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Inhibition of Aurora B Kinase Blocks Chromosome Segregation, Overrides the Spindle Checkpoint, and Perturbs Microtubule Dynamics in Mitosis

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Summary

How kinetochores correct improper microtubule attachments and regulate the spindle checkpoint signal is unclear. In budding yeast, kinetochores harboring mutations in the mitotic kinase Ipl1 fail to bind chromosomes in a bipolar fashion. In *C. elegans* and *Drosophila*, inhibition of the Ipl1 homolog, Aurora B kinase, induces aberrant anaphase and cytokinesis. To study Aurora B kinase in vertebrates, we microinjected mitotic XTC cells with inhibitory antibody and found several related effects. After injection of the antibody, some chromosomes failed to congress to the metaphase plate, consistent with a conserved role for Aurora B in bipolar attachment of chromosomes. Injected cells exited mitosis with no evidence of anaphase or cytokinesis. Injection of anti-Xaurora B antibody also altered the microtubule network in mitotic cells with an extension of the astral microtubules and a reduction of kinetochore microtubules. Finally, inhibition of Aurora B in cultured cells and in cycling *Xenopus* egg extracts caused escape from the spindle checkpoint arrest induced by microtubule drugs. Our findings implicate Aurora B as a critical coordinator relating changes in microtubule dynamics in mitosis, chromosome movement in prometaphase and anaphase, signaling of the spindle checkpoint, and cytokinesis.

Results and Discussion

The Aurora kinases are conserved regulators of mitosis and cytokinesis (for reviews, see [1–4]). Budding yeast have a single isoform, Ipl1 (Increase in ploidy-1) [5]. Recent studies suggest that Ipl1 may be involved in sensing and releasing inappropriate kinetochore-microtubule attachments [6, 7]. Metazoans have two ubiquitous Aurora kinase isoforms, Aurora A, which localizes mainly to centrosomes, and Aurora B, which associates with the inner centromere in prometaphase and metaphase.

To investigate the function of the vertebrate Aurora B kinase during mitosis, we microinjected function-blocking anti-Xaurora B antibody into *Xenopus* tissue culture cells (XTC). (For antibody characterization, see [8] and Figures S1 and S2 in the Supplementary Material available with this article online.) In mitotic cells injected with anti-Xaurora B antibody ($n = 15$), specific defects occurred (Figure 1A; Table 1; and Movies 1–3 in the Supplementary Material available with this article online). Some chromosomes near the spindle poles failed to achieve metaphase alignment. The injected cells also prematurely exited mitosis in the presence of unaligned chromosomes without detectable initiation of anaphase (Figure 1A). Although cells underwent the surface cytoplasmic blebbing characteristically seen during normal anaphase, no initiation of a cytokinetic furrow was observed, and cells reformed polyploid interphase nuclei. Cells injected with anti-Xaurora B remained in M phase for 77.0 ± 17.5 min, (range 48–100 min), which is not significantly longer than the times measured for cells injected with control IgG (61.7 ± 7.5 min; range 50–71 min; $n = 8$; $p = 0.076$) or for noninjected cells (62.8 ± 13.9 min; range 42–80 min; $n = 10$; $p = 0.151$).

Normally, cells exit M phase through ubiquitin-mediated proteolysis of mitotic regulators. This step is inhibited by the spindle checkpoint in cells with improper microtubule-kinetochore attachments (reviewed in [9]). XTC cells were microinjected with anti-Xaurora B antibody in the presence of MG132, a potent inhibitor of the 26S proteasome, and were followed by time-lapse microscopy. All of the injected cells ($n = 8$) remained arrested in M phase for at least 4 hr (Figure 1B, Table 1). This result indicates that inhibition of Aurora B antibody induces M phase exit by overriding the spindle checkpoint.

Because XTC cells round up in prolonged M phase, chromosome orientation in the living cells in MG132 could not be observed. Another group of mitotic cells ($n = 30$) was injected with anti-Xaurora B antibody in the presence of MG132. The cells were incubated for 4 hr and were fixed, and the chromatin was stained with DAPI. A total of 23 out of 30 cells injected (77%) at early mitosis contained multiple unaligned chromosomes after ~4 hr in MG132 (Table 1). In nearly every uninjected mitotic cell, all chromosomes were aligned at the metaphase plate. Thus, failure of chromosomes to align in cells injected with anti-Xaurora B antibody was not due simply to the accelerated exit from M phase. Instead, Aurora B is required for the proper movement of chromosomes to the metaphase plate.

While the injection of anti-Xaurora B into normal mitotic XTC cells stranded many chromosomes near the poles, such cells also showed many chromosomes near the metaphase plate. We suspect that, because our injections were performed on prophase and prometaphase cells, the antibodies may not have had sufficient time to inhibit Aurora B kinase activity before many chromosomes were bipolarly attached. We sought to experimentally increase the pool of mono-oriented chromosomes that would need to reorient after injection of cells

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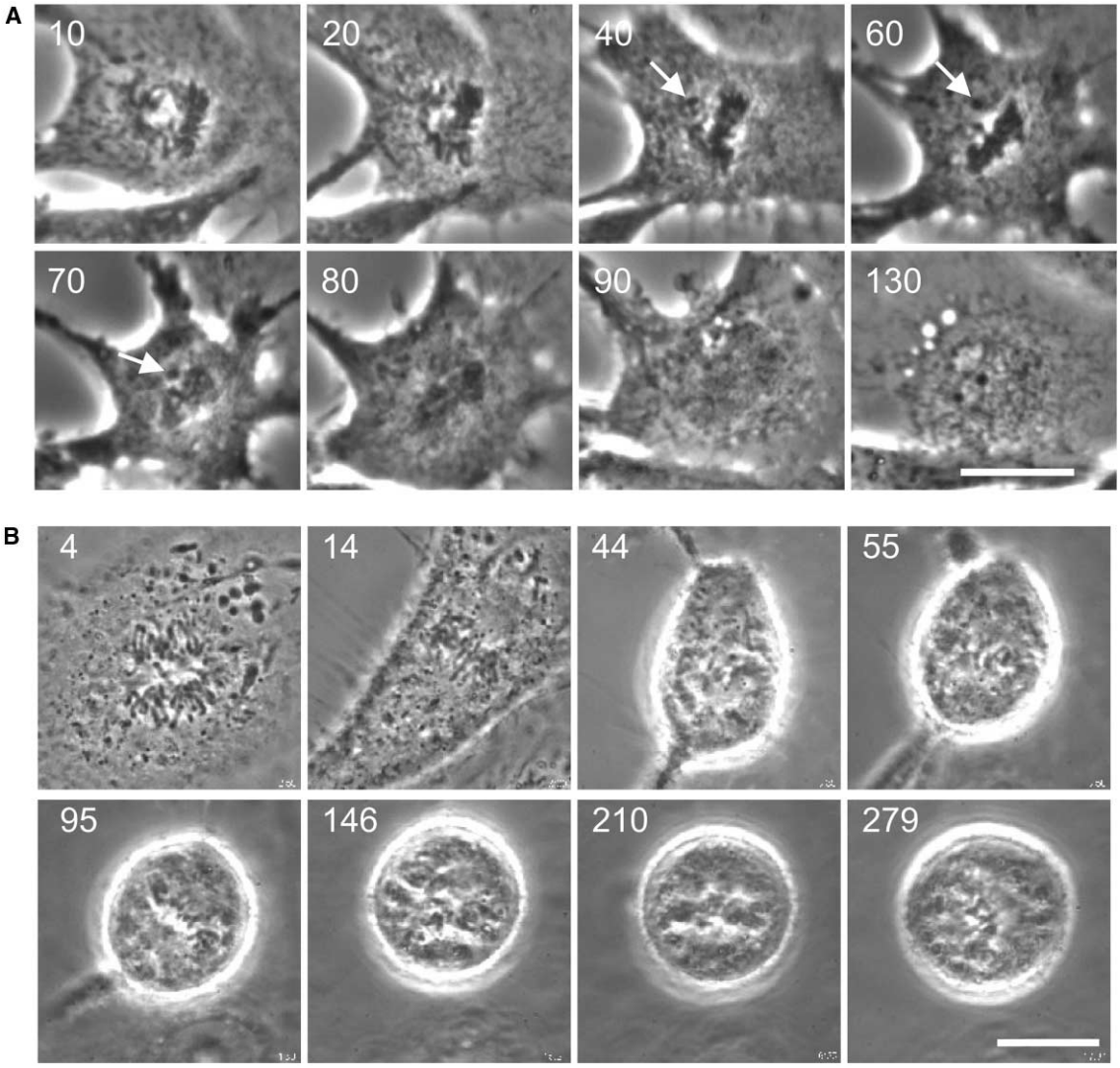

Figure 1. Microinjection of Mitotic XTC Cells with Anti-Xaurora B Antibody Abrogates the Spindle Checkpoint, Causing Premature Exit from M Phase without Anaphase and Cytokinesis

(A) An XTC cell injected at late prophase using a needle containing 8.0 mg/ml anti-Xaurora B antibody (time point of 0 min). Some of the chromosomes located near the spindle poles failed to move to the metaphase plate (arrow). The chromosomes began to decondense at ~80 min postinjection. The cell then exited M phase without evidence of anaphase chromosome movement. No cytokinesis occurred, and the cell formed a polyploid progeny cell with a fragmented nucleus. A QuickTime movie (Movie 1) corresponding to the still images of Figure 1A is included in the Supplementary Material available with this article online. Additional examples of injected cells (Movies 2 and 3) displaying the effects of anti-Xaurora B antibody microinjection are also available as Supplementary Material.

(B) A time-lapse video sequence of a cell treated with the proteasome inhibitor MG132 (25 μM; added 30 min before injection), then injected with anti-Xaurora B antibody. In the continued presence of MG132, anti-Xaurora B antibody does not induce exit from M phase for the duration of the experiment (~280 min). Thus, proteasome activity is required for mitotic exit when Aurora B kinase is inhibited. The numbers indicate minutes after the injection.

The scale bars represent 10 μm.

with anti-Xaurora B antibody. Thus, we first treated cells with nocodazole to disrupt the mitotic spindle, injected them with anti-Xaurora B antibody, then washed out the nocodazole but left the cells in the proteasome inhibitor MG132 to block exit from mitosis. A total of 120 min after the removal of nocodazole, the cells were fixed and the chromosomes were visualized. Of 27 cells so treated, 24 (89%) retained multiple mono-oriented chromosomes at the poles (Figure 2, Table 1). On the same coverslips, at the end of the experiment, only 6 of the 50 (12%) noninjected mitotic cells contained chromosomes that were not aligned at the metaphase plate. We conclude that inhibiting vertebrate Aurora B kinase interferes with the ability of chromosomes to align properly. In the cells injected with anti-Xaurora B antibody, some chromosomes were found to have congressed to the spindle midplane. This observation may be due to incomplete inactivation of kinase activity in some injected

Table 1. Cell Division Effects Induced by Microinjection of Anti-Xaurora B Antibody

Antibody	Treatment	Number of Cells Injected	Aberrant Exit from M Phase	Normal Exit from M Phase	Cells with Unaligned Chromosomes
Control IgG					
	none	8	0	8	0
Anti-Xaurora B					
	none	15	15	0	13
	MG132	38	0	0	23[a]
	nocodazole	12	7[b]	0	–
	taxol	26	15[b]	0	–
	noc/MG132→MG132[c]	27	0	0	24[d]

[a] Number of cells with unaligned chromosomes after 4 hr in MG132.
[b] Number of cells exiting M phase within 140 min after anti-Xaurora B antibody injection.
[c] Cells were treated with 400 nM nocodazole and 25 μM MG132 for 5–60 min before the microinjection after which nocodazole, but no MG132, was removed by extensive washes.
[d] Number of cells with unaligned chromosomes 80–120 min after nocodazole wash out.

cells. Alternatively, it is consistent with the idea that Aurora B kinase is not strictly required for chromosome movement of bipolarly attached chromosomes or that redundant systems may participate in moving chromosomes to the metaphase plate [10, 11].

To test whether the inhibition of Aurora B kinase affected mitotic spindle structure, we injected cells with anti-Xaurora B antibody, incubated cells at room temperature for a short time, lysed and fixed cells, and then labeled them for microtubules. Most cells showed

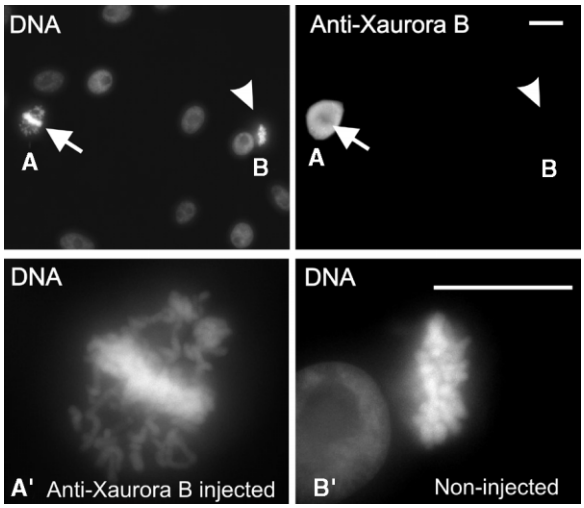

Figure 2. Introduction of Anti-Xaurora B Antibody into Mitotic XTC Cells Inhibits Chromosome Alignment

Mono-oriented chromosomes induced by release from nocodazole fail to congress to the metaphase plate if Aurora B is inhibited. Mitotic XTC cells were treated with nocodazole and MG132 and were then injected with anti-Xaurora B antibody. The nocodazole was washed out to allow reassembly of the spindle, but MG132 was retained to prevent cells from exiting mitosis. Two hours after washout of the nocodazole, cells were fixed and labeled for DNA with fluorescent secondary antibody to identify injected cells. In cells injected with anti-Xaurora B antibody (arrows and [A′]), many chromosomes failed to congress to the metaphase plate. In uninjected cells (arrowheads and [B′]), all chromosomes congressed to the metaphase plate. Images in (A′) and (B′) are maximum projections of through focus series collected through the entire cell. The scale bars represent 10 μm.

significant extension of the astral fiber microtubules even when fixed before the injected antibody had caused them to exit M phase. To test whether the extension of the astral microtubules might simply be an early consequence of driving cells out of M phase as a result of Aurora B kinase inhibition, we injected cells in early mitosis and maintained them in proteasome inhibitor MG132 to block anaphase onset and mitotic exit. Again, most cells, even those with most chromosomes near the spindle equator, showed elaboration of the astral array (Figure S3A). In some cases, the entire cytosol was filled with extended curving microtubules (Figure S3B). To study microtubule effects in cells with many monopolar chromosomes retained as a consequence of Aurora B inhibition, we again treated cells with nocodazole, injected them with anti-Xaurora B antibody, and then washed the cells free of nocodazole into medium containing MG132. This approach again allowed us to inhibit Aurora B kinase before the formation of the spindle and resulted in a greater proportion of mono-oriented chromosomes. Over 50% of the mitotic spindles of the treated cells revealed marked extensions of the cytoplasmic astral microtubule array (Figures 3C and S3D). None of the uninjected cells or cells injected with control IgG were similarly affected.

During the studies above, we noted that, in addition to the extension of the astral array, many cells injected with anti-Xaurora B antibody appeared to have greatly reduced kinetochore fibers. To test whether anti-Xaurora B antibody affected the microtubule stability of kinetochore-attached microtubules, we used the method of inducing preferential disassembly of non-kinetochore-bound microtubules by cell lysis in ice-cold buffer containing 80 μM calcium [12]. In cells injected with anti-Xaurora B antibody, kinetochore fiber bundles were significantly reduced in comparison to cells injected with nonimmune IgG (Figures S4A and S4B). Moreover, nine out of ten cells injected with anti-Xaurora B antibody retained long microtubules in the cytoplasm outside the central spindle, suggesting that some cytoplasmic microtubules were also unusually stable (Figure S4A). Together, all these studies indicate that inhibition of Aurora B kinase leads to alterations in mitotic microtubule dynamics with an elaboration of astral arrays, a marked decrease in

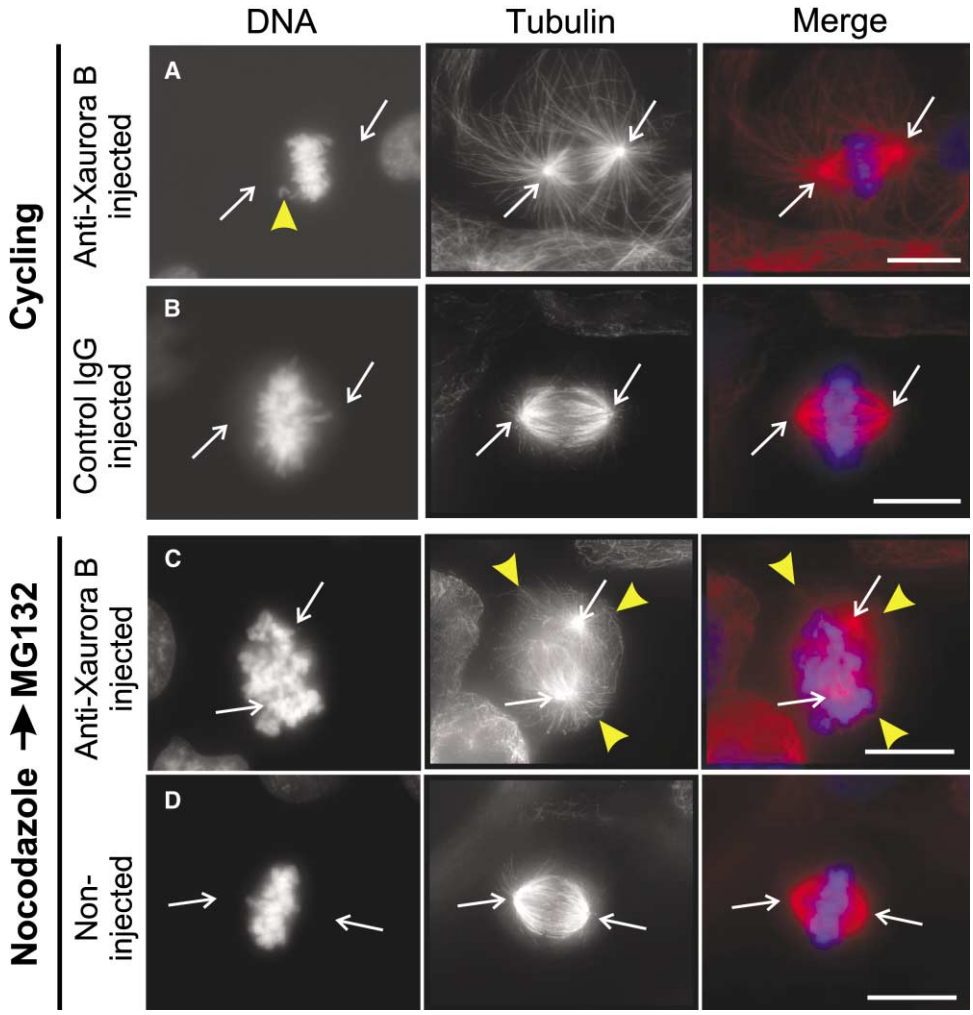

Figure 3. Inhibition of Aurora B Kinase Induces Changes in the Microtubule Cytoskeleton in Mitotic Cells

XTC cells were injected with anti-Xaurora B antibody or control IgG, incubated for 30 min, lysed, fixed, and labeled with anti-tubulin antibody.

(A) Cells injected with anti-Xaurora B antibody revealed enhanced astral microtubules. Because this cell was injected in prometaphase after many chromosomes had already achieved bipolar attachment, most of the chromosomes have successfully congressed to the metaphase plate. However, at least one chromosome remained off the plate at the time of fixation (yellow arrowhead).

(B) Cells injected with nonimmune IgG show typical short astral microtubules.

(C) In another experiment, cells were treated with nocodazole, injected with anti-Xaurora B antibodies, and incubated for 10–30 min. The cells were extensively washed to remove nocodazole and were further incubated in the presence of the proteasome inhibitor MG132 for 60 min. The cells were then detergent lysed, fixed, and labeled for DNA and tubulin. An anti-Xaurora B antibody-injected cell shows many mono-oriented chromosomes, particularly on all sides of the lower pole. At the same time, it shows extension of the astral/cytoplasmic microtubules into regions of the cytoplasm beyond the chromosomes (yellow arrowheads).

(D) An uninjected cell on the same coverslip shows alignment of all chromosomes and short astral microtubules. A second example of a cell from this experimental series is available in Figure S3D. The images are maximum projections of through focus series collected through the entire cell. Merged images of DNA (blue) and microtubules (red) are shown in color. The open white arrows denote the locations of the spindle poles.

The scale bars represent 10 μm.

kinetochore fiber microtubules, and an increase in the stability of some cytoplasmic microtubules. No effects on the microtubule networks of interphase cells were detected after microinjection (data not shown).

We speculate that Aurora B might regulate the dynamics of microtubules in mitosis either by promoting the catastrophe rate or by activating a microtubule-severing activity. In the cytoplasm, Aurora B might normally induce the formation of the short astral microtubules characteristic of cells in mitosis. By regulating microtubule instability at kinetochores, Aurora B may also play an essential role in regulating the end-on attachment of kinetochores to microtubules that is essential for bipolar orientation.

Studies in yeast suggest that inhibition of the Ipl1 kinase abrogates the spindle checkpoint when it is induced by mono-oriented chromosomes, but not when it is induced by spindle damage [6]. However, we found that, for cells incubated in either nocodazole ($n = 12$) or taxol ($n = 26$), 58% of cells in each case exited M phase prematurely (Table 1). For four cells incubated in taxol and continuously monitored, cells exited M phase at 81,

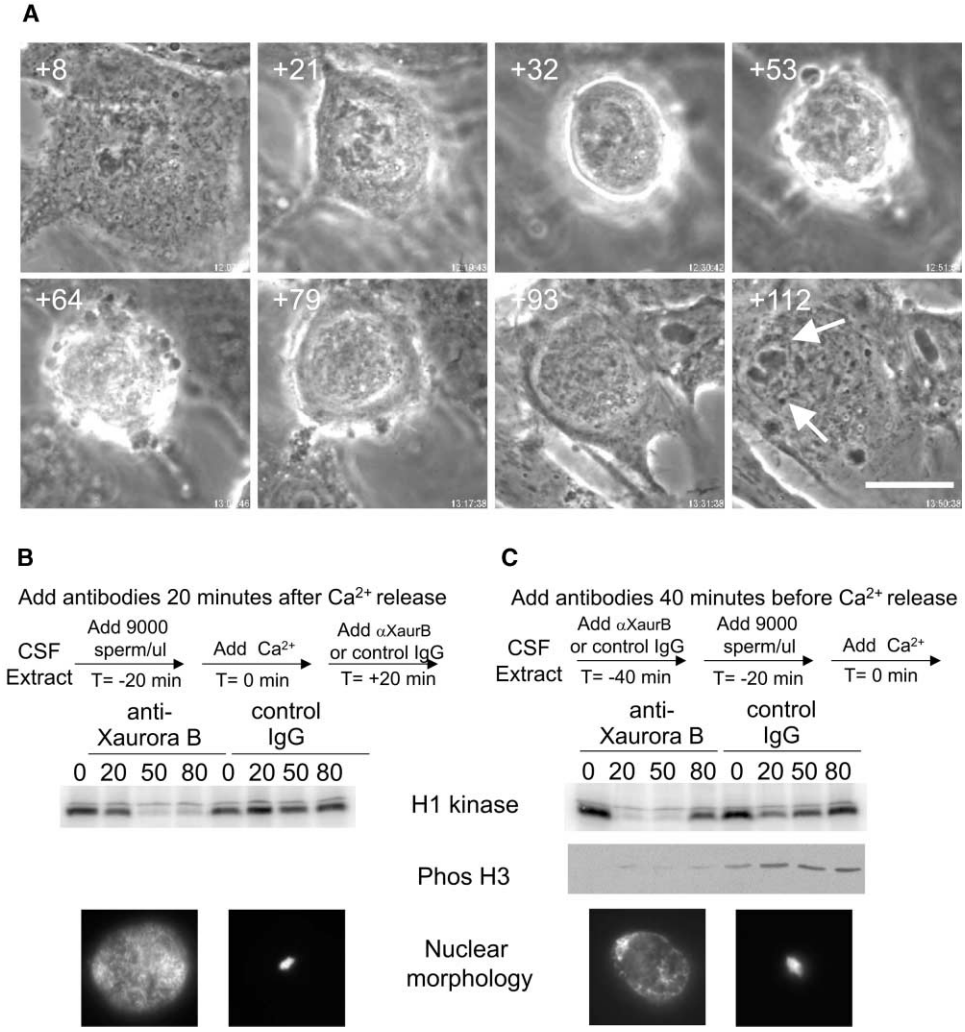

Figure 4. Aurora B Kinase Is Required for Spindle Checkpoint Signaling in Response to Microtubule Drugs in Living XTC Cells and in *Xenopus* Extracts

(A) A mitotic XTC cell that was treated with taxol and injected with anti-Xaurora B antibody (5.0 mg/ml in the microinjection needle) in early prometaphase (t = 0). After a temporary arrest during which the cell became rounded, the cell exited M phase, flattened onto the substratum, and reconstituted a polyploid interphase nucleus (arrows) with a prominent nucleolus. The scale bar represents 10 μm. Numbers indicate minutes after microinjection. A QuickTime movie (Movie 4) corresponding to another cell treated similarly is available in the Supplementary Material available with this article online.

(B) Inhibition of Aurora B kinase blocks maintenance of the spindle checkpoint in *Xenopus* extracts. Sperm and nocodazole were added to cytostatic factor (CSF) extracts to activate the spindle checkpoint. To inactivate CSF, calcium was added. Then, 20 min later, anti-Aurora B antibody or control IgG were added. Extract receiving anti-Xaurora B antibody did not maintain the checkpoint and exited M phase. Extract receiving control IgG remained arrested in M phase.

(C) Inhibition of Aurora B kinase blocks the establishment of the spindle checkpoint in *Xenopus* extracts. Anti-Xaurora B antibody or control IgG (final concentration 0.8 mg/ml) were added to unactivated egg extracts (CSF extracts). Then, sperm and nocodazole were added to initiate the spindle checkpoint signal. Finally, calcium was added to inactivate CSF. In extracts to which anti-Aurora B was added, the spindle checkpoint is not activated and the extract exits M phase, as indicated by the decline of H1 kinase activity and the decondensed interphase nuclear morphology. The phosphorylation of H3 at serine 10, a marker for Aurora B kinase activity, is inhibited in the extract supplemented with anti-Xaurora B antibody (Phos H3). With control IgG, the extracts remain in M phase with high H1 kinase activity, high Aurora B kinase activity against H3, and condensed sperm chromatin.

83, 113, and 134 min after microinjection (Figure 4A, Movie 4). Thus, unlike Ipl1 in budding yeast, Aurora B kinase is required for maintenance of the spindle checkpoint in mitotic vertebrate cells with disrupted spindles.

To determine if Aurora B is also required to maintain the spindle checkpoint once it is established, we turned to *Xenopus* extracts [13]. Anti-Xaurora B antibody was added to extracts that had been previously arrested in mitosis by the spindle checkpoint for 20 min (Figure 4B). A total of 30 min after adding the anti-Xaurora B antibody, the extracts had exited mitosis, while extracts to which control IgG had been added remained checkpoint arrested. If Aurora B antibodies were added before the spindle checkpoint was initiated, the extracts could

also not maintain a mitotic arrest (Figure 4C). These findings indicate that Aurora B is required to both establish and maintain the spindle checkpoint in response to a lack of microtubule attachment by the kinetochore.

Here, we provide evidence for versatile roles for the Aurora B kinase in both structural and regulatory events of mitosis. Previous reports in *C. elegans*, *Drosophila*, and in mammalian Mv1Lu cells suggested that compromising Aurora B function results in incomplete anaphase chromosome segregation and in abortive cytokinesis [14–19]. In contrast, our approach, inhibiting Aurora B by microinjection of function-blocking antibody, resulted in the total absence of detectable anaphase and cytokinesis. In addition, we found that normal microtubule dynamics, chromosome movements, and the spindle checkpoint are profoundly disturbed in mitotic cells injected with anti-Xaurora B antibody.

How kinetochores sense microtubule attachment and initiate the spindle checkpoint signal is still mysterious. Our results suggest that Aurora B regulates progression from monopolar to bipolar attachment of chromosomes and is required to send the checkpoint signal. We propose that Aurora B is a critical mitotic coordinator of several events in mitosis, such as linking microtubule dynamics to the proper attachment of kinetochores, the movements of chromosomes in prometaphase and anaphase, and the regulation of the spindle checkpoint and cytokinesis.

Supplementary Material

Supplementary Material including Movies 1–4, Figures S1–S4, and the Experimental Procedures is available at http://images.cellpress.com/supmat/supmatin.htm.

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