Aurora B kinase and protein phosphatase 1 have opposing roles in modulating kinetochore assembly

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The outer kinetochore binds microtubules to control chromosome movement. Outer kinetochore assembly is restricted to mitosis, whereas the inner kinetochore remains tethered to centromeres throughout the cell cycle. The cues that regulate this transient assembly are unknown. We find that inhibition of Aurora B kinase significantly reduces outer kinetochore assembly in Xenopus laevis and human tissue culture cells, frog egg extracts, and budding yeast. In X. laevis M phase extracts, preassembled kinetochores disassemble after inhibiting Aurora B activity with either drugs or antibodies. Kinetochore disassembly, induced by Aurora B inhibition, is rescued by restraining protein phosphatase 1 (PP1) activity. PP1 is necessary for kinetochores to disassemble at the exit from M phase, and purified enzyme is sufficient to cause disassembly on isolated mitotic nuclei. These data demonstrate that Aurora B activity is required for kinetochore maintenance and that PP1 is necessary and sufficient to disassemble kinetochores. We suggest that Aurora B and PP1 coordinate cell cycle–dependent changes in kinetochore assembly through phosphorylation of kinetochore substrates.

Introduction

Kinetochore assembly onto centromeres during mitosis, where they coordinate the chromosome movements that segregate DNA replication products into two daughter cells. They also resolve improper microtubule attachments and generate spindle checkpoint signals. The inner kinetochore, which organizes the underlying chromatin, is generated from several proteins including centromere protein C (Cenp-C) and a modified nucleosome containing the histone H3 variant Cenp-A (Cse4 in yeast). The inner kinetochore proteins are constitutively associated with the centromere throughout the cell cycle. The outer kinetochore, which directly contacts and regulates microtubules, is assembled onto the inner kinetochore during mitosis. In vertebrates, outer kinetochore proteins begin to assemble during prophase and kinetochores are fully assembled by early prometaphase.

The outer kinetochore proteins that assemble in mitosis are numerous. They include the KMN network that is believed to constitute the outer kinetochore core microtubule binding activity and contains the Knl1/Blinkin (Knl1) protein together with the hetero-tetrameric subcomplexes Mis12 (Mis12, Nsl1, Nnf1, and Dsn1) and Ndc80 (Ndc80, Nuf2, Spc24, and Spc25; Wigge and Kilmartin, 2001; Deluca et al., 2002, 2006; McCleland et al., 2003, 2004; Cheeseman et al., 2004, 2006; Obuse et al., 2004; Kline et al., 2006; Kiyomitsu et al., 2007). There are several microtubule motors and microtubule-binding proteins that play critical roles in chromosome alignment and segregation, including dynein/dynactin, Cenp-E, and Cep57 (Yen et al., 1991; Sharp et al., 2000; Emanuele and Stukenberg, 2007). Proteins required for spindle checkpoint signaling (Bub1, BubR1, Bub3, Mad1, Mad2, MPS1, Rod, Zw10, and Zwilch) also assemble onto kinetochores in prophase/prometaphase (for review see Musacchio and Salmon, 2007). The interdependencies of these proteins for assembly has been studied (for review see Maiato et al., 2004).

The effectors that trigger outer kinetochore assembly and disassembly at the beginning and end of mitosis remain unclear. Kinetochores begin to assemble in prophase when cyclin A/Cdk1 accumulates in the nucleus. Also in prophase, the Aurora B kinase, a member of a four-protein complex called the chromosome passenger complex, is activated and relocalizes from mitotic chromatin to the inner centromere. Aurora B has been implicated in kinetochore assembly. Overexpression of a kinase–dead Aurora B mutant displaces Cenp-E and dynein from kinetochores (Murata-Hori and Wang, 2002a). Treatment of HeLa cells with the small molecule inhibitor of Aurora B, X. leavis M phase extracts, and budding yeast.

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ZM447439, displaces the spindle checkpoint proteins BubR1 and Mad2 and the kinesin motor Cep152 (Ditchfield et al., 2003). Surprisingly, treatment with a second compound, hesperadin, or siRNA knockdown of Aurora B only affects BubR1 localization (Hauf et al., 2003; Liu et al., 2006). Yeast cells deficient for the Aurora B orthologue Ipl1 cannot localize the Dam1/DASH complex and Mad2 to centromeres but most other proteins have not been examined for dependence on Ipl1 activity (Cheeseman et al., 2002; Gillett et al., 2004).

Aurora B has been implicated in many kinetochore functions and it is unclear if the phenotypes are caused by direct regulation of assembled kinetochores or if they are the indirect consequence of improper kinetochore assembly. Ipl1-deficient yeast cells cluster centromeres close to spindle poles and cannot biorient chromosomes or signal the spindle checkpoint in response to a lack of tension (Biggins and Murray, 2001; Tanaka et al., 2002). In vertebrates, Aurora B kinase is required for checkpoint signaling, chromosome alignment, and release of syntelic and merotelic attachments (Kallio et al., 2002; Ditchfield et al., 2003; Hauf et al., 2003; Cimini et al., 2006; Knowlton et al., 2006). Aurora B phosphorylates a serine (S10 in yeast, frogs, and humans) on the N-terminal tail of histone H3 (Hsu et al., 2000). Phosphorylation of S10 on histone H3 (pH3S10) marks mitotic cells under normal growth conditions and provides an indicator of Aurora B activity.

Aurora B/Ipl1 kinase is essential for viability in budding yeast, and the viability of ipl1 mutant cells is rescued by specific mutant alleles of the protein phosphatase 1 (PP1) orthologue Glc7 (Francisco et al., 1994). The phosphatase activity of PP1/Glc7 is repressed potently and specifically by Glc8 (Inhibitor-2 [I-2] in vertebrates) in vitro, and ipl1 mutants can also be rescued by overexpression of Glc8 (Tung et al., 1995). In vertebrates, Aurora B activity and I-2 levels peak in mitosis and subsequently decrease in telophase (Brautigan et al., 1990).

Immunodepletion of Aurora B from frog egg extracts blocked the accumulation of Mis12, Ndc80, Zwint, and dynein/dynactin at kinetochores (Emanuele et al., 2005). This effect was more severe than that observed in human cells when Aurora B was inhibited with drugs. In this study, we have further explored the role of Aurora B kinase in kinetochore assembly. We show that inhibition of Ipl1 in budding yeast, using temperature-sensitive mutants in both Ipl1 and Sli15 (an inner centromere protein [INCENP] homologue), disrupts accumulation of Ndc80 and Zwint and dynactin at kinetochores. Inhibition of Aurora B using small molecule inhibitors also affected the accumulation of Ndc80 at kinetochores in human and Xenopus laevis tissue culture cells. In addition, we show a novel role for Aurora B kinase activity in maintaining outer but not inner kinetochore proteins at the centromere. In frog egg extracts, Aurora B inhibition causes rapid disassembly of the outer kinetochore. Inhibition of PP1 simultaneously with Aurora B rescues this effect, demonstrating that these enzymes work in opposition to one another with respect to kinetochore assembly. This is not caused by a reactivation of Aurora B after repression of phosphatases. Furthermore, extracts exiting M phase undergo PP1-dependent kinetochore disassembly as they enter interphase. Finally, purified PP1 alone is sufficient to disassemble kinetochores on nuclei isolated from egg extracts. We conclude that Aurora B and PP1 play an important role in the cell cycle-dependent assembly and disassembly of the kinetochore.

Results

Kinetochore assembly depends on Aurora B/Ipl1

To examine the role of Aurora B kinase in kinetochore assembly, X. laevis–derived S3 tissue culture cells were treated with the Aurora kinase inhibitor hesperadin and prepared for immunofluorescence. Because many proteins are removed from kinetochores after microtubule attachment, we added nocodazole 15 min before fixing cells. Hesperadin treatment depleted pH3S10 staining on chromatin, indicating the effectiveness of the drug treatment (Fig. 1 A). In DMSO controls, 90% of mitotic cells that had progressed beyond prophase had Ndc80 kinetochore staining on chromatin. After treatment with 250 nM hesperadin, 49% of cells had visibly undetectable levels of Ndc80 and 47% showed strongly reduced Ndc80 staining. Quantification of immunofluorescent intensity at centromeres shows that Ndc80 kinetochore staining was reduced 76% in cells treated with 250 nM hesperadin (Fig. 1 A, right). Immunostaining with antibodies to Zwilch and the dynactin subunit p150Glued produced similar results (unpublished data). These experiments also revealed that Zwilch and Ndc80 appear at kinetochores after Aurora B has been activated, which is indicated by costaining for pH3S10 (Fig. S1, available at http://www.jcb.org/cgi/content/full/jcb.200710019/DC1). This suggests that Aurora B kinase activity is required for the localization of Ndc80, Zwilch, and dynactin at kinetochores in X. laevis tissue culture cells.

Treatment of human tissue culture cells with hesperadin or ZM447439 alone did not effect Ndc80 recruitment to kinetochores, as has been found previously (unpublished data: Ditchfield et al., 2003; Hauf et al., 2003; Lampson et al., 2004). However, these treatments do not completely inhibit Aurora kinase activity. To more fully inhibit Aurora B enzymatic activity but avoid amplifying off-target effects caused by treatment with high drug concentrations, human tissue culture cells were treated simultaneously with both hesperadin and ZM447439. Double drug treatment additively inhibits the kinase activity of the purified Aurora B complex relative to using either drug alone (Fig. S2, available at http://www.jcb.org/cgi/content/full/jcb.200710019/DC1).

Treatment of human A549 lung epithelial cells with 250 nM hesperadin and 500 nM ZM447439 together caused a 70% reduction in the amount Ndc80 localized at kinetochores compared with control treated cells (Fig. 1 B). Ndc80 levels were quantified relative to the amount of anti-centromere antigen (ACA) staining in the same region, as described previously (Ditchfield et al., 2003). An identical drug treatment of human primary foreskin fibroblasts caused a 38% reduction in Ndc80 staining at kinetochores (Fig. 1 B, right). Further, hesperadin- and ZM447439-treated A549 cells were unable to maintain a nocodazole-induced mitotic arrest. The mitotic index of nocodazole-treated A549 cells was reduced from 18.3% (control)
to 5.3% after double drug treatment (n > 500 cells). These data suggest that the kinase activity of Aurora B is required for outer kinetochore assembly in human cultured cells. Interestingly, HeLa cells treated simultaneously with both drugs showed no effect on Ndc80 recruitment to kinetochores (unpublished data).

Aurora B/Ipl1 activity in yeast can be compromised with temperature-sensitive mutants in either Ipl1 or its activator Sli15. Asynchronous cultures of sli15-3 or wild-type yeast were shifted to the restrictive temperature (37°C) for 3 h and their ability to recruit and/or maintain kinetochore proteins to the centromere was measured by chromatin immunoprecipitation (ChIP; Fig. 1C). When grown at the restrictive temperature, the ability of Ndc80, Mtw1 (a Mis12 orthologue), Kip1 (a kinesin motor), Bim1 (an EB-1 orthologue), Ipl1, and Dam1 to be cross-linked to centromeres is either totally abolished or greatly reduced in sli15-3 cells, thus indicating that the abundance of these proteins at centromeres is reduced in the absence of Ipl1 activity. The inner kinetochore protein Cse4 (Cenp-A orthologue) and the central kinetochore protein Ctf19
of hesperadin (Fig. 2A, right). In a functional assay for Aurora B activity, 2 μM hesperadin inhibited spindle checkpoint signaling in nocodazole-treated extracts (Fig. S3, available at http://www.jcb.org/cgi/content/full/jcb.200710019/DC1). Aurora inhibition does not drive the extracts out of M phase. Histone H1 kinase activity, a readout of Cdk1 activity, remained high, and nuclear morphology appeared condensed for at least 60 min after hesperadin addition, demonstrating that it does not affect cell cycle state in egg extracts (Fig. S4).

We confirmed that Aurora B activity is required for kinetochore assembly in X. laevis CSF extracts. Hesperadin was titrated into CSF extracts, and sperm nuclei and nocodazole were added to initiate kinetochore assembly. X. laevis sperm have 18 chromosomes, and the assembly of proteins onto kinetochores can be detected by immunostaining, in which they appear as discrete spots on the chromatin mass. Hesperadin displaced the kinetochore/spindle checkpoint protein Zwilch from chromatin at the same concentration required to abolish pH3S10 (2 μM; Fig. 2B). After hesperadin treatment, <3% of nuclei have any discernable Zwilch kinetochore staining.

Figure 2. **Aurora B activity is required for kinetochore assembly in X. laevis CSF extracts.** (A and B) Hesperadin was titrated into X. laevis CSF egg extracts. Extracts were supplemented with nocodazole and sperm nuclei and, after 45 min, fixed and processed for immunofluorescence (A, top). (A) Nuclei stained with antibodies to pH3S10 and the nuclear stain Hoechst33342. The intensity of pH3S10 staining relative to the nuclear stain is graphed (right). Graphs show mean plus standard deviation. (B) Nuclei immunostained for the kinetochore protein Zwilch. Kinetochore assembly is inhibited at 2 μM hesperadin. (C) Kinetochores were assembled in CSF extracts preincubated with either anti–Aurora A or B antibodies. Nuclei were stained with antibodies to dynactin subunit p150Glucl. Bars, 5 μm.

**Aurora B activity drives kinetochore assembly in frog egg extracts**

To further dissect the role of Aurora B kinase in kinetochore assembly, we used X. laevis egg extracts that assemble functional kinetochores onto centromeres of sperm chromatin. Hesperadin was titrated into cytostatic factor (CSF)-arrested M phase extracts. Sperm nuclei were added for 45 min (Fig. 2A, top) and then reactions were fixed and examined by immunofluorescence. Aurora kinase was inhibited by 2 μM hesperadin as determined by pH3S10 staining (Fig. 2A; Zhang et al., 2007). Staining for pH3S10 was quantified as a ratio to a nuclear stain (Hoechst 33342) in the presence of increasing concentrations of hesperadin (Fig. 2A, right). In a functional assay for Aurora B activity, 2 μM hesperadin inhibited spindle checkpoint signaling in nocodazole-treated extracts (Fig. S3, available at http://www.jcb.org/cgi/content/full/jcb.200710019/DC1). Aurora inhibition does not drive the extracts out of M phase. Histone H1 kinase activity, a readout of Cdk1 activity, remained high, and nuclear morphology appeared condensed for at least 60 min after hesperadin addition, demonstrating that it does not affect cell cycle state in egg extracts (Fig. S4).

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In DMSO controls, >95% of nuclei stain positive for Zwilch (n > 100 nuclei per condition). These data suggest a role for Aurora kinase activity in kinetochore assembly. Importantly, this system provided us with a rapid, robust, and biochemically amenable assay for dissecting the contributions of Aurora B to kinetochore assembly.

Because hesperadin can inhibit both Aurora A and B kinases, we confirmed that the effect of hesperadin on kinetochore assembly is caused by inhibition of Aurora B. Kinetochore were assembled in CSF extracts preincubated with antibodies to either kinase. We examined kinetochore assembly with a mouse monoclonal antibody to the p150\textsuperscript{Glued} subunit of dynactin to avoid cross-reaction of our secondary antibody with the Aurora B antibodies. Treatment with the Aurora B antibody blocked accumulation of p150\textsuperscript{Glued} at kinetochores (Fig. 2 C), whereas adding Aurora A antibodies had no effect. We used a concentration of Aurora A antibody that disrupts microtubule aster formation (unpublished data). We conclude that the affect of hesperadin on kinetochore assembly is caused by inhibition of Aurora B and not Aurora A. These data are further supported by our previous demonstration that extracts immunodepleted of Aurora B are unable to assemble outer kinetochore proteins (Emanuele et al., 2005).

**Aurora B activity maintains kinetochore assembly status**

We next tested if continuous Aurora B activity is required to maintain assembly of the kinetochore. In vitro egg extracts are well suited for this because they allow us to temporally control Aurora B activity. We preassembled kinetochores on sperm chromatin in extracts for 30 min and then added hesperadin (Fig. 3 A, top). Reactions were fixed at 5-min intervals and immunostained with Ndc80 antibodies (Fig. 3 A). The ratio of Ndc80 to Cenp-A staining on the same centromeres was calculated as described previously (Ditchfield et al., 2003). Ndc80 staining was reduced >90% after 5 min and >95% after 10 min (Fig. 3 A). Identical effects were seen after adding anti–Aurora B (Fig. 3 B) or anti-INCENP (not depicted) antibodies instead of hesperadin. Therefore, Aurora B is required to both establish and maintain the Ndc80 protein at centromeres.

We used a panel of antibodies to identify the proteins that require Aurora B activity to be maintained at kinetochores. All outer kinetochore proteins tested, including Knl1, Ndc80, Zwint, Cep57, Dsn1 (Mis12 complex), CLIP-170, p150\textsuperscript{Glued} (dynactin complex), and the spindle checkpoint components Mad1, Mad2, and BubR1 (Fig. 3 C and Fig. S5, available at http://www.jcb.org/cgi/content/full/jcb.200710019/DC1) were displaced from kinetochores within 15 min of hesperadin treatment. In contrast, the inner kinetochore components Cenp-A and Cenp-C are retained at centromeres (Fig. 3 C). We conclude that in CSF extracts, Aurora B is critical for the maintenance of the outer kinetochore.

To confirm that Aurora B was also required to maintain kinetochore assembly on replicated chromosomes, CSF extracts containing sperm were cycled through S phase and rearrested in the subsequent mitosis with fresh CSF extract. Knl1, Mis12 (Fig. 4, A and B), Ndc80, Zwilch, and p150\textsuperscript{Glued} (not depicted) were displaced after hesperadin treatment. Together, our data demonstrate that kinetochores require persistent Aurora B kinase activity.

Treatment of CSF extracts with hesperadin, after kinetochore assembly, resulted in a loss of Aurora B staining on chromatin (Fig. 4 C, left). Identical results were obtained in cycled extracts immunostained for Dsra A and INCENP (Fig. 4 C, middle; and not depicted) and in X. laevis tissue culture cells (Fig. 4 C, right). These data suggest that Aurora B kinase activity is required for its association with the centromere.

**PP1 opposes Aurora B in kinetochore assembly**

If phosphatases act directly on the Aurora B substrates that are critical for kinetochore assembly, then phosphatase inhibition should rescue the kinetochore disassembly defects caused by hesperadin. To test this, kinetochores were preassembled on nuclei in extracts (Fig. 5 A, top). Hesperadin and the phosphatase inhibitor okadaic acid were added alone or in combination. After 15 min, kinetochore status was assayed by immunostaining for Zwilch (Fig. 5 A). Because the RZZ (Rod, Zw10, and Zwilch) complex requires Ndc80 for assembly and is itself essential for dynein/dynactin recruitment, it is an excellent indicator of kinetochore assembly status (Starr et al., 1998; Emanuele et al., 2005). We found that simultaneous addition of okadaic acid rescued the kinetochore disassembly caused by hesperadin (Fig. 5 A).

We tested if okadaic acid treatment was reactivating Aurora B despite the presence of its inhibitor (hesperadin). In an order-of-addition experiment, kinetochores were first preassembled onto sperm nuclei. Hesperadin was added, disassembling kinetochores, and then phosphatases were inhibited by adding okadaic acid. No discernable pH3S10 or kinetochore staining was observed after treatment with both drugs (Fig. 5 B), which suggests that Aurora B was not reactivated by phosphatase inhibition.

The concentration of okadaic acid required (1.0 μM; Fig. 5 A) suggested that PP1 and not PP2A was modulating kinetochore assembly. To test this hypothesis, we added hesperadin and the PP1-specific inhibitory protein I-2 to extracts containing preassembled kinetochores (Fig. 6). Zwilch, Ndc80, Knl1, Dsn1, and p150\textsuperscript{Glued} were retained at kinetochores after simultaneous treatment with hesperadin and I-2 (Fig. 6, A–C). After the addition of hesperadin and I-2, Aurora B remains dissociated from centromeres, whereas the kinetochore remains intact (Fig. 6 C). Thus, Aurora B localization at centromeres is not essential for maintaining kinetochore assembly in the absence of PP1 activity. This also suggests that Aurora B does not play a structural role at the centromere for the recruitment of kinetochore proteins. We conclude that in the kinetochore assembly pathway, Aurora B is opposed by PP1.

**PP1 and kinetochore disassembly**

We asked whether PP1 is important for kinetochore disassembly after mitotic exit. Kinetochore were assembled onto sperm nuclei in CSF extracts containing nocodazole. Extracts were driven out of M phase by the addition of calcium in the presence or absence of I-2. Histone H1 kinase activity was lost 10 min after calcium addition in BSA control and I-2–treated extracts (Fig. 7 A). In control extracts, immunostaining for Ndc80 was reduced after 10 min and was undetectable after 20 min (Fig. 7 B). In I-2–treated extracts, where PP1 is inactivated,
Figure 3. **Aurora B activity is required for kinetochore maintenance in CSF extracts.** (A) Kinetochore maintenance was assessed after hesperadin treatment in extracts containing nuclei with preassembled kinetochores. The relative intensity of Ndc80 to Cenp-A immunostaining on the same kinetochore was determined and graphed (bottom; \(n = 60\) individual kinetochores on four nuclei per condition). Graphs show mean plus standard deviation. (B) The addition of anti–Aurora B antibodies caused preassembled kinetochores to disassemble in an identical manner. (C) A panel of antibodies was used to assess the sensitivity of outer kinetochore proteins to hesperadin treatment in extracts with preassembled kinetochores. The outer kinetochore proteins Dsn1, Knl1, Ndc80, and Zwint and the checkpoint proteins BubR1, Mad1, and Mad2 are displaced after hesperadin treatment. The inner kinetochore proteins Cenp-A and Cenp-C are unaffected. Bars, 5 μm.
that these kinetochore assembly events are regulated by Aurora B kinase activity. Initial observations suggested that Aurora B was important for kinetochore assembly after immunodepletion from frog egg extracts (Emanuele et al., 2005). In this study, we demonstrate that Aurora B activity is required for assembly and maintenance of the outer kinetochore from yeast to humans. Aurora B inhibition, using multiple methods, leads to rapid disassembly of the kinetochore. Every outer kinetochore protein we examined was sensitive to Aurora B inhibition. In contrast, the inner kinetochore proteins, which include Cenp-A (Cse4) and Cenp-C, assemble independently of Aurora B activity. Importantly, the recruitment of the KMN network (Knl1, Mis12, and Ndc80 complexes), which is part of the kinetochores’ core microtubule binding machinery, is compromised after Aurora inhibition. These data suggest that Aurora B is a cell cycle regulator of outer kinetochore assembly.

Discussion

Cell cycle regulation of kinetochore assembly

The outer kinetochore proteins assemble in prophase/prometaphase and are disassembled in late anaphase. We have shown that these kinetochore assembly events are regulated by Aurora B activity. Initial observations suggested that Aurora B was important for kinetochore assembly after immunodepletion from frog egg extracts (Emanuele et al., 2005). In this study, we demonstrate that Aurora B activity is required for assembly and maintenance of the outer kinetochore from yeast to humans. Aurora B inhibition, using multiple methods, leads to rapid disassembly of the kinetochore. Every outer kinetochore protein we examined was sensitive to Aurora B inhibition. In contrast, the inner kinetochore proteins, which include Cenp-A (Cse4) and Cenp-C, assemble independently of Aurora B activity. Importantly, the recruitment of the KMN network (Knl1, Mis12, and Ndc80 complexes), which is part of the kinetochores’ core microtubule binding machinery, is compromised after Aurora inhibition. These data suggest that Aurora B is a cell cycle regulator of outer kinetochore assembly.

In budding yeast, Ipl1 mutants are rescued by specific mutant alleles of Glc7/PP1, demonstrating that their activities oppose each other. We have shown here that Aurora B’s role in kinetochore assembly is opposed by PP1 in vertebrates. Restraining PP1 activity using the small inhibitory protein I-2 protected the kinetochore from disassembly after Aurora B inhibition. PP1 activity was required for kinetochore disassembly at mitotic exit.

Figure 4. **Aurora B activity is required for kinetochore maintenance in cycled extracts.** (A and B) Cycled *X. laevis* extracts containing replicated chromatids and assembled kinetochores were tested for their sensitivity to hesperadin (or DMSO). After 15 min in hesperadin, Knl1 (A) and Mis12 (B) were displaced from kinetochores. (C) Maintenance reactions were repeated in CSF and cycled egg extracts. Aurora B complex proteins were displaced from chromatin by the addition of hesperadin after assembly. *X. laevis* S3 cells also lost Aurora B staining on chromatin after treatment with hesperadin. Bars, 5 μm.

The levels of Ndc80 staining on chromatin remain unchanged for 30 min after triggering M phase exit (Fig. 7 B). Both BSA- and I-2–treated extracts displayed interphase chromatin morphology by 45 min (unpublished data). We conclude that PP1 activity is necessary for kinetochore disassembly after exit from M phase.

To determine whether PP1 is sufficient to disassemble the kinetochore, nuclei with assembled kinetochores were isolated through a glycerol cushion, washed thoroughly, and mixed with purified PP1. Nuclei incubated with buffer and BSA alone retained Ndc80 at kinetochores and stained positive for pH3S10. Treatment with purified PP1 enzyme for 15 min caused dephosphorylation of H3S10 and displacement of the Ndc80 protein. We conclude that PP1 dephosphorylates either a chromatin- or kinetochore-bound substrate to induce kinetochore disassembly.
Inhibition of Aurora B and PP1 in extracts before the addition of sperm nuclei does not rescue kinetochore assembly, demonstrating that Aurora B activity is essential for initial assembly of the outer kinetochore even in the absence of PP1 activity (unpublished data). These data suggest that PP1 is a regulator of kinetochore assembly, as purified enzyme is sufficient to disassemble kinetochores on isolated nuclei. PP1 and Aurora B affect assembly of an identical subset of kinetochore proteins. Cenp-A and Cenp-C are insensitive to changes in Aurora B and PP1 activity, as they remain centromere associated throughout the cell cycle.

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kinetochore disassembly at mitotic exit. We conclude that Aurora B and PP1 act in opposition to each other as cell cycle-dependent regulators of outer kinetochore assembly.

A model depicting the activities of Aurora B and PP1 during the somatic cell cycle relative to the times of kinetochore assembly and disassembly is depicted in Fig. 8. The various
steps of the mitotic cell cycle, starting in late G2 and proceeding through telophase, are shown relative to Aurora B and PP1 activity. In prophase, Aurora B becomes activated and, by prometaphase, Aurora B is localized to inner centromere. At this time, the outer kinetochore proteins are recruited and assembled onto centromeres. At the metaphase-to-anaphase transition, Aurora B is released from the inner centromere but remains catalytically active in a gradient of activity concentrated on the spindle midzone and diffuses throughout the cell (unpublished data). We hypothesize that one role for the gradient of Aurora B activity is to keep kinetochores assembled in anaphase. By anaphase B, kinetochores pass beyond the periphery of Aurora B’s zone of activity, and outer kinetochore proteins are displaced from the centromere. Recent work has demonstrated that Ncd80 levels are reduced 5–10-fold at kinetochores in anaphase B (McAinsh et al., 2006). In late anaphase B and telophase, 1-2 levels, which remain high throughout mitosis, begin to decrease, which is concomitant with the complete loss of outer kinetochore proteins at centromeres, a decrease in Aurora B activity, and, finally, mitotic exit.

We propose a model wherein two mechanisms are essential for outer kinetochore assembly in vertebrates. The first mechanism spatially directs kinetochore assembly to a specific site, the centromere. This is accomplished by Cenp-A loading at centromeres. However, from yeast to vertebrates, Cenp-A localization is insufficient to induce kinetochore assembly. In yeast
cells, Cse4, but not the outer kinetochore proteins, is loaded at centromeres in Ipl1 mutants. Similarly, in frog extracts and cells, Cenp-A is properly localized but outer kinetochore proteins fail to assemble in the absence of Aurora B activity. Thus, the second mechanism driving outer kinetochore assembly is the activation of Aurora B, which temporally constrains outer kinetochore assembly to mitosis. Aurora B localizes to the centromere in late prophase, when both Cdk1 and Aurora B are activated. In addition, I-2 levels peak in mitosis, keeping PP1 inactive and preventing disassembly. Because Aurora B is necessary for assembly and is spatially localized to the kinetochore assembly site, we predict that its role will be direct. A large number of kinetochore proteins contain Aurora B phosphoconsensus motifs, including several conserved sites on proteins in the Ndc80 and Mis12 complexes. Identification and characterization of kinetochore substrates of Aurora B is an important future direction.

Because inhibition of Aurora B reduced Ndc80 levels at kinetochores in yeast cells, X. laevis cells and extracts, and in most human cells tested, we conclude that the role of Aurora B kinase activity in outer kinetochore assembly is evolutionarily conserved. In HeLa cells, we were unable to affect Ndc80 recruitment to centromeres even after prolonged treatment (24 h) with high concentrations of the Aurora inhibitors hesperadin, ZM447439, and VE-645 (a VX-680 derivative, Aurora B selective, small molecule inhibitor) used alone and in combination (unpublished data). These results are consistent with previously published findings (Hauf et al., 2003; Lampson et al., 2004; Meraldi and Sorger, 2005). It is disconcerting that our results in A549 and PFF cells and those found in HeLa cells by us and others could be attributed to changes that have occurred in the phospho-signaling cascades in this immortalized cell line. Importantly, treatment of human cells with either ZM447439 or hesperadin alone can represses pH3S10 staining but not outer kinetochore assembly, which suggests that only a small amount of Aurora B kinase activity is required for outer kinetochore assembly in human cultured cells.

Are the phenotypes resulting from reduced Aurora B/Ipl1 activity caused by improper kinetochore assembly?

It is important to reexamine the phenotypes of Aurora B/Ipl1 in light of its critical role for assembling kinetochores. Centromeres in ipl1 mutants cluster close to spindle poles and do not oscillate or breathe on the metaphase plate (He et al., 2001; Tanaka et al., 2002). These experiments suggested that Ipl1 was required for kinetochore biorientation because of a role in releasing improperly attached (syntelic) microtubules. The ipl1 phenotype is clearly different than kinetochore null mutants (ndc10-1) where chromosomes randomly float throughout the nucleus (He et al., 2001; Tanaka et al., 2002). Our data suggest that centromeres remain associated with spindle poles in ipl1 mutant cells with little or no outer kinetochore protein at centromeres. Thus, the kinetochore–microtubule attachments in Ipl1 mutants are mediated through some inner kinetochore protein or complex whose centromere association is refractory to Ipl1 activity. Therefore, biorientation may be the role of the outer kinetochore proteins that are recruited by Ipl1 rather than Ipl1 regulating outer kinetochore proteins to generate biorientation.

Interestingly, there is a discrepancy in the literature with respect to the localization of kinetochore proteins using ChIP versus fluorescent imaging. In yeast harboring ipl1 mutants, Ndc80 and Mtw1 appear properly localized at centromeres at the restrictive temperature when assayed by fluorescence (Pinsky et al., 2006; Maure et al., 2007). However, we have shown that these proteins are mislocalized under similar conditions when assayed by ChIP (Fig. 1). Furthermore, the same laboratory has reported that in ipl1 mutants, Dam1 localizes to centromeres by fluorescence imaging but is mislocalized by ChIP (Kang et al., 2001; Cheeseman et al., 2002). These differing results could be a result of many factors, including different mutants, the amount of time cells spend at the restrictive temperature, differing growth conditions, or some inherent, unexplained difference between the sensitivity of ChIP and fluorescent imaging of kinetochore proteins. Moving forward, it will be critical to analyze the

Figure 8. Model of Aurora B and PP1 activity relative to kinetochore assembly in the mitosis. G2 and the mitotic phases (prophase, prometaphase, metaphase, anaphase A and B, and telophase) are depicted. Microtubules are displayed in green, chromosomes in blue, kinetochores in yellow, and active Aurora B kinase in red. The times at which Aurora B (red lines) and PP1 (green lines) are activated are shown relative to the morphological changes of mitosis. In prophase, Aurora B is activated and begins to move from chromosome arms to centromeres. By prometaphase, activated Aurora B is fully localized to the inner centromere and outer kinetochore proteins are assembled. Complete chromosome alignment occurs in metaphase, at which point the spindle checkpoint signal is extinguished and sister chromatids separate and begin poleward anaphase movements. Aurora B dissociates from the inner centromere in anaphase and relocates to overlapping spindle midzone microtubules, where it remains active in a gradient of kinase activity that spreads from out from the spindle midzone (fading red). Kinetochores disassemble as they move outside of the zone of Aurora B activity in anaphase B. PP1 is initially activated in anaphase and, its small protein inhibitor I-2 is further degraded in anaphase B and telophase. Active PP1, in the absence of Aurora B, leads to outer kinetochore disassembly in telophase before the ensuing G1.
kinetochores’ assembly status by multiple methods under the conditions used to draw these conclusions. Importantly, a role for Ipl1 in kinetochore assembly and microtubule release is not mutually exclusive.

Poor outer kinetochore assembly can explain the phenotypes of chromosome passenger complex knockdown in tissue culture cells. Overexpression of kinase-dead Aurora B generated prometaphase spindles lacking any sign of K-fiber formation or chromosome congression (Murata-Hori and Wang, 2002b). RNAi knockdown of either Aurora B or INCENP in Drosophila melanogaster cells “abolished the ability of cells to achieve a metaphase chromosome alignment,” generating “anaphase-like melting” (Adams et al., 2001). Injection of Aurora B antibodies into X. laevis XTC cells generated severe chromosome misalignment defects and the absence of K-fibers, as judged by cold-calcium lysis (Kallio et al., 2002). Aurora B RNAi in human tissue culture cells caused “the absence of kinetochore–microtubule interactions” (Ditchfield et al., 2003). These phenotypes are easily explained by the lack of proper outer kinetochore formation. In fact, it has already been shown that Aurora is required for the affiliation of BubR1, CENP-E, and dynein with centromeres in HeLa cells, where the requirement for Aurora in assembly is diminished. Our results support the hypothesis espoused by Johnson et al. (2004), that Aurora B may provide a priming phosphorylation on specific substrates to both establish and maintain proteins on kinetochores. Corroborating the data presented here, a recent study has also shown that recruitment of the RZZ complex to kinetochores that are not under tension is required for proper assembly of outer kinetochore proteins.

Is it reasonable to conclude that Aurora B is required to release improper microtubule attachments in vertebrate cells? Syntelic attachments require Aurora B activity to be resolved in HeLa cells, where we do not see a dramatic effect on kinetochore assembly (Hauf et al., 2003; Lampson et al., 2004). In X. laevis S3 cells, Aurora B is enriched at merotelic kinetochore attachment sites and is required to either prevent or resolve them (Knowlton et al., 2006). These experiments were performed in the same X. laevis S3 cells used in this study but with short 30-min incubations in hesperadin. This treatment generates merotelic microtubule attachments but does not affect Ndc80 levels at kinetochores. We conclude that Aurora B has a role in both building kinetochores and resolving microtubule attachments in vertebrates. However, it is not clear if the role of Aurora B in resolving improper attachments is direct or through the recruitment of other known or unknown outer kinetochore proteins other than Ndc80 and the KMN. In support of the latter hypothesis, treatment of human cells with ZM447439 alone causes to the dissociation of BubR1 and Cenp-E from kinetochores, both of which are important factors in generating productive kinetochore–microtubule attachments. There are likely to be specific substrates that are phosphorylated by Aurora B in response to its multiple roles in mitosis. The identification of the specific kinetochore substrates that control these activities is an important area of future study.
glycerol + 0.1 M α-ketoisocaproic acid. Cushions were washed with CSF-XB and coverslips were blocked in CSF-XB + 5% BSA + 1 μM α-ketoisocaproic acid for 45 min at room temperature. Coverslips were washed once in CSF-XB and turned up-sided-down in a solution of PPI enzyme (10 U/ml) or buffer alone (purified and provided by D. Brautigam, University of Virginia, Charlottesville, VA). Coverslips were returned to culture dishes, washed once in CSF-XB, and fixed with BBR80 + 3.7% formaldehyde for 15 min. Reactions were postfixed in ice-cold methanol and processed for immunofluorescence. PPI enzyme was purified and used as described previously (Brautigam et al., 1985).

**Online supplemental material**

Fig. S1 demonstrates that Aurora B is activated in the nucleus, before kineto- chore assembly, during prophase. Fig. S2 shows that ZM447439 and hes- peradin additively inhibit the kinase activity of Aurora B in vitro. Fig. S3 shows that Aurora B inhibition, using hesperadin, blocks spindle checkpoint signaling in X. laevis egg extracts. Fig. S4 shows that the addition of hesper- adin to egg extracts does not affect the CSF-controlled cell cycle state of the extract. Fig. S5 demonstrates that the kinetochore proteins CUP170, Cep57, and p150/Glued require Aurora B kinase activity for their maintenance at preassembled kinetochores. Table S1 describes all antibodies used in this study. Table S2 describes all yeast strains generated for and used in yeast ChIP experiments. Online supplemental material is available at http://www.

**References**


### Table S1. Antibodies used for immunofluorescence

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The table describes the antibodies used for immunofluorescence in this study. All mouse antibodies are monoclonal and all rabbit antibodies are polyclonal. AP, affinity purified; h, human; NA, not applicable; x, X. laevis.


### Table S2. Yeast strains used in this study

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