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Aurora-A kinase-inactive mutants disrupt the interaction with Ajuba and cause defects in mitotic spindle formation and G2/M phase arrest in HeLa cells

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Aurora-A is a centrosome-localized serine/threonine kinase that is overexpressed in multiple human cancers. We previously reported an intramolecular inhibitory regulation of Aurora-A between its N-terminal regulatory domain (Nt, amino acids [aa] 1-128) and the C-terminal catalytic domain (Cd, aa 129-403). Here, we demonstrate that although both Aurora-A mutants (AurA-K250G and AurA-D294G/Y295G) lacked interactions between the Nt and Cd, they also failed to interact with Ajuba, an essential activator of Aurora-A, leading to loss of kinase activity. Additionally, overexpression of either of the mutants resulted in centrosome amplification and mitotic spindle formation defects. Both mutants were also able to cause G2/M arrest and apoptosis. These results indicate that both K250 and D294/Y295 are critical for direct interaction between Aurora-A and Ajuba and the function of the Aurora-A complex in cell cycle progression. [BMB Reports 2014; 47(11): 631-636]

INTRODUCTION

Aurora-A is a serine/threonine kinase in the Aurora kinase family, which includes members involved in regulating centrosome function, bipolar spindle assembly, and chromosome segregation processes (1). Aurora-A localizes to centrosomes and spindle microtubules proximal to centrosomes during mitosis (2). The expression and the activity of Aurora-A kinase are regulated in a cell cycle-dependent manner; levels are low in G1/S, upregulated during G2/M, and rapidly reduced after mitosis (3). The ectopic overexpression of Aurora-A induces oncogenic transformation (4, 5), and inhibition of Aurora kinase activity in mammalian cells leads to abnormal cell growth and polyploidy centrosome dysfunction (6-8). Thus, the Aurora kinases may be good molecular therapeutic targets for cancer.

Aurora-A kinase has a C-terminal catalytic domain (Cd) that is highly conserved within the Aurora kinase family and is regulated by phosphorylation in a cell cycle-dependent manner. This phosphorylation, which is required for kinase activity, occurs on a conserved residue, threonine 288, within the activation loop (aa 274-299) of the kinase's catalytic domain and significantly increases enzymatic activity. Aurora-A's kinase activity is regulated by one or more unknown kinases (9) and by association with the activators Ajuba (10), human enhancer of filamentation 1 (HEF1) (11), targeting protein for Xklp2 (TPX2) (12), etc.

We previously reported an intramolecular inhibitory regulation of Aurora-A between its N-terminal regulatory domain (Nt, aa 1-128) and the Cd (aa 129-403). The non-Aurora box (aa 64-128) in the Nt maintains Aurora-A in an auto-inhibited conformation to prevent phosphorylation and activation of the Cd in unstimulated cells. Once other proteins bind the Aurora box, the inhibitory conformation opens or becomes more closed, depending on the structure and function of the binding proteins (13).

We performed mutational analysis and found that two mutations (AurA-K250G, AurA-D294G/Y295G) in the conserved acidic aa residues in the Cd of Aurora-A both abolished the interaction of the Nt and Cd. However, kinase activity did not increase according to the auto-inhibitory model of Aurora-A. Furthermore, these mutations also abolished the interaction between Aurora-A and Ajuba. Overexpression of either mutant caused abnormal centrosome characteristics and G2/M arrest in HeLa cells. These results indicate that the mutation of these residues affects the direct interaction of Aurora-A and Ajuba to cause abnormal cell division.

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RESULTS

The Aurora-A mutants (AurA-K250G and AurA-D294G/Y295G) abolish the Nt and Cd interaction

We previously determined that the Nt domain of Aurora-A binds (aa 1-128) to the Cd (aa 129-403). Computer modeling studies predicted that four residues might affect the interaction between the Nt and Cd. Arg246 (R246), Lys250 (K250), Arg255 (R255), and Asp294Tyr295 (D294/Y295) are all located within or near the Aurora-A activation loop. Each of these residues was independently mutated to Gly to determine whether any of these amino acids affect the Nt-Cd interaction.

We examined whether the R246G, K250G, R255G, and D294G/Y295G mutants affected the binding ability of Nt and Cd. 293T cells were transfected with Myc-tagged Cd and Myc-tagged Cd mutants separately, and lysates from these cells were incubated with purified glutathione (GST)-Nt fusion proteins or GST and then subjected to immunoblotting analysis with anti-Myc antibody to reveal any coprecipitation. The results showed that Myc-AurA-R246G and Myc-AurA-R255G could bind to GST-Aurora-A-Nt similarly to Myc-Aurora-A but not to GST. However, AurA-K250G and AurA-D294G/Y295G mutants failed to interact with Aurora-A Nt (Fig. 1). This result strongly demonstrated that the two mutated residues, K250G and D294G/Y295G, contribute to the interaction between the Nt and Cd.

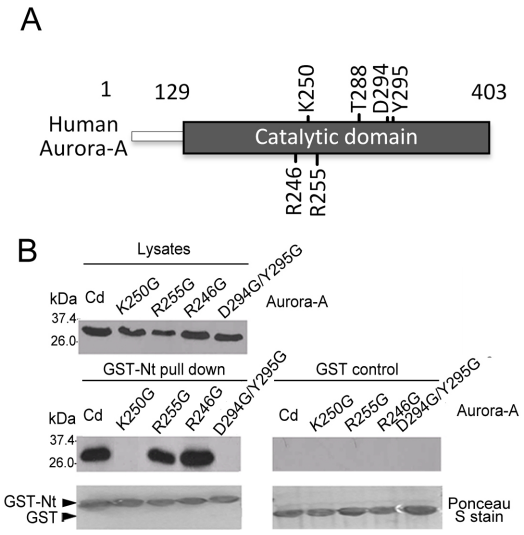

Fig. 1. The Aurora-A mutants abolish the interaction between the Nt and Cd. (A) Mutation positions close to T288 residue. (B) 293T cells transiently expressed Myc-Cd and its four mutants. After 48 h, cell lysates were incubated with GST alone or GST- Nt, which were prebound to GST agarose beads. These beads were lysed and subjected to immunoblot analyses with an anti- Myc antibody. GST-Nt could bind to Myc-tagged Cd, AurA- R246G, and AurA-R255G mutants but not to Myc- D294G/Y295G or Myc-K250G. The GST-tag control could not bind any Myc-tagged proteins.

The Aurora-A mutants fail to bind to Ajuba and reduce kinase activity

We found that the Aurora-A mutants (AurA-K250G and AurA-D294G/Y295G) affected the binding ability of the Nt (aa 1-128) and Cd (aa 129-403). Next, we examined whether these mutants influenced kinase activity.

The recombinant mutant kinases were assayed to determine whether their kinase activity toward myelin basic protein (MBP) was affected. Fig. 2A shows that mutants AurA-K250G and AurA-D294G/Y295G did not exhibit kinase activity, which is consistent with the results obtained with the kinase-dead form (K162R) of Aurora-A.

The phosphorylation of Aurora-A on Thr288 occurred during the G2-M phase, which is coincident with enzymatic activation. The phosphorylation of Thr288 thus provides an index of Aurora-A enzymatic activation. Given that Thr288 phosphorylation appears to be critical for Aurora-A kinase activity, we further determined whether the mutation would influence the autophosphorylation of Aurora-A on Thr288. We purified His-tagged wild-type Aurora-A and the mutants from *Escherichia coli* and examined the phosphorylation state of Thr288 by immunoblot analysis with an antibody specific for Aurora-A phosphorylated on this residue (T288P). His-tagged Aurora-A, which is highly phosphorylated, was detected on the T288P blot, whereas AurA-K250G and AurA-D294G/Y295G were not, similar to the kinase-inactive mutant AurA-K162R (Fig. 2B).

Ajuba was identified as a binding protein of Aurora-A, and the Aurora-A-Ajuba interaction contributes to the autophosphorylation and consequent activation of Aurora-A (10). Given that Ajuba appears to be required for the activation of Aurora-A, we next examined whether Ajuba influenced the activity of Aurora-A mutants. 293T cells were co-transfected with hemagglutinin (HA)-tagged Ajuba and Myc-tagged Aurora-A mutants (AurA-K250G, AurA-D294G/Y295G). Cell lysates were subjected to immunoblot analysis with an anti-T288P antibody. As shown in Fig. 2C, Ajuba enhanced the autophosphorylation of WT Aurora-A but did not affect AurA-K250G and AurA-D294G/Y295G, in which the mutation sites lie in or near the activation loop of Aurora-A. So can K250G and D294G/Y295G mutants maintain the interaction with Ajuba? To answer this question, we performed *in vitro* GST-pull down assays. Lysates from 293T cells expressing Myc-tagged Ajuba and GST fusion Aurora-A mutants were analyzed for their binding. Only direct binding of Ajuba with Aurora-A (WT) was observed (Fig. 2D). Moreover, immunoprecipitation assays were employed to assess the association of Ajuba with Aurora-A or the mutants *in vivo*. Consistently, when immunoprecipitated with anti-Myc antibody, endogenous Ajuba was only detected in the immunoprecipitated complex from Myc-Aurora-A-transfected cells (Fig. 2E). Taken together, the results indicate that both mutants affected the interaction between Ajuba and Aurora-A and abolished the auto-phosphorylation and consequent activation of Aurora-A.

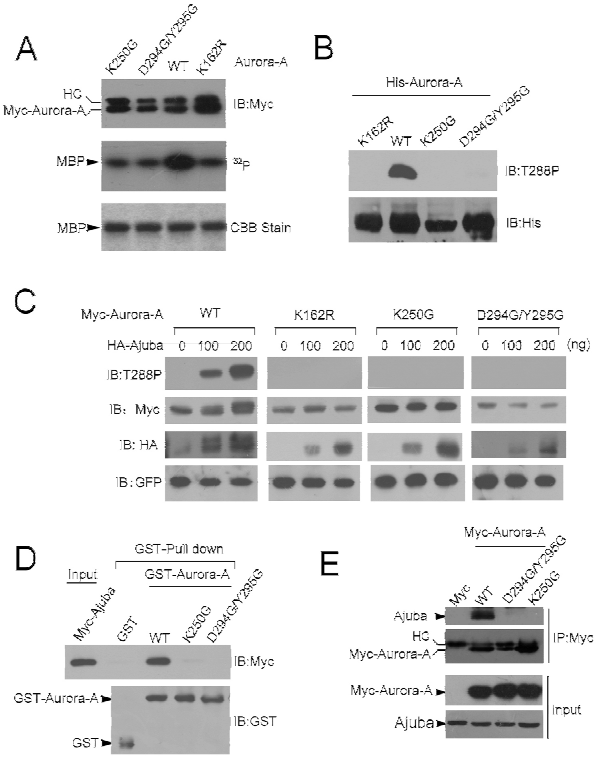

Fig. 2. The Aurora-A mutants lack kinase activity and fail to interact with Ajuba. (A) 293T cells were transfected with expression plasmids for wild-type (WT) Myc-Aurora-A, Myc-K250G, or Myc-D294G/Y295G. The kinase-dead mutant Myc-K162R was used as a control. After 48 h, cell lysates were immunoprecipitated (IP) with an anti-Myc antibody. Aliquots of the immunoprecipitates were probed with an anti-Myc antibody (top panels) and subjected to in vitro kinase assays with MBP and [γ-^{32}P] ATP as substrates. Aliquots of immunoprecipitates were separated by 12% SDS-PAGE followed by autoradiography. HC indicates antibody heavy chain. (B) The mutants influenced autophosphorylation of Aurora-A on Thr288. His-tagged WT Aurora-A and the mutants expressed from *E. coli* were examined for the phosphorylation state of Thr288 by immunoblot analysis with an antibody specific for Aurora-A phosphorylation on this residue (T288P). (C) Ajuba could not induce Aurora-A mutant autophosphorylation on Thr288. Myc-tagged Aurora-A, Myc-K250G, Myc-D294G/Y295G, or Myc-K162R were coexpressed with the indicated concentrations of HA-Ajuba. After 48 h, the lysates were collected, and the extent of Aurora-A phosphorylation was determined by immunoblot analysis with a T288P antibody. pEGFP-C1 cotransfection served as a control for transfection efficiency, and GFP levels were assessed using anti-GFP antibodies. (D) AurA-K250G, AurA-D294G/Y295G mutants abolished Ajuba binding. Cells expressing Ajuba were incubated with purified GST alone, GST-Aurora-A, or GST-Aurora-A mutants prebound to GST agarose beads. These beads were lysed and analyzed by western blotting. Ajuba bound to GST-Aurora-A but not to GST-tag or the mutants. (E) Neither AurA-D294G/295G nor AurA-K250G could interact with Ajuba in vivo. 293T cells were transfected to transiently express Myc-Aurora-A, Myc-Aurora-A-D294G/Y295G, or Myc Aurora-A-K250G. After 48 h, the cell lysates were immunoprecipitated (IP) with anti-Myc agarose, and the precipitates were subjected to immunoblot analysis with either anti-Myc or anti-Ajuba antibody.

Overexpression of either mutant leads to defects in mitotic spindle formation

As Aurora-A is involved in the regulation of centrosome function, bipolar spindle assembly, and chromosome segregation processes, the effects of the mutants on spindle organization and chromosome alignment were explored using immunofluorescence labeling. HeLa cells were transfected with Myc-tagged Aurora-A (WT), AurA-K250G, or AurA-D294G/Y295G mutants, and pCMV-Myc was used as a control. HeLa cells were synchronized by double treatment with thymidine to enrich G2/M phase cells and then harvested for immunofluorescence analysis. The Myc- or Myc-Aurora-A-transfected

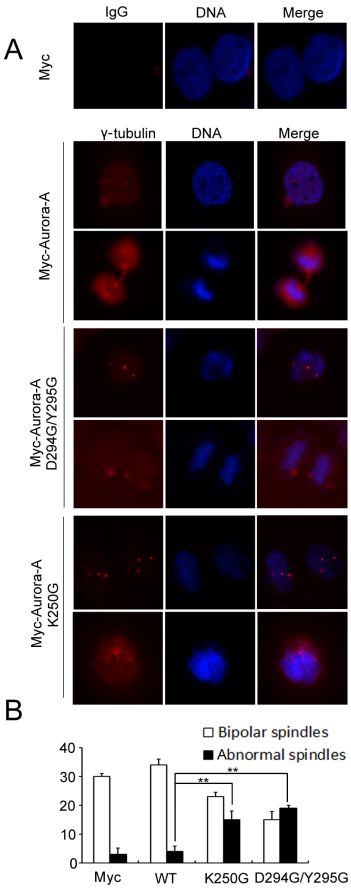

Fig. 3. Overexpression of either mutant results in abnormal mitotic spindle formation. (A) Representative immunofluorescent images of HeLa cells. Synchronized HeLa cells were transfected with Myc, Myc-Aurora-A, Myc-K250G, or Myc-D294G/Y295G between the two thymidine blocks. Cells were subjected to immunofluorescence microscopy 9 h after release. Overlapped images were obtained from cells labeled with an anti-γ-tubulin mouse antibody (tubulin, red) and DAPI (DNA, blue). (B) The percentage of spindle structures was averaged from three independent experiments, and at least 100 spindle structures were counted. The p values demonstrate a significant difference between the control and mutant-transfected cells for the incidence of abnormal spindles. **P $<$ 0.01.

cells displayed normal bipolar mitotic spindles. However, cells that overexpressed either AurA-K250G or AurA-D294G/Y295G mutants showed a variety of spindle organization defects, including multipolarity, severe chromosome alignment defects, or centrosome amplification (Fig. 3A). The percentages of cells with abnormal mitotic spindles were quantified (Fig. 3B). Control samples only displayed 3% abnormal mitotic spindles, whereas the mutant-transfected cells exhibited about 20% abnormal mitotic spindles, which is consistent with a known Aurora-A inhibition phenotype (14, 15).

Overexpression of either mutant induces G2/M arrest and apoptosis

Previous findings implied the involvement of Aurora-A in regulating cell cycle progression. To explore the roles of the mutants in cell cycle regulation, Myc-tagged Aurora-A (WT), AurA-K250G, or AurA-D294G/Y295G plasmids were transfected with the pBB14-GFP plasmid into HeLa cells. The population percentage of HeLa cells transfected with control vector was 8.26% in the G2/M phase, and it decreased to 6.65% when transfected with Myc-Aurora-A (WT), which could promote cell cycle progression. However, we observed increases in G2/M populations in HeLa cells that were transfected with either AurA-K250G or AurA-D294G/Y295G plasmids (18.77%, 18.12%) compared with control (8.26%) (Fig. 4A). The results imply that both mutants are able to induce arrest in the G2/M phase.

In addition, it has been reported that treatment with an Aurora-A kinase inhibitor causes apoptosis. Therefore, we carried out a standard cell apoptosis assay by labeling cells with Annexin V. Myc-tagged Aurora-A (WT), AurA-K250G, or AurA-D294G/Y295G plasmids were transfected with the pBB14-GFP plasmid into HeLa cells and subjected to Annexin V analysis

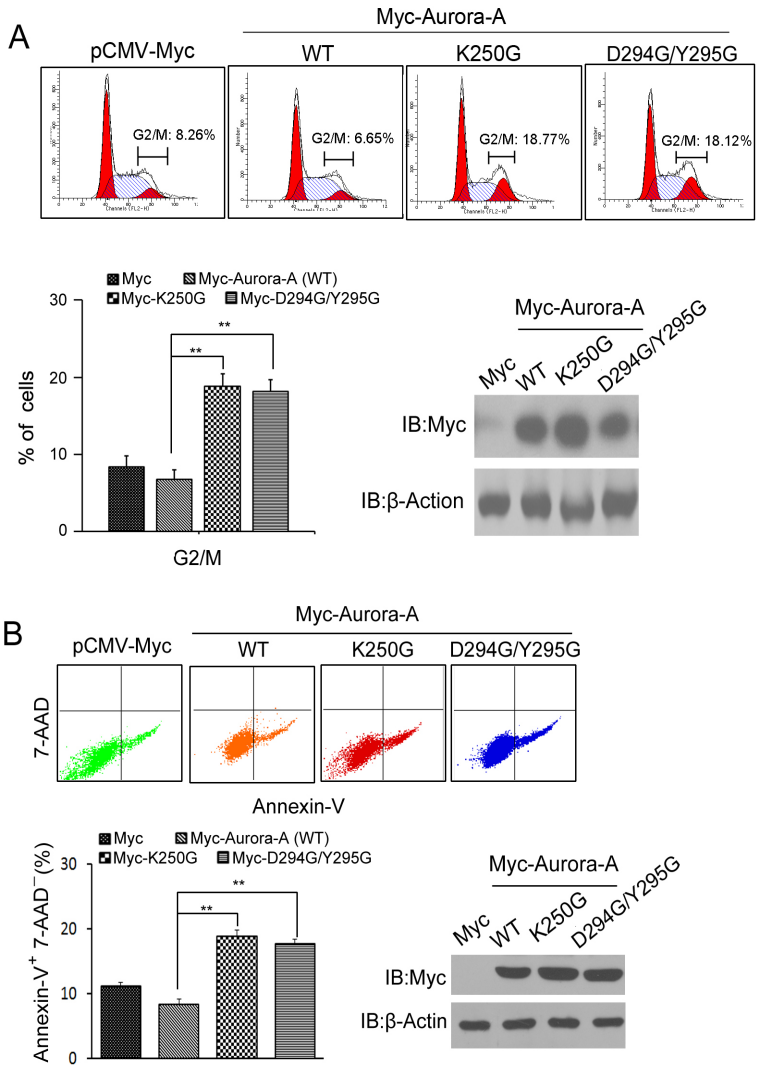

Fig. 4. Overexpression of either mutant increases G2/M populations and causes apoptosis. (A) Control, Aurora- A (WT) or mutant plasmids were cotransfected with pBB14-GFP plasmid into HeLa cells. After 48 h, cells were harvested, and the percentages of cells in individual cell cycle phases were determined by flow cytometry. The pCMV-Myc vector was used as a control. Values are given as mean ± SD, n=3. **P < 0.01. Cells described in panel A and B were analyzed by western blot using a Myc antibody, β-Actin was used as a loading control. (B) Annexin-V analysis showed that both mutants exerted an apoptotic effect. Apoptosis was calculated as the percentage of Annexin-V-positive and 7-AAD-negative cells compared to the total number of cells and is shown as mean ± SD; n = 3

48 h later. In Fig. 4B, the fraction of Annexin V-positive and 7-aminoactinomycin D (7-AAD)-negative cells is indicated, representing the apoptotic cell population. A moderate increase in the apoptotic cell population was observed in cells expressing AurA-K250G and AurA-D294G/Y295G compared to that in control cells, demonstrating that mutant expression could induce apoptosis.

DISCUSSION

Aurora-A is a centrosome kinase that contributes to the regulation of mitosis. The initial activation of Aurora-A in the late G2 phase of the cell cycle requires interaction with Ajuba. Previously, we found that the Nt can inhibit C-terminal kinase activity both *in vitro* and *in vivo* via a direct interaction. In this study, we characterized two Aurora-A mutants, K250G and D294G/Y295G. We found that mutation of the conserved amino acid residues in the acidic patch on Aurora-A abolished the binding of the Nt to Cd. We speculated that the mutant kinase activity should increase because the Nt's inability to bind to the Cd of the mutants would open the inhibitory conformation, resulting in increased kinase activity. However, the kinase activities of Aurora-A mutants did not increase according to the auto-inhibitory model. Additionally, we found that the mutants lost their ability to bind to Ajuba, which is required for Aurora-A activation; this is probably the reason why both mutants lost their kinase activity. Furthermore, overexpression of either of the Aurora-A mutants (AurA-K250G and AurA-D294G/Y295G) severely disturbed mitotic spindle formation and resulted in G2/M arrest and apoptosis in HeLa cells.

How could the expression of the Aurora-A mutants (AurA-K250G and AurA-D294G/Y295G) mediate defects in mitotic spindle formation? Aurora-A has crucial roles in mitotic spindle formation and centrosome maturation. Several reports have demonstrated that Aurora-A can interact with and regulate the activities of many important cellular proteins associated with cell cycle and cell division, including Ajuba, TPX2, HEF1, PAK, cyclin B, and Cdc2 (16). Although we have demonstrated that the K250 and D294/Y295 residues are crucial for the interaction between Ajuba and Aurora-A, it remains to be determined whether the mutants could affect the interaction of Aurora-A with other activators or substrates. Overexpression of either of the mutants may result in competitive binding with the activators or substrates of the endogenous Aurora-A, leading to abnormalities in spindle or centrosome regulation.

Why did mutation of K250 and D294/Y295 affect Aurora-A's binding to Ajuba and its activation? An obvious explanation may be that mutation of Asp294 and Tyr295, which lie in the activation loop of Aurora-A, may induce the conformational change of Aurora-A and result in the failure of Ajuba binding. Although Lys250 is not in the activation loop, this residue is particularly close to His280 and Pro282, which appear to be the pivot points of the conformational change of the activation loop (17). A small change at the pivot points (H280, P282) could result in a large conformational change and impair Ajuba binding, which is required for Aurora-A activation.

In summary, we firstly identified the distinct sites that are essential for Aurora-A activity. Our data provide a better understanding of Aurora-A regulation and offer insight into the development of specific small molecule inhibitors (18).

MATERIALS AND METHODS

Materials and Methods, were provided in Supplementary data: http://www. bmbreports.org/jbmb_by_volume.html?vol=47.

Cell culture, synchronization, and transfection

293T and HeLa cells were cultivated in DMEM (Gibco-BRL/Life Technologies, Carlsbad, CA, USA), which was supplemented with 10% (v/v) fetal calf serum (Gibco-BRL) at 37°C in 5% CO_2-humidified atmosphere. HeLa cells were blocked for 20 h with thymidine (final concentration 2 mM), washed three times with phosphate-buffered saline (PBS) and then incubated with fresh medium. After 8 h, thymidine was added again, and the cells were incubated for an additional 16 h. Plates were then washed, and fresh medium was added. The majority of cells were in G2/M phase 9 h later, as determined by fluorescence-activated cell sorting (FACS) analysis, and the cells were collected for study. For transfection, 293T or HeLa cells at 80% confluence were transfected with plasmids using Lipofectamine (Life Technologies) in serum-free medium. After 5 h of incubation, the medium was replaced with fresh complete medium. At 48-h post transfection, cells were subject to immunoblotting or immunofluorescence microscopy.

Immunofluorescence microscopy

Cells grown on coverslips were fixed in 4% formaldehyde in PBS for 10 min and resolved by 0.2% Triton X-100 for 15 min at room temperature. The permeabilized cells were blocked with 10% normal horse serum plus 1% bovine serum albumin (BSA) for 1 h. A drop of the diluted monoclonal antibody against γ-tubulin (1:500 Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) was placed on the coverslips overnight and then with Alexa 546 (red fluorescence)-conjugated goat anti-mouse secondary antibody (Life Technologies) for 1 h. The nuclei were stained with 4',6-diamidino-2-phenylindole (DAPI, 1:1000 dilution) for 10 min. Images were acquired using a LEICA DC 500 camera on a microscope equipped with LEICA DMRA2 fluorescent optics (Leica, Wetzlar, Germany).

Flow cytometry

HeLa cells were transfected with the pCMV-Myc vector, Myc-Aurora-A, or the two Myc-tagged mutants, and each plate was also transfected with the pBB14 plasmid, which was 10% of the Aurora-A plasmids. At 48 h after transfection, the cells were washed in PBS and fixed in cold 70% ethanol overnight at 40°C. After another wash in PBS, cells were incubated with 50 mg/ml propidium iodide (Sigma-Aldrich, St. Louis, MO,

USA) and 100 mg/ml RNaseA (Sigma-Aldrich) for 30 min at room temperature. Fluorescence from the propidium iodide-DNA complex was measured using a FACSCalibur flow cytometer (Becton Dickinson, Franklin Lakes, NJ, USA), and the percentage of cells in each cell cycle phase was quantified using the ModFit program.

Annexin-V staining

Apoptosis was analyzed with the Annexin-V-PE apoptosis detection kit I (BD Pharmingen, San Diego, CA, USA). According to manufacturer's protocol, cells were harvested, washed, and labeled with Annexin-V-PE for 15 min in dark at room temperature. A total of 10,000 events for each sample were collected and analyzed with a FACSCalibur flow cytometer (Becton Dickinson). The data are presented as the percentage of apoptotic cells (Annexin-V positive and 7-AAD negative cells) with respect to the total number of cells, which were collected for analysis 48 h after transfection.

GST pull-down assay

Mutant-expressing 293T cells were harvested in cell lysis buffer for 30 min at 4°C. GST fusion proteins were mixed with 40 μl glutathione-Sepharose 4B beads (Amersham Biosciences). After incubation, the beads were collected by centrifugation, washed three times with wash buffer (20 mM Tris/HCl [pH 7.4], 100 mM NaCl, 2 mM EDTA, 0.5% Triton X-100, 5 mM $MgCl_2$, 1 μg/ml leupeptin, and 1 mM PMSF) and the beads were incubated with lysates of transfected 293T cells with gentle rocking for 4 h at 4°C. After washing five times with wash buffer, the beads were suspended in sample buffer. The bound proteins were subjected to SDS-PAGE.

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