AIBp regulates mitotic entry and mitotic spindle assembly by controlling activation of both Aurora-A and Plk1

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We previously reported that Aurora-A and the hNinein binding protein AIBp facilitate centrosomal structure maintenance and contribute to spindle formation. Here, we report that AIBp also interacts with Plk1, raising the possibility of functional similarity to Bora, which subsequently promotes Aurora-A–mediated Plk1 activation at Thr210 as well as Aurora-A activation at Thr288. In kinase assays, AIBp acts not only as a substrate but also as a positive regulator of both Aurora-A and Plk1. However, AIBp functions as a negative regulator to block phosphorylation of hNinein mediated by Aurora-A and Plk1. These findings suggest a novel AIBp-dependent regulatory machinery that controls mitotic entry. Additionally, knockdown of hNinein caused failure of AIBp to target the centrosome, whereas depletion of AIBp did not affect the localization of hNinein and microtubule nucleation. Notably, knockdown of AIBp in HeLa cells impaired both Aurora-A and Plk1 kinase, resulting in phenotypes with multiple spindle pole formation and chromosome misalignment. Our data show that depletion of AIBp results in the mis-localization of TACC3 and ch-TOG, but not CEP192 and CEP215, suggesting that loss of AIBp dominantly affects the Aurora-A substrate to cause mitotic aberrations. Collectively, our data demonstrate that AIBp contributes to mitotic entry and bipolar spindle assembly and may partially control localization, phosphorylation, and activation of both Aurora-A and Plk1 via hNinein during mitotic progression.

Introduction

The centrosome is the primary microtubule-organizing center (MTOC) in animal cells and consists of a pair of centrioles surrounded by pericentriolar material (PCM). The PCM proteins responsible for microtubule nucleation, centrosome maturation, and organization include γ-tubulin, pericentrin, ninein, centrin, pericetrin, AKAP450, C-Nap 1, and Nek2. Centrosomes play an important role in the formation of bipolar spindles, spindle pole assembly, and mitotic entry. Mitosis involves the sequential activation of several protein kinases. Recent studies indicate that cyclin-dependent kinase 1 (CDK1), polo-like kinase (PLK), Nek kinase, and proteins in the Aurora family may be involved in regulating the centrosome cycle and formation of the mitotic spindle. Cell cycle and cell division mitotic checkpoints are controlled by the activity of kinase, phosphatases, and their substrates. Therefore, the organization of the centrosome is governed largely by the interaction of centrosomal adapter proteins and their coordinate bound kinases that regulate centrosome dynamics. Centrosome abnormalities result in the formation of multipolar spindles that promote chromosome segregation errors and genomic instability, contributing to the generation of aneuploidy and tumorigenesis.

Aurora-A has been implicated in centrosome duplication, separation, maturation, and spindle assembly. This protein recruits pericentriolar material (PCM) proteins, including LATS2, TACC, and NDEL1, thereby governing centrosome maturation and spindle assembly. Phosphorylation of TACC by Aurora-A induces formation of the ch-TOG/CKAP5/TACC complex, which promotes microtubule growth. Activation of Aurora-A occurs through the phosphorylation of Thr288. Bora was the first protein found to bind Aurora-A in...
Plk1 plays a role in regulating mitotic entry, centrosome separation and maturation, formation of bipolar spindles, and cytokinesis. At the onset of mitosis, Plk1 phosphorylates Nlp, negatively regulating the association between Nlp and dynein. NLP then dissociates from the centrosome, which promotes the localization of γ-tubulin and other mitosis-specific PCM components to the centrosome. This process increases the microtubule nucleation capacity of the mitotic centrosome. Plk1 is required for the incorporation of the PCM to organize microtubules for centrosome maturation. Plk1 phosphorylates pericentrin to initiate centrosomal accumulation of PCM, including NEDD1, Cep192, γ-tubulin, Aurora A, and Plk1 itself.

Plk1 is composed of an N-terminal kinase domain and a C-terminal Polo-box domain (PBD). The PBD binds to target proteins containing the consensus sequence for phosphorylation. PBD interaction with the kinase domain of Plk1 suppresses Plk1 activity, critically inhibiting the basal kinase activity. Strikingly, activation of Plk1 requires phosphorylation of a conserved threonine residue (Thr 210) in the T-loop of the kinase domain. Aurora-A kinase directly phosphorylates Plk1 on Thr 210, and the activity of Aurora-A toward Plk1 is greatly enhanced by Bora. Aurora-A and hNinein interact with the N-terminal region of AIBp1–268. These data indicate that AIBp binds to Plk1 and Aurora-A via its C-terminal region including amino acids 269–371, while hNinein interacted with the N-terminal region of AIBp1–187 (Fig. S1). Representative results are shown in Figs. 1C and D, where endogenous Plk1 and Aurora-A co-immunoprecipitated with the C-terminals of AIBp and AIBp-Aurora-A, while hNinein interacted with the N-terminal region of AIBp1–187, indicating that AIBp interacts with Aurora-A and hNinein at the centrosome in interphase and was co-localized with Aurora-A and Plk1 during mitotic entry. The overlap of the Plk1 and Aurora-A binding regions in AIBp implies temporal or spatial regulation of the hNinein/Aurora-A/Plk1 complex during mitotic entry.
Figure 1. Interaction of AIBp with PLK1 and Aurora-A. (A) Schematic diagram showing full-length AIBp and its truncated variants generated in this study. (B) Interaction of full-length AIBp and its truncated variants with respect to PLK1 and Aurora-A based on results from a yeast 2-hybrid system and co-immunoprecipitation. The results of interactions are shown as “+” or “−”. (C) and (D) The IP-PLK1, IP-Aurora-A and IP-GFP represents the co-precipitated proteins respects to antibodies used. Results of co-immunoprecipitation showing possible representative interactions between AIBp truncated variants, PLK1, and Aurora-A.
pro-metaphase to telophase (Fig. 2 and Fig. S2). AIBp co-localized with Aurora-A on the centrosome during interphase and to the spindle and spindle pole from prophase to metaphase (Fig. 2, right panel). Plk1 co-localized with AIBp to the centrosome from prophase to metaphase and to the spindle midzone during anaphase and telophase (Fig. 2 and Fig. S2). Taken together, these data indicate that AIBp co-localizes with Aurora-A and Plk1 on the centrosome and spindle poles during early mitosis and metaphase. Further, AIBp, Plk1, and Aurora-A co-localize to the spindle poles, suggesting that these proteins are involved in spatiotemporal regulation during mitotic entry.

AIBp acts as both a substrate and a positive regulator of Aurora-A and Plk1

Since AIBp interacts with Plk1, we performed an in vitro kinase assay to examine whether Plk1 could phosphorylate AIBp. Our data showed that the N-terminus (amino acids 1–187) of AIBp was phosphorylated by Plk1 (Fig. S3). Plk1 is a Ser/Thr kinase that phosphorylates substrates harboring the consensus sequence (E/D/Q-X-S/T). Four putative sites of AIBp1–187 (Ser64, Ser83, Ser154, and Ser182) were chosen and mutated to alanine residues. The S182A mutant effectively down-regulated the Plk1-mediated phosphorylation compared to wild-type AIBp1–187; however, the mutation of Ser182 to alanine in full-length AIBp did not affect its binding to Plk1 in a yeast 2-hybrid system (data not shown). To further explore the role of AIBp in controlling Plk1 function, we alternatively examined whether AIBp modulates Plk1 activity toward its substrates. In a kinase assay, AIBp acted as a positive regulator of Plk1 to phosphorylate histone H3 and MBP (Fig. 3A). Moreover, AIBp also increased Aurora-A activity toward histone H3 and MBP, which is consistent with our previous study31 (Fig. 3B). Interestingly, similar to Aurora-A, Plk1 phosphorylated hNinein2010–2090, and this phosphorylation was blocked by AIBp (Fig. 3, bottom bands).

AIBp promotes Aurora-A–mediated Plk1 activation

Since Bora promotes Aurora-A–mediated Plk1 activity as described above, we investigated whether AIBp has a similar role to Bora in promoting Aurora-A–mediated Plk1 activation. HA-Aurora-A, HA-Plk1, and GFP-AIBp were co-transfected into HEK293 cells. Co-immunoprecipitation assays revealed that AIBp alone had a minor influence on Plk1 activation, whereas Aurora-A was found to elevate Plk1 activity 2-fold compared to AIBp alone. When Aurora-A was present, AIBp caused an elevation of Plk Thr210 activation, up to 12.3-fold compared to untransfected controls (Fig. 4A). In contrast, overexpression of Plk1 alone did not cause significant variations in Aurora-A Thr288 phosphorylation (~1.1-fold), but AIBp alone increased phosphorylation of Aurora-A Thr288 up to 2-fold compared to controls. When both Plk1 and AIBp were overexpressed, the phosphorylation level of Aurora-A Thr288 was not further enhanced compared to the AIBp-alone group. This result indicates that Plk1 does not directly trigger any modulating effect on Aurora-A activation (Fig. 4B). Furthermore, the AIBp-depletion in Aurora-A-overexpressing cells showed the significant decrease (~2-fold) in Plk1 Thr210 phosphorylation (Fig. 4C, lanes 2 and 4; also compares to Fig. 4A). In sum, these data show that AIBp is an activator of Aurora-A and enhances the activity of Plk1 by Aurora-A–mediated phosphorylation, suggesting that AIBp may have a dual role in regulating the phosphorylation of Plk1 by (1) directly activating Aurora-A to phosphorylate Plk1 Thr210 and (2) binding to Plk1 and enhancing conformational accessibility for Aurora-A kinase.

**Figure 2.** AIBp, PLK1, and Aurora-A co-localize to the spindle poles in early mitosis. Left panel: HeLa cells stained with anti-AIBp (green), anti-Plk1 (red) antibody, and DAPI (blue). AIBp and Plk1 co-localized at the spindle pole in early mitosis, and were particularly abundantly expressed in pro-metaphase and metaphase. Right panel: HeLa cells stained with anti-AIBp (green), anti-Plk1 (red) antibody and DAPI (blue). AIBp and Aurora-A co-localized at the centrosome and spindle pole from interphase to metaphase. DAPI-stained area represents the nucleus, and light-yellow regions in the merged images indicate co-localization of AIBp and Plk1 and/or Aurora-A. Scale bar equals 10 μm.
Knockdown of hNinein blocked centrosomal targeting of AIBp, whereas depletion of AIBp did not affect the localization of hNinein and microtubule nucleation.

Since AIBp also binds to hNinein, we further determined whether knockdown of AIBp affects the localization and function of hNinein. Our data show that knockdown of AIBp did not alter the localization of hNinein in the centrosome; on the contrary, AIBp failed to localize to the centrosomes in hNinein-depleted cells (Figs. 5A and B). In addition, since hNinein plays a regulatory role in centrosomal microtubule nucleation and microtubule anchoring in interphase cells, we performed a microtubule regrowth experiment to examine whether hNinein could recruit γ-tubulin. The data show that whether AIBp was depleted or not, the microtubules started regrowth at the centrosome at 5 and 15 min after the removal of nocodazole. The microtubule asters were visible in both control and AIBp-depleted cells (Fig. 5C). These results indicate that microtubule assembly was not significantly affected. We concluded that hNinein is involved in recruiting AIBp to the centrosomes, whereas AIBp is not involved in microtubule nucleation during interphase.

AIBp depletion decreased phosphorylation of Plk1 Thr210 and Aurora-A Thr288

To determine whether AIBp affects both Aurora-A and Plk1 activity, we further depleted AIBp expression and determined the Thr210 phosphorylation levels of Plk1 and Thr288 phosphorylation levels of Aurora-A during mitosis. After administering siRNA duplexes of AIBp for 24 hours, AIBp mRNA expression was downregulated as expected in HeLa cells, western blot, and RT-PCR and real-time PCR results show that the AIBp mRNA and protein level decreased significantly (Fig. 6A and B). For immunoblotting, HeLa cells were treated with siRNAs in a time-dependent manner and further synchronized with nocodazole. When AIBp-specific siRNA treatment was prolonged up to 48 hours, the phosphorylation levels of Thr210 of Plk1 (left panel) and Thr288
of Aurora-A (right panel) decreased significantly (Fig. 6C). These data reveal that depleted AIBp expression leads to decreasing phosphorylation levels with respect to Plk1Thr210 and Aurora-A Thr288. To further confirm this finding we determined the phosphorylation status of Plk1 and Aurora-A on centrosomes during mitosis using immunofluorescent staining. AIBp-depleted cells were sequentially stained with Plk1, Aurora-A, and α-tubulin antibodies, revealing that Plk and Aurora-A were still localized to abnormal spindle poles and spindles, respectively (Fig. 6D and E). Consistent with the immunoblotting data, depletion of AIBp by RNAi decreased the Plk1 Thr210 and Aurora-A Thr288 signal on spindle poles during mitosis (Fig. 6F and G). Notably, phosphor-antibody against Aurora-A Thr288 also detected Aurora-B Thr232 phosphorylation on centromeres, but this phosphorylation event was not affected in AIBp-depleted cells (Fig. 6G).

Depletion of AIBp caused asymmetrical spindle poles, multipolar spindles, donut-shaped chromosomes, and chromosome misalignment

To further explore the function of AIBp during mitosis, we used siRNA to deplete AIBp expression in mitotic HeLa cells. Afterwards, HeLa cells were subjected to anti-α-tubulin antibody, anti-γ-tubulin, and DAPI. Knockdown of AIBp causes asymmetrical spindle poles, multipolar spindles, donut-shaped chromosomes, and chromosome misalignment, as in our previous study41 (Fig. 7A and B). Multipolar spindle (30.5%) and chromosome misalignment (15.5%) were observed as dominant phenotypes consequent to AIBp depletion. In addition, phenotype of donut-like shape (12%) and asymmetrical spindle poles (7.5%) were also observed, which is consistent with the monopolar spindle due to reduced hNin9nine levels and/or loss interaction between astrin and hNin9nine.39,41
AIBp was linked to mislocalization of TACC3 and ch-TOG to centrosomes

To stabilize the mitotic spindle, Aurora-A reportedly phosphorylates the centrosomal adaptor TACC3 and targets it to the mitotic centrosome. To further characterize the machinery by which AIBp maintains centrosome structure, we characterized the downstream substrates of Aurora-A. AIBp expression leads to down-regulation of Aurora-A Thr288 phosphorylation, and dysregulation of Aurora-A, ch-TOG, and AIBp all resulted in the same phenotype of multipolar spindles. We thus aimed to clarify whether AIBp is functionally involved in the Aurora A-TACC3/ch-TOG complex that regulates the mitotic spindle. As shown in Fig. 8A, the TACC3 signal on the mitotic spindle was somewhat decreased (29%) in AIBp-depleted cells compared to controls. Downregulation of AIBp also led to unsuccessful spindle pole formation and spindle targeting of ch-TOG (the signal decreased ~70%, Fig. 8B). These results indicate that AIBp is involved in regulation of the Aurora A/TACC3/ch-TOG complex that...
maintains centrosome structure. Plk1 is also critical for centrosome maturation, since inhibition of Plk1 activity results in monopolar spindles with reduced microtubule-organizing activity.43,44 Since pericentrin (PCNT), CEP192, and CEP215 are required for centrosome maturation and are also involved in recruitment of γ-tubulin into the spindle poles,45,46 we investigated whether localization of CEP192 and CEP215 was affected by AIBp depletion. Our data show that the subcellular localization and spindle pole abnormalities after knockdown of AIBp expression. Scale bar represents 10 μm.

**Figure 7.** Depletion of AIBp caused formation of multipolar spindle poles, asymmetrical spindle poles, donut-shaped chromosomes, and chromosome misalignment. For immunofluorescence, HeLa cells were treated for 48 h with an AIBp-specific siRNA duplex, synchronized with nocodazole, and then fixed. (A) Cells were stained with anti-γ-tubulin antibody (red) and DAPI. (B) Cells were probed with anti-α-tubulin antibody (red) and DAPI. Numbers indicate the percentage of centrosome structure and spindle pole abnormalities after knockdown of AIBp expression. Scale bar represents 10 μm.

In this study, we showed AIBp binds and activates Plk1. These findings suggest a novel AIBp-dependent regulatory machinery that may control mitotic entry. Additionally, knockdown AIBp in HeLa cells impairs both Aurora-A and Plk1 kinase, resulting in phenotypes of multiple spindle pole formation and chromosome misalignment. These findings strongly suggest that AIBp interacts with centrosomal hNinein, Aurora-A, and Plk1 to govern centrosome maturation and maintain mitotic spindle assembly.

**Discussion**

In this study, we showed AIBp binds and activates Plk1. These findings suggest a novel AIBp-dependent regulatory machinery that may control mitotic entry. Additionally, knockdown AIBp in HeLa cells impairs both Aurora-A and Plk1 kinase, resulting in phenotypes of multiple spindle pole formation and chromosome misalignment. These findings strongly suggest that AIBp interacts with centrosomal hNinein, Aurora-A, and Plk1 to govern centrosome maturation and maintain mitotic spindle assembly.

**AIBp acts as a positive and negative regulator toward both Aurora-A and Plk1**

As shown in Figure 3, AIBp not only acts as a substrate but a positive regulator toward both Aurora-A and Plk1. However, AIBp functions as a negative regulator to block the phosphorylation of hNinein mediated by Aurora-A and Plk1. The opposite function of AIBp for Plk1 and Aurora-A may be due to allosteric modification by protein conformational changes after additional binding of a regulatory protein such as hNinein.41(Figure S1). It should also be noted that, unlike hNinein targeting of γ-tubulin in microtubule nucleation,34,35 overexpressed AIBp appears to affect Aurora-A and Plk1 function in hNinein phosphorylation, which may indirectly prevent cells from entering mitosis (Fig. 3, bottom bands and unpublished data). The emerging evidence of complex collaboration between hNinein, AIBp, Aurora-A, and Plk1 uncovers a novel view of their regulatory network for both temporal and spatial control of the cell cycle. Of note, Plk1 did phosphorylate hNinein (Fig. 3), which is inconsistent with a previously report by Casenghiet et al.25

**AIBp is similar to Bora, but facilitates its role by interacting with hNinein and is involved in regulation of centrosomal microtubule signaling**

To date, 4 different proteins, Bora, Furry, Cep192, and AIBp, have exhibited the ability to bind both Aurora-A and Plk1. Initial activation of Plk1 in late G2 is induced by the cooperative action of Aurora-A and Bora. Bora binds to Plk1 and promotes Aurora-A–mediated Plk1 activation.17,18 Once Plk1 is activated, Bora is phosphorylated by Cdk1 and Plk1 and thereafter degraded by ubiquitin-assisted proteolysis at the entry step of mitosis.19,20 Cep192 is reported to recruit pericentrin, whereas AIBp is involved in hNinein-related signaling. In addition, Cep192 has no regulatory role affecting Plk1 activity or promoting Aurora-A–mediated Plk1 activation.47 Once Plk1 is activated, Bora is phosphorylated by Cdk1 and Plk1 and thereafter degraded by ubiquitin-assisted proteolysis at the entry step of mitosis.19,20 Cep192 is reported to recruit pericentrin, whereas AIBp is involved in hNinein-related signaling. In addition, Cep192 has no regulatory role affecting Plk1 activity or promoting Aurora-A–mediated Plk1 activation.47 Once Plk1 is activated, Bora is phosphorylated by Cdk1 and Plk1 and thereafter degraded by ubiquitin-assisted proteolysis at the entry step of mitosis.19,20 Cep192 is reported to recruit pericentrin, whereas AIBp is involved in hNinein-related signaling. In addition, Cep192 has no regulatory role affecting Plk1 activity or promoting Aurora-A–mediated Plk1 activation.47 Once Plk1 is activated, Bora is phosphorylated by Cdk1 and Plk1 and thereafter degraded by ubiquitin-assisted proteolysis at the entry step of mitosis.19,20 Cep192 is reported to recruit pericentrin, whereas AIBp is involved in hNinein-related signaling. In addition, Cep192 has no regulatory role affecting Plk1 activity or promoting Aurora-A–mediated Plk1 activation.47 Once Plk1 is activated, Bora is phosphorylated by Cdk1 and Plk1 and thereafter degraded by ubiquitin-assisted proteolysis at the entry step of mitosis.19,20 Cep192 is reported to recruit pericentrin, whereas AIBp is involved in hNinein-related signaling. In addition, Cep192 has no regulatory role affecting Plk1 activity or promoting Aurora-A–mediated Plk1 activation.47 Once Plk1 is activated, Bora is phosphorylated by Cdk1 and Plk1 and thereafter degraded by ubiquitin-assisted proteolysis at the entry step of mitosis.19,20 Cep192 is reported to recruit pericentrin, whereas AIBp is involved in hNinein-related signaling. In addition, Cep192 has no regulatory role affecting Plk1 activity or promoting Aurora-A–mediated Plk1 activation.47 Once Plk1 is activated, Bora is phosphorylated by Cdk1 and Plk1 and thereafter degraded by ubiquitin-assisted proteolysis at the entry step of mitosis.19,20 Cep192 is reported to recruit pericentrin, whereas AIBp is involved in hNinein-related signaling. In addition, Cep192 has no regulatory role affecting Plk1 activity or promoting Aurora-A–mediated Plk1 activation.47 Once Plk1 is activated, Bora is phosphorylated by Cdk1 and Plk1 and thereafter degraded by ubiquitin-assisted proteolysis at the entry step of mitosis.19,20 Cep192 is reported to recruit pericentrin, whereas AIBp is involved in hNinein-related signaling. In addition, Cep192 has no regulatory role affecting Plk1 activity or promoting Aurora-A–mediated Plk1 activation.47 Once Plk1 is activated, Bora is phosphorylated by Cdk1 and Plk1 and thereafter degraded by ubiquitin-assisted proteolysis at the entry step of mitosis.19,20 Cep192 is reported to recruit pericentrin, whereas AIBp is involved in hNinein-related signaling. In addition, Cep192 has no regulatory role affecting Plk1 activity or promoting Aurora-A–mediated Plk1 activation.47 Once Plk1 is activated, Bora is phosphorylated by Cdk1 and Plk1 and thereafter degraded by ubiquitin-assisted proteolysis at the entry step of mitosis.19,20 Cep192 is reported to recruit pericentrin, whereas AIBp is involved in hNinein-related signaling. In addition, Cep192 has no regulatory role affecting Plk1 activity or promoting Aurora-A–mediated Plk1 activation.47 Once Plk1 is activated, Bora is phosphorylated by Cdk1 and Plk1 and thereafter degraded by ubiquitin-assisted proteolysis at the entry step of mitosis.
after mitotic entry. As shown in Figure S2, AIBp partially co-localized with Plk1 during anaphase and telophase).

Formation of multiple spindle poles
Centrosome defects cause the assembly of multipolar spindles. To date at least 3 different routes are known to lead to multipolar spindle formation: centrosome amplification, premature centriole splitting, and fragmented centrosomal PCM. Depletion of the following proteins has been reported to cause the formation of multiple spindle poles: 1) Knockdown of Aurora-A or inhibition of Aurora-A by antibody microinjection results in multipolar spindle formation; 2) Depletion of Bora frequently revealed a phenotype of multipolar spindles in mitosis compared to cells treated with a control siRNA; 3) In ch-TOG-depleted mitotic cells, the spindle microtubules did not appear to be dramatically destabilized, but the spindles were usually highly disorganized; 4) Silencing of astrin in HeLa cells by RNA interference resulted in growth arrest, with formation of multipolar and highly disordered spindles.

In this study, we clearly showed that knockdown of AIBp in HeLa cells resulted in phenotypes with multiple spindle poles. Down-regulation of AIBp with siRNA resulted in partial unsuccessful mitotic spindle-targeting of TACC3 and a decreased ch-TOG signal in spindle poles, but not CEP 192 and CEP215 (Fig. 8C and D), suggesting that loss of AIBp dominantly affects the Aurora-A target protein and causes mitotic aberrations. Another study has reported that depletion of Aurora-A leads to mislocalization of ch-TOG, which abnormally accumulates at spindle poles and also exhibits multipolarity. More recently, the centrosomal adaptor TACC3 and the microtubule polymerase ch-TOG have been suggested to interact via defined C-terminal subdomains in an Aurora-A-kinase-independent manner. On the other hand, Plk1 has also been reported to maintain spindle pole integrity by phosphorylating...
centrosomal protein Kizuna at Thr 379. Further studies to determine whether AIBp plays a role in controlling Kizuna are needed. Based on our observations and current knowledge of centrosome biology, we propose a model demonstrating the interplay of hNinein, AIBp, Aurora-A and Plk1 complex in centrosomal maturation and spindle pole organization (Fig. 9).

In conclusion, we provide evidence to show that AIBp has a crucial role in spindle pole integrity, partially via hNinein, which may control the localization, phosphorylation, and activation of both Aurora-A and Plk1 during mitotic entry and mitotic spindle assembly.

Figure 9. The interplay of hNinein, AIBp, Aurora-A, and Plk1 in centrosomal maturation and spindle pole organization. hNinein recruits AIBp to the centrosome. AIBp is a positive regulator of Aurora-A and Plk1 and enhances their kinase activity. However, AIBp functions as a negative regulator to block the phosphorylation of hNinein mediated by Aurora-A and Plk1. The Aurora-A-TACC3-chTOG signaling axis may impact mitotic spindle integrity, whereas the Plk1-PCMs (CEP192, CEP215, Aurora-A, Pericentrin, NEDD1, and γ-tubulin) signaling axis is more likely responsible for centrosome maturation and spindle assembly. Knockdown of AIBp resulted in phenotypes showing multiple spindle pole formation and chromosome mis-alignment. Depletion of AIBp appeared to cause mis-localization of TACC3 and ch-TOG, but not CEP192 and CEP215, suggesting that loss of AIBp dominantly affects Aurora-A target proteins and subsequently causes mitotic aberration. Solid lines represent confirmed signaling pathways; dashed lines indicate uncertain pathways.

Materials and Methods

Plasmid Construction

To construct full length pAS2-1 or pACT2-AIBp and PLK1 for the yeast 2-hybrid working assay, DNA fragments encoding AIBp or PLK1 were amplified by PCR using the Taq polymerase (TaKaRa/Clontech, Mountain View, CA, USA). The PCR fragments were then inserted into the pAS2-1 or pACT2 (Clontech) vector. The pET32a vector was used as the backbone to generate full-length AIBp and the other truncated AIBp fragments for recombinant protein expression and purification. Full length pEGFP-C1-AIBp and various truncated AIBp fragments, full-length pcDNA-HA-PLK1 and pcDNA-HA-Aurora-A were constructed for co-immunoprecipitation. DNA fragments encoding AIBp, PLK1 or Aurora-A were amplified by PCR using the Taq polymerase (TaKaRa). The PCR products of full length and truncated AIBp were then inserted into the SalI and BamHI site of the pEGFP-C1 (Clontech) vector. The PCR fragment corresponding to full length PLK1 was cloned into the KpnI/BamHI site of pcDNA-HA. The truncated AIBp fragments were also inserted into the SalI/BamHI I site of pET32a (Novagen/Merck KGaA, Darmstadt, Germany).

Yeast two-hybrid system

Standard techniques were used for yeast 2-hybrid screening. Briefly, the AIBp gene was cloned in-frame with the Gal4 DNA-activation domain in the pACT2 vector (MARCHMAKER Two-Hybrid System 2, Clontech) to yield the pACT2-AIBp plasmid. YRG2 was transformed with (Stratagene, La Jolla, CA, USA) pAS2-1-PLK11–603 a.a bait plasmid DNA and pACT2 AIBp1–371 and other fragments (Clontech). The positive clones were able to grow on Trp, Leu, and His dropout media supplemented with the HIS3 inhibitor 3-aminotriazole (3-AT) and turned blue in a β-galactosidase filter assay.

Protein purification

To generate His-tagged AIBp and its truncated His-tagged protein, AIBp was introduced into the pET-32a vector (Novagen). DNA fragments encoding AIBp were amplified by PCR and inserted into the SalI/BamHI I site of the pET32a vector. The pET32a-AIBp plasmid was then transformed into E. coli BL21 cells (DE3) harboring the different constructs (designated e.g., pET-32a-AIBp) were cultured in 50 mL of LB medium at 37°C to mid-log phase. Isopropyl-β-D-galactopyranoside (IPTG) was then added to a final concentration of 0.5 mM to induce the expression of His-tagged fusion proteins. After incubation

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for 3 hours, cells were pelleted by centrifugation and suspended in 5 mL binding buffer containing 0.1% lysozyme and protease inhibitor (Roche, Basel, Switzerland). The bacterial suspension was then sonicated until clear and centrifuged at 14000 rpm for 30 minutes at 4°C. Ni-charged resins were added to the supernatant, and the mixture was incubated in an end-over-end shaker for 16 hours at 4°C. The resins were washed 3 times with binding buffer (no protease inhibitor) and wash buffer. After washing, His-tagged proteins were released from the beads with elution buffer. Site-directed mutagenesis was performed using pET32a-AI Bp as a parental vector to generate the mutants. All mutants were sequenced to confirm that only the intended point mutations were introduced. All proteins used in this study were purified by the same methodology.

Site-directed mutagenesis
Specific primers for mutagenesis (Quality Systems Biotech) were synthesized. Mutant AIBp constructs were generated according to the manufacturer’s instructions with minor modifications (QuickChange, Stratagene). Briefly, after amplification by PCR, 1.5 μL Dpn-I (10 U/μL) was added for parental DNA digestion at 37°C for 10 minutes. During digestion, competent cells were prepared by gently thawing XL10-Gold-ultra-competent cells on ice. β-mercaptoethanol (2 μL) was added to competent cells (45 μL), followed by thorough mixing every 2 minutes for 10 minutes. Dpn I-treated DNA (2 μL) was added to prepared competent cells (45 μL) and gently swirled to mix. Transformation reactants were then incubated on ice for 30 minutes, followed by a heat pulse at 42°C for 30 seconds and immediate incubation on ice for 2 minutes. LB medium (500 μL) was added and incubated at 37°C on an orbital shaker at 220 rpm for 1 hour. After incubation, the cells were spread onto selective plates containing antibiotics (0.05 mg/mL ampicillin or kanamycin), and incubated at 37°C for 16–18 hours. Several colonies were then picked for digestion and the DNA sequenced.

In vitro kinase assay
Briefly, His-tagged AIBp and its variants were purified and then incubated with Aurora-A, Plk1 (Invitrogen), and GSK3β (25 units) (NEB, Ipswich, MA, USA). The mixtures were further incubated with Histone 3, Tau, and hNinein in kinase buffer [200 mM Na3VO4, 100 mM dithiothreitol, 50 mM EGTA, 2M Tris (pH 7.2), 1M MgCl2, 10 mM ATP, 100 mM PMSF, 100% glycerol, and 10% glycerol with [γ-32P]ATP (PerkinElmer, Santa Clara, CA, USA), 3000 Ci/mM]. The assays were carried out for 60 minutes at 30°C and terminated by adding 5×SDS sample buffer and heating at 95°C for 10 minutes. The reaction mixtures were resolved by SDS-PAGE, and radioactive signaling was detected by autoradiography.

Cell culture, synchronization, RNA interference and transfection
HeLa and HEK293 cells were grown at 37°C in DMEM supplemented with 10% FBS and penicillin with streptomycin (100 IU/mL). For synchronization, cells were treated with 200 ng/mL nocodazole (Sigma). The following siRNA duplexes were applied: AIBp: sense, 5’GGAAGAGUGU UUCAUCUCAUAGAA3’ and antisense, 5’UUUGGUAUG GAGAUGAAACACUUCC3’ (Invitrogen); hNinein: sense, 5’UAUGAGCAAUGAGGCAAGCt and antisense, 5’UCUG CCUAAGCUCUAAtt (Ambion/Life Technologies, Carlsbad, CA, USA). A scrambled siRNA duplex was used as the negative control (Invitrogen). For transient transfection studies, HeLa cells were seeded onto glass coverslips at a density of 8×10⁵ cells/well in 12-well plates. Transfection with plasmid DNA and duplex RNA was carried out using Lipofectamine 2000 (Invitrogen).

Immunofluorescence
After transfection, the cells were washed with PBS and fixed in PBS containing 4% formaldehyde for 5 min at 25°C followed by immersion in methanol at −20°C for 5 minutes. The fixed cells were probed with rabbit anti–Aurora-A serum (1:500, pure preparation), mouse anti-Plk1 antibody (1:400) (Abcam, Cambridge, England), rabbit anti-AIBp antibody (1:250, our preparation), mouse anti-γ-tubulin antibody (1:1000) (GTU-88, Sigma), mouse anti-α-tubulin antibody (1:1000) (DM1A, Sigma), rabbit anti-hNinein antibody (1:500, our preparation ), rabbit anti-CEP215 and anti-CEP192 antibody (1:500) (Bethyl Laboratories, Montgomery, Texas, USA), rabbit anti-ch-TOG (1:250) (Novus, St. Louis, MO, USA), rabbit anti-TACC3 (1:500) (Santa Cruz Biotechnologies, Santa Cruz, CA, USA), rabbit anti-phospho-Plk1 (T210) (1:300, Cell Signaling Technology, Beverly, MA, USA), and rabbit anti–phospho-Aurora-A (T288/Aurora-B (T232)/Aurora-C (T198) (1:300) (Cell Signaling Technology). The secondary antibodies were rhodamine-conjugated goat anti-rabbit or goat anti-mouse antibodies (1:300) (Invitrogen) and FITC-conjugated goat anti-rabbit or anti-mouse antibodies (1:500) (Invitrogen). DNA was stained with 4, 6-diamidino-2-phenylindole (DAPI, 2 μg/mL) as previously described. Immunofluorescent imaging was performed using a Fluoview 1000 confocal laser scanning microscope (Olympus, Tokyo, Japan).

Western blot analysis
For Western blot analysis, HeLa and HEK293 cells were maintained in DMEM supplemented with 10% FBS. Cells were harvested 24 hours after transfection and washed once in HBSS. The cells were then centrifuged at 1,000 rpm for 5 minutes at room temperature, re-suspended in lysis buffer (50 mM Tris-HCl [pH 7.8], 150 mM NaCl, 5 mM EDTA, 0.5% Triton X-100, 0.5% Nonidet P-40, 0.1% deoxycholate, and leupeptin, aprotinin, and 4-[2-aminoethyl] benzenesulfonyl fluoride [10 μg/mL each]). Samples were sonicated and then centrifuged at 14 000 rpm for 15 minutes at 4°C. The supernatant was then transferred into a fresh centrifuge tube, and protein sample buffer was added and further heated at 95°C for 5 minutes. Proteins were separated by 10–12% SDS-PAGE, transferred to Hybond PVDF membranes, blocked in TBS with 5% skim milk and 0.1% Tween 20 for 1 hour, then washed 3 times in TBS with 0.1% Tween 20, 5–10 minutes per wash. The membranes were incubated with primary antibodies including anti-AIBp, anti-Aurora-A (Abcam), anti-phosphorylated Aurora-A (Thr288)}
Co-immunoprecipitation

pcDNA-Aurora A (HA-tagged Aurora A), pEGFP-C1-AlBp (GFP-tagged AlBp) and pcDNA-Plk1 (HA-tagged Plk1) were overexpressed in HeLa cells, and transfectants were collected from one 10-cm² dish and then re-suspended in 1 mL of mRIPA buffer (50 mM Tris-HCL, pH 7.8, 150 mM NaCl, 5 mM EDTA, 0.5% Triton X-100, 0.5% Nonidet P-40, 0.1% deoxycholate, and 10 μM each of leupeptin, aprotinin, and 4-(2-aminoethyl) benzenesulfonyl fluoride). Total protein lysates (50 μL) were subjected to co-immunoprecipitation assay. Anti-Plk1 antibody was added to the protein lysates and further incubated at 4°C overnight. Protein-A/G-agarose beads (30 μL) (Calbiochem, Davis, CA, USA) were added to the lysates and the mixtures further incubated with gentle shaking for 10 minutes at room temperature. Immunoprecipitates were washed 3 times with mRIPA buffer. Proteins bound to the beads were eluted and analyzed by adding 20 μL of 5 × loading dye and resolved by immunoblotting with anti-HA antibody, anti-GFP antibody, anti-Plk1-Thr210, anti-Aurora A-Thr288, or anti-Aurora A and Plk1 antibody.

Statistical analysis

All the experiments were performed at least 3 times, and the representative results are shown. The results are presented as mean ± standard deviation. Statistical significance between the groups was examined using Student’s t-test. p-Values less than 0.05 were considered statistically significant.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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Supplemental Material

Supplemental data for this article can be accessed on the publisher’s website.

References

1. Bettenourt-Dias M, Glover DM. Centrosome biogenesis and function: centrosomies bring new understand- ing. Nat Rev Mol Cell Biol 2007; 8:451-63; PMID:17505520; http://dx.doi.org/10.1038/nrm2180
2. Bornens M. Centrosome composition and microtubule anchoring mechanisms. Curr Opin Cell Biol 2002; 14:25-34; PMID:11779254; http://dx.doi.org/10.1016/S0959-4405(01)00290-3
3. Meraldi P, Honda R, Nigg EA. Aurora kinases link chromosome segregation and cell division to cancer susceptibility. Curr Opin Genet Dev 2004; 14:29-36; PMID:15108802; http://dx.doi.org/10.1016/j.coe. gde.2003.11.006
4. Azimzadeh J, Bornens M. Structure and duplication of the centrosome. J Cell Sci 2007; 120:2139-42; PMID:17591686; http://dx.doi.org/10.1242/jcs.005231
5. Wang G, Jiang Q, Zhang C. The role of mitotic kinases in coupling the centrosome cycle with the assembly of the mitotic spindle. J Cell Sci 2014; 127:4111-22; PMID:25128564; http://dx.doi.org/10.1242/jcs.151753
6. Doxsey S. Duplicating dangerously: linking centrosome duplication and aneuploidy. Mol Cell 2002; 10:439- 40; PMID:12408813; http://dx.doi.org/10.1016/S1097-2765(02)00654-8
7. Holland AJ, Cleveland DW. Boveri revisited: chromo- somal instability, aneuploidy and tumorigenesis. Nat Rev Mol Cell Biol 2009; 10:439-50; PMID:19546858; http://dx.doi.org/10.1038/nrm2718
8. Carmina M, Earnshaw WC. The cellular geography of aurora kinases.Nat Rev Mol Cell Biol 2003; 4:842-54; PMID:14625535; http://dx.doi.org/10.1038/nrm1245
2776 Volume 14 Issue 17

Cell Cycle

32. Joukov V, Walter JC, De Nicolo A. The Cep192-orga-

31. Ikeda M, Chiba S, Ohashi K, Mizuno K. Furry

30. Elia AE, Cantley LC, Yaffe MB. Proteomic screen

29. Lee K, Rhee K. PLK1 phosphorylation of pericentrin

28. Casenghi M, Barr FA, Nigg EA. Phosphorylation of

27. Lee K, Rhee K. CEP215 is involved in the dynein-

26. Caienghi M, Barr FA, Nigg EA. Phosphorylation of

25. Caienghi M, Meraldi P, Weinhart U, Duncan PI,

24. Petronczki M, Lenart P, Peters JM. Polo on the Rise-

from Microrna Entry to Cytokinesis with Plk1. Dev Cell

23. Barr FA, Sillje HH, Nigg EA. Polo-like kinases and the

22. Archambault V, Glover DM. Polo-like kinases: conser-

21. Kufer TA, Sillje HH, Korner R, Graus OF, Meraldi P,

20. Kafer TA, Sillje HH, Korner R, Graus OF, Meraldi P,

19. Joukov V, Walter JC, De Nicolo A. The Cep192-orga-

18. Archambault V, Glover DM. Polo-like kinases: conser-

17. Ikeda M, Chiba S, Ohashi K, Mizuno K. Furry

16. Elia AE, Cantley LC, Yaffe MB. Proteomic screen

15. Caienghi M, Meraldi P, Weinhart U, Duncan PI,

14. Petronczki M, Lenart P, Peters JM. Polo on the Rise-

from Microrna Entry to Cytokinesis with Plk1. Dev Cell

13. Ikeeda M, Chiba S, Ohashi K, Mizuno K. Furry protein

promotes aurora A-mediated Polo-like kinase 1 activation. J Biol Chem 2012; 287:27670-

12. Caienghi M, Barr FA, Nigg EA. Phosphorylation of

11. Lee K, Rhee K. CEP215 is involved in the dynein-

10. Caienghi M, Meraldi P, Weinhart U, Duncan PI,

9. Caienghi M, Meraldi P, Weinhart U, Duncan PI,

8. Caienghi M, Meraldi P, Weinhart U, Duncan PI,

7. Caienghi M, Meraldi P, Weinhart U, Duncan PI,

6. Caienghi M, Meraldi P, Weinhart U, Duncan PI,

5. Caienghi M, Meraldi P, Weinhart U, Duncan PI,

4. Caienghi M, Meraldi P, Weinhart U, Duncan PI,

3. Caienghi M, Meraldi P, Weinhart U, Duncan PI,

2. Caienghi M, Meraldi P, Weinhart U, Duncan PI,