

A single amino acid change converts Aurora-A into Aurora-B-like kinase in terms of partner specificity and cellular function

Jingyan Fu^a, Minglei Bian^a, Junjun Liu^b, Qing Jiang^a, and Chuanmao Zhang^{a,1}

^aThe Education Ministry Key Laboratory of Cell Proliferation and Differentiation and the State Key Laboratory of Bio-membrane and Membrane Bio-engineering, College of Life Sciences, Peking University, Beijing 100871, China; and ^bDepartment of Biological Sciences, California State Polytechnic University, Pomona, CA 91768

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Aurora kinase-A and -B are key regulators of the cell cycle and tumorigenesis. It has remained a mystery why these 2 Aurora kinases, although highly similar in protein sequence and structure, are distinct in subcellular localization and function. Here, we report the striking finding that a single amino acid residue is responsible for these differences. We replaced the Gly-198 of Aurora-A with the equivalent residue Asn-142 of Aurora-B and found that in HeLa cells, Aurora-A^{G198N} was recruited to the inner centromere in metaphase and the midzone in anaphase, reminiscent of the Aurora-B localization. Moreover, Aurora-A^{G198N} compensated for the loss of Aurora-B in chromosome misalignment and cell premature exit from mitosis. Furthermore, Aurora-A^{G198N} formed a complex with the Aurora-B partners, INCENP and Survivin, and its localization depended on this interaction. Aurora-A^{G198N} phosphorylated the Aurora-B substrates INCENP and Survivin *in vitro*. Therefore, we propose that the presence of Gly or Asn at a single site assigns Aurora-A and -B to their respective partners and thus to their distinctive subcellular localizations and functions.

single amino acid mutation | TPX2 | INCENP | Survivin

Aurora kinases have been implicated in several vital events at the spindle pole and the equatorial region during mitosis (reviewed in refs. 1–8). Such diverse roles are accomplished by Aurora-A and -B, which share 70% homology in the catalytic domain and yet are functionally distinct (2–7). In mitotic cells, Aurora-A localizes to the spindle pole and the microtubule, and is mainly involved in centrosome maturation, mitotic entry, and spindle assembly (9–11). On the other hand, Aurora-B persists at the inner centromere from prometaphase to metaphase and relocates to the midzone in anaphase, where it participates in chromatin modification, microtubule–kinetochore attachment, the spindle checkpoint, and cytokinesis (12, 13).

To fulfill their routine functions in cell cycle control, both Aurora-A and -B are tightly regulated by their respective partner proteins. TPX2 binding targets Aurora-A to spindle microtubules, enhances its autophosphorylation, and protects the activation site from PP1 dephosphorylation (14–20). Conversely, localization of Aurora-B depends on an intact chromosomal passenger complex containing at least 3 other proteins, INCENP, Survivin, and Borealin (21–26), and the activity of Aurora-B is stimulated mainly by INCENP (27, 28).

To investigate how Aurora-A and -B, despite great similarity in protein sequence and structure, interact with such disparate cellular machinery, we conducted a strategy in which the regulation of Aurora-A or -B by its partner proteins was impaired. When G198 within the human Aurora-A catalytic domain (G205 in *Xenopus*) is substituted by the equivalent residue N142 in human Aurora-B (N158 in *Xenopus*), the binding affinity and the activation of Aurora-A by recombinant TPX2 is reduced *in vitro* (29, 30). Given this, we introduced the Aurora-A^{G198N} mutant into HeLa cells and found that Aurora-A^{G198N} colocalized with Aurora-B at the centromere and the midzone in mitosis, and lost

the original Aurora-A localization at the spindle. Moreover, Aurora-A^{G198N} prevented the chromosome misalignment and cell premature exit from mitosis caused by Aurora-B knockdown, indicating functional substitution for Aurora-B in cell cycle regulation.

Results

Aurora-A^{G198N} Colocalizes with Aurora-B at Centromere and Midzone. We fused the mutants Aurora-A^{G198N} and Aurora-B^{N142G} (Fig. 1A) with GFP and transiently expressed them in HeLa cells [supporting information (SI) Fig. S1A]. Through immunoprecipitation, we found that Aurora-A^{G198N} bound less TPX2 than Aurora-A^{WT} (Fig. 1B), and that Aurora-B^{N142G} bound less INCENP and Survivin than Aurora-B^{WT} (Fig. S2A). Consistent with previous reports (29, 30), the kinase activity of Aurora-A^{G198N} was reduced by 10-fold compared with that of Aurora-A^{WT} (Fig. S3).

To evaluate the behaviors of Aurora-A^{G198N} and Aurora-B^{N142G} in the cell, HeLa cells were transfected with GFP-tagged Aurora-A^{WT}, Aurora-A^{G198N}, Aurora-B^{N142G}, and Aurora-B^{WT} and harvested for immunofluorescence microscopy. Whereas Aurora-A^{WT} was concentrated at the centrosome and spindle microtubule as reported (9–11), Aurora-A^{G198N} exhibited a different localization (Fig. 1C). Besides a signal at the centrosome and a diffuse distribution in the cytoplasm, Aurora-A^{G198N} was observed at the centromere in ≈63% of metaphase cells and at the midzone in 91% of anaphase cells (Fig. 1D), reminiscent of Aurora-B localization (12, 13). Similar results were seen with living cell microscopy (Fig. S4). Confocal images further confirmed that Aurora-A^{G198N} colocalized with Aurora-B at the inner centromere and the midzone but not with Hec1 at the outer kinetochore (Fig. 1E). Like Aurora-A, Aurora-B^{N142G} displayed localization at the centrosome and the spindle, in addition to a signal at the centromere and the midzone (Fig. S2B–D). These results demonstrate that a single amino acid change causes Aurora-A to exhibit a cellular distribution similar to that of Aurora-B apart from remnant at the spindle poles and leads Aurora-B to deviate from its wild-type localization to that of Aurora-A. We next focused on Aurora-A^{G198N} to study the mechanism of the transformation between Aurora-A and -B.

Aurora-A^{G198N} Prevents Chromosome Misalignment and Cell Premature Exit from Mitosis Caused by Aurora-B Knockdown. We assessed whether Aurora-A^{G198N} was functional by monitoring cell cycle

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¹To whom correspondence should be addressed. E-mail: zhangcm@pku.edu.cn.

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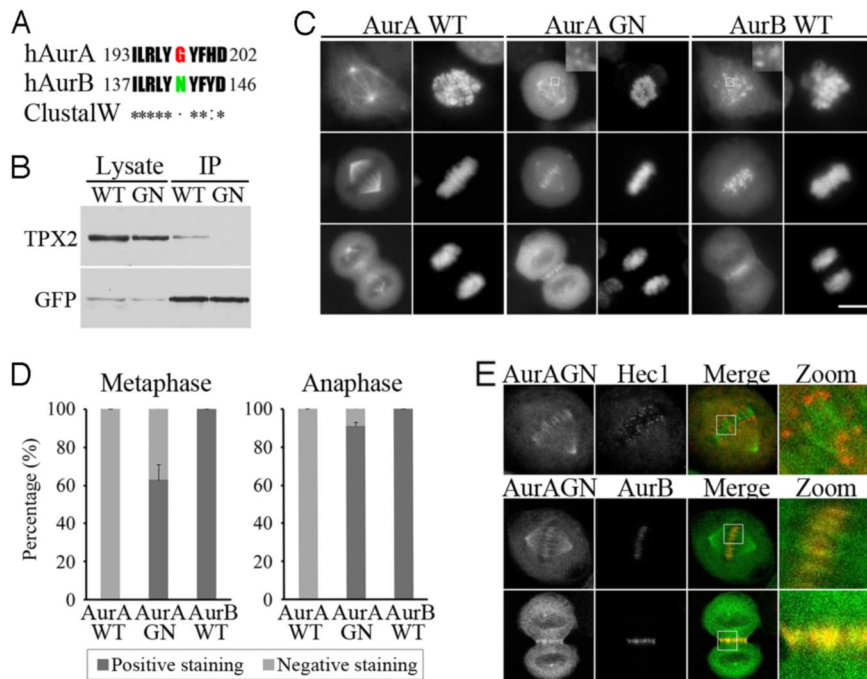


Fig. 1. Aurora-A^{G198N} colocalizes with Aurora-B at inner centromere and midzone in mitosis. (A) Scheme depicting the mutation domain of Aurora-A and relative sequence of Aurora-B. G198 (red) in human Aurora-A was altered to the equivalent residue N142 (green) in Aurora-B. Homology of mutation domain is presented by ClustalW. (B) GFP-Aurora-A^{WT} or GFP-Aurora-A^{G198N} was transfected into HeLa cells and immunoprecipitated from cell lysate with GFP antibody. The immunoprecipitated complexes were blotted for TPX2 (Upper) or GFP (Lower). Aurora-A^{G198N} bound less TPX2 compared with Aurora-A^{WT}. (C) Immunofluorescence photomicrographs of HeLa cells transfected with GFP-tagged Aurora-A^{WT}, Aurora-A^{G198N}, or Aurora-B^{WT} (Left). Aurora-A^{G198N} and Aurora-B^{WT} localized to centromere and midzone, whereas Aurora-A^{WT} did not. DNA labeled with DAPI (Right). (Scale bar, 10 μm.) (D) Quantitative characterization of Aurora-A^{G198N} localization in C. Dark-gray bars represent the percentage of cells positively stained with Aurora-A^{G198N} at centromere and midzone, and light-gray bars represent the percentage unstained at these locations. Each data point represents 3 independent experiments, each measuring 250 mitotic cells, and error bars indicate SD (*P* < 0.01). (E) Confocal images of HeLa cells transfected with GFP-Aurora-A^{G198N} (green) and stained with Hec1 or Aurora-B antibody (red). Aurora-A^{G198N} colocalized with Aurora-B at inner centromere and midzone but not with Hec1 at outer kinetochore.

progression after simultaneous Aurora-B knockdown and Aurora-A^{G198N} expression. For this purpose, 2 cell lines were generated using the tet-on system to express Aurora-A^{WT} or Aurora-A^{G198N} in the presence of tetracycline (Fig. S1B). Cells treated with or without tetracycline were cotransfected with Aurora-B RNAi vector and RFP-H2B (ratio 40:1), which is a marker for Aurora-B knockdown and chromosome morphology (Fig. 2A and Fig. S5A). By time-course imaging, we found that Aurora-B knockdown cells could not properly align their chromosomes to the equatorial plate and prematurely exited mitosis from prometaphase without chromosome separation and cytokinesis (Fig. 2B and Movie S1, Movie S2, Movie S3, Movie S4, Movie S5, and Movie S6), indicating compromise of the spindle checkpoint consistent with previous reports (31–34). Expression of Aurora-A^{WT} by tetracycline inducement did not affect progression of the cell cycle (Fig. 2B and Movie S7). In contrast, expression of Aurora-A^{G198N} largely restored the mitotic progression after Aurora-B knockdown (Fig. 2B and Movie S8). RNAi treatment caused a 21% increase in the proportion of prophase to metaphase cells and a corresponding decrease in that of anaphase cells (Fig. 2C), in agreement with previous reports (31, 33). Simultaneous expression of Aurora-A^{G198N} but not Aurora-A^{WT} reduced this increase by 13% (Fig. 2C). Together, these results show that Aurora-A^{G198N} partially compensated for the loss of Aurora-B function in mitosis.

Aurora-A^{G198N} Interacts with INCENP and Survivin, and Its Localization Depends on This Interaction. We asked how Aurora-A^{G198N} was recruited to the inner centromere and the midzone like Aurora-B. Since Aurora-B relies on an intact chromosomal passenger complex for proper localization, we hypothesized that this might also be

necessary for Aurora-A^{G198N}. To address this, HeLa cells were synchronized, transfected with GFP-tagged Aurora-A^{WT}, Aurora-A^{G198N}, and Aurora-B^{WT}, and harvested for immunoprecipitation with GFP antibody. Biochemical analysis of the immunoprecipitated complexes revealed that Aurora-A^{G198N} was in a complex with INCENP and Survivin, and this complex did not contain Aurora-B (Fig. 3A). We also tested the interaction between Aurora-A^{G198N} and INCENP *in vitro* by GST pull-down assay using recombinant proteins. Consistent with the *in vivo* immunoprecipitation result, Aurora-A^{G198N} bound INCENP^{826–919}, as did Aurora-B, either in the presence or absence of TPX2^{1–43} (Fig. 3E). Surprisingly, we found that Aurora-A^{WT} also bound INCENP^{826–919} *in vitro*, but this interaction was remarkably reduced by the addition of TPX2^{1–43} (Fig. 3E), suggesting that TPX2 has higher affinity to Aurora-A than INCENP. Our results show that Aurora-A^{G198N} forms a complex with INCENP and Survivin both *in vivo* and *in vitro*.

We then examined the impact of INCENP and Survivin on Aurora-A^{G198N} localization. In the presence of tetracycline, Aurora-A^{G198N} tet-on cells were depleted of endogenous INCENP or Survivin by RNAi (Fig. S5B and C) and harvested for immunofluorescence microscopy. Both INCENP and Survivin knockdown completely displaced Aurora-A^{G198N} from the inner centromere and the midzone (Fig. 3B–D and Fig. S5D). Thus, the localization of Aurora-A^{G198N} to the inner centromere and the midzone is due to its association with INCENP and Survivin as a result of the G198N mutation.

Aurora-A^{G198N} Phosphorylates INCENP and Survivin *In Vitro*, and Its Kinase Activity Is Enhanced in the Presence of INCENP. We sought to resolve how Aurora-A^{G198N} compensated for the loss of

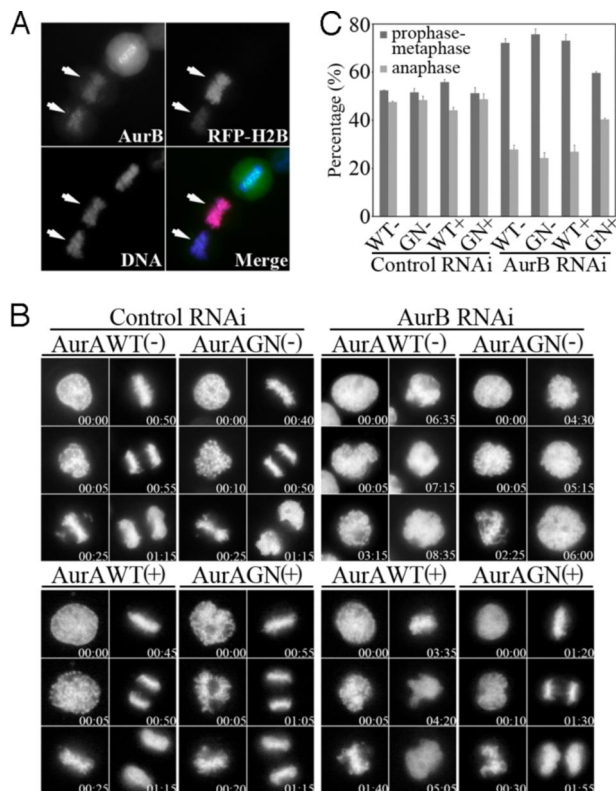


Fig. 2. Aurora-A^{G198N} prevents chromosome misalignment and cell premature exit from mitosis caused by Aurora-B knockdown. (A) Immunofluorescence photomicrographs of HeLa cells cotransfected with Aurora-B RNAi and RFP-H2B (red) at a ratio of 40:1 and labeled with Aurora-B antibody (green). Note that those cells expressing RFP-H2B showed disperse staining of Aurora-B with decreased fluorescence intensity, as indicated by arrows. Thus, in addition to chromosome morphology, RFP-H2B was a marker for Aurora-B knockdown. DNA labeled with DAPI (blue). (B) HeLa cells induced by tetracycline to express Aurora-A^{WT} or Aurora-A^{G198N} were cotransfected with Aurora-B RNAi and RFP-H2B at a ratio of 40:1, and subjected to time-course fluorescence microscopy. Chromosome morphology, visualized by RFP-H2B staining, was filmed every 5 min. With Aurora-B knockdown, cells could not properly align the chromosomes and exited mitosis without chromosome separation and cytokinesis. Induced expression of Aurora-A^{G198N}, but not Aurora-A^{WT}, prevented this abnormality. Representative cells can be viewed as movies or still images in [Movie S1](#), [Movie S2](#), [Movie S3](#), [Movie S4](#), [Movie S5](#), [Movie S6](#), [Movie S7](#), and [Movie S8](#). (C) Quantitative characterization of mitotic HeLa cells treated as in B. Dark-gray bars represent the percentage of cells from prophase to metaphase, and light-gray bars represent the percentage in anaphase. Cells were classified by chromosome morphology visualized by RFP-H2B. Each data point represents 3 independent experiments, each measuring 250 mitotic cells, and error bars indicate SD ($P < 0.01$).

Aurora-B in mitosis. As a kinase, Aurora-B functions primarily by phosphorylating its substrates. INCENP is phosphorylated by Aurora-B at 3 consecutive residues T893/S894/S895 (28), and Survivin at T117 (35). Thus, we tested if Aurora-A^{G198N} could phosphorylate these proteins. Recombinant Aurora-A^{G198N} was incubated with GST, GST-INCENP⁸²⁶⁻⁹¹⁹, and Survivin in the presence of γ^{32} ATP. Both INCENP⁸²⁶⁻⁹¹⁹ and Survivin were phosphorylated by Aurora-A^{G198N}, Aurora-B^{WT} (Fig. 4A), and Aurora-A^{WT} (36). To test whether Aurora-A^{G198N} could be activated by INCENP as is Aurora-B, kinase assay was performed using MBP as the substrate. The phosphorylation of MBP by Aurora-A^{G198N} was enhanced by the addition of INCENP⁸²⁶⁻⁹¹⁹ (Fig. 4B). These findings suggest that Aurora-A^{G198N} is capable of phosphorylating Aurora-B substrates, and its kinase activity is enhanced by INCENP.

Both Elongation and Hydrophilicity of the Side Chain at Aurora-A^{G198} Are Necessary to Convert Aurora-A into Aurora-B-Like Kinase. We further explored the molecular explanation of how a single amino acid change caused such tremendous transformation of Aurora-A in partner specificity and protein function. According to the crystal structure of human Aurora-A¹²²⁻⁴⁰³/TPX2¹⁻⁴³ (14), the C α atom of G198 in Aurora-A interacts so closely with TPX2^{P13} that a longer side chain is intolerable (Fig. 5A *i-iv*), but the situation is different within the equivalent domain of the Aurora-B complex. According to the structure of *Xenopus* Aurora-B⁷⁸⁻³⁶¹/INCENP⁷⁹⁸⁻⁸⁴⁰ (37), the side chain of N158 in Aurora-B interacts properly with INCENP^{Y825/1828/D829/S830} in a hydrophilic state; it also forms hydrogen bonds with INCENP^{Y825/1828} to strengthen the interaction (Fig. 5A *v* and *vi*). In our hypothesis, the side chain of N198 in Aurora-A^{G198N} prevents attachment to TPX2, leaving perfect space for INCENP. Moreover, the potential hydrogen bond with human INCENP¹⁸⁷² further stabilizes the complex of Aurora-A^{G198N}:INCENP (Fig. 5A *vii* and *viii*).

To test this speculation, we generated a series of Aurora-A mutants by replacing G198 with various amino acids (Fig. 5B). Aurora-A^{G198A} localized to the spindle, as did Aurora-A^{WT} (Fig. 5C). However, when the side chain of this residue was elongated to the length of that of V or L, Aurora-A did not localize to the spindle (Fig. 5C). Furthermore, when G198 was replaced by N or Q, similar in length to V and L but hydrophilic, Aurora-A localized to the centromere and the midzone (Fig. 5C). To quantitatively analyze the INCENP-binding activity of all mutants, HeLa cells were synchronized, transfected with GFP-tagged Aurora-A^{G198A}, Aurora-A^{G198V}, Aurora-A^{G198L}, Aurora-A^{G198N}, and Aurora-A^{G198Q}, and harvested for immunoprecipitation with GFP antibody. Biochemical analysis revealed that Aurora-A^{G198N} and Aurora-A^{G198Q} formed complexes with INCENP, whereas Aurora-A^{G198A}, Aurora-A^{G198V}, and Aurora-A^{G198L} did not (Fig. 5D). Together, these results show that the elongation and the hydrophilicity of the side chain at Aurora-A^{G198} determine its interaction with INCENP instead of TPX2 and, therefore, are required for its conversion into Aurora-B-like kinase.

Discussion

Our study reveals that G198 of Aurora-A is a key residue in terms of discriminating the behaviors of Aurora-A and -B in the cell. Because the protein sequences of their catalytic domains are very similar, we are interested in understanding how distinct localization and function are determined. Although it has been reported that G198 in human Aurora-A (29) [G205 in *Xenopus* (30)] is a determinant for its regulation by TPX2 in vitro, the molecular mechanism and the biological effects of the G-N mutation were unknown. Here, we propose that the G-N mutation converts Aurora-A into Aurora-B-like kinase in cell (Fig. 6). It is achieved through the alteration of partner specificity, which in turn, defines subcellular localization and substrate phosphorylation. First, the G-N mutation enables Aurora-A to interact with INCENP instead of TPX2. Because INCENP, Survivin, and Borealin associate with each other through a tight 3-helical bundle (38), once Aurora-A is connected to INCENP, it is brought into the chromosomal passenger complex and is localized where this complex settles. Finally, Aurora-A^{G198N} phosphorylates the Aurora-B substrates (Fig. 4A); thus, it probably works as an enzymatic component in the complex substituting for Aurora-B. Accordingly, the cell cycle proceeds successfully even when Aurora-B is absent (Fig. 6). To our surprise, recombinant Aurora-A, purified from either *Escherichia coli* or Sf21 insect cells, binds INCENP⁸²⁶⁻⁹¹⁹ in a GST pull-down assay (Fig. 3E), and phosphorylates INCENP⁸²⁶⁻⁹¹⁹ and Survivin in vitro (36), indicating the potential of Aurora-A to act like Aurora-B.

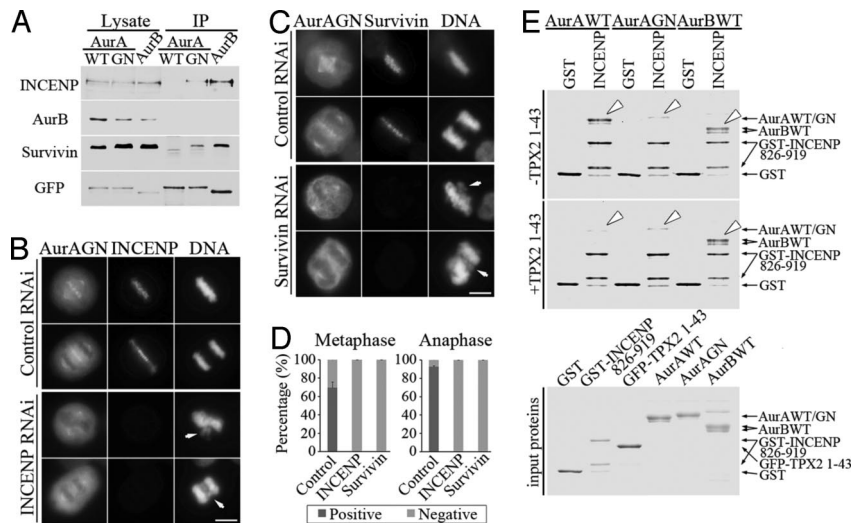


Fig. 3. Aurora-A^{G198N} physically interacts with INCENP and Survivin, and its localization depends on them. (A) GFP-tagged Aurora-A^{WT}, Aurora-A^{G198N}, or Aurora-B^{WT} was transfected into HeLa cells and immunoprecipitated from cell lysate with GFP antibody. The immunoprecipitated complexes were blotted for INCENP, endogenous Aurora-B, Survivin, or GFP. Aurora-A^{G198N} bound INCENP and Survivin, not Aurora-B. (B and C) Photomicrographs of tet-on HeLa cells transfected with INCENP RNAi (B) or Survivin RNAi (C) in the presence of tetracycline and labeled with INCENP (B) or Survivin (C) antibody. Knockdown of INCENP or Survivin displaced Aurora-A^{G198N} from centromere and midzone. Arrows indicate lagging chromosomes. (Scale bars, 10 μ m.) (D) Quantitative characterization of Aurora-A^{G198N} localization after INCENP or Survivin RNAi. Dark-gray bars represent the percentage of cells positively stained with Aurora-A^{G198N} at centromere and midzone, and light-gray bars represent the percentage unstained at these locations. Each data point represents 3 independent experiments, each measuring 250 mitotic cells, and error bars indicate SD ($P < 0.01$). (E) GST pull-down assay for recombinant Aurora-A^{WT}, Aurora-A^{G198N}, or Aurora-B^{WT} using GST-INCENP⁸²⁶⁻⁹¹⁹, in the presence or absence of GFP-TPX2¹⁻⁴³. Aurora-A^{G198N} bound INCENP⁸²⁶⁻⁹¹⁹ regardless of TPX2¹⁻⁴³ addition (Top and Middle). Although Aurora-A^{WT} bound INCENP⁸²⁶⁻⁹¹⁹ (Top), this interaction was remarkably reduced when TPX2¹⁻⁴³ was added (Middle). Input proteins were shown in separate lane with corresponding amount (Bottom). Arrowheads indicate Aurora-A^{WT}, Aurora-A^{G198N}, and Aurora-B^{WT}.

Addition of TPX2¹⁻⁴³ eliminates the interaction between Aurora-A and INCENP in the GST pull-down assay (Fig. 3E), suggesting that in vivo, this potential of Aurora-A is probably

restrained due to the anchoring effect by TPX2 that keeps Aurora-A to the spindle and away from the centromere and the midzone.

We also observed in our experiments that when the equivalent residue N142 of human Aurora-B is replaced with G to make an Aurora-A-like mutant, it exhibits additional localization to the centrosome and the spindle, deviating from the characteristics of Aurora-B to those of Aurora-A. The affinity of this mutant to INCENP and Survivin is lower than that of Aurora-B^{WT}, although it still maintains a high level. This result suggests that there might be other residues responsible for the interaction between Aurora-B and its partners. Because Aurora-B^{N142G} has a strong signal at the centromere and the midzone like Aurora-B^{WT}, it is difficult to further analyze the different protein interactions between them by biochemical approaches. Thus, our experiments concentrated on understanding how Aurora-A is converted into Aurora-B-like kinase. In vertebrates, there is a distinction between Aurora-A and -B within this particular site, with Gs in Aurora-A and Ns in Aurora-B. However, in those organisms with only 1 Aurora, such as *Saccharomyces cerevisiae* and *Saccharomyces pombe*, whose Aurora works like Aurora-B (39–40), or *Dictyostelium*, whose Aurora has the property of both Aurora-A and -B (41), there are Gs instead of Ns, suggesting broader application of G in this site during evolution. Because a single amino acid change imparts Aurora-B-like behavior on Aurora-A and confers some Aurora-A-like properties on Aurora-B, we propose that our observations might be of evolutionary significance, suggesting that a new protein might be generated from its precursor by point mutation and evolutionary selection.

In conclusion, we demonstrate that a single amino acid change within the Aurora-A catalytic domain is sufficient to convert it into an Aurora-B-like kinase in terms of partner specificity and function in the cell. Through delicate differentiation in the local

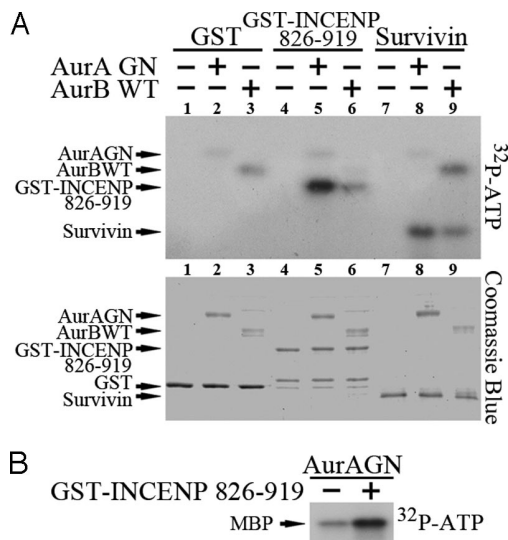


Fig. 4. Aurora-A^{G198N} phosphorylates INCENP and Survivin in vitro, and its activity is enhanced by INCENP. (A) In vitro kinase assay for recombinant Aurora-A^{G198N} using GST, GST-INCENP⁸²⁶⁻⁹¹⁹, and Survivin as substrates. Phosphorylated INCENP and Survivin were detected by autoradiography (Top), and total inputs were visualized by Coomassie blue staining (Middle). Lane 1–3, GST; lane 4–6, GST-INCENP⁸²⁶⁻⁹¹⁹; lane 7–9, Survivin. Lanes 1, 4, and 7, without kinase; lanes 2, 5, and 8, with Aurora-A^{G198N}; lanes 3, 6, and 9, with Aurora-B^{WT}. (B) In vitro kinase assay for recombinant Aurora-A^{G198N} using MBP as substrate, in the presence or absence of GST-INCENP⁸²⁶⁻⁹¹⁹. Phosphorylated MBP was detected by autoradiography. INCENP⁸²⁶⁻⁹¹⁹ stimulated phosphorylation of MBP by Aurora-A^{G198N}.

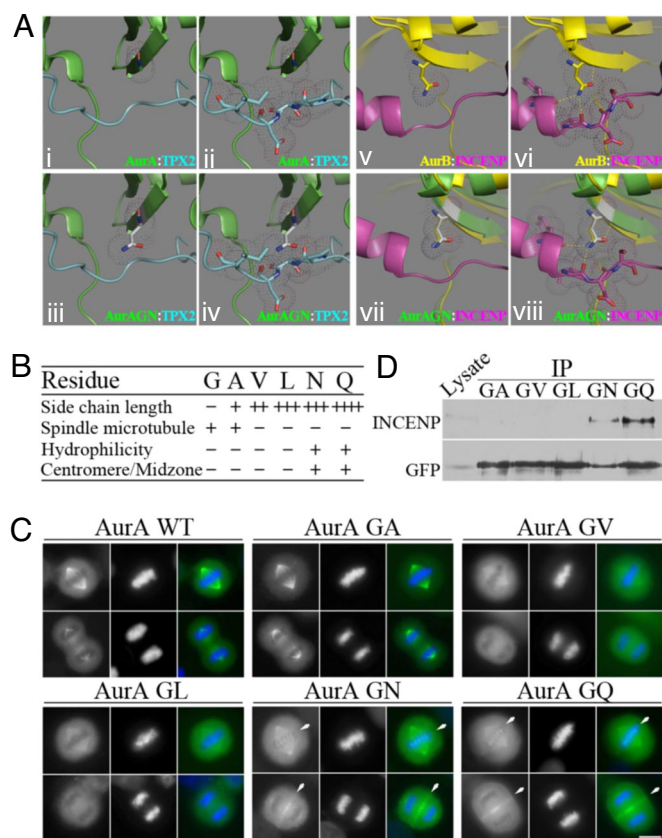


Fig. 5. Both elongation and hydrophilicity of the side chain at residue 198 are required to convert Aurora-A into Aurora-B-like kinase. (A) Aurora-A and -B are distinguished by different partner proteins through local structural change at a single amino acid. (*i* and *ii*) The complex of human Aurora-A¹²²⁻⁴⁰³/TPX2¹⁻⁴³ (PDB ID code 1OL5 in ref. 14). (*v* and *vi*) The complex of *Xenopus* Aurora-B⁷⁸⁻³⁶¹/INCENP⁷⁹⁸⁻⁸⁴⁰ (PDB ID code 2BFY in ref. 37). (*iii*, *iv*, *vii*, and *viii*) Predicted by point mutation using PyMol (www.pymol.org). Structurally, G198 of Aurora-A (green) interacts with TPX2¹⁻⁴³ (blue) in 3-dimensional space (*i* and *ii*). When G198 is replaced by N, the side chain is elongated, and TPX2 is prevented from Aurora-A (*iii* and *iv*). In contrast, Aurora-B (yellow) interacts with INCENP (purple) in a looser way within the equivalent domain (*v* and *vi*). The side chain of N158 in *Xenopus* Aurora-B interacts perfectly with INCENP^{Y825/I828/D829/S830} and forms hydrogen bonds with INCENP^{Y825/I828}. Thus, Aurora-A^{G198N} can interact with INCENP through its N198, which not only fits the 3-dimensional structure but probably builds hydrogen bonds with human INCENP^{I872} (*vii* and *viii*). (B) Overview of Aurora-A localization after substituting G198 with various amino acids. When the length of side chain reached that of V and L, Aurora-A was prevented from spindle. Hydrophilic side chains N and Q localized Aurora-A to centromere and midzone. (C) HeLa cells were transfected with GFP-tagged Aurora-A^{WT}, Aurora-A^{G198A}, Aurora-A^{G198V}, Aurora-A^{G198L}, Aurora-A^{G198N}, or Aurora-A^{G198Q} (Left, and green in Right), and subjected to immunofluorescence microscopy. DNA labeled with DAPI (Center, and blue in Right). Arrows indicate centromere and midzone. (Scale bar, 10 μ m.) (D) GFP-tagged Aurora-A^{G198A}, Aurora-A^{G198V}, Aurora-A^{G198L}, Aurora-A^{G198N}, or Aurora-A^{G198Q} was transfected into HeLa cells and immunoprecipitated from cell lysate with GFP antibody. The immunoprecipitated complexes were blotted for INCENP (Upper) or GFP (Lower). Aurora-A^{G198N} and Aurora-A^{G198Q} bound INCENP, whereas Aurora-A^{G198A}, Aurora-A^{G198V}, and Aurora-A^{G198L} did not.

structure, generated by the presence of either Gly or Asn, Aurora-A and -B are matched with different sets of partner proteins, brought to different subcellular locations, and assigned distinct functions.

Materials and Methods

For additional information on materials and methods used, see *SI Text*.

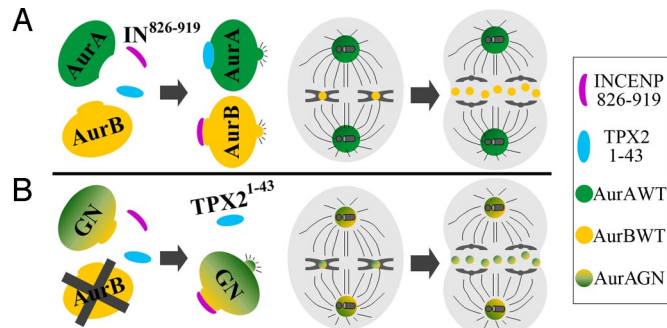


Fig. 6. Model depicting the relationship between Aurora-A and -B: Aurora-A substitutes for Aurora-B in mitosis when it is displaced to the location of Aurora-B. (A) Under conventional condition, Aurora-A (green) binds TPX2¹⁻⁴³ (blue) and localizes to spindle microtubule, and Aurora-B (yellow) binds INCENP⁸²⁶⁻⁹¹⁹ (purple) and localizes to centromere and midzone. (B) When Aurora-A is altered at G198 by N, it binds INCENP instead of TPX2. In this complex containing at least Aurora-A^{G198N}, INCENP, and Survivin, Aurora-A^{G198N} is activated and phosphorylates Aurora-B substrates. Hence, when Aurora-B is absent from the cell, Aurora-A^{G198N} maintains normal progression of the cell cycle.

Molecular Cloning, Protein Purification, and Antibody Preparation. Wild-type Aurora-A, -B, Survivin, TPX2, and PRC1 were cloned by RT-PCR from HeLa cell lysate. Mutants of Aurora-A and -B were generated by point mutation PCR. GFP was cloned by PCR from pEGFP vector. For overexpression in HeLa cells, wild type and mutants of Aurora-A and -B were inserted into pEGFP-C. For expression of recombinant proteins in BL21 *E. coli*, Aurora-A^{WT}, Aurora-A^{G198N}, Aurora-B^{WT}, Survivin, TPX2, and PRC1 were inserted into pET28a. Proteins were affinity purified using nickel beads (Qiagen). Recombinant Aurora-A (active, from Sf21 cells) and GST-INCENP⁸²⁶⁻⁹¹⁹ were from Upstate Biotechnology. Antibodies to Aurora-A, TPX2, GFP, and PRC1 were raised in rabbits or mice. Antibodies to INCENP and Hec1 were from Abcam, antibody to Aurora-B from BD Biosciences, antibodies to Survivin and actin from Santa Cruz Biotechnology, and antibody to phospho-histone H3 (Ser-10) from Upstate Biotechnology.

Cell Line Generation and Cell Culture. Tet-on cell lines were generated according to the supplier's instructions (plasmids and drugs from Invitrogen). HeLa cells were transfected with pcDNA6/TR, and cultured in the presence of 10 μ g/mL blasticidin for 2 weeks. Positive clones were selected for further transfection of pcDNA4/TO-GFP-tagged Aurora-A^{WT}, Aurora-A^{G198N}, Aurora-B^{WT}, or Aurora-B^{N142G} and cultured in the presence of 200 μ g/mL Zeocin for 2 more weeks. Again, positive clones were selected and cultured for use. GFP-tagged Aurora-A^{WT}, Aurora-A^{G198N}, Aurora-B^{WT}, or Aurora-B^{N142G} was expressed when 1 μ g/mL tetracycline was added.

For immunofluorescence, cells were plated on coverslips 1 day before transfection by using a standard calcium phosphate transfection protocol and subjected to immunofluorescence microscopy 48 h later. For living-cell imaging, cells were plated in glass-bottomed culture dishes and observed 48 h after transfection. DNA was labeled with 100 ng/mL Hoechst 33342 for 30 min at 37 $^{\circ}$ C before observation. In time-lapse experiments, images were acquired every 5 min by using a Zeiss Axiovert 200M microscope and AxioVision image acquisition software.

Immunoprecipitation. Fifteen microliters of Protein-A Sepharose beads (GE Healthcare) were washed 3 times with PBS, diluted into 500 μ L and incubated with 5 μ g of rabbit-anti-GFP antibody for 1 h at 4 $^{\circ}$ C on a rotator. Beads were then washed 3 times with lysis buffer, consisting of 0.5% Nonidet P-40, 20 mM Tris-HCl (pH 7.4), 500 mM NaCl, 0.5 mM EGTA, 10 mM NaF, 2 mM sodium vanadate, 10 mM β -glycerophosphate, 10 μ g/mL leupeptin, 0.7 μ g/mL pepstatin A, 1.5 μ g/mL aprotinin, and 1 mM PMSF. Mitotic HeLa cells transfected with GFP-tagged Aurora-A^{WT}, Aurora-A^{G198N}, Aurora-B^{WT}, or Aurora-B^{N142G} were shaken off the culture dish 11 h after release from double thymidine block. For immunoprecipitation, the cells were lysed on ice for 30 min with lysis buffer. Lysates were centrifuged at 16,000 \times g for 15 min, and supernatants were incubated with beads for 1 h at 4 $^{\circ}$ C on the rotator. After 6 washes with lysis buffer, the bound proteins were analyzed by Western blotting.

Immunofluorescence Microscopy. Cells grown on coverslips were fixed with precooled methanol for 6 min on ice. After rehydration in TBS, the cells were incubated with the primary antibody overnight at 4 °C. Then they were washed 3 times in TBS and incubated with the secondary antibody for 45 min at room temperature. Coverslips were mounted onto slides by using Mowiol containing 1 μ g/mL DAPI and analyzed under a Zeiss Axiovert 200M microscope. Images were captured with a Zeiss MRM CCD camera and Axiovision image acquisition software. The antibodies were diluted in 3% BSA-TBS before use: Hec1 (1:100; Abcam), Aurora-B (1:100; BD Biosciences), INCENP (1:1,000; Abcam), Survivin (1:100; Santa Cruz), PRC1 (1:100), Alexa Fluor 488 donkey anti-mouse (1:100; Invitrogen), Alexa Fluor 549 donkey anti-mouse (1:100; Invitrogen), and TRITC-conjugated anti-rabbit (1:100; Jackson ImmunoResearch).

RNA Interference. pSUPER mammalian expression vector was used to direct the synthesis of siRNA-like transcripts. Oligonucleotides for suppressing INCENP, Aurora-B, and Survivin were synthesized as previously reported (42–44): INCENP, 5'-gatccccgaagagacggattctattctcaagagaataagaatccgtctcttttta-3' and 5'-agctt-aaaagaagagacggattctattcttgaataagaatccgtctctctggg-3'; Aurora-B, 5'-gatcccc-

gagcgtctcaccatctgttcaagagacagatgggggtgacagggctcttttgaaa-3' and 5'-agctttt-ccaaaagagcgtctcaccatctgttcttgaacagatgggggtgacagggctctggg-3'; and Survivin, 5'-gatccccgagcgtctcattctcaagagaagtgatgaagccagctcttttta-3' and 5'-agcttaa-aagagcgtctcattctcacttcttgaagtgatgaagccagcctctggg-3'.

Oligonucleotides were annealed and ligated into the BglII/HindIII restriction sites of digested pSUPER vector (45).

Kinase Assays. To measure the kinase activity of Aurora-A^{G198N}, 1 μ g of recombinant Aurora-A^{G198N} was incubated with 1 μ g of GST, GST-INCENP^{826–919} or Survivin for 20 min at 30 °C. Kinase assays were performed in 50 mM Tris-HCl (pH 7.4), 50 mM NaCl, 10 mM MgCl₂, 1 mM DTT, and 100 μ M ATP containing 3,000 Ci/mmol [γ -³²P]ATP. Reactions were quenched with SDS sample buffer and analyzed by SDS/PAGE and autoradiography.

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