Cdk1 controls many aspects of mitotic chromosome behavior and spindle microtubule (MT) dynamics to ensure accurate chromosome segregation. In this paper, we characterize a new kinetochore substrate of fission yeast Cdk1, Nsk1, which promotes proper kinetochore-MT (k-MT) interactions and chromosome movements in a phosphoregulated manner. Cdk1 phosphorylation of Nsk1 antagonizes Nsk1 kinetochore and spindle localization during early mitosis. A nonphosphorylatable Nsk1 mutant binds prematurely to kinetochores and spindle, cementing improper k-MT attachments and leading to high rates of lagging chromosomes that misregate. Accordingly, cells lacking nsk1 exhibit synthetic growth defects with mutations that disturb MT dynamics and/or kinetochore structure, and lack of proper phosphorylation leads to even more severe defects. Intriguingly, Nsk1 is stabilized by binding directly to the dynein light chain Dlc1 independently of the dynein motor, and Nsk1–Dlc1 forms chainlike structures in vitro. Our findings establish new roles for Cdk1 and the Nsk1–Dlc1 complex in regulating the k-MT interface and chromosome segregation.

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Abbreviations used in this paper: ChIP, chromatin immunoprecipitation; CPC, chromosomal passenger complex; DLC, dynein light chain; k-MT, kinetochore-MT; MBP, maltose-binding protein; MS, mass spectrometry; MT, microtubule; SAC, spindle assembly checkpoint; SPB, spindle pole body; TAP, tandem affinity purification; TBZ, thiabendazole; YE, yeast extract.

Introduction

The forces that drive chromosome motions during mitosis derive from the persistent interaction of dynamic microtubule (MT) plus-ends with kinetochores (Rieder and Salmon, 1998; Joglekar et al., 2010). Whereas stable kinetochore–MT (k-MT) attachments require the functions of the evolutionarily conserved Ndc80, KNL-1, and Mis12 complexes (Santaguida and Musacchio, 2009), other proteins act on k-MT plus-ends to regulate their stability and/or dynamics. For example, MT plus-end–tracking proteins, such as EB1 and CLASP, and members of the kinesin-8 family impact k-MT dynamics positively and negatively, respectively, and thereby influence the overall positioning of chromosomes within the mitotic spindle by defining their motile properties (Walczak et al., 2010).

Given the dynamic nature of k-MT interactions, it follows that k-MT regulators must be carefully controlled through post-translational modification. In particular, Aurora B kinase, the catalytic subunit of the chromosomal passenger complex (CPC), is crucial for modulating the activity of kinetochore proteins to influence the stability of k-MT attachments (Lampson and Cheeseman, 2011). Other mitotic kinases, including Cdk1 and the Polo-like kinase 1, have also been implicated in regulating k-MT interactions, but few relevant kinetochore substrates are known (Petronczki et al., 2008; Ma and Poon, 2011).

Results and discussion

Nsk1 is a novel kinetochore and spindle protein

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Cdc14 phosphatases antagonize Cdk1 in yeasts (Stegmeier and Amon, 2004; Mocciaro and Schiebel, 2010). To identify new Cdk1 substrates relevant to chromosome dynamics, tandem affinity purification (TAP) of a substrate-trapping mutant of the fission yeast Cdc14 phosphatase, Clp1, was performed from mitotic cells, and associated proteins were identified by 2D liquid chromatography mass spectrometry (MS). A top hit was an uncharacterized protein (Fig. S1 A) that we named Nsk1.
(nucleolus spindle kinetochore 1) to reflect its localization pattern. This pattern was determined by imaging Nsk1 that had been tagged endogenously with GFP. During interphase, Nsk1-GFP was nucleolar (Fig. S1 B). During prometaphase/metaphase, Nsk1-GFP distributed throughout the nucleus but was also observed at puncta between spindle pole bodies (SPBs; Fig. 1 A) that colocalized with NuF2-RFP, an outer kinetochore component (Fig. 1 B). During anaphase, Nsk1-GFP increased at kinetochores and also decorated the spindle, although it was excluded from the spindle midzone (Fig. 1, A and B).

We verified the kinetochore localization of Nsk1 using chromatin immunoprecipitation (ChIP). Anti–Nsk1-GFP immuno-precipitates enriched centromeric sequences, with preference for the central core and innermost repeats over a segment of the outer repeats, relative to a euchromatic control (Fig. 1 D; Santaguida and Musacchio, 2009). Because Nsk1 also localized to spindles, we tested whether it bound MTs using copelleting and microscopy assays. Nsk1 bound MTs with an apparent $K_d$ of 0.23 µM (Fig. 1 E) and also bundled MTs (Fig. 1 F), suggesting that Nsk1 may localize to the spindle via a direct interaction with MTs.

**Nsk1 promotes chromosome alignment and proper chromosome segregation**

To determine the mitotic function of Nsk1, we examined cells lacking it. $nsl1$Δ cells were viable over a range of temperatures (Fig. S1 C), and their length at division was normal, indicating that $nsl1$Δ had generally unperturbed cell cycle control (Fig. S1 D). However, consistent with Nsk1 acting at kinetochores and spindle during mitosis, $nsl1$Δ cells exhibited mild sensitivity to thiamazole (TBZ), an MT-destabilizing agent (Fig. S1 E) that was exacerbated by the deletion of Alp7/Mia1, a factor that promotes spindle integrity (Fig. S1 F; Oliferenko and Balasubramanian, 2002; Sato et al., 2004). Reciprocally, $nsl1$Δ suppressed the TBZ resistance of the $klp5$Δ/kinesin-8 deletion, which hyperstabilizes MTs (Fig. S1 G; West et al., 2001). $nsl1$Δ also showed strong negative genetic interactions with a variety of mutations affecting kinetochore proteins (Fig. S1 H) and lost minichromosomes at an elevated rate (0.6% vs. 0% for wild type; Fig. 2 A), comparable with loss rates of mutants affecting centromere gene silencing and DNA repair (Ekwall et al., 1996; Nakagawa et al., 2002; Greenall et al., 2006).

To understand why chromosomes missegregate in $nsl1$Δ, we used time-lapse microscopy to follow spindle dynamics, which in fission yeast occur in three phases (Hagan, 1998; Nabeshima et al., 1998). In phase 1, the spindle assembles. During phase 2, spindle length is constant, and, in phase 3, the pole-to-pole distance increases as anaphase B ensues. $nsl1$Δ cells spent significantly longer than wild-type cells in phase 2 (Fig. 2, B–D), which corresponds primarily to late prometaphase or metaphase.

A phase 2 delay can be caused by defects in kinetochore capture by spindle MTs that trigger activation of the spindle assembly checkpoint (SAC; Musacchio and Salmon, 2007). We did observe an elevated percentage of Mad2- and Mad3-GFP–positive kinetochores in $nsl1$Δ relative to wild type (Fig. 2 E), and phase 2 timing was similar to wild type in SAC-deficient $nsl1$Δ $mhp$Δ cells (Figs. 2 C and S1 I). Thus, the phase 2 delay in $nsl1$Δ cells is a result of SAC activity. However, SAC mutants did not affect $nsl1$Δ cell growth significantly (Fig. S1 J), as would be expected if there were appreciable defects in kinetochore capture or biorientation. This suggested that $nsl1$Δ cells had defective k-MT interactions after biorientation. Interestingly, kinesin-8 $klp5$Δ mutants also display a SAC-dependent phase 2 delay and are viable with $mad2$Δ highlighting a commonality between Nsk1 and another factor that influences k-MTs after biorientation (Garcia et al., 2002).

Next, we tracked a single centromere (Cen2) at 10-s intervals to examine chromosome movements. In wild-type cells, sister kinetochores seldom separated, and their movements were coordinated. In contrast, $nsl1$Δ sister kinetochores moved apart from each other (Fig. 2, F–H) and also collided with SPBs (Fig. 2 I) more frequently. Accordingly, the mean interkinetochore distance in $nsl1$Δ cells was increased compared with wild type (Fig. 2 J), although spindle length was not (Fig. 2, B and D). These data suggest that k-MT plus-ends undergo catastrophe at higher frequencies in $nsl1$Δ cells, leading to defects in chromosome movements and segregation.

**Nsk1 is a phosphoprotein regulated by Cdk1 and Clp1**

Given that Nsk1 was identified as a potential Cdk1 substrate, we tested whether Nsk1 was phosphorylated during mitosis to affect its function. Nsk1-GFP was phosphorylated at a prometaphase arrest and then dephosphorylated as cells progressed through mitosis (Fig. 3, A and B). In $clp1$Δ, Nsk1 was hyperphosphorylated in prometaphase-arrested cells, which is consistent with Nsk1 being a Clp1 substrate during early mitosis (Fig. 3, A and B). However, Nsk1 was partially dephosphorylated in $clp1$Δ cells progressing through mitosis (Fig. 3 A), suggesting that another phosphatase contributes to Nsk1 dephosphorylation at these stages. Next, we tested Nsk1 as a target for Cdk1 and Clp1 in vitro. Cdk1, but not catalytically inactive Cdk1, efficiently phosphorylated recombinant maltose-binding protein (MBP)–Nsk1 but not MBP (Figs. 3 C and S1 K). After washing out Cdk1, purified MBP-Clp1 but not catalytically inactive MBP-Clp1-C286S dephosphorylated Nsk1 (Fig. 3 C). Thus, Nsk1 is a substrate of both Cdk1 and Clp1.

To determine whether Cdk1 phosphorylation affects Nsk1 function, we first tested the effect of Cdk1 inhibition on Nsk1 localization using a Cdk1 analogue–sensitive mutant (Dischinger et al., 2008). Nsk1 localized to kinetochores of nda3-arrested cells after Cdk1 inhibition but not before (Fig. 3, D and E). In nda3 cdc2-as cells that had progressed to metaphase, as judged by SPB separation, 1NMPP1 treatment led to Nsk1-GFP localization between SPBs, indicative of kinetochore and/or spindle localization (Fig. 3 F). These data suggest that Nsk1 may bind kinetochores independently of MTs and show that Cdk1 antagonizes Nsk1 kinetochore and spindle targeting.
nsk1-GFP sid4-RFP

Interphase | Metaphase | Anaphase | Septation
-----------|-----------|----------|-----------
Nsk1        | Sid4      | Merge    | BF Merge  

Figure 1. Nsk1 is a kinetochore-localized MT-binding protein. (A) Live-cell images of nsk1-GFP sid4-RFP. BF, brightfield. (B) Nsk1-GFP Nuf2-RFP localization in fixed cells. Fixation eliminated diffuse nucleoplasmic Nsk1, allowing kinetochore detection in early mitosis. The left two columns show inverted images, whereas the right column shows merged images. (C) Anti-GFP ChIPs were performed on nsk1-GFP and untagged cells synchronized by block and release of nda3-KM311. Real-time PCR was used to analyze association of anti-GFP immunoprecipitates with centromeric loci and with the euchromatic adh1+ control at the times indicated. Data represent the mean relative enrichment of centromeric sites over adh1+ in Nsk1-GFP immunoprecipitate samples, relative to the input material, and normalized to the immunoprecipitates of untagged strains. Error bars represent SEM (n = 4). Culture synchronicity was monitored by antitubulin staining of cells processed in parallel. The mean number of spindle-containing cells (±SEM) is shown. cnt, central core; dh, outer repeat; imr, innermost repeat. (D) Localization of Nsk1-GFP to kinetochores (KTs) was measured in ≥48 mitotic wild-type, spc7-23, or mis6-302 cells after shifting to 36°C for 3.5 h in a representative experiment of two. Representative images of fixed cells for each strain are shown. (E) Recombinant MBP-Nsk1 was incubated with paclitaxel-stabilized MTs and ultracentrifuged. The percentage of Nsk1 in pellet fractions is shown. A representative experiment of three is shown. (F) Nsk1 bundles MTs in vitro. Rhodamine-labeled GMP-CPP MTs were incubated with no protein or recombinant MBP-Nsk1 for 15 min and imaged by fluorescence microscopy. Bars: (A, B, and D) 5 μm; (F) 10 μm.
Proper Nsk1 phosphoregulation is essential for accurate chromosome segregation

We used MS to identify 18 phosphorylated Cdk1 consensus sites on Nsk1 isolated from mitotic cells (see Materials and methods). When these 18 residues were mutated to alanines, Cdk1 phosphorylation of Nsk1 in vitro was abolished (Fig. S1 K).

Strains were constructed in which the endogenous nsk1+ locus was replaced with nsk1-18A or a mutant in which the 18 serine/threonine residues had been converted to aspartic or glutamic acids, respectively, nsk1-18D. The mobility shift of Nsk1-GFP...
As a result of hyperphosphorylation in clp1Δ, the mutation abolished the 1NMPP1-induced phase 2 kinetics observed in wild-type cells. Nsk1-18A isolated from mitotic cells comigrated with dephosphorylated Nsk1 from interphase cells (Fig. S1 L), indicating that the vast majority, if not all, Cdk1 phosphorylation sites in Nsk1 had been ablated.

During mitosis, nsk1-18A cells had phase 2 kinetics similar to wild type (Figs. 2 C and 3 H). However, lagging chromosomes were observed in 3% of nsk1-18A cells (n = 41), whereas they were not detected in wild-type cells (n = 71; Fig. S2, A and B).

Accordingly, minichromosomes were lost at a high rate (10.3%) in nsk1-18A cells (Fig. 2 A). In contrast to wild type (Fig. 4 A), Nsk1-18A-GFP localized strongly to kinetochores and the spindle at the onset of its assembly and remained there until the end of mitosis (Fig. 4, B–D). Also, in >50% of nda3-arrested cells, Nsk1-18A-GFP colocalized to kinetochores (Fig. S2 D) and SPBs (Fig. 4 E), to which kinetochores associate at this mitotic stage. These results are consistent with Cdk1 and Clp1 competitively controlling Nsk1 kinetochore localization, with Clp1...
to a high incidence of lagging chromosomes and chromosome loss in the absence of a phase 2 delay, as we have seen (Figs. 2 [A and C] and 3 H). Consistent with merotelic attachments causing lagging chromosomes in nsk1-18A cells, we observed a high incidence of stretched and split kinetochores (Fig. S2 F). This differs from nsk1Δ cells, in which attachments are defective.

nsk1-18D had phase 2 timing similar to wild type (Figs. 2 C and 3 I), but its chromosome loss rate was higher than the

promoting kinetochore targeting (Fig. 3, A and B). We propose that kinetochore localization of Nsk1 in prometaphase is dynamic, a property that would allow destabilization of incorrect k-MT attachments. In this model, premature binding of Nsk1-18A would hamper correction mechanisms by inappropriately stabilizing k-MT interactions. Because merotelic attachments are frequent during prometaphase and unrecognized by the SAC (Gregan et al., 2011), their stabilization by Nsk1-18A would lead to a high incidence of lagging chromosomes and chromosome loss in the absence of a phase 2 delay, as we have seen (Figs. 2 [A and C] and 3 H). Consistent with merotelic attachments causing lagging chromosomes in nsk1-18A cells, we observed a high incidence of stretched and split kinetochores (Fig. S2 F). This differs from nsk1Δ cells, in which attachments are defective.

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Figure 4. Nsk1 localization is phosphoregulated. (A, B, and F) Time-lapse microscopy of the indicated strains. (C and G) Images from the indicated strains. The left panels show inverted images, whereas the right panels show merged images. (D, E, H, and I) Representative images of fixed cells from the indicated strains. Bars, 5 µm.
nsk1Δ strain (Fig. 2 A). Like the Nsk1-18A mutant, Nsk1-18D-GFP was enriched on kinetochores relative to wild type during early stages of mitosis, but its localization to the spindle was attenuated (Fig. 4, F–H). Nsk1-18D-GFP also colocalized at SPBs (Fig. 4 I) and kinetochores (Fig. S2 E) in >50% of nsa3-arrested cells, although the signals were typically less intense. The behavior of the Nsk1-18D mutant suggests that it is a weak phosphomimetic, not recapitulating the results of either full phosphorylation (that prevents kinetochore and spindle localization) or dephosphorylation (impelling both kinetochore and spindle association), although it might have defects unrelated to phosphoryregulation. However, because there is no phase 2 delay in either nsk1-18A or nsk1-18D cells in contrast to nsk1Δ cells and both Nsk1-18A and Nsk1-18D are present on kinetochores, Nsk1 must impact spindle dynamics primarily at kinetochores.

Given the aforementioned data showing that Nsk1 promotes proper coupling between kinetochores and MT plus-ends, we tested whether Nsk1 opposes Aurora kinase (Ark1) function, which destabilizes incorrect k-MT attachments (Kelly and Funabiki, 2009; Liu and Lampson, 2009). As predicted, arkl-T7 nsk1Δ grew better at semipermissive temperatures (27 and 29°C) than arkl-T7 alone (Fig. S3 A). Furthermore, Nsk1-18D, which is enriched at kinetochores during early mitosis relative to Nsk1, exacerbated the arkl-T7 phenotype (Fig. S3 A). Reciprocally, two mutations in protein phosphatase 1, dis2cs and dis2Δ, which oppose Ark1, displayed negative genetic interactions with nsk1Δ but not nsk1-18D (Fig. S3 B). The high chromosome loss rate in nsk1-18A cells precluded meaningful genetic analysis with this strain. These genetic data support the proposed Nsk1 function in proper k-MT interactions.

### Dynein light chain (DLC) promotes Nsk1 oligomerization and stability

Interestingly, DLC 1 (Dlc1) was identified by 2D liquid chromatography MS as a top hit in both the Clp1-TAP (Fig. S1 A) and Nsk1 purifications from mitotic cells (Fig. S3 C). A structural study of the human DLCs TcTex1 and LC8/DYNLL1 in complex with a fragment of dynein intermediate chain shows that they are sandwiched in a configuration that is incompatible with binding other partners (Williams et al., 2007). These structures extended evidence that DLCs form dynein motor–independent complexes with, for example, Myosin Va and Nup159 (Hödi et al., 2006; Stelter et al., 2007; Barbar, 2008). Given the lack of Dhc1 in our purifications, we tested whether a discrete Nsk1–Dlc1 complex could exist; indeed, we were able to reconstitute it by coexpression in Escherichia coli (Fig. 5 A). By negative-stain EM, the Nsk1–Dlc1 complex was observed in chainlike structures of variable lengths and twists (Fig. 5 B) that are remarkably similar to structures formed by a fragment of budding yeast Nup159 in complex with Dyn2 (Stelter et al., 2007). Alignment and classification of relatively straight regions of the Nsk1–Dlc1 chains revealed discrete globular domains stacked into chains (Fig. 5 B, inset). Dlc1 or Nsk1 alone was globular (Fig. 5, C and D), indicating that the chainlike structures represent oligomerized Nsk1–Dlc1 complex. It is intriguing to note that fibrils, whose molecular composition are unknown, emanating from the kinetochore outer plate contact MT plus-ends in vertebrate cells (McIntosh et al., 2008). It will be interesting to learn whether DLCs, in complex with factors analogous to Nsk1, contribute to the formation of such fibrils and, in so doing, comprise functional units of the k-MT interface.

To determine how Dlc1 binding affects Nsk1, we examined Nsk1 localization in dlc1Δ cells. Nsk1 fluorescence was weak in both interphase and mitotic dlc1Δ cells (Fig. 5, E–G). This was also true for Nsk1-18A and Nsk1-18D, which in dlc1Δ- cells displayed stronger kinetochore and/or spindle targeting (Fig. S3, D and E). Consistent with diminished fluorescence intensity, Nsk1 levels were significantly reduced in dlc1Δ cells (Fig. 5 H), suggesting that complex formation with Dlc1 stabilizes Nsk1. In contrast, Nsk1 localization was unaffected by dhlc1Δ (Fig. 5, E–G). Thus, we confirmed the prediction of a Dhc1-independent role for Dlc1 based on the finding that the double mutant dlc1Δ dhc1Δ has more severe meiotic defects than the dhc1Δ mutant alone (Miki et al., 2002). How many distinct complexes Dlc1 and other light chains participate in is not known, but the growing list of heavy chain–independent light chain interactions suggests that caution should be used in assigning dynein motor functions based on phenotypes caused by the loss of DLCs. In this regard, it is noteworthy that although it is not known whether it interacts directly with these proteins, LC8/DYNLL1 copurified with astrin and its binding partner kinastrin/small kinetochore-associated protein (Schmidt et al., 2010; Dunsch et al., 2011), human proteins with analogous functions to those of Nsk1 in binding kinetochores and spindle, stabilizing bioriented attachments, and facilitating proper chromosome movements (Yuan et al., 2009; Manning et al., 2010; Schmidt et al., 2010; Dunsch et al., 2011). Future studies will be useful in determining the extent of the functional and structural similarity between these proteins.

In conclusion, despite a principle role played by the CPC/PP1 system in regulating k-MT attachments (Lampson and Cheeseman, 2011), our results indicate the importance of fine tuning the phosphostatus of a Cdk1 substrate, Nsk1, that affects the proper coupling of kinetochores with MTs, ensuring that it interacts stably with the kinetochore and spindle only at the correct time during mitosis (Fig. 5 I). Nsk1 shares this property of carefully timed phosphoregulated redistribution with other Cdk1/Clp1 targets, namely monopolin (Choi et al., 2009; Rumpf et al., 2010), Klp9 (Fu et al., 2009), and the CPC (Tsukahara et al., 2010), although the specificities of timing and localization differ. Our evidence indicates that Cdk1 regulation of Nsk1–Dlc1 at the k-MT interface is crucial for protecting the fidelity of chromosome segregation.

### Materials and methods

#### Strains and general yeast methods

The strains used in this study are listed in Table S1. Yeast strains were grown in yeast extract (YE) media or Edinburgh minimal media with supplements. nsk1, nsk1-18A, nsk1-18D, nuf2, and sid4 were tagged endogenously at the 3′ end with GFP::kan6, GFP::hyg2, linker-GFP::kan6, TAP::kan6, RFP::kan6, and RFP::hyg2 cassettes as previously described (Bähler et al., 1998). A lithium acetate method (Keeney and Boeke, 1994) was used in Schizosaccharomyces pombe transformations for tag insertions.
Construction of nsk1Δ, nsk1-18A, and nsk1-18D strains

Deletion of nsk1Δ was achieved by PCR-based homologous recombination. In brief, the DNA sequence of 600 bp upstream of the nsk1Δ start codon was cloned in pSK-ura4 (pKG700) with BamHI-PstI. The 600-bp sequence downstream of the nsk1Δ stop codon was cloned in the same plasmid with XhoI–KpnI. The entire insert was then amplified by PCR and transformed into diploid strain ade6-M210/ade6-M216 ura4D18/ura4D18 leu1-32/leu1-32 h/h+. Stable
integrants were selected, and the deletion was confirmed while whole-cell PCR with primers corresponding to 800 bp upstream or downstream of the nsk1 ORF and inside the ura4 gene. After sporation, tetracyl analysis yielded a haploid nsk1::ura4 strain (nsk1Δ). nsk1::kan was subsequently obtained by a marker switch approach (Sato et al., 2005).

Using pREP1GFP-nsk1-18A or -18D as a template, the PCR product containing nsk1-18A or -18D with 100 bp of genomic flanking sequences was transfected into nsk1::ura4 cells, and the transfected cells were selected by plating on YE containing 1 mg/ml 5FOA. Correct replacements were verified by whole-cell PCR. The DNA sequences of endogenous nsk1-18A and -18D were confirmed by whole-cell PCR followed by DNA sequencing. Introduction of nsk1-18A or -18D into other strains was verified by whole-cell PCR followed by restriction digestion. Nol was used to differentiate wild type (1.4 kb) from nsk1-18A (582 and 807 bp). Accl was used for wild type (582 and 807 bp) and nsk1-18D (240, 510, and 636 bp).

ChIp
Untagged nda3-KM311 and nsk1::GFP nda3-KM311 strains were grown in YE media to exponential growth phase at 32°C and then arrested by growing for 6.5 h at 18°C. Cells were fixed with 3% formaldehyde for 18 min at room temperature at 0, 15, and 30 min after release of cells to 32°C. Aliquots were taken for spindle analysis by immunofluorescence. ChIP was performed as previously described (Petrie et al., 2005). In short, fixed cells were resuspended in 400 µl of ChIP lysis buffer and disrupted by bead beating (2 x 1 min). Chromatin was then sheared by probe sonication (3 x 20 s) in an ultrasonic-upercut-impregnated with 0.75 µg monochromatic anti-GFP antibody (a gift from K. Gull, University of Oxford, Oxford, England, UK; Woods et al., 1989) followed by Texas red batting the cells first with anti-TAT1 (provided by K. Gull, University of Oxford, Oxford, England, UK; Woods et al., 1989) and then incubating with 1 mg/ml zymolyase for 90 min in PEMS buffer (100 mM NaCl, 15 mM imidazole, 5 mM 2-Mercaptoethanol, and 1% Triton X-100). After washing the cells with 1 ml of buffer A, the supernatants were incubated with Ni-NTA agarose (QIAGEN) for 30 min at 4°C. The beads were washed twice with 0.5 ml wash buffer (same with buffer A, except that the imidazole concentration was reduced to 50 mM) and then incubated with 0.5 ml elution buffer (same with buffer A, except that the imidazole concentration was increased to 300 mM) for 5 min.

Immunoprecipitations and immunoblotting
Whole-cell S. pombe lysates were prepared in NP-40 buffer (McDonald et al., 1999), and immunoprecipitations were performed using anti-GFP antibodies as previously described (McDonald et al., 1999), with minor modifications in that only 2 µg of antibody was used in each immunoprecipitation. Immunoprecipitation was performed with 0.4 µg/ml anti-GFP antibodies. Immunoblotting was performed using the infrared imaging system and protocol provided by LI-COR (Lincoln, NE), with peroxidase as the secondary antibody.
In vitro kinase and phosphatase assays

In vitro kinase assays were conducted as previously described (Yoon et al., 2006), with the following modifications. After immunoprecipitation from denatured cell lysates, sepharose-bound proteins were washed three times with 1 ml NP-40 buffer and two times with 1 ml of phosphatase buffer (25 mM Hepes-NaOH, pH 7.4, 150 mM NaCl, and 0.1 mg/ml BSA), divided in half, and pulsed down, and the supernatant was aspirated off. 20-µl reactions composed of 1 x phosphatase buffer, 2 mM MgCl₂, 1 µg of MBP-Clp1 or MBP-Clp1-C286S were then incubated at 30°C for 30 min with gentle mixing. The reactions were terminated by adding 5 µl of 5x SDS sample buffer. When the phosphatase was Clp1, ~100 ng MBP-Clp1 or MBP-Clp1-C286S was used, and the phosphatase buffer for wash and assay was changed to 50 mM imidazole, pH 6.9, 1 mM EDTA, and 1 mM DTT.

MT methods

For cosedimentation experiments, varying concentrations of monomeric tubulin or paclitaxel-stabilized MTs were incubated with 50 ng of preclari-fied wild-type MBP-Nsk1 in BRB80 + 100 mM KCl. After 1 hr at room temperature, the reactions were pipetted onto 100 µl of BRB80 + 40% sucrose and pelleted for 20 min at 60,000 rpm in a TL-A100 rotor at 22°C. Cushions for polymeric tubulin reactions also contained 20 µM paclitaxel.

For complex supernatant, 40 µl was withdrawn from the top of the tube and mixed with 40 µl of sample buffer. The pellet was resuspended in 80 µl of sample buffer. Equal amounts of supernatant and pellet fractions were subjected to SDS-PAGE and processed for immunoblotting using anti-MBP to detect Nsk1 or Coomassie blue staining to visualize tubulin. Quantitation of Nsk1 in supernatant and pellet fractions was performed using the Odyssey imaging system.

MT bundling by Nsk1 proteins was analyzed using a microscopy assay. Rhodamine-labeled GMP-CPP MTs (500 nM tubulin) were incubated with 1 µg MBP-Nsk1 in BRB80 for 15 min at room temperature. Reactions were visualized by widefield fluorescence microscopy using a 100x NA 1.4 objective (Nikon).

EM and image processing

Uranyl formate (0.7% w/v) was used for conventional negative staining as previously described (Ohl, 2004). Images of Dlc1 and Nsk1 alone were recorded using a transmission electron microscope [Morgagni; FEI Company] run at 100 kV at a 36,000x magnification. Images were collected on 1K x 1K charge-coupled device camera (Advanced Microscopy Imaging Systems). Images of the Nsk1–Dlc1 oligomers were collected on an electron microscope [Tecnai F20; FEI company] equipped with a field emission electron source and operated at an acceleration voltage of 120 kV under low-dose conditions at a magnification of 62,000x and a defocus value of ~1.5 mm. Images were collected using a 4K x 4K charge-coupled device camera (Gatan, Inc). Charge-coupled device images were converted to mixed raster content format and binned by a factor of two, resulting in final images with 3.46 A/pixel. Particle images of the Nsk1–Dlc1 complex were selected with Boxer software (Ludtke et al., 1999) and win-dowed with 120-pixel side lengths. Only Nsk1–Dlc1 chains that contained relatively straight regions were chosen. Image analysis was performed with SPIDER (System for Processing Image Data from Electron microscopy and Related fields) and the associated display program WEB (Frank et al., 1996). 4,005 images of Nsk1–Dlc1 oligomers were rotationally and translationally aligned and subjected to 10 cycles of multireference alignment and K-means classification. Particles were grouped into 40 classes. From the 40 class means, 10 representative projections were chosen and used as references for another cycle of multireference alignment.

Online supplemental material

Fig. S1 presents additional characterization of nsk1 ΔA, nsk1 ΔA genetic interactions, and Nsk1 phosphoregulation. Fig. S2 presents images of chromosome segregation in nsk1 ΔA, nsk1-18A, and nsk1-18D cells. Fig. S3 presents additional characterization of nsk1 phosphomutants and MS results. Table S1 presents the strains used in this study. Online supplemental material is available at http://www.jcb.org/cgi/content/full/jcb.201105074/DC1.

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