Aurora B controls kinetochore–microtubule attachments by inhibiting Ska complex–KMN network interaction

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Introduction

Chromosome alignment and segregation require that all kinetochores (KTs) establish stable bioriented attachments to spindle microtubules (MTs). Central to this process and providing the core KT–MT attachment interface at outer KTs is the conserved KMN network, composed of the KNL1 protein and the four-subunit Mis12 (Mis12, Mis13, Mis14, and Nnf1) and Ndc80 (Hec1, Nuf2, Spc24, and Spc25) complexes (Santaguida and Musacchio, 2009). Both the Ndc80 complex and KNL1 bind directly to MTs in vitro, whereas the Mis12 complex serves as a scaffold to form the KMN network and enhances the MT binding activities of the other components (Cheeseman et al., 2006). KTs are also important for discriminating bipolar from inappropriate MT attachments and for generating a signal that controls the timing of anaphase onset through the spindle assembly checkpoint (Musacchio and Salmon, 2007). The mitotic kinase Aurora B is critical for both error correction through destabilization of incorrect attachments (Lampson et al., 2004; Nezi and Musacchio, 2009) and for checkpoint signaling (Maresca and Salmon, 2010; Santaguida et al., 2011). Other proteins localized to the KT–MT interface during mitosis also contribute to the formation of stable and functional connections. Prominent among these is the Ska complex, composed of Ska1, 2, and 3, which has been proposed to be required for stable KT–MT attachments (Hanisch et al., 2006; Gaitanos et al., 2009; Raaijmakers et al., 2009; Theis et al., 2009; Welburn et al., 2009). The Ska complex has also been implicated in silencing of the spindle checkpoint (Hanisch et al., 2006; Daum et al., 2009) and in maintenance of sister chromatid cohesion (Daum et al., 2009; Theis et al., 2009). It comprises two copies of each subunit and directly binds to MTs in vitro (Welburn et al., 2009). Efficient depletion of the Ska complex leads to severe attachment defects and unstable K-fibers, reminiscent of the KT-null phenotype observed upon Ndc80 depletion (Gaitanos et al., 2009; Raaijmakers et al., 2009; Welburn et al., 2009; however, see Daum et al. [2009]). As recruitment of the Ska complex to KTs has been shown to depend on the Ndc80 complex (Gaitanos et al., 2009; Raaijmakers et al., 2009; Welburn et al., 2009), the phenotype observed in Ndc80-depleted cells might reflect the loss of both complexes.

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Abbreviations used in this paper: KT, kinetochore; MT, microtubule; NEBD, nuclear envelope breakdown; WT, wild type.

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The available evidence indicates that the Ska complex acts in concert with the KMN network for generating stable end-on attachments. However, no interaction between the KMN network and the Ska complex has been described, and how the KT recruitment of the Ska complex is regulated remains unknown. To understand how functional KT–MT attachments are stabilized in mitosis, it is therefore critical to determine whether and how the Ska complex interacts with members of the KMN network and how this interaction is regulated in time and space.

Results and discussion

To obtain insight into the regulation of KT–MT attachment by the Ska complex, we first investigated how its KT localization is controlled. In line with a dependency on MT occupancy at KTs (Hanisch et al., 2006; Gaitanos et al., 2009), Ska3 staining intensity was about twofold higher at metaphase KTs than prometaphase KTs (Fig. 1, A and B). Moreover, Ska levels were clearly reduced at misaligned KTs when compared with aligned KTs (Fig. S1 A), indicating that the Ska complex preferentially accumulates at fully attached and bioriented KTs. As phosphorylation of Aurora B substrates at outer KTs decreases as KTs become bioriented and tension is established (Liu et al., 2009; Welburn et al., 2010), we asked whether Aurora B might be responsible for removing the Ska complex from unattached KTs.

We monitored Ska3 localization in nocodazole-treated cells incubated with the Aurora B inhibitor ZM447439 (Ditchfield et al., 2003). In parallel, we also tested the effect of inhibiting the mitotic kinases Mps1 (by Mps1-IN-1 [Kwiatkowski et al., 2010] or reversine [Santaguida et al., 2010]) or Plk1 (by ZK-thiazolidinone [TAL]; Santamaria et al., 2007). Cold-treated cells were included as a positive control, as exposure to low temperature restores the KT localization of Ska proteins in nocodazole-treated cells (Hanisch et al., 2006; Gaitanos et al., 2009). Indeed, inhibition...
of Aurora B, but not Mps1 or Plk1, restored 70% of the Ska3 KT signal detected in metaphase cells (Figs. 1 [A and B] and S1 [B and C]). Similar results were obtained when GFP-labeled Ska2 and endogenous Ska1 were examined (Figs. 1 C and S1 D). Together, these results strongly suggest that Aurora B negatively regulates the association of the Ska complex with KTs, reminiscent of recent data on the Astrin–small kinetochore-associated protein complex (Schmidt et al., 2010). They further indicate that Aurora B plays a major role in regulating Ska recruitment to KTs, whereas KT–MT attachment seems to contribute to Ska recruitment independently of Aurora B’s effect. To further strengthen this notion, we expressed a Mis12-INCENP (inner centromere protein) fusion protein, shown to increase phosphorylation of Aurora B substrates by recruiting additional Aurora B to outer KTs (Liu et al., 2009). Expression of Mis12-INCENP, but not a Mis12-INCENP (TAA) mutant that is unable to activate Aurora B (Sessa et al., 2005), resulted in a twofold reduction of Ska3 staining at KTs (Fig. 1, D and E).

Next, we investigated how Aurora B influences the KT recruitment of the Ska complex. As it has been shown that KT localization of Ska proteins depends on the Ndc80 complex (Hanisch et al., 2006; Gaitanos et al., 2009; Raaijmakers et al., 2009; Welburn et al., 2009), we asked whether other KMN components, notably KNL1 and the Mis12 complex, are also required. Indeed, depletion of not only Hec1 but also KNL1 or Mis13 abolished the KT localization of Ska3 without affecting Ska protein levels (Fig. S1, E–G). To exclude that this phenotype was a result of an inability of cells to form proper KT–MT attachments, cells depleted of KNL1, Mis13, Hec1, or GL2 (Photinus pyralis luciferase gene; as a control) were treated with nocodazole followed by ZM447439. Importantly, Aurora B inhibition did not restore Ska3 to the KT in KNL1, Mis13, or Hec1-depleted cells, although it did so in control (GL2 treated) cells (Fig. 2, A and B). This strongly argues that the KMN network is directly involved in recruiting the Ska complex to KTs and that Aurora B regulates the interaction between the KMN and Ska complexes. To corroborate this conclusion, we investigated whether KMN components could be coimmunoprecipitated with Ska proteins and whether Aurora B inhibition might strengthen this interaction. Indeed, Mis12 could readily be

Figure 2. Aurora B regulates the interaction between Ska and KMN. (A) HeLa S3 cells were treated for 48 h with GL2, KNL1, or Hec1 siRNAs or for 72 h with Mis13 siRNA before treatment with 3.3 μM nocodazole (Noc) for 1 h and a 2-h incubation with DMSO or ZM. Bar, 10 μm. (B) A bar graph showing the quantification of Ska3 staining intensity, as in Fig. 1 E, of cells treated as in A (>100 KTs from five cells; SD of five cells). (C) 0.33 μM nocodazole-arrested mitotic cells was treated with MG132 or MG132 plus ZM. After 2 h, cells were collected, and immunoprecipitations (IP) were performed with either rabbit IgGs (Rb IgG; as a control) or anti-Ska1 antibodies. Molecular mass is indicated in kilodaltons. (D) A bar graph showing the quantification of the intensities of coprecipitated proteins upon ZM treatment (relative to treatment without ZM; SD of three independent experiments).
Aurora B sites in Ska proteins have been shown to be phosphorylated in vivo in phosphoproteomics studies (Fig. 3 C; Nousiainen et al., 2006; Sui et al., 2008; Olsen et al., 2010; Santamaria et al., 2011). Thus, we generated mutants covering the potential Aurora B sites in Ska proteins. Kinase assays performed on nonphosphorylatable mutants (denoted as Ska14A and Ska33A) confirmed that these sites represent major targets of Aurora B in Ska1 and Ska3 (Fig. 3 D).

To assess the role of Aurora B phosphorylation on the Ska complex in vivo, we investigated the functionality of the phosphomimetic (to aspartate) and nonphosphorylatable (to alanine) mutants of Ska1 and Ska3 in HeLa S3 cells stably expressing H2B-GFP by combining an siRNA-based complementation approach with live-cell imaging (Figs. 4 A and S3, B–D). Consistent with previous work (Gaitanos et al., 2009), codepletion of Ska1 and Ska3 yielded a significant increase in mitotic timing (mean duration of 338 min from nuclear envelope breakdown [NEBD] to anaphase onset or mitotic cell death), and a significant proportion of cells (47.6%) died after prolonged arrest in mitosis but before anaphase. Of all Ska-depleted cells, 62% showed prolonged prometaphase with obvious chromosome congression defects (Figs. 4 A and S3, B–D), whereas the remaining cells experienced a delay without obviously misaligned chromosomes (Fig. S3 D). For comparison, GL2-treated control cells proceeded rapidly from NEBD to anaphase (mean of 42 min), only 3% displayed misalignment, and virtually no mitotic cell death was observed (Figs. 4 A and S3, B–D; and Videos 1 and 2). Although prolonged mitosis can lead to chromosomes scattering as a result of cohesion fatigue (Daum et al., 2009, 2011; Gassmann et al., 2010), we emphasize that the misalignment defects in Aurora B sites in Ska proteins have been shown to be phosphorylated in vivo in phosphoproteomics studies (Fig. 3 C; Nousiainen et al., 2006; Sui et al., 2008; Olsen et al., 2010; Santamaria et al., 2011). Thus, we generated mutants covering the potential Aurora B sites in Ska1 and Ska3. [A] In vitro Aurora B phosphorylates Ska1 and Ska3 in vitro. [B] Sequence alignment of the potential Aurora B sites in Ska1 and Ska3 from the indicated species; alignments were performed with CLUSTALW [network protein sequence analysis]. Potential Aurora B sites conserved across species are marked with a rectangle. [C] A table summarizing collected information for the seven potential Aurora B sites mutated in Ska1 and Ska3. [D] In vitro Aurora B kinase assays of Ska1WT-His and His-Ska3WT and the corresponding nonphosphorylatable mutants. An autoradiogram and Coomassie-stained gel [top and bottom, respectively] are shown.

Following up on earlier evidence that mitotic phosphorylation of Ska3 is partly Aurora B dependent (Theis et al., 2009), we assayed the ability of Aurora B to phosphorylate a reconstituted Ska complex. Under these in vitro conditions, Ska1 and Ska3, but not Ska2, were readily phosphorylated by Aurora B (Fig. 3 A). This phosphorylation was completely abolished by ZM447439 treatment, attesting to its specificity (Fig. 3 A). Examination of the Ska1 and Ska3 protein sequences revealed four conserved Aurora B consensus sites (K/R-X-T/S) in Ska1 (T157, S185, T205, and S242) and two in Ska3 (S87 and S110; Fig. 3, B and C). Furthermore, mass spectrometric analysis of the in vitro phosphorylated Ska complex identified a highly conserved phosphorylation site in Ska3 (S159). Although this site does not match the canonical Aurora B consensus (Fig. 3, B and C), a peptide spanning S159 of Ska3 was clearly phosphorylated by Aurora B (Fig. S3 A). In addition, several of the putative co-immunoprecipitated with the Ska complex, and the interaction was clearly enhanced when cells were treated with ZM447439 (Fig. 2, C and D). Reciprocally, the Ska complex could also be co-immunoprecipitated with Mis12 (Fig. S2 A). Aurora B inhibition also revealed a faint interaction between the Ska complex and Hec1 (Fig. 2, C), but no KNL1 could be detected in our Ska1 immunoprecipitates (not depicted). To see whether any of the subunits of the Mis12 and Ndc80 complexes can interact with the Ska proteins, directed yeast two-hybrid experiments were performed. Importantly, we observed multiple interactions between members of the Ndc80 and Mis12 complexes and the Ska complex, namely Hec1-Ska1, Mis13-Ska2, and Spc24-Ska3 (Fig. S2, B–E). Furthermore, we could confirm binding between Ska2 and Mis13 by GST pull-down experiments (Fig. S2 F).
As Aurora B activity controls Ska localization at KTs (Fig. 1), we next examined the localization of the aforementioned mutant proteins. The phosphomimetic mutants (Ska14D + Ska33D) failed to localize to KTs (Fig. 4 B), confirming that phosphorylation by Aurora B significantly reduces binding of the Ska complex to KTs. However, they still interacted with Hec1 and Spc24 in yeast two-hybrid assays, indicating they do not effectively mimic phosphorylation in the latter context (unpublished data). In contrast, the nonphosphorylatable Ska mutants (Ska14A + Ska33A) localized to KTs identically to their WT counterparts. Considering that the association of the Ska complex with KTs is both highly dynamic (Raaijmakers et al., 2009) and maximal during metaphase (Fig. 1 A), we reasoned that the absence of Aurora B phosphorylation on the Ska14A and Ska33A mutants might induce premature KT recruitment (or prevent normal turnover) of the Ska complex at KTs. To test whether the nonphosphorylatable Ska mutants would resist Aurora B–dependent removal from KTs, Ska1 and Ska3 constructs were cotransfected with Mis12-INCENP WT or TAA plasmids (Fig. 4 C). In contrast to the SkaWT proteins, Ska-depleted cells could be observed within 2 h after NEBD and before formation of metaphase plates (see example in Fig. S3 B). Furthermore, in Ska1 + Ska3–depleted cells, positive Mad1 signals could be seen not only on unaligned KTs but also on some apparently aligned KTs (unpublished data), which is in line with the notion that KT–MT attachments failed to fully stabilize. These defects were largely rescued by coexpressing wildtype (WT) Ska1 (Ska1WT) and Ska3WT (mean of 85 min for mitotic timing, with only 3% cell death and 14% of cells with misaligned chromosomes; Figs. 4 A and S3 B and Video 3). In contrast, expression of neither the nonphosphorylatable mutants (Ska14A + Ska33A) nor the phosphomimetic mutants (Ska14D + Ska33D) rescued the observed depletion phenotypes (mean of 246 min for mitotic timing, 23 and 38% of cell death and misalignment, respectively, for Ska14A + Ska33A; mean of 297 min for mitotic timing, 31 and 40% of cell death and misalignment, respectively, for Ska14D + Ska33D; Figs. 4 A and S3, B and D; and Videos 4 and 5). These results suggest that precise temporal control of Ska1/Ska3 phosphorylation/dephosphorylation is essential for mitotic progression.
were displaced from KTs in cells expressing Mis12-INCENP, the nonphosphorylatable mutants persisted at KTs even in the presence of increased Aurora B activity at outer KTs (Fig. 4 C), clearly indicating that mutations preventing Aurora B phosphorylation on the Ska complex severely compromise its Aurora B–dependent removal from KTs.

As shown previously (Gaitanos et al., 2009), K-fiber stability is lost upon Ska depletion. Thus, we assayed the stability of K-fibers after exposure of cells expressing either Ska WT or phosphorylation site mutants to cold. As expected, expression of SkaWT proteins in Ska-depleted cells rescued K-fiber stability (Fig. 5, A–C). In contrast, the phosphomimetic mutants failed to restore K-fiber stability, which is in line with their reduced KT recruitment and inability to rescue the Ska depletion phenotype. However, the nonphosphorylatable mutants not only supported stable K-fiber formation (Fig. 5, A–C) but also generated inter-KT tension comparable with that observed in cells expressing SkaWT (inter-KT distance of 1.25 and 1.21 µm for Ska14A + Ska33A and SkaWT, respectively; Fig. S3, E and F). This then raised the question of why the expression of these mutants in cells caused
severe mitotic defects. Given that the nonphosphorylatable mutants persisted at KTs with high Aurora B activity (Fig. 4 C), we considered it plausible that these proteins might produce hyper-stable KT–MT attachments. Thus, we conducted monastral washout assays to test the ability of these cells to correct syntenic attachments (Kapoor et al., 2000). 1 h after monastral washout, only 30% of cells expressing the nonphosphorylatable Ska mutants had achieved metaphase, whereas >60% of control (GL2 treated) and Ska WT-expressing cells had done so (Fig. 5, D and E). These results support the view that nonphosphorylatable Ska mutants cause premature and/or excessive stabilization of KT–MT attachments, which in turn interferes with error correction and gives rise to mitotic defects. Finally, we tested whether Aurora B might also modulate the MT binding affinity of the Ska complex. Aurora B did not detectably influence the whether Aurora B might also modulate the MT binding affinity of the Ska complex. Aurora B did not detectably influence the amount of Ska protein pelleted with MTs (Fig. S3 G), although it strongly reduced the amount of Ndc80 examined for control (Ciferri et al., 2008). Together, these results demonstrate that Aurora B–mediated regulation of Ska complex recruitment to KTs is crucial to stabilize KT–MT interactions.

Phosphorylation of the KMN subunits Hec1, Mis13, and KNL1 by Aurora B has previously been shown to reduce the KMN’s affinity for MT binding (Cheeseman et al., 2006; DeLuca et al., 2006; Ciferri et al., 2008; Guimaraes et al., 2008; Welburn et al., 2010). Here, we describe an additional mechanism for how Aurora B can negatively regulate KT–MT attachments. We propose that the Ska complex is recruited to KTs to stabilize end-on attachments mediated by the KMN network and that Aurora B regulates this recruitment. At poorly attached and/ or tensionless KTs, the Ska complex is spatially close to Aurora B and therefore readily phosphorylated (Liu et al., 2009; Wang et al., 2011). This then interferes with KMN–Ska interactions and reduces the recruitment of the Ska complex to KTs. Once sister chromatids are bioriented and tension is established, members of the KMN and Ska complexes are dephosphorylated, leading to full stabilization of the KT–MT attachments. We do not exclude that Aurora B phosphorylation on Ska proteins could produce additional effects, such as influencing Ndc80 activity or preventing Ska proteins from interacting with proteins other than KMN. Likewise, Aurora B may phosphorylate additional targets that contribute to the interaction between KMN and Ska.

The cooperation of the KMN network and the Ska complex in the formation of stable end-on attachments is reminiscent of the relationship between the Ndc80 and Dam1 complexes in budding yeast. Thus, the Ska complex has been proposed to be the functional counterpart of the fungal Dam1 complex in metazoan organisms (Hanisch et al., 2006; Gaitanos et al., 2009; Welburn et al., 2009). Although this view is far from being universally accepted, our present results strengthen the view that different organisms use an evolutionarily conserved mechanism to form and maintain KT–MT attachments. Similar to the results reported here, the interaction between Dam1 and Ndc80 is also regulated by Aurora B in vitro (Lampert et al., 2010; Tien et al., 2010), and Ndc80 mutants that cannot bind Dam1 show defects in forming end-on attachments in Saccharomyces cerevisiae (Maure et al., 2011). Surprisingly, Dam1 in Schizosaccharomyces pombe and Ska3 in chicken DT40 cells are nonessential for viability (Sanchez-Perez et al., 2005; Ohta et al., 2010), arguing that in these cells, the KMN network may be sufficient, albeit perhaps less efficient, to support functional KT–MT attachments. In the future, it will be interesting to determine whether the Ska complex is essential in mammalian cells. Also, structural information on KMN network–Ska complex interactions will hopefully contribute to our molecular understanding of how these proteins cooperate to perform their functions at metazoan KTs.

Materials and methods

Cloning, plasmid, and siRNA transfection

The cDNAs of Ska1 and Ska3 were cloned into pcDNA5/FRT/TO or pcDNA3.1 vectors (Invitrogen) encoding an N-terminal myc tag or an N-terminal mCherry tag. An siRNA-resistant version of Ska1 containing six silent mutations and the phosphorylation site mutants of Ska1 and Ska3 were generated using the QuikChange site-directed mutagenesis kit (Agilent Technologies). Mis12-INCENP-GFP and Mis12-INCENP [TAA]-GFP plasmids (Liu et al., 2009) were provided by S.M. Lens (University Medical Center Utrecht, Utrecht, Netherlands). Plasmid transfections were performed using TransIT-LT1 reagent (Mirus Bio Corporation) according to the manufacturer’s instructions. siRNA duplexes were transfected using Oligofectamine (Invitrogen) according to the manufacturer’s instructions. siRNA duplexes for GL2 (5’-CGTACGGGAAAATCTCGGA-3’), Ska1 (5’-CCCGCTAACCTATATACAA-3’; Hanisch et al., 2006), Ska3 (5’-AGA-CAAAATGAAACTAA-3’; Gaitanos et al., 2009), and Ska1 (5’-GGAGAATTAAAGAATTTA-3’; Heic1 (5’-GTCAAAAGCTGATGATC-3’; Chan et al., 2009), KNL1 (5’-GGAAATCCATGTCAAGGA-3’; Liu et al., 2010), and Mis13 (5’-GGCGCTTCCAGAGGAAAAG-3’; Obuse et al., 2004) were previously described.

Protein purification, GST pull-down, and kinase assays

All His6-tagged proteins or GST-Ska2 were expressed in Escherichia coli and purified by Ni-nitrilotriacetic acid agarose beads (Qiagen) or glutathione Sepharose (GE Healthcare), respectively. The His6 encoding His6-tagged Aurora B (Yang et al., 2008) was a gift from X. Yao (University of Science and Technology of China). The full-length Ska complex comprising Ska1-His6, untagged Ska2 (a gift from A.M. Jeyaprakash and E. Conti, Max Planck Institute of Biochemistry, Martinsried, Germany), and His6-Ska3 was purified as previously described (Gaitanos et al., 2009). In brief, cells transformed with Ska1-His6 and Ska2 were mixed with cells transformed with His6-Ska3. Cells were then lysed in buffer containing 10 mM Tris-HCl, pH 8, 500 mM NaCl, 1 mM DTT, 0.05% NP-40, and 1 mM EDTA. The protein complexes were isolated by Ni-nitrilotriacetic acid beads and further purified by RESOURCE Q and Superdex 200 columns using fast protein liquid chromatography [AKTA Explorer; GE Healthcare]. Myc-Mis13 was produced using the TNT T7 coupled transcription/translation system (Promega) and incubated with glutathione Sepharose coupled with GST-Ska2 or GST alone for 1 h. The Sepharose beads were then washed five times with PBS, and bound species were resolved by SDS-PAGE and analyzed by Western blotting. In vitro kinase assays on recombinant proteins were performed at 30°C in buffer [25 mM Hepes, 50 mM NaCl, 1 mM DTT, 2 mM EGTA, 5 mM MgSO4, 10 μM ATP, and 5 μC γ[32P]ATP] for 30 min. Samples were then resolved by SDS-PAGE and visualized by autoradiography. For kinase assays on membranes, 12-mer peptides containing putative Aurora B sites (S/T) of Ska1 and Ska3 at position 7 were generated using standard Fmoc (N-(9-fluorenyl)methoxycarbonyl) chemistry on a Multispot robotic spotter (Intavis) according to the manufacturer’s directions and immobilized on cellulose membranes. The membranes were then incubated with Aurora B in the aforementioned kinase buffer, and kinase assays were performed as previously described (Santamaria et al., 2011).

Cell culture and synchronization

HeLa cells were cultured in a 5% CO2 atmosphere in DMEM (Invitrogen) supplemented with 10% heat-inactivated FCS and penicillin-streptomycin (100 IU/ml and 100 μg/ml, respectively). Nocodazole (0.33 or 3.3 μM), thymidine (2 mM), and monastrol (150 μM) were obtained from Sigma-Aldrich, and ZM447439 (10 μM) was obtained from Tocris Bioscience. MG132 (10 μM) was obtained from EMD. Reversine (0.5 μM) was obtained from Cayman Chemical. Mps1-IN-1 (2 μM; Kwiatkowski et al., 2010) was a gift from N.S. Gray (Dana-Farber Cancer Institute, Boston, MA).
Cell lysates, immunoprecipitation, and Western blot analysis

Cell lysates were prepared with Heps lysis buffer (50 mM Heps, 150 mM NaCl, 0.5% Triton X-100, 1 mM DTT, 30 g/mL DNase, 30 mg/mL RNase, and protease and phosphatase inhibitors), and Western blotting was performed as previously described (Chan et al., 2009). For coimmunoprecipitation, lysates were prepared using Heps lysis buffer (buffers containing 0.1% or no Triton X-100 were used for Ska1 or Mis12 coimmunoprecipitation, respectively). Immunoprecipitations on cell lysates were performed using 5 µl of solid beads (Affi-Prep Protein A Matrix, Bio-Rad Laboratories) chemically cross-linked to 2–3 µg/µl of antibody against 2 mg of clarified lysates for 2 h at 4°C. The beads were washed four times with Heps lysis buffer, and bound species were resolved by SDS-PAGE and analyzed by Western blotting. Image (National Institutes of Health) was used to quantify the relative intensities of the coprecipitated proteins in Ska1 immunoprecipitations. First, we subtracted the intensity of the measured protein precipitated with control IgGs from that precipitated with Ska1 antibodies. Intensities were then normalized by dividing them by the mean intensity of the inputs. The value from the Noc + MG132 samples was set as 1.

Immunofluorescence microscopy

HeLa S3 cells or HeLa cells stably expressing GFP-metaSka2 (a gift from J.M. Cheeseman, Whitehead Institute for Biomedical Research, Cambridge, MA; Welburn et al., 2009) were grown on coverslips and simultaneously immunostained with rabbit anti-Ska3 (1:1,000; Gaitanos et al., 2009), rabbit anti-mCherry (1:1,000 for E. coli; Hübner et al., 2010), human CREST autoantibodies (1:500; ImmunoVision, Inc.), rabbit anti-Myc (1:5; 9E10 tissue culture supernatant). For immunofluorescence analysis, primary antibodies were detected with Cy2-, Cy3-, and Cy5-conjugated donkey anti-mouse, –rabbit, or –human IgGs (1:1,000; Dianova). Directed yeast two-hybrid analysis
cDNAs encoding the respective prey or bait proteins were cloned in-frame with the GAL activation domain of pACT2 (Spz24) or pGAD-C1 (in the case of Ska1, Sk2, and Sk3a) vectors or the GAL-binding domain of pBF19 vector (in the case of Hec1, Nuf2, Spz24, and Spz25). All vectors were obtained from Takara Bio Inc. Plasmids encoding members of the Mis12 complex (Mis12, Mis13, Mis14, and Nuf1; Kiyomitsu et al., 2010) were a gift from M. Yanagida (Kyoto University, Kyoto, Japan). Directed yeast two-hybrid experiments were performed as previously described (Hanisch et al., 2006).

Timeline microscopy

After siRNA and plasmid transfection, HeLa S3 cells stably expressing hH2B-GFP (Sillje et al., 2006) were imaged using a microscope (Eclipse Ti; Nikon) equipped with a CoollED d1 excitation system and a 20×/0.75 air Plan Apochromat objective (Nikon) at 37°C. Images were acquired at multiple positions every 3 min for 18 h. GFP signal was acquired every time point with a 30-ms exposure time. MetaMorph software (7.7; Molecular Devices) was used to collect and process the data. The results are displayed as box and whisker plots; boxes represent 25–75% of the cells, the line within the box indicates the median, top and bottom whiskers represent the 10th and 90th percentiles, and dots represent the 5th and 95th percentiles, respectively.

MT pelleting assay

MT cosedimentation assays were performed as reported elsewhere (Ciferri et al., 2008). In a typical reaction, 3 µM Ska complex or Ndc80 complex (provided by A. Musacchio, Max Planck Institute for Molecular Physiology, Dortmund, Germany; purified as reported in Ciferri et al. [2008]) was incubated with increasing concentration of Aurora B and 10 mM ATP and 20 mM MgSO4 for 30 min at 30°C. 6 µM taxol-stabilized MTs was added, and incubation was continued for a further 10 min at room temperature. Reactions were transferred onto 100 µl of cushion buffer (BRBB0 buffer containing 30% glycerol and 50 µM taxol) and ultracentrifuged for 10 min at 80,000 rpm using a TLA-100 rotor (Beckman Coulter). Equal volumes of pellets and supernatants were analyzed by SDS-PAGE.

Online supplemental material

Fig. S1 shows the localization of Ska1 in hSpindly-depleted or Aurora B–inhibited cells and Ska3 staining in mitotic kinase–inhibited and KMN-depleted cells. Fig. S2 shows the results from the Mis12 coimmunoprecipitation, directed yeast two-hybrid experiments, and GST pull down. Fig. S3 shows the information from the live-cell imaging experiments, the measurement of inter-KT distances, and the MT pelleting assay. Videos 1–5 show the mitotic progression of cells treated with Ska1 + Ska3 siRNAs followed by transfection with vector control, WT, or mutant Ska plasmids. Online supplemental material is available at http://www.jcb.org/cgi/content/full/jcb.201109001/D1C1.

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References

Andrews, P.D., Y. Ovechkin, N. Morrice, M. Wagenbach, K. Duncan, L. Wordeman, and J.R. Swedlow. 2004. Aurora B regulates MCAK at the mitotic centromere. Dev. Cell. 6:253–268. http://dx.doi.org/10.1016/S1534-8007(04)00025-5

Chan, Y.W., L.L. Fava, A. Uldschmid, M.H. Schmitz, D.W. Gerlich, E.A. Nigg, and A. Santamaria. 2009. Mitotic control of kinetochore-associated dynein and spindle orientation by human Spindly. J. Cell Biol. 185:859–874. http://dx.doi.org/10.1083/jcb.200812167

Cheeseman, I.M., J.S. Chappie, E.M. Wilson-Kubalek, and A. Desai. 2006. The conserved KMN network constitutes the core microtubule-binding site of the kinetochore. Cell. 127:983–997. http://dx.doi.org/10.1016/j.cell.2006.09.039

Ciferri, C., S. Pasqualotto, E. Screpanti, G. Varetti, S. Santaguida, G. Dos Reis, A. Masiolica, J. Polka, J.G. De Luca, P. De Wulf, et al. 2008. Implications for kinetochore-microtubule attachment from the structure of an engineered Ndc80 complex. Cell. 133:427–439. http://dx.doi.org/10.1016/j.cell.2008.03.020

Daum, J.R., J.D. Wren, J.J. Daniel, S. Sivakumar, J.N. McAvoy, T.A. Potapova, and G.J. Gorbsky. 2009. Ska3 is required for spindle checkpoint silencing and the maintenance of chromosome cohesion in mitosis. Curr. Biol. 19:1467–1472. http://dx.doi.org/10.1016/j.cub.2009.07.017

Daum, J.R., T.A. Potapova, S. Sivakumar, J.J. Daniel, J.N. Flynn, S. Rankin, and G.J. Gorbsky. 2011. Cohesion fatigue induces chromatid separation in cells delayed at metaphase. Curr. Biol. 21:1018–1024. http://dx.doi.org/10.1016/j.cub.2011.05.012

DeLuca, J.G., W.E. Gall, C. Ciferri, D. Cimini, A. Musacchio, and E.D. Salmon. 2006. Kinetochore microtubule dynamics and attachment stability are regulated by Hec1. Cell. 127:969–982. http://dx.doi.org/10.1016/j.cell.2006.09.047

Ditchfield, C., V.L. Johnson, A. Tighe, R. Eliston, C. Haworth, T. Johnson, A. Mortlock, N. Keen, and S.S. Taylor. 2003. Aurora B couples chromosome...
alignment with anaphase by targeting ByBk1, Mad2, and Cenp-E to kineto-

Gaitanos, T.N., A. Santamaria, A.A. Jayaprakash, B. Wang, E. Conti, and E.A. Nigg. 2009. Stable kinetochore-microtubule interactions depend on the Ska complex and its new component Ska3/130p3. EMBO J. 28:1442–

Gassmann, R., A.J. Holland, D. Varma, X. Wan, F. Civril, D.W. Cleveland, K. Oegema, E.D. Salmon, and A. Desai. 2010. Removal of Spindly from microtubule-attached kinetochores controls spindle checkpoint silencing in human cells. genes Dev. 24:957–971. http://dx.doi.org/10.1101/gad.1886810

Guimarães, G.J., Y. Dong, B.F. McEwen, and J.G. Deluca. 2008. Kinetochore- microtubule attachment relies on the dimerized N-terminal tail domain of Hecl. Curr. Biol. 18:1778–1784. http://dx.doi.org/10.1016/j.cub.2008.08.012

Hanisch, A., H.H. Silljé, and E.A. Nigg. 2006. Timely anaphase onset requires a novel spindle and kinetochore complex comprising Skal and Sk2a. EMBO J. 25:5504–5515. http://dx.doi.org/10.1038/sj.emboj.7600426

Hübner, N.C., L.H. Wang, M. Kaulich, P. Descombes, I. Poser, and E.A. Nigg. 2010. Re-examination of siRNA specificity questions role of PICH and Taol in the spindle checkpoint and identifies Mad2 as a sensitive target for small RNAs. Chromosoma. 119:149–165. http://dx.doi.org/10.1007/s00412-009-0244-2

Kapoor, T.M., T.U. Mayer, M.L. Coughlin, and T.J. Mitchison. 2000. Probing kinetochore substrates. Cell. 114:1–10. http://dx.doi.org/10.1016/S0092-8674(00)00045-2

Kiyomitsu, T., O. Iwasaki, C. Obuse, and M. Yanagida. 2010. Inner centromere protein recruitment requires KNL1, an inner centromere protein that specifically recruits HP1 to human chromosomes. J. Cell Biol. 188:791–807. http://dx.doi.org/10.1038/jcb.200908096

Kwiatkowski, N., N. Jelluma, P. Filippakopoulos, P. Sourdaarajani, M.S. Manak, M. Kwon, H.G. Choi, T. Sim, Q.L. Deveraux, S. Rottmann, et al. 2011. Small-molecule kinase inhibitors provide insight into Mps1 cell cycle function. Nat. Chem. Biol. 7:359–368.

Lampert, F., P. Hornung, and S. Westermann. 2010. The Dam1 complex confers microtubule plus-end-tracking activity to the Ndc80 kinetochore complex. J. Cell Biol. 189:641–649.

Lampsen, M.A., K. Renduchintala, A. Khodjakov, and T.M. Kapoor. 2004. Correcting improper chromosome-spindle attachments during cell division. Nat. Cell Biol. 6:232–237.

Lan, W., X. Zhang, S.L. Kline-Smith, S.E. Rosasco, G.A. Barrett-Wilt, J. Shahabanowitz, D.F. Hunt, C.E. Walczak, and P.T. Stukenberg. 2004. Aurora B phosphorylates centromeric MCAK and regulates its localization and microtubule depolymerization activity. Curr. Biol. 14:273–286.

Liu, D., G. Vader, M.J. Vromans, M.A. Lampson, and S.M. Lens. 2009. Sensing chromosome bi-orientation by spatial separation of aurora B kinase from kinetochore substrates. Science. 323:1350–1353.

Liu, D., M. Vleugel, C.B. Backer, T. Hori, T. Fukagawa, I.M. Cheeseman, and M.A. Lampson. 2010. Regulated targeting of protein phosphatase 1 to the outer kinetochore opposes Aurora B kinase. J. Cell Biol. 188:809–820. http://dx.doi.org/10.1038/jcb.201001006

Maresca, T.J., and E.D. Salmon. 2010. Welcome to a new kind of tension: Evidence that Aurora B is implicated in spindle checkpoint signalling in- dependently of error correction. EMBO J. 30:1508–1519. http://dx.doi.org/10.1038/emboj.2011.70

Santamaria, A., R. Neef, U. Eberspaicher, K. Eis, M. Huesemann, M. Dumberg, S. Prechtel, V. Schulze, G. Siemeister, L. Wortmann, et al. 2007. Use of the novel Plk1 inhibitor ZK-thiazolidinedione to elucidate functions of Plk1 in early and late stages of mitosis. Mol. Cell. Biol. 18:4024–4036. http://dx.doi.org/10.1128/MCB.005517-09

Santamaria, A., B. Wang, S. Elowe, R. Malik, F. Zhang, M. Bauer, A. Schmidt, H.H. Silljé, R. Körner, and E.A. Nigg. 2011. The Plk1-dependent phospho-
proteome of the early mitotic spindle. Mol. Cell. Proteomics. 10:3160–3145.

Scheidt, J.C., T. Kiyomitsu, T. Hori, C.B. Backer, T. Fukagawa, and I.M. Cheeseman. 2010. Aurora B kinase controls the targeting of the Astrin-SKAP complex to bi-oriented kinetochores. J. Cell Biol. 191:269–280.

Sessa, F., M. Mapelli, C. Ciferri, C. Tarricone, L.B. Areces, T.R. Schneider, P.T. Stukenberg, and A. Musacchio. 2005. Mechanism of Aurora B activation by INCENP and inhibition by hesperadin. Mol. Cell. 18:379–391.

Silljé, H.H., S. Nagel, R. Körner, and E.A. Nigg. 2006. HURP is a Ran-importin that controls spindle checkpoint silencing in fission yeast. Curr. Biol. 16:731–742.

Sui, S., J. Wang, B. Yang, L. Song, J. Zhang, M. Chen, J. Liu, Z. Lu, Y. Cai, S. Chen, et al. 2008. Phosphoproteome analysis of the human Chac liver cells using SCX and a complementary mass spectrometric strategy. Proteomics. 8:2024–2034.

Thes, M., M. Slabicki, M. Junqueira, M. Paszkowski-Rogacz, J. Sontheimer, R. Kiitter, A.K. Heninger, T. Glatter, K. Kruusmaa, I. Poser, et al. 2009. Comparative profiling identifies C13orf3 as a component of the Skak complex required for mammalian cell division. EMBO J. 28:1453–1465. http://dx.doi.org/10.1038/emboj.2009.114

Tien, J.F.T., U. Fleig, D.F. Hudson, B. Sessa, F., M. Mapelli, C. Ciferri, C. Tarricone, L.B. Areces, T.R. Schneider, P.T. Sessa, F., M. Mapelli, C. Ciferri, C. Tarricone, L.B. Areces, T.R. Schneider, P.T. Stukenberg, and A. Musacchio. 2005. Mechanism of Aurora B activation by INCENP and inhibition by hesperadin. Mol. Cell. 18:379–391.

Wang, E., E.R. Ballister, and M.A. Lampson. 2011. Aurora B dynamics at centromeres create a diffusion-based phosphorylation gradient. J. Cell Biol. 194:539–549. http://dx.doi.org/10.1083/jcb.201103044

Welburn, J.P., E.L. Grishchuk, C.B. Backer, E.M. Wilson-Kubaleza, J.R. Yates III, and I.M. Cheeseman. 2009. The human kinetochore Skl complex facilitates microtubule depolymerization-coupled motility. Dev. Cell. 17:374–385. http://dx.doi.org/10.1016/j.devcel.200911.011

Yang, Y., F. Wu, T. Ward, F. Yan, Q. Wu, Z. Wang, T. McGlothen, W. Peng, T. You, M, Sun, et al. 2008. Phosphorylation of H2Av13 by Aurora B kinase is essential for assembly of functional kinetochore. J. Biol. Chem. 283:26726–26736. http://dx.doi.org/10.1074/jbc.M804207200