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The microtubule-binding protein Cep170 promotes the targeting of the kinesin-13 depolymerase Kif2b to the mitotic spindle

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Abstract
Microtubule dynamics are essential throughout mitosis to ensure correct chromosome segregation. Microtubule depolymerization is controlled in part by microtubule depolymerases, including the kinesin-13 family of proteins. In humans, there are three closely related kinesin-13 isoforms (Kif2a, Kif2b, and Kif2c/MCAK) that are highly conserved in their primary sequences, but display distinct localization and non-overlapping functions. Here, we demonstrate that the N-terminus is a primary determinant of kinesin-13 localization. However, we also find that differences in the C-terminus alter the properties of kinesin-13, in part by facilitating unique protein-protein interactions. We identify the spindle-localized proteins Cep170 and Cep170R (KIAA0284) as specifically associating with Kif2b. Cep170 binds to microtubules in vitro and provides Kif2b with a second microtubule binding site to target it to the spindle. Thus, the intrinsic properties of kinesin-13s and extrinsic factors such as their associated proteins result in the diversity and specificity within the kinesin-13 depolymerase family.

Introduction
The mitotic spindle is a complex structure composed of dynamic microtubule polymers that is essential to ensure correct chromosome segregation. The dynamic instability of
microtubules is modulated by microtubule-associated proteins that impart stabilizing or destabilizing properties. The control of these microtubule-associated proteins is critical for the formation and maintenance of a bipolar spindle, the alignment and oscillation of the sister chromatids, and the movement of chromosomes to daughter cells in anaphase (Goshima and Scholey, 2010). The kinesin-13 family of microtubule depolymerases, composed of Kif2a, Kif2b, and Kif2c/MCAK in humans, plays an important role in each of these microtubule-based mitotic processes to spatially and temporally control microtubule dynamics. Kinesin-13 depletion results in defects in spindle bipolarity and length, chromosome segregation, and microtubule dynamics (Bakhoum et al., 2009; Ganem and Compton, 2004; Goshima and Vale, 2003; Goshima et al., 2007; Goshima et al., 2005; Kline-Smith et al., 2004; Maney et al., 1998; Manning et al., 2007; Rogers et al., 2004).

Despite roughly similar sequences, Kif2a, Kif2b, and Kif2c display distinct localization and non-overlapping functions during mitosis. Kif2a localizes to centrosomes (Ganem and Compton, 2004) and has been observed weakly at kinetochores (Cameron et al., 2006). Kif2b localizes to spindles, centrosomes, and unaligned kinetochores (Manning et al., 2007). MCAK/Kif2c localizes primarily to kinetochores and weakly to centrosomes during mitosis (Maney et al., 1998; Walczak et al., 2002; Walczak et al., 1996). In addition, MCAK/Kif2c associates with EB1 and Kif18b to target to microtubule plus ends (Honnappa et al., 2009; Tanenbaum et al., 2011). In contrast, the mechanisms controlling Kif2a and Kif2b localization remain less clear. Although the general roles and functions of kinesin motor domains and their interactions with microtubules are well established (reviewed in Hirokawa and Takemura, 2004; Miki et al., 2005; Wordeman, 2010), the molecular basis for their differences in targeting to the correct subcellular locations remains ill-defined. In addition, it is not known whether these three closely related proteins display functional specificity beyond their differential localization.
Here, we conducted a comparative analysis of the kinesin-13 proteins in human cells. We define the divergent regions flanking the motor domains of Kif2a and Kif2c as the regions responsible for specifying kinesin-13 targeting to distinct microtubule-based structures. In addition, we find that Kif2b, but not Kif2a or Kif2c, stably associates with the microtubule binding protein Cep170. The Cep170-Kif2b interaction provides a second extrinsic microtubule binding activity to Kif2b to target it to the mitotic spindle. Thus, the intrinsic features of a kinesin as well as specific extrinsic associations define its localization and activities.

Results

The N-terminal regions of kinesin-13 proteins specifies their localization

The three closely related kinesin-13 proteins each play distinct roles in chromosome segregation. The enzymatic depolymerase kinesin “motor” domain and the neck region (together denoted as “M” in this paper) are well conserved amongst the kinesin-13 isoforms (Figure 1A), whereas the N- and C-terminal regions (denoted “N” and “C” respectively) show greater diversity (Figure 1A,B). To assess the relative contributions of these domains, we first generated clonal cell lines stably expressing low to moderate levels of Kif2a, Kif2b, and Kif2c/MCAK as GFP fusions (Figure 1C). Each kinesin targeted to distinct microtubule-based structures throughout mitosis (Figure S1A), similar to previous reports (Ganem and Compton, 2004; Manning et al., 2007; Wordeman and Mitchison, 1995). To dissect the contributions of the N, M, and C regions to the localization of each kinesin-13 during mitosis, we first expressed GFP fusions lacking the N-terminus. The three kinesin-13 “MC” constructs (also denoted as ΔNT) each displayed identical localization to microtubules during interphase (not shown) and to spindle microtubules during mitosis with an accumulation at centrosomes (Figure 1C). Thus, the localization of kinesin-13 proteins to specific cellular sites and microtubule subpopulations requires their N-terminal regions.
Although the kinesin-13 catalytic domains are highly conserved among the isoforms, the N- and C-terminal regions show greater divergence (Figure 1A) and are likely to be involved in creating specificity and diversity among the kinesin-13 family. The essential contribution of the N-terminus of MCAK/Kif2c to its localization to kinetochores is well established (Maney et al., 1998; Walczak et al., 2002). The N-terminal region of MCAK also contains the majority of the established regulatory sites in MCAK/Kif2c (Andrews et al., 2004; Lan et al., 2004) and the EB1 binding motif that targets MCAK to microtubule plus ends (Honnappa et al., 2009; Figure 1B). To test the contribution of the N-terminal region of the kinesin-13 proteins to their distinct localization, we expressed their N-terminal regions alone (“N”, also labeled ∆MC). In contrast to previous studies (Maney et al., 1998), the N-terminal region of Kif2c was sufficient to target to kinetochores in human cells (Figure 1C). Like full length Kif2a, the N-terminal region of Kif2a localized to centrosomes (Figure 1C). In contrast, the N-terminal region of Kif2b did not localize to specific structures (Figure 1C). These results indicate that the divergent N-terminal regions define the localization of kinesin-13 proteins, but that Kif2b localization requires additional contributions from its motor and C-terminal domains.

Sequences in the enzymatic and C-terminal regions alter targeting of the kinesin-13 N-terminus

Although the N-terminal region is an important determinant for kinesin-13 localization, we next sought to test whether there is additional functional specificity between the three kinesin-13 proteins arising from the catalytic or C-terminal domains. To test this, we generated chimeric constructs in which the N-terminal domain of each kinesin was fused to the enzymatic and C-terminal domains of the three isoforms (Figure 1D). To designate these nine fusion proteins, we refer to these as NₓMᵧCₜ, with x, y, z indicating the corresponding kinesin isoform. Importantly, following transient transfection in HeLa cells, each control chimera constructed using this cloning strategy (NₐMₐCₐ, NₐMₐCₐ,
and N_BM_CB) displayed localization identical to the corresponding wild type protein (Figure 1D). The subcellular localization of each chimera and fusion protein is summarized in Figure S1B. In the majority of cases, we found that the localization of the chimera was determined primarily by the identity of the N-terminal region and displayed similar localization to the control kinesins. For example, N_AM_BC and N_AM_CC both displayed strong localization to the spindle poles and very weak localization to the kinetochores, similar to Kif2a (Figure 1D). In addition to its recruitment to kinetochores in mitosis, N_CM_AC could track the plus end of microtubules in a similar manner to Kif2c/MCAK in interphase (Supplemental Movie 1). In addition, N_CM_CA was enriched at microtubules plus ends, similarly to EB1 (Figure S1C).

However, we also observed some notable differences with respect to the localization of other chimeras. Unlike Kif2b (N_BM_CB), which localized only to the spindle during metaphase (Figure 1D), N_AM_CC and N_BMC_C also localized to metaphase kinetochores and centrosomes in the majority of cells (Figure 1D,E; Figure S1D). Despite the EB1 binding motif present in the N_C domain, N_CM_CB did not track microtubules plus ends during interphase, but instead bound to microtubules along their length (Figure S1E). In addition, when moderately expressed, N_CM_BC localized primarily to the spindle in metaphase (Figure 1D) and targeted to astral microtubules in anaphase and telophase (Supplemental Movie 2), whereas N_CM_CC/Kif2c did not, relocating from kinetochores to the midzone (Figure 1D, Figure S1A). When overexpressed, N_CM_BC localized to kinetochores, centrosomes, and spindles (Figure 1F), similarly to N_CM_CC/Kif2c (Figure 1D).

Taken together, these results indicate that chimeras containing the enzymatic domain and C-terminal region of Kif2b localize more robustly to microtubules regardless of the presence of a domain that would normally target the protein to kinetochores or microtubule plus ends. This suggests that the N-terminal region of kinesin-13s is a
major determinant for targeting the catalytic domain, but that the C-terminal and enzymatic domains also contribute to its localization.

**Kif2b stably associates with unique interacting partners**

As described above, intrinsic factors related to specific kinesin sequences contribute to the specificity of each kinesin’s localization. However, extrinsic factors such as association with distinct partners could also result in differences in kinesin-13 regulation or activity. To define the unique associations of kinesin-13s, we isolated Kif2a, Kif2b and Kif2c in separate one step affinity purifications from human cells using our established procedures (Cheeseman and Desai, 2005). We did not identify any stable interacting partners for Kif2c/MCAK (Figure S2A), suggesting the previously identified interactions with EB1 and Sgo2 (Honnappa et al., 2009; Tanno et al., 2010) do not persist under our purification conditions. In reciprocal purifications, we found that GFP-EB3 interacted with EB1 and EB2 as established by pervious work (De Groot et al., 2010), but we did not isolate Kif2c (Figure S2B). Similarly, we did not identify interacting partners for Kif2a (Figure S2A). Kif2a has been reported to bind to DDa3 (Jang et al., 2008). However, we also did not identify Kif2a in reciprocal affinity purifications using GFP-DDa3 (Figure S2A). We note that DDa3 and Kif2a do not display identical subcellular localization (Figure S2C), suggesting these proteins may also function independently of each other.

In contrast, GFP-Kif2b purifications isolated three interacting proteins (Figure 2A): Cep170, an uncharacterized protein KIAA0284/Fam68C that shows strong sequence similarity to Cep170, and Suppressor of IKK Epsilon (SIKE1; Huang et al., 2005). In contrast to recent reports (Manning et al., 2010), we did not detect CLASP1 in Kif2b purifications. Although we consistently isolated SIKE1 in Kif2b purifications, we were unable to validate the significance of this interaction in downstream analyses (data not shown). Thus, we chose to focus on the interaction of Kif2b with Cep170 and KIAA0284.
Cep170 and KIAA0284 possess the same domain structure with an FHA phospho-binding domain at the N-terminus and uncharacterized, but similar C-termini (Figure S3). Due to their sequence similarity, we will refer to KIAA0284/Fam68C as Cep170-Related (Cep170R). Cep170 has been shown previously to localize to centrosomes and microtubules (Guarguaglini et al., 2005). Recent large-scale analyses also identified Cep170 as interacting with the kinesin-14 Kifc3 (Hutchins et al., 2010), an interaction that we confirmed using affinity purifications of GFP-Kifc3 from HeLa cells (Figure S2A).

To determine which domains of Kif2b support the interaction with Cep170 and Cep170R, we purified the Kif2b/Kif2c chimeras from human cells. We did not identify any associated proteins from N_bM_cC_c purifications (Figure 2B), similar to Kif2c purifications. However, Cep170 co-purified with N_cM_bC_b and N_cM_bC_b (Figure 2 D, E), but not N_cM_bC_c (Figure 2F), suggesting that Cep170 interacts with Kif2b via the C-terminal domain. We note that we did not identify Cep170R in any of the purifications with the chimeric Kif2b proteins, suggesting either that multiple regions are required for this interaction, or that additional regulation of this interaction is occurring. Thus, Kif2b displays specific interactions with Cep170.

**The Kif2b-interacting proteins Cep170 and Cep170R localize to microtubules**

We next analyzed the localization of Cep170 and Cep170R. Cep170 has been reported previously to localize to centrosomes and the mitotic spindle (Guarguaglini et al., 2005; Hutchins et al., 2010), similar to Kif2b (Figure 1C). Consistent with this, based on antibodies against endogenous Cep170 and a Cep170-GFP fusion, we found that Cep170 localized to microtubules and centriole structures during interphase (Figure S4A; data not shown) and to spindle microtubules during metaphase (Figure 3A, B). Based on antibodies against the endogenous protein and GFP fusions to both the full length protein and C-terminus (CT; residues 921-1553), Cep170R also localized to microtubule-based structures including microtubules and centrioles during interphase
(Figure S4A), centrosomes during metaphase (Figure S4B), and the midzone during telophase (Figure S4C). However, in contrast to Cep170, Cep170R localized only weakly to the spindle in metaphase (Figure S4B, D), but became strongly enriched on microtubules in the late anaphase (Figure S4D).

The change in Cep170R localization at anaphase onset suggests that it maybe controlled by CDK activity. To test this, we added the CDK inhibitor flavopiridol to cells that had been arrested in mitosis using the MG132 protease inhibitor. Cep170R rapidly localized to spindle and astral microtubules upon the addition of flavopiridol (Figure S4E), suggesting that Cep170R localization to microtubules is negatively regulated by CDK1. Both Cep170 and Cep170R have multiple potential S/T-P CDK consensus sites in their C-terminus, which are known to be phosphorylated in vivo based on large scale analyses (Dephoure et al., 2008; Malik et al., 2009; Nousiainen et al., 2006) and the identification of these phosphorylation sites in our affinity purifications (data not shown). Consistent with this, we found that purified Cep170 (709-1486) is directly phosphorylated by CDK2/cyclinA in vitro (data not shown). However, although both proteins are likely to be phosphorylated by CDK, only the localization of Cep170R is altered by this phosphorylation. Thus, despite their primary sequence similarity, the association of Cep170 and Cep170R with microtubules is differentially regulated during mitosis.

**Cep170 binds to microtubules with high affinity**

To determine the functional contribution of the Kif2b-interacting proteins, we next tested their biochemical properties. Overexpression of Cep170 leads to microtubule bundling in interphase cells (Guarguaglini et al., 2005). Based on the localization of Cep170 to microtubules and this bundling activity, we tested the microtubule binding properties of Cep170. The C-terminus of Cep170 is comprised of three predicted globular domains. Two of the three domains - Cep170^{709-1009} and Cep170^{1010-1209} - displayed independent binding to microtubules in microtubule co-sedimentation assays (Figure 3C). To
determine the affinity of Cep170 for microtubules, we expressed the entire C-terminus of Cep170\textsuperscript{709-1486} containing both microtubule-binding domains. Under the tested conditions, Cep170\textsuperscript{709-1486} fully bound to 0.25 \(\mu\)M microtubules indicating that the apparent affinity was significantly stronger than 0.25 \(\mu\)M (Figure 3D). This high affinity is likely due to the cooperativity between the two microtubule-binding domains. We were unable to purify a similar fragment of Cep170R preventing us from directly testing its microtubule binding activity. However, based on the sequence similarity between Cep170 and Cep170R, and the localization of both proteins to microtubules, we suspect that both proteins bind microtubules. Thus, Cep170 possesses an intrinsic microtubule-binding activity, thereby providing a second non-motor binding site to the Kif2b complex.

**Cep170 association with the C-terminus of Kif2b enhances localization of Kif2b to the spindle**

Based on the localization of the kinesin-13 chimeras described above, the C-terminal region of Kif2b is important for its localization to the spindle (Figure 1). The Kif2b C-terminus is also the region that interacts with Cep170 (Figure 2), which possesses its own microtubule binding activity (Figure 4C, D). We hypothesized that Cep170 may provide a second extrinsic microtubule binding activity to target Kif2b to spindle microtubules. To test this, we co-depleted Cep170 and Cep170R from cell lines expressing low to moderate amounts of mCherry-Kif2b. Indeed, we observed that the levels of Kif2b on the spindle were decreased by 80% upon individual depletion of Cep170 (P-value= 1.1 \(10^{-5}\); Figure 4A, B) or co-depletion of Cep170/Cep170R (P-value = 4.3\(10^{-17}\); Figure 4A, B). Similarly, upon Cep170/Cep170R co-depletion, the targeting of the N\textsubscript{C}M\textsubscript{C}C\textsubscript{B} chimera, which associates with Cep170 (Figure 2C), to spindles was also severely reduced (P-value = 3\(10^{-4}\); Figure 4C, D). In contrast, the protein levels of mCherry-Kif2b as assessed by Western blotting were unaffected following depletion of Cep170 or Cep170R (Figure S5A). These data suggest that Cep170 is required to target Kif2b to the mitotic spindle.
In reciprocal experiments, Cep170 localization to the spindle was not affected by the depletion of Kif2b or the other Kif2b-interacting proteins (Figure S5B) suggesting that these proteins function upstream of Kif2b for spindle localization. Importantly, the localization of Kifc3, which also interacts with Cep170 (Figure S2A; (Hutchins et al., 2010), to spindles was also strongly reduced upon depletion of Cep170 (Figure 4E, F; P-value = 5.4x10^{-10}). However, Kifc3 depletion did not affect Cep170 localization (Figure S5C). In total, these results suggest that Cep170 targets at least the two kinesins - Kif2b and Kifc3 - to the spindle by providing a second microtubule binding activity to these complexes.

**Cep170 is required for Kif2b-induced microtubule depolymerization**

The microtubule-associated protein Cep170 is necessary to target Kif2b to the mitotic spindle (Figure 4). Next, we sought to test whether Cep170 contributes to Kif2b-induced microtubule depolymerization. Endogenous Kif2b is present at low abundance protein in HeLa cells and other tissue culture systems, but shows similar localization to that of GFP-Kif2b (Manning et al., 2010; Manning et al., 2007). Its low abundance and potential redundancy with other pathways prevented us from assessing the activity of endogenous Kif2b. Thus, we examined the depolymerase activity of Kif2b in a Kif2b-inducible cell line. Upon overexpression, Kif2b accumulated at kinetochores of bi-orientated chromosomes (Figure S6A) and resulted in spindle defects such as monopolar spindles and short spindles (Figure 5A, B; data not shown). These observations are a hallmark of kinesin-13 hyperactivity and are similar to the spindle defects caused by MCAK overexpression (Figure S6B, C; Maney et al., 1998). When Cep170 was depleted from cells overexpressing Kif2b, we observed a significant decrease in spindle defects relative to control cells overexpressing Kif2b (Figure 5A,B). To test if the spindle defects were due to excessive Kif2b depolymerase activity, we quantified the levels of polymerized tubulin in cells overexpressing Kif2b in presence or absence of Cep170. We found that there were increased levels of tubulin polymer when Cep170 was
depleted (Figure 5C, D), consistent with reduced levels of depolymerase activity in the absence of Cep170. In total, these data suggest that Cep170 targets Kif2b to spindles. Future biochemical analyses will be required to determine whether Cep170 modulates Kif2b activity in addition to promoting its spindle localization.

**Discussion**

**Extrinsic factors control the targeting and depolymerase activity of Kif2b**

Here, we demonstrated that Kif2b, but not other kinesin-13 proteins, interacts with Cep170 to target to the mitotic spindle. The identification of Kif2b-associated proteins that display their own microtubule binding activity presents a paradigm for the control of kinesin microtubule depolymerases. The EB1 family of proteins has been shown to target Kif2c/MCAK to microtubule plus ends by interacting with the Kif2c N-terminal region. Our work demonstrates that Cep170 uses its microtubule-binding properties to increase the association of Kif2b with microtubules via the C-terminal region of Kif2b. Upon association with Cep170, the enhanced association of Kif2b with the microtubule lattice could facilitate its depolymerase activity by increasing its concentration on microtubules.

The function of Kif2b and the context for its microtubule depolymerase activity has been investigated primarily in tissue culture systems and remains under debate, due in part to its low level of expression and the redundancy with other pathways controlling microtubule dynamics during mitosis (Manning et al., 2010; Tanenbaum et al., 2009). In addition to controlling spindle assembly and chromosome movement, kinesin-13 family proteins have also been reported to contribute to regulating centriole length (Delgehyr et al., 2012; Kobayashi et al., 2011), and it remains possible that Kif2b could contribute to diverse processes in humans. Our work suggests that Kif2b does possess a microtubule depolymerase activity based on the decreased levels of microtubules observed when Kif2b is overexpressed, and demonstrates that extrinsic interacting partners contribute to the proper localization of Kif2b to the spindle. Cep170
is widely expressed in diverse cell types (Novartis Gene Atlas) and can act as a potent activator of Kif2b depolymerase based on the work presented here. Low levels of Kif2b are found in most cell types, with higher levels in testes (Su et al., 2004; Wu et al., 2009). To prevent excessive microtubule depolymerase activity, controlling Kif2b expression levels may be critical. It remains to be determined how the Kif2b-Cep170 interaction is regulated in cells. For example, MCAK levels are higher than Kif2b in cells (Wu et al., 2009), but MCAK activity and localization are tightly regulated by protein interactions and phosphorylation to prevent spindle abnormalities. Elevated levels of MCAK in cancer cells are associated with resistance to taxol (Ganguly et al., 2011). For Kif2b, the expression or protein levels of Kif2b appear to be a rate-limiting factor. Therefore, ensuring low levels of Kif2b may prevent microtubule defects that would be associated with the activity of the Kif2b depolymerase that is promoted by targeting to the spindle by Cep170. It has also been suggested recently that Plk1 phosphorylation of Kif2b regulates its activity in vivo (Hood et al., 2012). Future work will be needed to determine under which conditions Kif2b acts as a microtubule depolymerase and whether Cep170 interactions or phosphorylation contributes to Kif2b activation using biochemical approaches.

**Addition of a second microtubule binding site alters the targeting and functional properties of kinesins**

The kinesin-13 depolymerases play important and non-redundant roles in regulating microtubule dynamics (Mennella et al., 2005; Rogers et al., 2004). Here, we dissected the properties that make each member of the kinesin-13 family unique. Kinesins have an intrinsic microtubule binding site in the catalytic domain. Most kinesin studies to date have focused exclusively on the motor domain and its interactions with microtubules. However, recent work has identified cases in which a second non-motor microtubule binding site is necessary for correct kinesin function. For example, the kinesin-5 Eg5 has been reported to have a second microtubule binding site in addition to the motor
domain (Weinger et al., 2011). This second site in the C-terminal tail increases the association of kinesin-5 with microtubules to ultimately increase the processivity of the motor. Similarly, kinesin-8 uses a second microtubule-binding site to target to the correct subcellular structure (Stumpff et al., 2011; Su et al., 2011; Weaver et al., 2011). This non-motor microtubule-binding domain enhances the processivity to allow targeting to microtubule plus ends. Kinesins also associate with other microtubule-binding proteins, such as EB1, to promote recruitment of the kinesin to a particular cellular localization (Honnappa et al., 2009; Stout et al., 2011; Tanenbaum et al., 2011). The microtubule binding protein CHICA has been shown to associate with Kid/Kif22 to target it to the spindle (Santamaria et al., 2008). Here, we demonstrated that Cep170 can impart its microtubule binding activity to Kif2b and Kifc3 to target these kinesins to the mitotic spindle. Future work will be required to test the role of Cep170 as a general microtubule targeting factor for kinesins. The Cep170 interaction partner Kifc3 is overexpressed in cancer cell lines that are resistant to taxol (De et al., 2009). Therefore, it will be interesting to test whether the stability of Kifc3 and the resistance to taxol in these cancer types can be modulated by Cep170 function. This work points to a generalized mechanism for how kinesins use a second intrinsic or extrinsic microtubule binding site to function appropriately.

Materials and Methods

Molecular biology and cell culture
cDNAs were obtained as IMAGE clones. Stable clonal cells lines expressing GFPLAP fusions were generated in HeLa cells as described previously (Cheeseman et al., 2004). To generate the mCherry-Kif2b HeLa cell line, we used the inducible Flp-In™ system (Invitrogen). The Kif2b cDNA was inserted into a pcDNA5/FRT/TO-based mCherry-tagged vector and co-transfected into the HeLa FRT line along with a plasmid expressing the FLP recombinase (pOG44, Invitrogen) using LipofectAMINE™ Plus (Invitrogen). Cells were selected in 400 µg ml⁻¹ Hygromycin B (Roche) and colonies
were pooled and expanded. Protein expression was induced with 0.5-1 µg/ml tetracycline overnight. To achieve Kif2b overexpression, we transiently transfected mCherry-Kif2b into a mCherry-Kif2b expressing HeLa cell line.

A HeLa Kyoto cell line expressing Cep170-GFP (mouse) was obtained from Mitocheck (Hutchins et al., 2010). RNAi experiments were conducted using RNAi MAX transfection reagent (Invitrogen) according to the manufacturer’s guidelines. For experiments with small molecule inhibitors, the following final concentrations were used: MG132 (10 µM), flavopiridol (5 µM), STLC (10 µM) and ZM447439 (2 µM).

To evaluate the depletion efficiency of Kif2b siRNA, we tested two sets of siRNAs. With previously published Kif2b siRNAs (Bakhoum et al., 2009; Manning et al., 2007), we could not rescue the phenotype using a GFP-Kif2b resistant to the siRNAs (Figure S5D). We therefore used a different set of Kif2b siRNA oligos (Dharmacon SMART-pool siRNA, CGAAUGGGUUGCGAUGAU, GCUCAGAAACUCCACAUUAU, GCACAUUGAUCGAAGAUAU, CAAGGUGUAUGAUUUGUUG) and obtained full depletion of GFP-Kif2b. Pools of siRNAs for Cep170 (GAAGUAAGUAACGAAAUC, CGUAACACUCUCGGAUUUG, GAUUAUAUAGGCCCUGUUA, CGAUGUAGCAGGAGAGAUA) and Cep170R (GUACGGCGCUCAGCCAUAA, GGCAAGAGAGCUUCACUAA, GAAUGGGGACGCUGUGUUAA, GCUAGGUUCUCGGCGGAA) were obtained from Dharmacon. Successful depletion of Cep170 and Cep170R (Dharmacon SMART-pool siRNA) was confirmed by immunofluorescence cells using anti-Cep170 and Cep170R antibodies, respectively (Figure S5B). Cep170 depletion (Dharmacon SMART-pool siRNA) was also confirmed by Western blotting using anti-Cep170 antibodies (Figure S5E). Although the phenotypes observed following depletion of Cep170 appear specific and are consistent with previously published work (Guarguaglini et al., 2005), we note that we were unable to test the ability of an RNAi-resistant version of Cep170 to complement these defects
due to the large size of this cDNA which prevented the generation of full length “hardened” version of this cDNA and corresponding stable cell line.

**Immunofluorescence and microscopy**

Immunofluorescence was conducted as described previously (Kline et al., 2006). For immunofluorescence against microtubules, cells were fixed in methanol for 5 min and DM1α (Sigma-Aldrich) was used at 1:1000. For visualization of kinetochore proteins, we used mouse EB1 (BD), mouse anti-HEC1 (9G3; Abcam) and human anti-centromere antibodies (ACA; Antibodies, Inc.). Affinity-purified rabbit polyclonal antibodies were generated against Cep170\(^{1010-1200}\) and Cep170R\(^{1341-1553}\) as described previously (Desai et al., 2003). For time-lapse imaging, cells were maintained in CO\(_2\)-independent media (Invitrogen) at 37\(^\circ\)C, and imaged every 4-5 minutes. To visualize DNA in live cells, Hoechst was used at 100 ng/ml. Images were acquired on a DeltaVision Core microscope (Applied Precision) equipped with a CoolSnap HQ2 camera. 30 z sections were acquired at 0.2 \(\mu\)m steps using a 100X, 1.3 NA Olympus U-Plan Apochromat objective lens with 1x1 binning. For live cell imaging, 6-12 z sections were acquired at 0.5-1 \(\mu\)m steps. Images were deconvolved using the DeltaVision software. Images shown represent maximal intensity projections. Equivalent exposure conditions and scaling were used between controls and RNAi-depleted cells. To quantitate the fluorescence intensity of Kif2b on spindles, we measured the integrated intensity of Kif2b over a 21 by 21 pixel box on the spindle and in the background, using a mCherry-Kif2b cell line or transiently transfected GFP-N\(_C\)M\(_C\)C\(_B\). Rare cells for which the mean spindle intensity was three times greater than the average spindle average intensity in either controls or test conditions were removed from the analysis. Each experiment was repeated 3 times. To quantitate the fluorescence intensity, we measured the integrated fluorescence intensity of tubulin over the entire cell, as described previously (Stumpff et al., 2007). 40-50 cells were examined for each experiment. Each experiment was repeated 3 times.
Protein purification and biochemical assays

GFP\textsuperscript{LAP} tagged Kif2a, DDa3, Kif2b, Kif2c, Cep170FL, Cep170\textsuperscript{755-1486}, Cep170\textsuperscript{R\textsuperscript{259-1553}}, Cep170\textsuperscript{R\textsuperscript{982-1553}}, and N\textsubscript{C}M\textsubscript{B}C\textsubscript{C}, N\textsubscript{C}M\textsubscript{B}C\textsubscript{B} and N\textsubscript{C}M\textsubscript{C}B chimeras were isolated from HeLa cells as described previously (Cheeseman and Desai, 2005). Purified proteins were identified by mass spectrometry using an LTQ XL Ion trap mass spectrometer (Thermo Fisher Scientific) using SEQUEST software as described previously (Washburn et al., 2001).

For the expression and purification of the recombinant Cep170 fragments (709-1009, 1010-1200, 1200-1486, 709-1486), 6xHis-Cep170 fusions were generated in pET28a. His-Cep170\textsuperscript{R\textsuperscript{1341-1553}} was purified and used for antibody production. Proteins were purified using Ni-NTA agarose (QIAGEN) according to the manufacturer’s instructions, further purified by gel filtration and then desalted into S buffer (20 mM Hepes, pH 7.0, 50 mM NaCl, 1 mM EDTA, and 1 mM DTT). Microtubule binding assays using the purified proteins were conducted as described previously (Cheeseman et al., 2006) using equal volumes of microtubules in BRB80 and test protein in S buffer.

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Figure 1. The N-terminus of kinesin-13 is a primary determinant of kinesin localization. (A) Schematic diagram showing the kinesin-13 domains and their percentage similarity with respect to Kif2c/MCAK. (B) Sequence alignment of the N-terminus of human Kif2a, Kif2b and Kif2c. The sequences were aligned using the program T-coffee (EBI) and formatted with ESPRIPT (Gouet et al., 1999). Known phosphorylation sites for Kif2c/MCAK and the EB1 binding motif are highlighted. (C) Images of HeLa cells transiently expressing GFP fusions to full length and domains of Kif2a, Kif2b and Kif2c. The ΔN and ΔMC domains represent GFP-fusions lacking the N-terminus or the motor and C-terminal domains of the kinesins, respectively. (D) Images of mitotic HeLa cells transiently expressing the indicated GFP-tagged kinesin-13 chimeras. (E) Images of chimeric kinesin-13 GFP fusion constructs that display heterogeneous localization in the observed cells. Percentages represent the frequency with which the chimera shows localization to only spindle microtubules (left) or spindle...
microtubules and kinetochores (right). n = 100 cells. (F) Image of a cell transiently overexpressing GFP-N\textsubscript{CM6C6}. Scale bars, 10 \( \mu \text{m} \).
Figure 2. Kif2b associates with Cep170 and Cep170R. GFP\textsuperscript{LAP} tagged fusions were used to isolate Kif2b and kinesin-13 chimeras from stable cell lines using one step immunoprecipitations as described in (Cheeseman and Desai, 2005). (A) Top left, silver stained gel of the GFP-Kif2b immunoprecipitation. Top right, image of cell expressing GFP-Kif2b. Bottom, percent sequence coverage from the mass spectrometric analysis of the indicated samples showing the proteins identified in the Kif2b complex purifications, but not in unrelated control samples (including diverse samples that our lab has tested identical similar conditions; for example, see (Schmidt et al., 2010; Welburn et al., 2010)). (C, D, E, F) Left, images of the indicated kinesin-13 chimeras showing their localization during metaphase. Right, percent sequence coverage from the mass spectrometric analysis of the indicated samples showing the proteins identified in each complex and the number of peptides recovered. Note that both Kif2b and Kif2c were identified in these samples due to the peptides present in the chimeric proteins. The association of Cep170 with Kif2b requires the C-terminus. Scale bars, 10 \mu m.
Figure 3. Cep170 targets to the spindle and has microtubule binding activity. 

(A) Immunofluorescence images of metaphase cells expressing GFP-Centrin stained for Cep170 and microtubules. (B) Images of live cells expressing GFP-Kif2b and GFP-Cep170 at the indicated time points throughout mitosis. Cep170 and Kif2b localize to microtubules throughout mitosis. (C) Cep170 has two microtubule binding domains. Coomassie gel showing the co-sedimentation of the indicated Cep170 domains in the absence or presence of 5 µM microtubules. Cep170 fragments containing amino acids 709-1009 or 1010-1200 bound to microtubules. (D) Western blot showing the co-
sedimentation of 250 nM His-Cep170-CT (amino acids 709-1486) at the indicated microtubule concentrations. The Western blot was probed with anti-Cep170 antibodies.
Figure 4. Kif2b and Kifc3 spindle localization requires Cep170. (A) Images of live mitotic cells expressing mCherry-Kif2b under control conditions or following Cep170 or Cep170R depletion, or Cep170/Cep170R co-depletion by RNAi. (B) Quantification of mCherry-Kif2b fluorescence on the mitotic spindle from the cells in (A). (C) Images of live mitotic cells expressing GFP-N₃M₃C₃ under control conditions or following Cep170/Cep170R co-depletion by RNAi. (D) Quantification of GFP-N₃M₃C₃ fluorescence on the mitotic spindle from the cells in (C). (E) Images of live mitotic cells expressing GFP-Kifc3 under control conditions or following Cep170 depletion by RNAi. (F) Quantification of GFP-Kifc3 spindle-localized fluorescence in controls and Cep170-depleted cells. For all fluorescence localization experiments in this figure, each experiment was repeated three times with 50 cells analyzed for each experiment. The fluorescence intensity values indicate the average percent quantified fluorescence.
intensity (+/- sem) relative to controls. Statistical T-tests were performed for each experiment and the confidence interval is indicated by *. *** corresponds to a P-value <0.001. Scale bars, 10 µm.
**Figure 5. Cep170 promotes Kif2b activity in vivo.** (A) Images of HeLa cells transiently overexpressing mCherry-Kif2b, in presence or absence of Cep170. Representative examples of normal bi-polar and defective spindle structures present in these cells are shown. (B) Graph showing the frequency of cells displaying a bipolar or defective spindle morphology when Kif2b is overexpressed in control cells or following Cep170 depletion as in (A). Error bars represent the sem. (C) Immunofluorescence images showing the localization of tubulin/microtubules (using DM1α antibodies) in a cell line overexpressing mCherry-Kif2b in controls or cells depleted for Cep170 by RNAi. (D) Quantification of microtubule intensity for the cells in (C). Numbers indicate the average percent quantified spindle fluorescence intensity in the cells overexpressing μχηηρψ–Κιφ2β (+/− σεμ) relative to the cells overexpressing μχηηρψ–Κιφ2β in the absence of Cep170. Statistical T-tests were performed for each experimental condition and the differences were significant at p < 0.01. Each condition was repeated three times, with 30 cells counted for each condition. Scale bar, 10 μm.