Spatial Compartmentalization Specializes the Function of Aurora A and Aurora B

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Aurora kinase A and B share great similarity in sequences, structures, and phosphorylation motif, yet they show different localizations and play distinct roles. The factors that determine such differences are largely unknown. Here we targeted Aurora A to the localization of Aurora B and found that Aurora A phosphorylates the substrate of Aurora B and substitutes its function in spindle checkpoint. In return, the centrosome targeting of Aurora B substitutes the function of Aurora A in the mitotic entry. Expressing the chimera proteins of the Auroras with exchanged N termini in cells indicates that the divergent N termini are also important for their spatiotemporal localizations and functions. Collectively, we demonstrate that functional divergence of Aurora kinases is determined by spatial compartmentalization, and their divergent N termini also contribute to their spatial and functional differentiation.

The Aurora kinases belong to the serine/threonine kinase families and are essential for the cell cycle control in the eukaryotes (1, 2). Many low species have only one Aurora, whereas the mammals have at least three, Aurora A, B, and C, among which Aurora C resembles Aurora B but regulates meiosis and mitosis during early development (2–4). Aurora A and B display different localizations and functions during the cell cycle. Aurora A is located to the centrosomes throughout the cell cycle, spreads on the spindle microtubules in metaphase, and is relocated to the central spindle in anaphase and telophase (5–8). In contrast, Aurora B is mainly located on the centromere in early mitosis, where it serves as a component of the chromosome passenger complex, and on the midzone/midbody during the cytokinesis (3). In accordance with the spatiotemporal localization divergence, Aurora A and B perform distinct functions. Aurora A is required for centrosome maturation, mitotic entry, and centrosome separation, whereas Aurora B mainly regulates spindle assembly checkpoint, kinechores attachment, and cytokinesis (3, 9–11).

Both molecules of Aurora A and B have a divergent N terminus and a conserved catalytic domain-containing C terminus in sequence (12–14). Aurora B combines with INCENP, Survivin, and Borealin to form the chromosome passenger complex, which is required for activation and specialized localization of Aurora B (3). Aurora A interacts with a microtubule-associated protein TPX2 that not only regulates the localization but also the activation of Aurora A on the spindle in prometaphase and metaphase (15). Aurora A also interacts with a variety of binding partners/substrates including centrosome-localized Ajuba, Bora, and PAK1 and functions on the centrosomes (10, 16, 17).

In the known cases of yeast (Saccharomyces cerevisiae and Schizosaccharomyces pombe), mycetozoa (Dictyostelium discoideum), primitive deuterostome (starfish, ascidian, and urchin), and so on, the only Aurora kinase shows the localization and function of both Aurora A and B of high species (18–20). Phylogenetic analysis shows that both Aurora A and B are likely evolved from a primitive bifunctional Aurora kinase, although how the differentiation of their Auroras is formed remains largely unknown (21). It is well accepted that duplication and divergence are the primary means by which new proteins and pathways are created. There are many factors including the substrate specificity, the specified binding of proteins, and the spatial mechanisms like the scaffolds, the regulation of reactions, and the formation of macromolecular complexes that contribute to the evolution and divergence of the proteins (22, 23). Generally, fully conserved positions of proteins may confer the proteins’ common functional features, whereas less
conserved specificity-determining positions are related to their divergent functions. It has been found that both Aurora A and B may have similar optimal phosphorylation motifs (24) and that they may share some common substrates such as CENP-E, MCAK, Ki67, and RASSF1A on the spindles in cells (25–29). However, the paradox is why Aurora A and B show very different localizations and play distinct roles during the cell cycle with such a similarity in sequence and phosphorylation motif affinity.

In this study we set out to determine how Aurora A and B are spatially compartmentalized for their functional divergence. We found that, in addition to the catalytic conservative C termini, the divergent N termini also regulate their localization, and the resultant spatial compartmentalization further leads to functional divergence.

**Experimental Procedures**

**Molecular Cloning**—For construction of fusion proteins, GFP-H2B-Aurora A, GFP-CENPB-Aurora A, GFP-Hecl-Aurora A, GFP-PLK4-Aurora B, wild-type Aurora A, or Aurora B was inserted into pEGFP-C1 plasmid. Full-length H2B, CENPB-B aa1–158 targeting sequence, and full-length Hec1 were cloned by RT-PCR from HeLa cell lysate and inserted to the N terminus of Aurora A sequence. PLK4 aa570–820 centrosome-targeting sequence was inserted in the N-terminus of Aurora B sequence. Kinase dead mutation was generated by point mutation PCR. N-terminal and C-terminal truncation mutant of Aurora A and Aurora B was cloned to pEGFP-C1 plasmid. And fusion proteins GFP-Aurora A-B and GFP-Aurora B-A were generated as in Fig. 1 by overlapping PCR.

**Cell Culture and Drug Treatment**—HeLa cells were cultured at 37 °C under 5% CO2 in DMEM supplemented with 10% fetal bovine serum. To synchronize HeLa cell to mitosis, the cell cycle was blocked by double thymidine treatment to G1-S phase and then released for 10 h. To generate mitosis cells with misaligned chromosomes, HeLa cell dissociated by trypsin and then treated with 50 μM STLC for 20–25 min, and then released for 10 h. Kinase activity of Aurora A is inhibited by 50 μM STLC. Aurora B is preferentially phosphorylated by Aurora A in vitro (31), we set out to test whether special functions of Aurora A and B are determined by their distinct localizations. We speculated that, if the functional divergence of Aurora A and B is achieved by their spatial compartmentalization through specifically binding their substrates and binding partners, the forced localization exchange of the both would substitute the functions of each other. By fusing Aurora A with either histone H2B or the centromere protein truncate CENP-Baa1–158(30) tagged with GFP (GFP-H2B-Aurora A and GFP-CENPB-Aurora A) and transiently expressing these fusion proteins in cells, we found that the fusion protein GFP-CENPB-Aurora A was localized to the nucleus and primarily the centromere during the cell cycle, and a fraction of it was relocated to the spindle/poles as did the wild-type Aurora A from prophase to metaphase and that the fusion protein GFP-H2B-Aurora A was located primarily on the chromosome/chromosomes during the cell cycle (Fig. 1A and supplemental Movies S1 and S2). As it is known that Aurora B is localized in interphase nucleus and relocated to the centromere in early mitosis, we concluded that GFP-H2B-Aurora A and GFP-CENPB-Aurora A proteins had been localized to the areas to which Aurora B is preferentially localized. By probing the active phosphorylation status of Aurora A at Ser-232 using a phospho-specific antibody, we also found that these two fusion pro-
proteins were also activated on their T-loops like the wild-type Aurora A (data not shown). To evaluate whether these fusion proteins may substitute the functions of Aurora B, we eliminated the kinase activity of endogenous Aurora B by treating HeLa cells with a serial concentration of an Aurora B-specific inhibitor AZD1152. The inhibition efficiency was tested by detecting the active phosphorylation status of Aurora proteins using the phospho-specific antibody. The result showed that, in the presence of AZD1152 at 200 nM and above, the kinase activity of Aurora B was totally inhibited, whereas the kinase activity of GFP-CENPB-Aurora A was less affected by AZD1152 (Fig. 1B). Then the cells expressing GFP-H2B-Aurora A and GFP-CENPB-Aurora A were treated with 200 nM AZD1152 for 1 h and processed for immunofluorescence microscopy. The result showed that the Aurora B substrate histone H3 Ser-10 phosphorylation in almost all transfected cells by GFP-H2B-Aurora A regardless the period of the cell cycle (96.52%). In contrast, only a very small amount of cells transfected with GFP-Aurora A showed slight phosphorylation of histone H3 Ser-10. Moreover, in cells expressing GFP-CENPB-Aurora A that was located at the centromere, the phosphorylated histone H3 Ser-10 was only restricted at the centromere (Fig. 1, C and D). On the contrary, the kinase dead mutants of GFP-H2B-Aurora A and GFP-CENPB-Aurora A were treated with 200 nM AZD1152 for 1 h and processed for immunofluorescence microscopy. The result showed that the Aurora B substrate histone H3 Ser-10 was extensively phosphorylated in almost all transfected cells by GFP-H2B-Aurora A and GFP-CENPB-Aurora A expression resulted in phosphorylation of, and colocalized with histone H3 Ser-10 (H3pS10), whereas GFP-H2B-Aurora A-KD, GFP-CENPB-Aurora A-KD, and GFP-Aurora A did not induce histone H3 Ser-10 phosphorylation. Scale bar, 10 μm. D, quantitative characterization of HeLa cells treated as in C. Positive staining cells are defined as strongly staining with clear edge. Cells with no stain or weak vague staining are not counted as positive staining cells. Bars represent the percentage of the cells with histone H3 Ser-10 phosphorylation. Each data point represents three independent experiments with each measuring 100 cells, and error bars indicate S.D. E, histone H3 Ser(P)-10 immunofluorescence labeling of HeLa cells co-transfected with Aurora B siRNA to knock down endogenous Aurora B and GFP-H2B-Aurora B or RNAi resistant GFP-H2B-Aurora B. Note that both GFP-H2B-Aurora B and GFP-H2B-Aurora A phosphorylated histone H3 Ser-10 (H3pS10). F, quantitative characterization of HeLa cells treated as in E. Bars represented the percentage of the cells with histone H3 Ser-10 phosphorylation. Each data point represents three independent experiments with each measuring 100 cells, and error bars indicate S.D. Scale bar, 10 μm.
CENPB-Aurora A (GFP-H2B-Aurora A-KD and GFP-CENPB-Aurora A-KD) barely phosphorylated histone H3 Ser-10 (Fig. 1, C and D). Together, these results strongly indicated that histone H3 Ser-10 was specifically phosphorylated by GFP-H2B-Aurora A or GFP-CENPB-Aurora A. To further confirm whether GFP-H2B-Aurora A performed the same function as Aurora B, endogenous Aurora B was knocked down in HeLa cells by siRNA, and GFP-H2B-Aurora A or GFP-H2B-Aurora B was simultaneously expressed. By immunofluorescence microscopy, we found that histone H3 at Ser-10 was phosphorylated in almost all of the cells expressing GFP-H2B-Aurora A or GFP-H2B-Aurora B (Fig. 1, E and F). Collectively, these results demonstrated that, when forced to locate to the same place of Aurora B, Aurora A may phosphorylate the Aurora B substrate histone H3. Thus, we conclude that the specific phosphorylation of histone H3 Ser-10 by Aurora B rather than Aurora A is indeed caused by the spatial compartmentalization of the Auroras and that other substrates of Aurora B could also be phosphorylated by Aurora A provided that the spatial compartmentalization of these two Aurora kinases is eliminated.

**Kinetochoore-targeted Aurora A Performs the Function of Aurora B on the Spindle Checkpoint Regulation**—One of the most important functions of Aurora B in mitosis is to regulate the kinetochore-microtubule attachment and the spindle checkpoint. The activation of the spindle checkpoint is stimulated by Aurora B in two pathways, one of which is that Aurora B destabilizes erroneous kinetochore-microtubule attachment and indirectly induce spindle checkpoint. The centromere-located Aurora B gives rise to a phosphorylation gradient around the centromere, and the phosphorylation of the Aurora B substrates at the kinetochores depends on their distance from Aurora B at the centromere (32, 33). If microtubules from two opposite poles fail to bind the kinetochores correctly, the tension between the paired kinetochores is low, and Aurora B phosphorylates its multiple substrates of the KMN network (including Hec1, DSN1, Knl1) on the kinetochores, as the distance from the substrates to Aurora B is short and thus activates the spindle assembly checkpoint by releasing the microtubules from the kinetochores (34, 35). Another pathway is that Aurora B functions synergistically with MPS1 to induce kinetochore localization of spindle checkpoint proteins BubR1 and mad2 (36, 37). To further explore whether the forced localization of Aurora A on the centromere can substitute Aurora B for the function of the spindle checkpoint regulation, we generated and expressed fusion proteins of Aurora A or its kinase dead mutant with Hec1 tagged with GFP (GFP-Hec1-Aurora A and GFP-Hec1-Aurora A-KD) to target Aurora A to the kinetochores (Fig. 2A and supplemental Movie S3). Furthermore, we treated HeLa cells by STLC, a specific Eg5 inhibitor that weakens the interaction of Eg5 with microtubules resulting in the failure of bipolar spindle formation and mitotic arrest (38, 39), to synchronize the cells in prometaphase and then with 200 nM AZD1152 for 1 h to inhibit their endogenous Aurora B, and immunostained the cells for the spindle checkpoint protein BubR1. We observed that AZD1152 treatment abolished the kinetochore localization of this spindle checkpoint protein, and the chromosomes were misaligned, whereas the DMSO-treated control cells clearly had BubR1 on their kinetochores (Fig. 2, B and D). Then the cells expressing GFP-CENPB-Aurora A, GFP-Hec1-Aurora A, GFP-Hec1-Aurora A-KD, and GFP-Aurora A were synchronized to prometaphase by STLC and treated with 200 nM AZD1152 for 1 h. The results showed that when GFP-Hec1-Aurora A was expressed, the spindle checkpoint was rescued, as indicated by the localization of BubR1 on the kinetochores (Fig. 2, C and D). When GFP-Hec1-Aurora A-KD was expressed, BubR1 localization on the kinetochores was abolished in the presence of AZD1152, indicating no functions of GFP-Hec1-Aurora A-KD for the localization of BubR1. Moreover, expressing GFP-CENPB-Aurora A and GFP-Aurora A that could not localize to the kinetochores also could not rescue the localization of BubR1 on the kinetochores in the presence of AZD1152 (Fig. 2, C and D). Thus, the forced localization of functional Aurora A on the kinetochores rather than the other places, including the centromere, is sufficient to substitute Aurora B for the function of activating the spindle checkpoint.

We further knocked down Aurora B by siRNA to make the functional loss defect of Aurora B. The result showed that the Aurora B knockdown significantly reduced the localization of BubR1 on the kinetochores by ~70% in comparison with the irrelevant control knockdown (Fig. 2, E–G). By expressing exogenous Aurora B, we performed the rescue experiment and found that exogenous GFP-Aurora B expression could rescue the defect by up to 95%. When GFP-Hec1-Aurora B was expressed, it could rescue the defect by up to >95% (Fig. 2, E–G). We also expressed GFP-Hec1-Aurora A in Aurora B knockdown cells. Interestingly, we observed that GFP-Hec1-Aurora A could almost fully rescue the defect caused by Aurora B knockdown (Fig. 2, F and G). In contrast, GFP-CENPB-Aurora A expression was not able to rescue the defect (Fig. 2, C and D), indicating that the centromere-targeted GFP-CENPB-Aurora A could not be able to reach the substrates of Aurora B at the kinetochores. Together, these results demonstrate that the spindle checkpoint defect caused by Aurora B knockdown can be fully rescued by targeting Aurora A to the kinetochores to substitute endogenous Aurora B for regulating the kinetochore localization of BubR1.

**Centrosome-localized Aurora B Can Substitute Aurora A in Promoting the Mitotic Entry**—The active Aurora A at the centrosome is required for the timely mitotic entry. In G2 phase, Aurora A directly phosphorylates Cdc25B at Ser-353 and regulates its localization at the centrosome to activate CDK1 kinase (40). Aurora A, with its co-activator Bora at the centrosome, also activates Plk1 by phosphorylating it at Thr-210. Then, Plk1, Aurora A, Cdc25B, and Cdk1 form a feedback loop and positively regulate each other’s activity for the mitotic entry (41). To investigate whether Aurora B can substitute Aurora A on the centrosome to regulate the mitotic entry, we constructed a fusion protein GFP-PLK4<sup>Cts</sup>-Aurora B, which contains a PLK4 centrosome targeting sequence (CTS, aa 570–820) that may lead the protein to the centrosomes (42), and expressed it in HeLa cells. We observed that GFP-PLK4<sup>Cts</sup>-Aurora B was specifically localized to the centrosomes both in mitotic and interphase cells in addition to a fraction of it in the nucleus and the centromeres (Fig. 3A and supplemental Movie S4). The phosphorylation status identification of Aurora B at the T-loop indicated that GFP-PLK4<sup>Cts</sup>-Aurora B was autophosphorylated...
FIGURE 2. Forced kinetochore localization of Aurora A performs the function of Aurora B on the spindle checkpoint regulation. A, immunofluorescence photomicrographs of HeLa cells transfected with GFP-Aurora A or GFP-Hec1-Aurora A and stained with a CREST antibody. Note that Aurora A (Aura) and its kinase-dead mutant (KD) were localized to the spindle and centrosomes in mitosis, whereas part of Hec1-Aurora A and its relevant kinase-dead mutant were localized to the kinetochores. Scale bar, 10 μm. B, HeLa cells were synchronized to prometaphase by STLC and treated by 200 nM AZD1152 for 1 h followed by immunofluorescence labeling with an anti-BubR1 antibody. Note that inhibiting Aurora B kinase activity resulted in the loss of BubR1 on the kinetochores. Scale bar, 10 μm. C, HeLa cells were transfected with GFP-Hec1-Aurora A, GFP-Hec1-Aurora A-KD, GFP-CENPB-Aurora A, or GFP-Aurora A, synchronized to prometaphase by STLC and treated with 200 nM AZD1152 for 1 h followed by immunostaining with an anti-BubR1 antibody. Note that expressing GFP-Hec1-Aurora A could rescue the loss of BubR1 on the kinetochores induced by AZD1152 treatment, whereas GFP-Hec1-Aurora A-KD, GFP-CENPB-Aurora A, and GFP-Aurora A could not. Scale bar, 10 μm. D, quantitative characterization of HeLa cells treated as in B and C. Bars represented the percentage of the cells with BubR1 on kinetochores. Each data point represents three independent experiments with each measuring 50 cells, and error bars indicate S.D. E, Aurora B knockdown by siRNA resulted in the loss of BubR1 on kinetochores. HeLa cells were transfected with Aurora B siRNA and processed for immunostaining with an anti-BubR1 antibody. Scale bar, 10 μm. F, quantitative characterization of HeLa cells treated as in E and G. Bars represent the percentage of the cells with BubR1 on kinetochores. Each data point represents three independent experiments with each measuring 50 cells, and error bars indicate S.D. G, BubR1 immunofluorescence labeling of HeLa cells co-transfected with Aurora B siRNA and GFP-Hec1-Aurora A or RNAi-resistant GFP-Hec1-Aurora B. Note that expression of either GFP-Hec1-Aurora A or GFP-Hec1-Aurora B rescued the loss of BubR1 on kinetochores caused by endogenous Aurora B knockdown. Scale bar, 10 μm.
FIGURE 3. Forced localization of Aurora B to centrosome could perform the function of Aurora A in promoting the mitotic entry. A, immunofluorescence photomicrographs of HeLa cells transfected with GFP-Aurora B or GFP-PLK4^CTS^-Aurora B (GFP-PLK4^CTS^-AurB) and stained with a γ-tubulin (γ-tub) antibody. Note that GFP-PLK4^CTS^-Aurora B was localized to the centrosomes, whereas GFP-Aurora B was localized to the nucleus and the centromere in interphase and mitosis. Scale bar, 10 μm. B, HeLa cells transfected with GFP-PLK4^CTS^-Aurora B were synchronized to mitosis and treated with different concentrations of Aurora A inhibitor MLN8237 for 1 h. Cell extracts were subjected to immunoblot analysis with antibodies to phospho-Aurora A/Aurora B/Aurora-C and Aurora A. Note that the activity of Aurora A was inhibited by MLN8237 at 50 nM, whereas the activity of GFP-PLK4^CTS^-Aurora B was not affected. C, quantitative characterization of mitotic index of the cells expressed GFP-Aurora B, GFP-PLK4^CTS^-Aurora B, or GFP-PLK4^CTS^-Aurora B-KD. HeLa cells were synchronized to G1-S phase by thymidine block and released for 8–13 h. Before immunofluorescence analysis, 50 nM MLN8237 was added for 1 h. DMSO was used as solvent control. Each data point represents three independent experiments with each measuring 50 cells, and error bars indicate S.D. D and E, immunofluorescence staining and quantitative characterization of cyclin B1 nuclear localization in HeLa cells expressed GFP-Aurora B, GFP-PLK4^CTS^-Aurora B, or GFP-PLK4^CTS^-Aurora B-KD. HeLa cells were synchronized and treated with MLN8237 as in C and stained with cyclin B1 antibody. Cyclin B1 nuclear localization was defined by its staining and is stronger in the nucleus than in the cytoplasm. Each data point represents three independent experiments with each measuring 50 cells, and error bars indicate S.D. Scale bar, 10 μm. F, quantitative characterizations of time of mitotic entry. Cells expressed GFP-Aurora B, GFP-PLK4^CTS^-Aurora B, or GFP-PLK4^CTS^-Aurora B-KD were synchronized to G1-S phase by thymidine block and released followed by live cell imaging to measure the duration from the time point of G1-S phase release to nuclear envelope breakdown. n = 50 cells per group. *** p < 0.001. Error bars indicate S.D.
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and activated (Fig. 3B). The cells expressing the GFP-PLK4CTS-Aurora B fusion proteins were treated with the Aurora A-specific inhibitor MLN8237 to eliminate the kinase activity of endogenous Aurora A. The result showed that, in the presence of MLN8237 at the final concentration of 50 nM and above, the endogenous Aurora A kinase activity was totally inhibited, indicated by the phosphorylation of its T-loop, but the kinase activity of GFP-PLK4CTS-AurB was not affected (Fig. 3B). Then the cells expressing the fusion proteins were synchronized to G1-S phase and released for 8–13 h following the drug treatment. It was found that, in DMSO-treated control cells, the mitotic index peaked at 10 h after the release, whereas the mitotic index of MLN8237-treated cells reached the peak level at 12 h (Fig. 3C). These indicate that inhibiting Aurora A kinase activity delayed the mitotic entry. Interestingly, when the cells expressing GFP-PLK4CTS-Aurora B were treated with MLN8237, the mitotic delay by MLN8237 was largely rescued. In contrast, the kinase dead mutant GFP-PLK4CTS-Aurora B-KD, that had the similar centrosome localization and GFP-Aurora B, failed to alleviate the mitotic delay by MLN8237 treatment. In the cells expressing the fusion proteins and treated with MLN8237 we also checked the nuclear translocation of cyclin B1 that precedes the nuclear envelope breakdown and is required for the G2-M transition (43). Consistent with the mitotic index data (Fig. 3C), the timing of cyclin B1 nuclear translocation in MLN8237-treated cells was largely delayed, and the delay could be rescued by expressing GFP-PLK4CTS-Aurora B but not GFP-PLK4CTS-Aurora B-KD or GFP-Aurora B (Fig. 3, D and E). Furthermore, we directly determined the time of mitotic entry by examining the duration from the time point of G1-S phase release to nuclear envelope breakdown in cells expressing the fusion proteins and treated with MLN8237. Consistent with the results above, we observed that GFP-PLK4CTS-Aurora B indeed could rescue the mitotic delay (Fig. 3F). Together, these results demonstrate that when Aurora B is artificially forced to locate to the centrosomes, it can substitute Aurora A to regulate the mitotic entry.

Both N Termini of Aurora A and B Contribute to Their Spatial Localizations—It has been reported that the critical domains for localization and function of Aurora A and B reside within their C termini (2, 4), and that the C termini alone could localize to the equivalent sites of and function as the full-length Aurora A and B (44); however, the functions of the N termini remain unknown. To determine whether the N termini contribute to their localizations and functions, we constructed a series of GFP-tagged truncation mutants of the human Auroras and examined their localizations (Fig. 4A). The result showed that GFP-tagged truncates Aurora A1–121 (GFP-Aurora A aa 1–121) showed a diffuse pattern with a significant fraction on the spindle and spindle poles in HeLa cells, in comparison with the centrosomal localization of Xenopus Aurora A N-terminal truncate in Xenopus cells XL2 and egg extracts (45) and that Aurora B1–65 (GFP-Aurora B aa 1–65) was largely in the nucleus in G2/prophase and smeared in the cytoplasm but not in the centromere in mitosis (Fig. 4, B and C, and supplemental Movies S5 and S6). We also observed that the catalytic C-terminal truncates GFP-Aurora A aa 123-end and GFP-Aurora B 66-end showed the similar localizations with the wild-type Aurora A and B in mitosis, respectively (Fig. 4D), as reported (46). However, when the N terminus of Aurora A was substituted by the N terminus of Aurora B to construct a chimera protein GFP-Aurora B-A and expressed in cells, this chimera protein was found to be largely localized to the nucleus like Aurora B in G2/prophase and relocated to the area of the wild-type Aurora A in mitosis (Fig. 4, E and F, and supplemental Movie S7), indicating that the N terminus of Aurora B may contribute to its nuclear localization before the mitotic entry. In comparison, we fused the N terminus of Aurora A with the C terminus of Aurora B (GFP-Aurora A-B) and expressed it in cells. Surprisingly, we observed that this chimera protein showed the centrosomal localization in G2/prophase like Aurora A, and this localization was enhanced before the mitotic entry followed by a typical localization of Aurora B on the centromere and midzone/midbody in mitosis (Fig. 4, D and G, and supplemental Movie S8). Consistently, we also noticed that Aurora A depleted of its N terminus showed the reduced centrosomal localization and Aurora B depleted of its N terminus showed the reduced nuclear concentration (Fig. 4, D, F, and G). These indicate that the N terminus of Aurora A and B may contribute to their respective localization before the mitotic entry. Together, these results demonstrate that the divergent N termini of the Auroras may contribute to their localizations in interphase and that once the cell enters mitosis, the C termini may mainly determine the canonical localizations of the Aurora A and B proteins.

Next, we set to test the functions of the chimera proteins. By knocking down endogenous Aurora B and expressing GFP-tagged exogenous Aurora A, Aurora A, or Aurora B-A, we found that both exogenous Aurora B and Aurora B-A, but not Aurora A, could efficiently rescue the phosphorylation defect of histone H3 at Ser-10 in the nucleus caused by endogenous Aurora B knockdown (Fig. 4, H and I). This indicates that the N terminus of Aurora B may contribute its localization and function. By knocking down endogenous Aurora A and expressing GFP-tagged exogenous Aurora A, Aurora A, or Aurora A-B, we tested the function of the Aurora A N terminus through examining the mitotic index. We observed that the mitotic entry was delayed when Aurora A was knocked down and the delay could be rescued by GFP-Aurora A and GFP-Aurora A-B but not GFP-Aurora B (Fig. 4J). Furthermore, we measured the duration from the time point of G1-S phase release to nuclear envelope breakdown in these cells. Consistent with previous result, we found that, like GFP-Aurora A, GFP-Aurora A-B also rescues mitotic delay, indicating that the N terminus of Aurora A targeted the chimera protein GFP-Aurora A-B to the typical localization site of Aurora A, where kinase activity rescued the defect of Aurora A knockdown (Fig. 4K). Collectively, we conclude that the N termini of both Aurora A and B contribute to their localizations before mitosis and hence functions of the both kinases.

N-terminal Deletion of Aurora A Resulted in Prolonged Mitosis and Defect in Spindle Bipolarity—Next, we set to further study the roles of the N terminus of Aurora A by depleting endogenous Aurora A and rescuing it with different constructs. First, we knocked down Aurora A in HeLa cells that stably expressed GFP-α-tubulin and observed by time-lapse micros-
copy that, in comparison with the control cells, the spindle assembly in Aurora A knockdown cells was abnormal with extra poles, and the mitosis was prolonged (Fig. 5, A–D), consisting with previously reports (47, 48). Then, we depleted Aurora A from HeLa cells and transiently expressed full-length GFP-Aurora A. We observed that the cells were able to form normal bipolar spindle and divided normally. In contrast, the C terminus of Aurora A (GFP-Aurora A aa 123-end) could not efficiently rescue the defects caused by endogenous Aurora A knockdown (Fig. 5, C, E, and F). The cells assembled extra poles, the chromosomal congression was defective, the mitosis lasted significantly longer, and the cell division was abnormal (Fig. 5F). Taken together, we conclude that the N terminus contributes to the function of Aurora A in regulating the spindle bipolarity.

N-terminal Deletion of Aurora B Results in Reduced Phosphorylation of Hec1 and Abnormal Mitosis—We also further studied the function of the N terminus of Aurora B with similar knock-down and rescue experiments. When Aurora B was depleted...
from HeLa cells that stably expressed GFP-α-tubulin, bipolar spindles could form, but the chromosome congression was slow; the cells were finally committed to exit mitosis after a long arrest and without successful division (Fig. 6, A–D). These cells contained either a single large nucleus or several small nuclei. Then we performed the rescue experiments in HeLa cells using

FIGURE 5. N-terminal deletion of Aurora A results in prolonged mitosis and multipolar spindle assembly. A and B, HeLa cells stably expressing GFP-α-tubulin were transfected with irrelevant (A) or Aurora A (AurA) siRNA (B) and subjected to time-lapse microscopy. RFP-H2B was transiently expressed as a chromatin/chromosome indicator. Note that Aurora A depletion resulted in multipolar cell division. Scale bars, 10 μm. C and D, quantitative characterizations of the duration of mitosis with live cells (C) and percentage of the cells with abnormal spindle assembly with fixed cells (D) shown in (A, B, E, and F) (see below). *, p < 0.05; **, p < 0.01; ***, p < 0.0001. The duration of mitosis is determined from the nuclear envelope breakdown to two daughter cells formation. Each data point represents 3 independent experiments with each measuring 50 cells, and error bars indicate S.D. E and F, HeLa cells co-transfected with Aurora A siRNA-, RFP-H2B-, and RNAi-resistant GFP-Aurora A (E) or GFP-Aurora A aa123-end (F) were viewed by time-lapse microscopy. Note that Aurora A aa123-end could not rescue Aurora A depletion. Scale bars, 10 μm.
either the full-length GFP-Aurora B or the C terminus truncate aa 66-end of GFP-Aurora B. The results showed that the full-length GFP-Aurora B successfully rescued the defect caused by endogenous Aurora B knockdown; in contrast, the N terminus truncate was less efficient in rescuing these defects, and the chromosome congression still failed leading to one large nucleus or multinuclei formation (Fig. 6, C–F). We further examined the phosphorylation of Hec1 at Ser-55 on the kineto-
chores, which is thought to be regulated by Aurora B (42). We observed that when Aurora B was knocked down by siRNA, the phosphorylation of Hec1 Ser-55 on the kinetochores was significantly reduced and that this defect could be rescued by full-length wild-type Aurora B but not the C terminus truncate aa 66-end of GFP-Aurora B (Fig. 6, G–I). Together, we conclude that the N terminus is required for the full function of Aurora B in phosphorylating its substrates on the kinetochores, which is required for normal cell division. This also indicates that the N terminus of Aurora B may play an important role in bringing centromere located Aurora B to the kinetochores to phosphorylate its substrates such as Hec1, DSN1, and KNL1, suggesting that N terminus-mediated relocation of Aurora B from the centromere to kinetochores is needed for the regulation of kinetochore-microtubule connections.

Discussion

In this study we reveal the relations between the spatiotemporal compartmentalizations and functions of Aurora A and B and show that one may carry out the functions of the other when one is artificially localized to the places of the other. We also reveal that the divergent N termini of the Auroras have roles for their spatiotemporal localizations. When the N terminus of Aurora A was fused to the C terminus of Aurora B, the chimera proteins could localize and function like Aurora A in addition to like Aurora B and vice versa. Consistent with the previous reports, our results have shown that Aurora A and B have similar potential substrates and kinase activity, and their specific functions are likely determined by their different localizations. Therefore, the functional divergence of Aurora A and B is likely determined by the spatial compartmentalization of their binding partners that binds both the conservative C and the divergent N termini of the Aurora kinases.

Previously we and others have uncovered that the localizations of both Aurora A and B are mainly determined by their substrates and binding partners, and their functions rely on their catalytic C termini (4, 44). We revealed that a single amino acid change on residue Gly-198 can convert Aurora A into Aurora B-like kinase (4). The mutant Aurora AG198N exhibits the Aurora B-like localization on the centromere and midzone, where it interacts with components of the chromosome passenger complex and rescues the chromosome misalignment caused by Aurora B knockdown. A longer side chain on residue 198 of mutant Aurora AG198N prevents its attachment to TPX2, and increased hydrophilicity of residue 198 also promotes...
Aurora A<sup>G198N</sup> to interact with INCENP. This indicates that the slight difference in structure of Aurora A and B leads to differential interaction with the binding partners of each other and thus results in differentiation of localization and function. In this work we further find that the divergent N termini of both Aurora A and B may have their own binding partners and contribute to the distinct localizations. Without the N terminus, the ability of Aurora A in regulating the mitotic spindle bipolarity is impaired, whereas Aurora B cannot fully phosphorylate its substrates on kinetochores and regulate the kinetochore-microtubule connection required for faithful chromosome separation and cell division.

In the evolution process both Aurora A and B may originate from a single Aurora protein. Phylogenetic analysis suggests that the duplication of Aurora genes has independently occurred in vertebrates, invertebrates, and plants (21, 30). The polar Aurora kinases (Aurora A-like) and the equatorial Aurora kinases (Aurora B-like) in invertebrates (such as <i>Caenorhabditis elegans</i> and <i>Drosophila melanogaster</i>) have the similar localizations and functions with their vertebrate counterpart, although they are not orthologous with the respective vertebrate Aurora A and B. Interestingly, despite confusion in the phylogenetic trees, all polar Aurora kinases in model organisms share a common feature in that the amino acid sequence corresponding to human Aurora A residue 198 is glycine or alanine, which has a short and hydrophobic side chain (Fig. 7A). On the contrary, all equatorial Aurora kinases share an amino acid with a long and hydrophilic side chain such as asparagine or threonine at the residue corresponding to human Aurora A residue 198. Our previous and present studies together with others' have presented that Aurora A and B share great similarity in substrates and kinase activity, and the functional divergence of Aurora kinases is largely determined by their different localization defined by their binding affinity with specific binding partners. So, it seems that, after the gene duplication events in the evolution process, the mutation on a single amino acid of the Auroras confers the differential interaction affinity with their binding partners such as TPX2 and INCENP, and in turn, the Auroras are brought to the polar or equatorial localization by the differential binding partners, respectively, and thus led to the differentiation of their functions (Fig. 7B). Furthermore, their elongated and low conservative N termini suggest that they may have differential binding sites for their additional binding partners that may bring them to their special locations to perform their distinct functions. To fully understand the evolution and function of the Auroras, it is worthy identifying these additional binding partners in the future.

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