Aurora B in the absence of IK. These results imply that IK guides PP2A into Aurora B to suppress its activity in the interphase. On the other hand, IK depletion highly enhanced the INCENP-Aurora B interaction (Fig. 6a). This result suggests the possibility that IK could loosen the strength of INCENP and Aurora B interaction to prevent Aurora B activation during interphase.

Several studies have shown that Aurora B activity is regulated by phosphatases. Sds22 identified in fission yeast is a known modulator of PP1 activity during mitosis [40]. Sds22 binds to PP1 and positively regulates its activity during mitosis, which in turn plays a critical role in regulation of the levels of phospho–Aurora B at kinetochores [41, 42]. End binding 1 (EB1) plays a role in microtubule dynamics and interacts with Aurora B. This interaction prevents Aurora B inactivation from dephosphorylating of Aurora B by PP2A. Thus, EB1 acts as a positive regulator of Aurora B [34]. In addition to the prevention of Aurora B dephosphorylation by direct binding with Aurora B and the suppression of phosphatase by direct binding with phosphatase. The present study provides a new negative regulatory mechanism that IK recruits PP2A to inhibit Aurora B activity.

In summary, we reveal a previously unrecognized role for IK in the suppression of Aurora B kinase activity in the interphase. This is supported by the following findings: (1) IK forms a complex with PP2A and Aurora B; (2) IK depletion disrupts PP2A and Aurora B interaction, leading to the increase in Aurora B activity; and (3) IK guided-PP2A promotes Aurora B dephosphorylation in the interphase. Taken together, IK, as a scaffold protein, recruits PP2A to suppress Aurora B in the interphase.

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Compliance with ethical standards

Conflict of interest The authors declare that there are no conflicts of interest.

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