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Investigating the role of Aurora Kinases in RAS signaling

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ABSTRACT

Activating *ras* mutations are frequently found in malignant tumors of the pancreas, colon, lung and other tissues. RAS activates a number of downstream pathways that ultimately cause cellular transformation. Several recent studies suggested that one of those pathways involves Aurora kinases. Overexpression of Aurora-B kinase can augment transformation by oncogenic RAS, however the mechanism was not determined. The cooperative effect of high levels of Aurora kinase is important since this kinase is frequently overexpressed in human tumors. We have used two Aurora kinase inhibitors to test their effect on RAS signaling. We find that these inhibitors have no effect on the phosphorylation of MEK1/2 or MAPK in response to RAS. Furthermore, inhibiting Aurora kinases in human cancer cells with or without activated RAS did not change the length of the cell cycle nor induce apoptosis suggesting that these kinases do not play a direct role in these key cellular responses to activated RAS. Overexpression of Aurora B can cause cells to become polyploid. Also, inducing polyploidy with cytochalasin D was reported to induce neoplastic transformation, suggesting that Aurora overexpression may cooperate with RAS indirectly by inducing polyploidy. We find that inducing polyploidy with cytochalasin D or blebbistatin does not enhance transformation by oncogenic RAS. Our observations argue against a direct role for Aurora kinases in the RAS-MAPK pathway, and suggest that the polyploid state does not enhance transformation by RAS. J. Cell. Biochem. 106: 33–41, 2009.

KEY WORDS: MAPK; TRANSFORMATION; CYTOCHALASIN; POLYPLOIDY

Despite many recent advances in cancer prevention, diagnosis and treatment, this disease is still a major public health concern. Given that cancer is characterized by abnormal and excessive cell proliferation it is important to understand the pathways that control cell division. Extracellular growth factors that signal through the small GTPase RAS play a key role in controlling whether or not a cell enters the cell cycle [Vogelstein and Kinzler, 2004]. Activating RAS mutations occur in up to 90% of malignancies of the pancreas, 50% of the colon, 30% of the lung, 50% of the thymus and 30% of myeloid leukemias [Bos, 1989]. RAS regulates many cellular activities including communication via cell–cell junctions, golgi trafficking, vesicle formation, transcription, apoptosis, and cell cycle progression [Malumbres and Pellicer, 1998]. A number of the biological effects of RAS are mediated by the RAS/RAF/MEK/MAPK pathway including stimulation of the G1-S transition via upregulation of Cyclin D [Pruitt and Der, 2001].

The three members of the Aurora kinase family, Aurora A, B, and C play essential roles in cell division [Katayama et al., 2003; Bolanos-Garcia, 2005]. Aurora A associates with centrosomes and microtubules in prophase. It has also been found to bind to RASGAP, an enzyme that enhances the hydrolysis of GTP to GDP, leading to the inactivation of RAS [Katayama et al., 2003]. When overexpressed, Aurora A disrupts centrosome duplication [Katayama et al., 2003]. Aurora B, along with INCENP, Survivin and Borealin form the chromosomal passenger complex [Katayama et al., 2003; Bolanos-Garcia, 2005]. During metaphase, the CPC localizes to the inner centromere and controls the proper attachment of mitotic spindles to kinetochores. During anaphase, the CPC is localized to the spindle midzone where it helps to position the contractile ring. This process appears to involve the localized phosphorylation of MKLP1 by Aurora B, which then recruits additional proteins that activate cleavage furrow formation [Guse et al., 2005]. Overexpression of Aurora B disrupts chromosome segregation resulting in the production of aneuploid cells [Katayama et al., 2003]. Aurora C is less well studied but can function as a component of the CPC [Yan et al., 2005].

Overexpression of Aurora kinases can cooperate with activated RAS in cellular transformation and it was suggested that it might do

Abbreviations used: MLK3, mixed-lineage kinase 3; MAPK, mitogen activated protein kinase; DMSO, dimethyl sulfoxide; DMEM, Dulbecco's modified eagle media; PBS, phosphate buffered saline.

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so by enhancing oncogenic signaling by RAS [Kanda et al., 2005; Tatsuka et al., 2005]. We have investigated a potential role of Aurora kinases in RAS signaling using the inhibitors ZM447439 and VE-465. We find that Aurora kinase activity is not a major requirement for RAS-MAPK signaling. Cell cycle progression and cell death were unaffected by inhibiting Aurora kinases in cells with or without activated RAS. Also, inducing polyploidy, a known effect of Aurora overexpression, did not enhance transformation by activated RAS. Together, our studies suggest that Aurora kinases do not play a direct role in cell cycle progression and proliferation in response to RAS signaling, and that polyploidy induced by cleavage furrow failure does not predispose to RAS transformation.

MATERIALS AND METHODS

CELL LINES AND CULTURE CONDITIONS

10T1/2 mouse and REF52 rat fibroblast cells were used for focus formation assays. Parental NIH3T3 mouse fibroblasts were compared to cell lines transformed by either Ki-RAS or RAF-1 [Chadee and Kyriakis, 2004]. All cell lines were grown in a humidified atmosphere containing 10% CO_2 in medium containing penicillin (10,000 units/ml), and streptomycin (10,000 units/ml) (Cambrex Biosciences) at 37°C. Growth medium for 10T1/2 and NIH3T3-derived strains consisted of Dulbecco's modified eagle media (DMEM) containing 10% (v/v) cosmic calf serum (Gibco), while HT1080, WI38 and REF52 cells were grown in DMEM containing fetal calf serum (Gibco). H16N2 is an immortalized cell line derived by introducing the HPV-16 E5, E6 and E7 genes into human mammary epithelial cells [Band et al., 1990]. H16N2 were grown in Ham's F12 supplemented with 0.1% BSA, 0.5 mg/ml fungizone, 5 mg/ml gentamycin, 5 mM ethanolamine, 10 mM HEPES, 5 mg/ml transferrin, 10 mM T3, 50 mM selenium, 5 mg/ml insulin, 1 mg/ml hydrocortisone, and 10 ng/ml EGF. For starvation, H16N2 were incubated in Ham's F12 with 1% BSA, 0.5 mg/ml fungizone, and 5 mg/ml gentamycin for 48 h. EGF was then added to a final concentration of 20 ng/ml.

TRANSFECTION AND FOCUS FORMATION ASSAY

1.5×10^5 cells were seeded into 6-well plates. 6 μl of Fugene6 transfection reagent (Roche Applied Science) was added to 94 μl of DMEM without serum, penicillin or streptomycin. The mixture was incubated for 5 min at room temperature. A total of 1.7 μg of DNA was added to each tube followed by 20 min incubation at room temperature. Each mixture was added dropwise to the appropriate well. After 16 h, cells were trypsinized and split in two wells. One well remained untreated while the other well was exposed to cytochalasin-D at 2 μg/ml for 16 h. The wells were then washed with PBS and media supplemented with 10% cosmic calf serum was added and changed as needed for the next 2 weeks. Foci were stained with methylene blue. In a second type of experiment, cells were pretreated with either cytochalasin-D or blebbistatin for 18 h. The drugs were removed and the cells were allowed to recover for 1 week before being transfected with *ras*. Foci were assayed as described above.

WESTERN BLOT ANALYSIS

Cells were harvested after washing once with ice cold 1× PBS, by scraping into 1 ml of ice cold PBS. Cells were collected by centrifugation (4,500 rpm, 4°C) and then incubated in RIPA lysis buffer (supplemented with 1 μg/ml aprotinin, 2 μg/ml leupeptin, 1 μg/ml pepstatin A, 1 mM DTT, and 0.1 M PMSF) for 20 min on ice. The cell lysate was centrifuged at high speed for 30 min at 4°C. To ensure equal loading, protein concentrations of each lysate were determined using BSA Protein Assay Kit (Pierce). Proteins were then separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis and then transferred to polyvinylidene difluoride membranes [Taylor et al., 1999]. For phosphorylated primary antibodies, membranes were first blocked in 5% BSA-PBST (phosphate buffered saline plus 0.05% [v/v] Tween-20) solution and then probed overnight (4°C) using a 1:1,000 or 1:500 dilution. Phosphorylated antibodies include: phospho Histone H3 (Ser10) (Cell Signaling), phospho Cenp-A (Ser7) (Cell Signaling), phospho MLK3 (Thr277/Ser281) (Cell Signaling), phospho MEK1/2 (Ser221, 166F8) (Cell Signaling), and phospho ERK (E-4) (Santa Cruz). After incubation with primary antibody, the membranes were washed three times for 10 min in PBST. Goat-anti-mouse and goat-anti-rabbit secondary antibodies conjugated to horse-radish peroxidase (Santa Cruz) were used at a dilution of 1:1,000 in 5% nonfat dried milk and PBST for 45 min. Membranes were again washed three times for 10 min in PBST. Antibodies to p53 and p21 directly conjugated to horse-radish peroxidase (Santa Cruz) were used at a dilution of 1:1,000 for 1 h. Antibodies to actin (Neomarkers) were used at a dilution of 1:1,000 for 1 h. Antibodies for total MEK1 (12-B) (Santa Cruz), total ERK2 (C-14) (Santa Cruz), and total MLK3 (C-20) (Santa Cruz) were used at a dilution of 1:1,000. All membranes were treated with 2 ml of enhanced luminal reagent and 2 ml of oxidizing reagent (PerkinElmer Life Sciences) for 1 min prior to detection by exposure to film.

TIME-LAPSE MICROSCOPY

Cells were maintained in a sealed flask containing growth media pre-equilibrated to 10% CO_2 on a 37°C-heated stage on an inverted microscope. Images were captured with using the 40× microscope objective and an Olympus C740 digital camera controlled by Cam2Com software. Images were converted to stacks and navigated using ImageJ software. To analyze cell division in H16N2 cells, the cells were plated in normal growth medium and filmed for at least 18 h. We monitored the first 20 untreated cells that entered mitosis, and the first 50 ZM447439, or VE-465-treated cells that entered mitosis and determined their ability to divide upon mitotic exit.

PROLIFERATION ANALYSIS

The effects of Aurora and MAP kinase inhibitors on overall proliferation were tested by staining cells using methylene blue. 2×10^4 cells were seeded into each well of a 12-well plate and incubated for 16 h. Cells were then treated with either ZM447439 (2.5 μM) or PD98059 (25 μM). Untreated cells received an equal volume of the dimethyl sulfoxide (DMSO) vehicle. At the days indicated, growth medium was decanted and cells were fixed and stained by adding a saturated solution of methylene blue in 50% ethanol for 15 min. Excess stain was removed using tap water and

the cells were then air dried. Stain was extracted by adding 3 ml of phosphate-buffered saline containing 0.1 M HCl, and incubating at 37°C for 15 min. Samples were analyzed by spectrophotometry to determine absorbance at 630 nm.

RESULTS

EFFECT OF ZM447439 ON RAS/RAF/MEK/MAPK PATHWAY

Overexpression of Aurora kinases cooperate with activated RAS in transformation in vitro [Kanda et al., 2005; Tatsuka et al., 2005]. Also, suppressing the levels of Aurora B using RNA interference reduced transformation by RAS but not by RAF suggesting that Aurora B might play a role in the MAPK pathway [Kanda et al., 2005]. To test this idea, we exposed parental and RAS-transformed NIH3T3 cells to ZM447439, an inhibitor of Aurora A and B kinases, and measured the phosphorylation of kinases in the MAPK pathway by Western blotting. First, we tested the effect of ZM447439 on the phosphorylation of MEK1/2, downstream targets of RAF. ZM447439 had minimal effect on the phosphorylation of MEK1/2 in both the parental NIH3T3 cells and the RAS-transformed derivatives (Fig. 1). A reduced signal for phosphorylated MEK1/2 was observed in NIH3T3 cells exposed to DMSO for 4 h, however, less total MEK and Actin were observed in this sample indicating that the reduction was due to underloading of this sample. Similarly, exposure of NIH3T3 cells transformed by RAF to ZM447439 had no effect on the phosphorylation of MEK1/2 (our unpublished data). We next tested the effect of ZM447439 on the phosphorylation of MEK1/2 in the cell line HT1080. HT1080 were derived from a human fibrosarcoma and contain an activated *n-ras* [Brown et al., 1984]. Similar to the

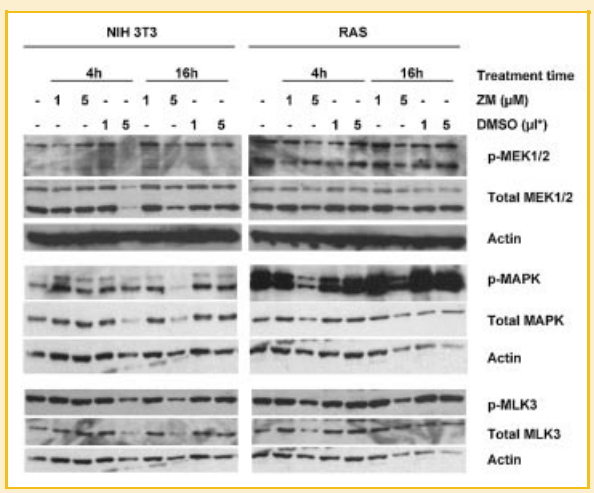

Fig. 1. Effect of ZM447439 on the phosphorylation of MEK, MAPK, and MLK3 in mouse fibroblasts. Parental or RAS-transformed NIH3T3 cells were treated with either ZM447439 (ZM) or DMSO for the indicated times. Two different doses, 1 or 5 μM were used. The asterisk (*) represents the addition of DMSO vehicle at equal volume to the corresponding ZM447439 treatment. Phospho- and total protein levels were measured by Western blot analysis. Membranes were first probed for the phospho-antigen, stripped and re-probed for total antigen, and then stripped and probed for β actin to determine total loading. Representative results from two independent experiments are shown.

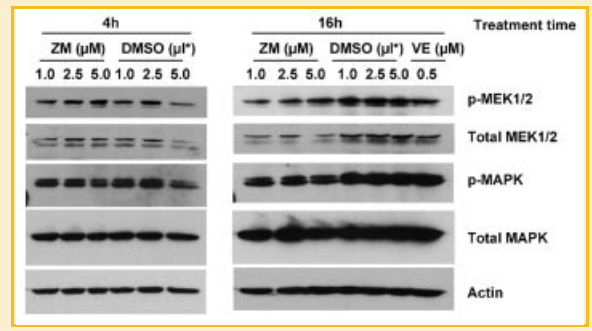

Fig. 2. Effect of ZM447439 and VE-465 on the phosphorylation of MEK and MAPK in human tumor cells. HT1080 human fibrosarcoma cells were exposed to ZM447439 (ZM) or VE-465 (VE) for the indicated times. Phosphorylation of MEK and MAPK was measured using Western blotting as described for Figure 1. The asterisk (*) represents the volume of DMSO vehicle equal to the corresponding ZM447439 treatment. Representative results from two independent experiments are shown.

mouse cells, exposure of human HT1080 cells to either 2.5 or 5.0 μM ZM447439 had no effect on the phosphorylation of MEK1/2 (Fig. 2). Exposure to VE-465, an inhibitor of Aurora A, B, and C kinases, also had no effect on the phosphorylation of MEK1/2 in HT1080 cells (Fig. 2)

Next we analyzed the effect of ZM447439 on the phosphorylation of MAPK, the target of MEK1/2. Exposure to 1μM ZM447439 had little effect on the phosphorylation of MAPK in parental or RAS-transformed NIH3T3 cells (Fig. 1). We did observe a reduction in phosphorylation of MAPK at the 5 μM dose of ZM447439, which was most evident in the RAS-transformed cells after 16 h of exposure (Fig. 1). Since MEK1/2 was not inhibited, this suggested that Aurora B might contribute to MAPK activity independently of MEK1/2. However, ZM447439 is able to directly inhibit MEK1 at high doses [Ditchfield et al., 2003]. Therefore, at this highest dose, direct inhibition of MEK1/2 by ZM447439 might be responsible for the reduction in the phosphorylation of MAPK in mouse cells. Exposure of human HT1080 cells to either 2.5 or 5.0 μM ZM447439 had no effect on the phosphorylation of MAPK (Fig. 2). Furthermore, VE-465 had no effect on the phosphorylation of MAPK in HT1080 cells (Fig. 2).

Next, we tested whether normal Ras signaling induced by EGF stimulation was affected by inhibiting Aurora kinases. The nontransformed mammary epithelial cell line H16N2 was incubated without growth factors to arrest them in G_0. The addition of EGF induced the phosphorylation of MAPK within 15 min even in the presence of either ZM447439 or VE-465 (Fig. 3A). To confirm that ZM447439 and VE-465 were capable of inhibiting Aurora kinases in these cells we monitored cell division by time-lapse microscopy. Cell division depends on active Aurora kinases [Ditchfield et al., 2003]. All of 20 untreated H16N2 cells that entered mitosis subsequently divided into two daughter cells. However, none of the 50 ZM447439 or VE-465-treated cells that entered mitosis were able to divide (our unpublished data). Therefore, both drugs appear capable of inhibiting Aurora kinases in H16N2 cells under the conditions we have used. All together, these results suggest that Aurora kinases are not critical components of the RAS-MAPK pathway. We also

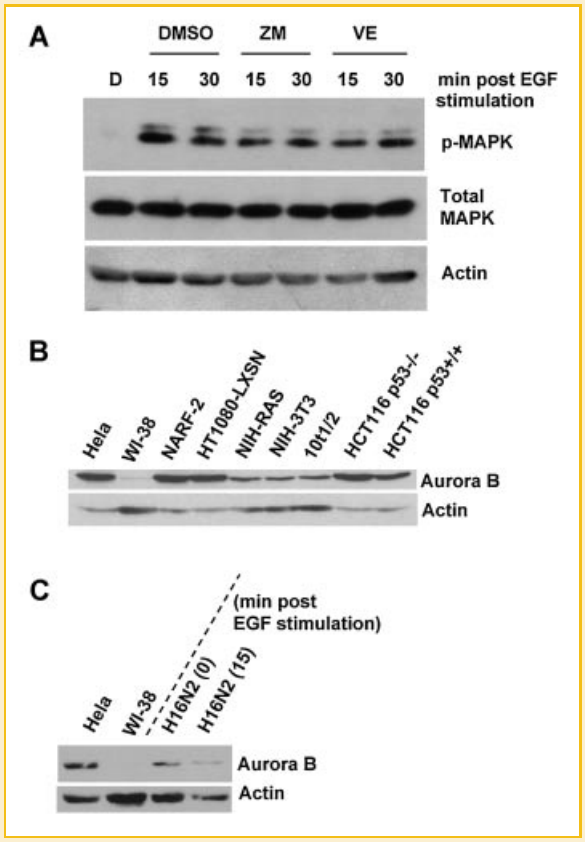

Fig. 3. Effect of ZM447439 and VE-465 on MAPK phosphorylation upon EGF stimulation and comparison of Aurora B levels. The phosphorylation of MAPK, as well as the levels of Aurora B were assessed using Western blotting. A: EGF stimulation of MAPK phosphorylation. H16N2 cells were starved of insulin and EGF for 48 h as described in Materials and Methods Section. Cell lysates were analyzed by Western blotting at various times after adding EGF. B,C: Comparison of Aurora B levels. Cell lines were grown asynchronously except for H16N2 which were starved or stimulated with EGF. Cells were lysed and analyzed by Western blotting to measure the levels of Aurora B protein.

compared the levels of Aurora B in a number of cell types including those used in this study. The normal diploid fibroblast WI38 and H16N2 cells expressed the lowest levels of Aurora B, while tumor-derived cell lines express higher levels (Fig. 3B,C).

EFFECT OF ZM447439 ON PHOSPHORYLATED MLK3

Mixed-lineage kinase 3 (MLK3) is a mitogen activated protein kinase kinase kinase (MAP3K) that is required for proliferation of normal fibroblasts and cell lines containing activated RAS [Chadee and Kyriakis, 2004]. MLK3 activates multiple MAPK pathways and is a downstream component in a pathway initiated by the small GTPase CDC42. CDC42 regulates actin polymerization in response to activated RAS [Burbelo et al., 1995; Teramoto et al., 1996]. Therefore, we analyzed the effect of Aurora kinase inhibition on the phosphorylation of MLK3 as a measure of CDC42 activity. ZM447439 had little effect on the phosphorylation of MLK3 (Fig. 1). Slight variations observed correspond to slight variations in loading, and similar results were observed in RAF-transformed NIH3T3 cells (Fig. 1 and our unpublished data).

PHOSPHORYLATION OF HISTONE H3 AND CENP-A IN CELLS EXPOSED TO AURORA KINASE INHIBITORS

Phosphorylation of histone H3 at serine 10 occurs to a limited extent during interphase but much more extensively as cells enter mitosis [Hsu et al., 2000; Dong and Bode, 2006]. Immunofluorescence assays have shown that Hela cells exposed to 2 μM ZM447439 enter mitosis with condensed chromosomes that do not contain histone H3 phosphorylated at serine 10 [Ditchfield et al., 2003]. Also, siRNA directed against Aurora B abolishes the phosphorylation of serine 10 of histone H3 during mitosis [Hauf et al., 2003]. This suggests that Aurora B is the primary mitotic H3 kinase responsible for phosphorylation of serine 10. We used immunofluorescence to determine whether histone H3 phosphorylation was inhibited in NIH3T3 cells under the conditions we have used. Phosphorylated histone H3 was still observed in mitotic NIH3T3 cells exposed to 1 μM ZM447439, however the intensity of staining was lower (Fig. 4A). In contrast, cells exposed to 5 μM of the drug entered mitosis with very low levels of phosphorylated histone H3 confirming that ZM447439 is capable of inhibiting mouse Aurora B (Fig. 4A). In human cells, we observed that histone H3 phosphorylation was very low after exposure to 2.5 μM ZM447439 (Fig. 4B).

During interphase, the activation of the RAS-MAPK signaling pathway leads to a rapid increase in the phosphorylation of histone H3 at serine 10 [Cano et al., 1995; Chadee et al., 1999]. If Aurora kinases were an essential component of the RAS pathway, they might be responsible for this phosphorylation event. In this case, inhibiting Aurora kinases in cells with activated RAS should reduce interphase phosphorylation of histone H3. To test this idea, we measured the intensity of staining using the antibody to phospho-S10 histone H3. We excluded mitotic cells based on chromatin morphology and observed that treatment with ZM447439 reduced the phosphorylation of histone H3 by approximately 30% (Fig. 4C). This reduction was statistically significant but not dramatic suggesting that kinases other than Aurora A and B play a major role in phosphorylating H3 during interphase in response to RAS signaling.

As an additional test of the effect of ZM447439 and VE-465 on Aurora kinase activity in vivo, we analyzed the phosphorylation of Cenp-A at serine 7. There is evidence that Aurora A, B, and C phosphorylate S7 of Cenp-A in mammalian cells [Zeitlin et al., 2001; Slattery et al., 2008]. Exposure to either ZM447439 or VE-465 abolished S7 phosphorylation of Cenp-A in mitotic cells (Fig. 5). These observations indicate that these chemicals are inhibiting Aurora kinases in vivo.

CELL CYCLE EFFECTS OF ZM447439

Using time-lapse microscopy we have analyzed the behavior of several human tumor cells exposed to ZM447439. Similarly to previous reports we observed that cytokinesis cannot be completed (Fig. 6A). We also observed that cells are capable of attempting mitosis multiple times in the presence of the drug providing an opportunity to analyze the interval between mitoses (i.e., interphase). The length of interphase in Hela cells was longer by

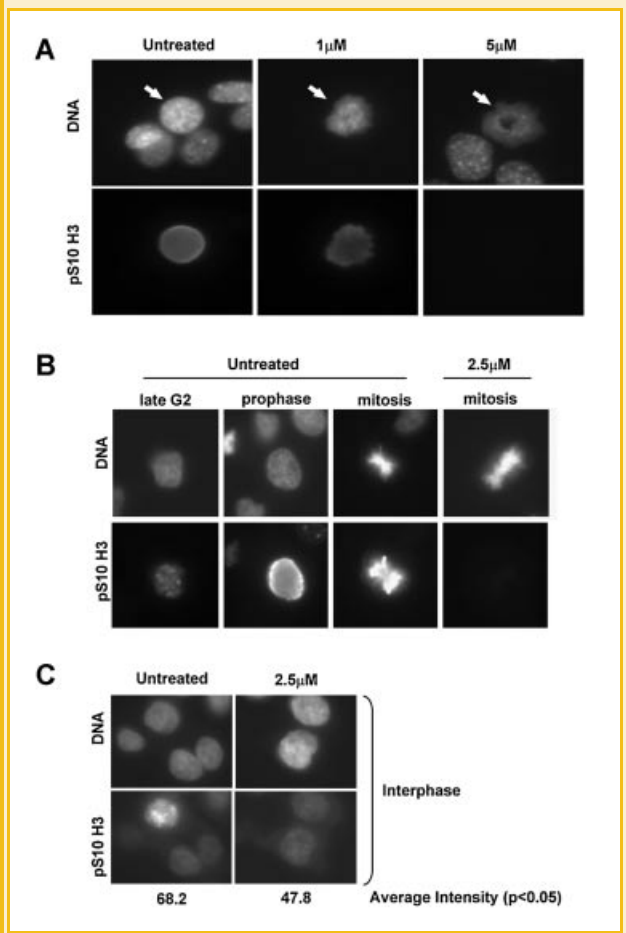

Fig. 4. Phosphorylation of histone H3 at serine 10 in cells exposed to ZM447439. Mouse NIH3T3 fibroblasts or human HT1080 tumor cells were exposed to ZM447439 for 16 h and the phosphorylation of histone H3 determined using immunofluorescence, as previously described (Kaur et al., 2007). A: Suppression of H3 phosphorylation in mitotic NIH3T3 cells. B: Suppression of H3 phosphorylation in mitotic HT1080 cells. C: Effect of ZM447439 on H3 phosphorylation during interphase in HT1080 cells. Interphase cells were identified based on chromatin morphology, and staining intensity measured by determining pixel intensities of digital images. Average pixel intensities of 69 untreated and 75 treated cells are shown.

several hours in the presence of ZM447439 compared to untreated cells (Fig. 6B). This difference is accounted for by the fact that mitosis is prolonged in the presence of ZM447439 (Fig. 6C). Hela cells contain wild-type RAS genes. HCT116 colon cancer cells, containing an activated K-RAS, spent the same amount of time in interphase in the presence or absence of ZM447439, despite being unable to complete cytokinesis (Fig. 6D; our unpublished data). Furthermore, exposure of either Hela or HCT116 cells to ZM447439 did not increase cell death which was lower than 1% for both cell types during a 48 h treatment period (our unpublished data).

As a second test of the effect of ZM447439 on overall proliferation, we used methylene blue staining. Exposure of HCT116 cells lacking p53 to ZM447439 blocks them from dividing but does not appear to reduce their movement through the cell cycle. In the absence of division these cells grow much larger than normal

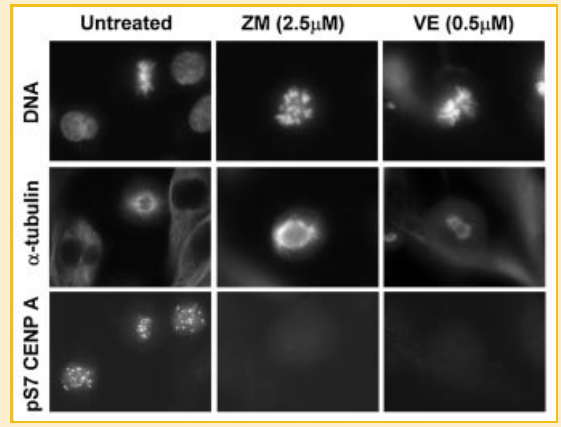

Fig. 5. Phosphorylation of Cenp-A at serine 7 in cells exposed to Aurora kinase inhibitors. HT1080 tumor cells were exposed to ZM447439 (ZM) or VE-465 (VE) for 18 h. Phosphorylation of S7 of Cenp-A was determined by immunofluorescence and examples of mitotic cells are shown. VE-465 suppressed microtubule staining, therefore a longer exposure was used to visualize the spindle. Otherwise, the exposures used to detect DNA were identical as were the exposures used to detect S7 Cenp-A.

(Fig. 7). In order to measure cell cycle progression under conditions where cell number does not increase, we stained cellular nucleic acids with methylene blue. Cells were plated in the presence of ZM447439, PD98059 or the DMSO vehicle and incubated for 4, 5, or 6 days. Exposing HCT116 to the MEK inhibitor PD98059 during the incubation period caused a ~2-fold reduction in methylene blue staining at 4 days after treatment, and ~3-fold reduction at 5 and

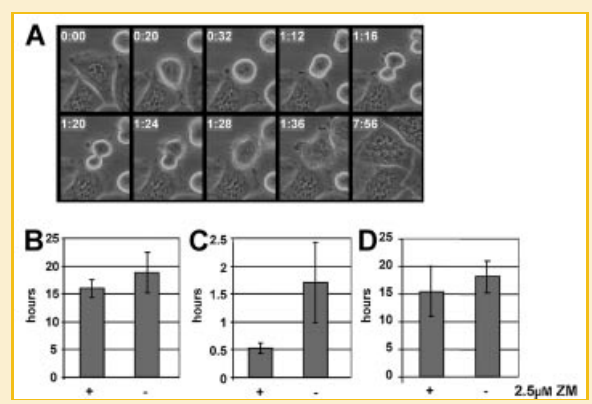

Fig. 6. Length of the cell cycle in cells exposed to ZM447439. Human tumor cells with or without activated RAS were exposed to ZM447439 (2.5 μM) and followed by time-lapse microscopy. Example of a Hela cells attempting mitosis in the presence of the drug is shown in (A). B: Effect of ZM447439 on the length of interphase in Hela cells. Hela cells that attempted mitosis twice during the filming period were used to measure the interval between attempts (i.e., interphase). C: Length of mitosis. The length of mitosis in Hela cells in the presence or absence of ZM447439 was analyzed by time-lapse phase-contrast microscopy. D: Length of interphase in HCT116 cells. Time-lapse analysis was used to measure the length of interphase in HCT116 cells lacking p53 in the presence and absence of ZM447439. Average and standard deviations are shown, and all movies were run twice with similar results.

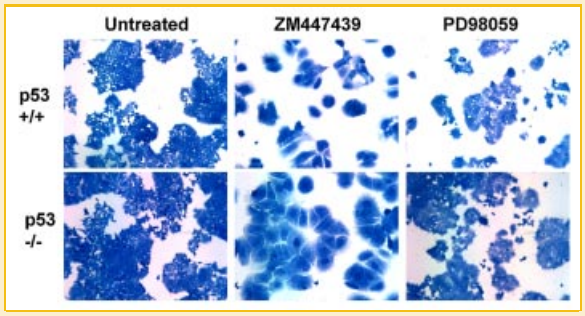

Fig. 7. Giant cell formation in response to ZM447439. HCT116 cells with or without p53 were exposed to ZM447439 (2.5 μM), PD98059 (20μM) or an equal volume of DMSO (untreated) for 4 days. Cells were stained with methylene blue and photographed. Formation of giant cells indicates continued progression through the cell cycle without successful cytokinesis. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

6 days after treatment (Fig. 8). This effect occurred regardless of p53 status. This indicates that inhibiting the RAS-MAPK pathway in HCT116 cells does reduce expansion of the cell culture, presumably as a result of reduced cell cycle progression and possibly increased apoptosis. In cells with functional p53, ZM447439 treatment reduced methylene blue staining to a similar extent as PD98059 at 4 and 5 days after treatment (Fig. 8). By 6 days, ZM447439 reduced methylene blue staining below the level induced by PD98059.

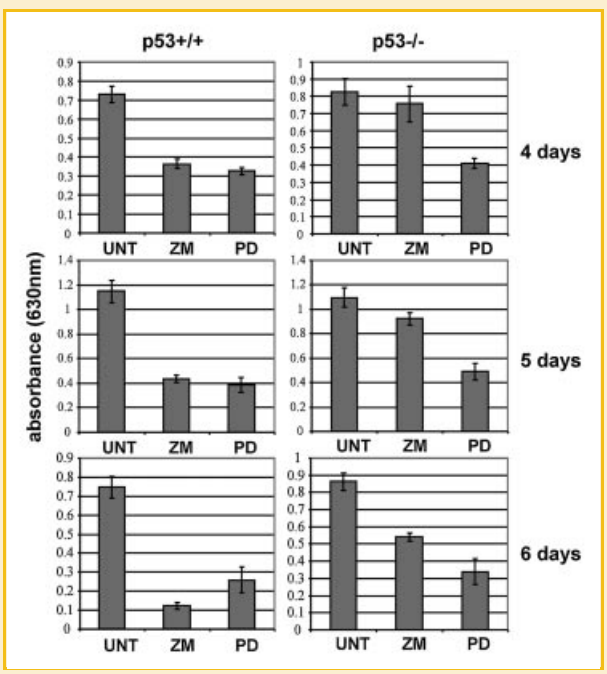

Fig. 8. Effect of ZM447439 and PD98059 on cell cycle progression. HCT116 cells with or without p53 were exposed to ZM447439 ("ZM" 2.5 μM), PD98059 ("PD" 25 μM) or an equal volume of DMSO ("UNT") for 4, 5, or 6 days. Cells were stained with methylene blue and the amount of dye retained by the cells was quantified by spectrophotometry. Averages and standard deviations are shown.

Interestingly, in HCT116 cells lacking p53, ZM447439 had minimal effects on methylene blue staining at 4 and 5 days after treatment (Fig. 8). Thus, HCT116 lacking p53 exposed to ZM447439 accumulate cellular contents at a rate similar to untreated cells at least for 5 days. By 6 days of treatment, methylene blue staining in the ZM447439-treated p53-null cells was lower than untreated cultures, but still not as low as that observed in cells exposed to PD98059 (Fig. 8). Exposure of HCT116 cells to ZM447439 induces DNA damage and upregulates p53 explaining the reduction in methylene blue staining over time in the cells with intact p53 (our unpublished data). Together, the lack of effect of ZM447439 on methylene blue staining in p53-null cells suggest that Aurora A and B do not play a critical role in cell proliferation or survival in human tumor cells with an activated *ras* allele.

POLYPLOIDY AND TRANSFORMATION BY RAS

Our studies suggested that Aurora B did not cooperate with RAS by regulating the RAS/MAPK pathway. We considered another possibility based on the fact that high levels of Aurora B can cause cytokinesis failure and that the tetraploid state has been linked to neoplastic transformation [Meraldi et al., 2002; Fujiwara et al., 2005]. Together, these observations suggested that transfection of cells with Aurora B might induce cells to become tetraploid which might enhance transformation by activated RAS. In order to test this idea we transfected 10T1/2 mouse fibroblast cells with activated RAS followed by treatment with cytochalasin-D to inhibit cleavage and induce binucleation. Plates treated with cytochalasin-D formed fewer foci than those transfected solely with RAS (Fig. 9A,B). Next, we pretreated cells for 18 h with cytochalasin-D or blebbistatin, a myosin inhibitor. Under these conditions, cytochalasin D caused 83% of 10T1/2 cells to become binucleated while blebbistatin induced 76% to become binucleated (1% of untreated 10T1/2 cells are binucleated; see Fig. 10 for examples). The drugs were removed and cultures allowed to recover for 1 week. Next, cells were trypsinized, counted, plated and transfected with activated RAS. Cells pretreated with blebbistatin formed the same number of foci after transfection with RAS, while cytochalasin-D treated cells formed fewer foci than untreated cells (Fig. 9C). 10T1/2 cells normally contain ~80 chromosomes, twice the normal diploid number. Although cytochalasin D and blebbistatin cause a relative doubling of chromosome number, the fact that these cells were originally tetraploid might have influenced our observations. Therefore, focus forming assays were repeated in the hyperdiploid cell line REF52 (average of 56 chromosomes/cell) [Stiegler et al., 1997]. REF52 cells behave very similarly to normal fibroblasts in at least two respects: (i) they require a cooperating oncogene to be transformed by Ras, (ii) they are unable to generate resistance to PALA, a feature closely associated with the normal functioning of p53-dependent pathways for growth arrest [Franza et al., 1986; Perry et al., 1992]. In three trials, we observed that Ras was unable to transform REF52 whether or not they were pretreated with either blebbistatin or cytochalasin D (for examples see Fig. 9). These results suggest that polyploidy induced by cytokinesis failure does not enhance transformation by activated RAS in a cell line permissive for transformation (10T1/2) nor does it render a nonpermissive cell line (REF52) susceptible to RAS transformation.

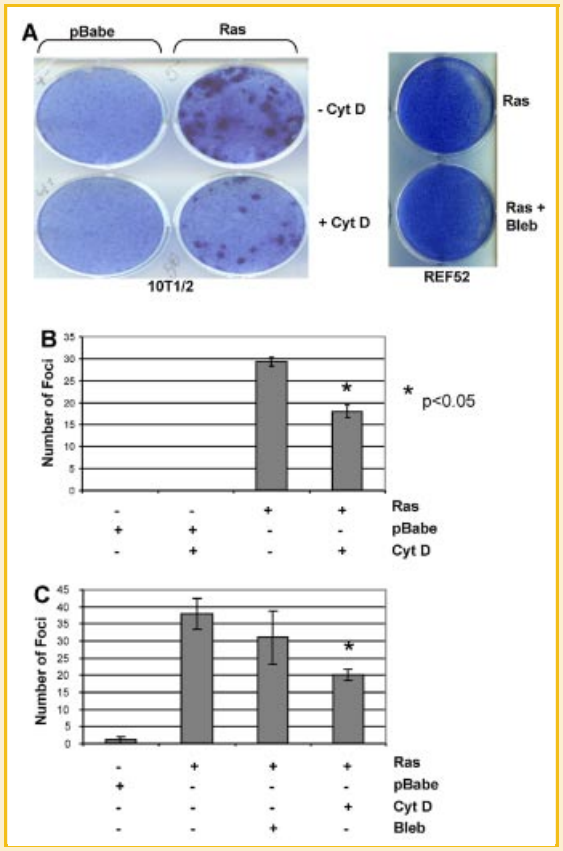

Fig. 9. Effect of tetraploidy on oncogenic transformation. 10T1/2 mouse fibroblast cells or REF52 fibroblasts were transfected with either pBabe-puro (plasmid control) or pH06Ti (T24-RAS) and foci were visualized by staining with methylene blue. Transfections were carried out in triplicate and repeated at least once. A: Foci formed in response to RAS. Typical foci that form after transfecting RAS into 10T1/2 cells are shown. Failure of RAS to transform REF52 cells is documented. B: Suppression of RAS transformation by treatment with cytochalasin-D. Cells were transfected with RAS, and then exposed to cytochalasin-D for 24 h. Cytochalasin-D was removed and plates were incubated for 2 weeks to allow foci to form. C: Suppression of RAS transformation in populations pretreated with cytochalasin-D or blebbistatin. 10T1/2 cells were exposed to cytochalasin-D or blebbistatin for 24 h, drugs removed and cells allowed to recover for 1 week. Populations were then replated and transfected with RAS. Foci were stained 2 weeks later. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

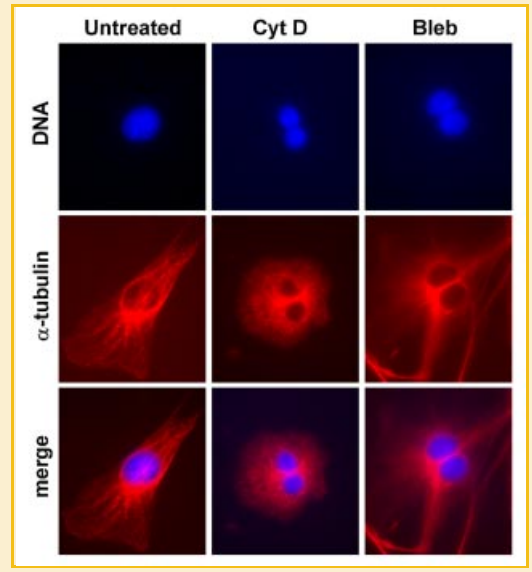

Fig. 10. Creation of binucleated cells by cytochalasin-D and Blebbistatin. Examples of a normal mononucleated cell in an untreated culture, as well as binucleated cells observed after exposure to cytochalasin-D or blebbistatin are shown. Cells were exposed to drugs for 18 h after which the drugs were removed for 2 h to allow cells to re-spread on coverslips before fixation. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

DISCUSSION

Recent publications suggest that, when overexpressed, Aurora kinases promote transformation by oncogenic RAS possibly by enhancing signaling and by converting cells with stable chromosome numbers to aneuploid cells [Kanda et al., 2005; Tatsuka et al., 2005]. In particular, *ras*-transfected cells with elevated levels of Aurora kinase formed more foci than cells transfected with *ras* alone. Also, short interfering RNA directed against Aurora B kinase reduced the number of foci formed in response to *ras* transfection but had no effect in cells transfected with *raf* [Kanda et al., 2005]. These observations were of significant interest given the fact that Aurora B is overexpressed in many types of human cancer [Giet et al., 2005; Sorrentino et al., 2005; Chieffi et al., 2006]. We used ZM447439, an inhibitor of Aurora A (IC_{50} 110 nM) and Aurora B (IC_{50} 130 nM) to analyze several signaling pathways activated by RAS. The fact that short interfering RNA directed against Aurora kinase had no effect on RAF-transformed cells suggested that Aurora B kinase might act upstream of RAF [Kanda et al., 2005]. Using ZM447439 to inhibit Aurora kinases, we observed that the phosphorylation of MEK1/2 and MAPK was unchanged. This was the case in human cells with activated K-RAS, in mouse NIH3T3 cells transformed with activated Ki-RAS and in nontransformed human epithelial cells in which RAS signaling was activated by EGF. RAS-transformed NIH3T3 cells did show a reduction in MAPK phosphorylation at a dose of ZM447439 that may be capable of directly inhibiting MEK1. The fact that the phosphorylation of neither MAPK or MEK1/2 was altered by a second inhibitor, VE-465, suggests that Aurora kinases are not required for the activation of the MAPK pathway by RAS. VE-465 is a structural analogue of MK-0457/VX-680. MK-0457 inhibits Aurora A (IC_{50} 0.6 nM), Aurora B (IC_{50} 18 nM) and Aurora C (IC_{50} 4.6 nM). Under the conditions of our assays, the phosphorylation of the Aurora kinase substrates histone H3 and Cenp-A were greatly reduced by the inhibitors used. Based upon these observations, we feel confident in stating that Aurora kinases were inhibited under the conditions we have used. It is conceivable that a small residual activity of Aurora kinases remained in the inhibitor-treated cells. Either way, if Aurora kinases were to play a major role in RAS-MAPK signaling, one would expect some reduction in these pathways under conditions where two other Aurora substrates were no long being phosphorylated. These observations argue that Aurora kinases do not play a major role in RAS-MAPK signaling.

We analyzed one additional pathway activated by RAS. RAS causes changes in the cytoskeleton in part by activating the small GTPases RHO, RAC, and CDC42. We analyzed CDC42 activity indirectly by measuring the phosphorylation of MLK3, a downstream target. Inhibiting Aurora kinases had no effect on the phosphorylation of MLK3 suggesting that they are not needed for this pathway. RAS activates many pathways that we have not directly analyzed. If Aurora kinases cooperate with RAS in cellular transformation by participating in RAS signaling pathways it is likely that those pathways would have some biological effect consistent with transformation. RAS-dependent pathways are well known to induce cell cycle progression (for example by upregulating D-type Cyclins) and suppress apoptosis (for example by inducing NFκB). We have used time-lapse microscopy to analyze both cell death and cell cycle progression in human tumor cells with and without activated RAS. Exposure of either Hela or HCT116 cells to ZM447439 effectively blocked cytokinesis but had no effect on the length of interphase, and did not induce apoptosis. Furthermore, in p53-null cells, the total increase in cellular nucleic acids, as measured by staining with methylene blue, was unaffected by ZM447439 under conditions where cytokinesis was blocked. In contrast, inhibiting the RAS-MAPK pathway with the MEK1 inhibitor PD98059 reduced the accumulation of nucleic acids. This suggests that in a cell line where proliferation is dependent on constant signaling via the RAS-MAPK pathway, inhibiting Aurora kinase activity does not affect movement through the cell cycle. These observations argue against a major role of Aurora kinase activity in RAS-signaling pathways that control cell cycle progression or cell death.

Another possible explanation for how Aurora B cooperates with RAS was provided by observations linking polyploidy with neoplastic transformation. Binucleated mammary epithelial cells created by treatment with cytochalasin-B, were separated and then exposed to carcinogen in vitro [Fujiwara et al., 2005]. These cells became capable of forming colonies in soft agar with high efficiency [Fujiwara et al., 2005]. Inducing tetraploidy with cytochalasin-D without exposure to carcinogen was enough to give rise to malignancies in mice injected subcutaneously [Fujiwara et al., 2005]. Overexpression of Aurora kinases can disrupt chromosome segregation leading to the production of aneuploid cells [Meraldi et al., 2002]. This suggested that Aurora kinase might enhance RAS transformation because its overexpression leads to chromosome instability and aneuploidy. We tested this hypothesis using a focus formation assay. We transfected 10T1/2 cells activated *H-ras* and monitored focus formation in cells treated with cytochalasin-D. In one type of experiment, Cytochalasin-D was added to the cells a day after transfection with *ras*. We found that treatment with cytochalasin-D resulted in the formation of fewer foci in response to RAS when compared to transfected cells that were not exposed to cytochalasin-D. In a second type of experiment, 10T1/2 cells were exposed to either cytochalasin-D or blebbistatin for 18 h, and then allowed to recover for 1 week. When these populations were transfected with RAS, we observed no effect of blebbistatin on focus formation, whereas cytochalasin-D reduced focus formation by RAS. Furthermore, neither blebbistatin nor cytochalasin-D were able to allow RAS to transform REF52 cells, a cell line that can be efficiently transformed by RAS only in the presence of a second cooperating oncogene. Thus, tetraploidy/polyploidy on its own does not enhance neoplastic growth induced by activated RAS.

It is not clear why cytochalasin-D suppresses transformation by RAS, whereas it can induce tumor formation on its own. It is possible that when added alone, cytochalasin-D produces a population of genetically heterogeneous cells. Most cells in this population may be less susceptible to neoplastic transformation when confronted with an activated oncogene. Along these lines, some tetraploid cells are predisposed to apoptosis. However, there may be rare variants in the population that are capable of forming a tumor when injected into mice. In our studies, cytochalasin-D reduced transformation by RAS, an effect that was not observed with blebbistatin. Cytochalasin-D inhibits actin polymerization while blebbistatin inhibits non-muscle myosin II. Although these drugs have different targets, this is unlikely to explain the suppression of transformation by cytochalasin-D, since this effect persisted even when cells were transfected 1 week after removing the drugs. What is clear however, is that the tetraploid state induced by either drug does not enhance transformation suggesting that Aurora does not cooperate with RAS by causing cytokinesis failure.

Previous work in cells transfected with Aurora kinase cDNAs implicated Aurora B in RAS signaling. Our studies with Aurora inhibitors indicate that endogenous levels of Aurora kinases do not participate in RAS signaling. Our experiments do not directly address the effect of high levels of Aurora kinases which might be achieved during transient transfection. Aurora kinases are known to be overexpressed in tumor cells. Consistent with this trend, we find that the fibrosarcoma-derived cell line HT1080 expresses much higher levels of Aurora B compared to normal diploid cell strain WI38. Inhibition of Aurora kinases in HT1080 failed to alter MAPK phosphorylation suggesting that the elevated levels of Aurora B in this cell line do not contribute to RAS-MAPK signaling. We favor an alternative hypothesis to explain cooperation between Aurora B and RAS in transformation. High levels of Aurora B may drive parallel pathways which enhance transformation by RAS. These pathways may be initiated by physiological substrates of Aurora B, or other substrates that are only phosphorylated when very high levels of Aurora B have accumulated. The identification of substrates phosphorylated in normal cells and cancer cells with high levels of Aurora B will help to resolve this question.

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