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Kinetochore–microtubule attachment throughout mitosis potentiated by the elongated stalk of the kinetochore kinesin CENP-E

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INTRODUCTION
Accurate chromosome segregation in mitosis depends on the dynamic interactions between the kinetochore and spindle microtubules. Kinetochore microtubule attachment throughout mitosis potentiated by the elongated stalk of the kinetochore kinesin CENP-E...
tail-mediated motor inhibition (Espeut et al., 2008). At mitosis onset, CENP-E also becomes recruited to the outer layer of each kinetochore via its kinetochore-binding site located near its C-terminus (Chan et al., 1998). A fully elongated extension of the active, kinetochore-bound CENP-E may help to increase the span of the amino-terminal motor domain from a bound kinetochore cargo, thereby potentiating capture of spindle microtubules during prometaphase (Kim et al., 2008, 2010; Gudimchuk et al., 2013). At the kinetochores of aligned chromosomes in metaphase, however, the CENP-E is not fully extended, and its conformation changes when the kinetochore is under tension (Wan et al., 2009).

Results of the genetic depletion of CENP-E (Putkey et al., 2002; Weaver et al., 2003), its removal from kinetochores (McEwen et al., 2001), and inhibition of its motor activity (Gudimchuk et al., 2013) implicate CENP-E as an important kinetochore component that contributes to high fidelity of chromosome segregation. CENP-E plays an essential role in transporting pole-proximal chromosomes to the spindle equator during prometaphase, a process called chromosome congression (Yen et al., 1991; Schaar et al., 1997; Wood et al., 1997). The initial congression of misaligned chromosomes takes place along already established kinetochore fibers and along the microtubules directed to the spindle equator (Kapoor et al., 2006; Cai et al., 2009). CENP-E-mediated chromosome motility requires that its motor domain is active (Kim et al., 2008, 2010), but whether other molecular features of this kinetochore protein have important physiological significance in not yet known. In addition to its transporting role during congression, CENP-E has been suggested to play a role in facilitating kinetochore–microtubule attachment during metaphase (Gudimchuk et al., 2013). Consistently, depletion of CENP-E results in 50% decrease in the density of kinetochore fibers in human and mouse cells (McEwen et al., 2001; Putkey et al., 2002).

Of interest, the stabilization of kinetochore–microtubule attachment also appears to require CENP-E-dependent localization of other proteins to the kinetochore, especially protein phosphatase 1 (PP1; Kim et al., 2010) and the checkpoint protein BubR1 (Guo et al., 2012). CENP-E-dependent localization of these (and potentially other kinetochore proteins) may help to regulate the dynamics of kinetochore–microtubule interactions and/or signal the state of these attachments to the cell cycle machinery. It has also been suggested that CENP-E is required for normal kinetochore–microtubule attachment during anaphase, since a genetic depletion of CENP-E from cells in regenerating mouse liver and embryonic fibroblasts in culture results in an increased frequency of lagging chromosomes (Putkey et al., 2002; Weaver et al., 2003).

Much of the molecular information about the CENP-E kinesin has also been learned from motility assays in vitro. Molecules of purified full-length CENP-E protein show heterogeneous behavior on a microtubule wall: whereas many CENP-E molecules bind microtubules transiently and show diffusive motion, some motors walk unidirectionally and processively (Gudimchuk et al., 2013). The heterogeneity of single CENP-E molecules is consistent with the idea that the CENP-E tail can inhibit its own motor domain and that these interactions are transient. Indeed, the reported dissociation constant of 3 μM for purified tail and head domains suggests weak binding (Espeut et al., 2008). Of importance, truncated versions of CENP-E, which completely lack the elongated stalk and C-terminal tail, can walk well along a microtubule and transport microbeads, thereby mimicking the activities of the active molecules of full-length CENP-E and indicating that the stalk and tail have little effect during CENP-E’s walking (Kim et al., 2008; Yardimci et al., 2008; Gudimchuk et al., 2013). However, whereas full-length CENP-E can associate processively with the ends of dynamic microtubules in vitro, the truncated motor falls off microtubule ends and fails to track (Gudimchuk et al., 2013). Of interest, when purified tail and motor domains are joined together using Quantum Dots, this produces conjugates that walk well along a microtubule wall and are able to recapitulate the processive association with the plus end of dynamic microtubules characteristic of full-length CENP-E (Gudimchuk et al., 2013). These experiments suggested that neither the tip-tracking activity nor the CENP-E–dependent transport along a microtubule wall in vitro requires the elongated stalk of CENP-E, raising a question about its contribution to CENP-E functions. Through creation of “Bonsai” versions of CENP-E in which its stalk is significantly shortened but wild-type motor and tail domains are retained, we now identify the biological role for the CENP-E stalk.

RESULTS

CENP-E coiled-coil stalk regulates motor function in vitro

Previous structural work visualizing purified CENP-E dimers with electron microscopy reported that soluble CENP-E can adopt many different conformations (Kim et al., 2008), consistent with a highly flexible coiled-coil region. Direct examination of full-length Xenopus CENP-E by electron microscopy using quick-freeze deep-etch and platinum replication (Heuser, 1989) identified a small proportion of CENP-E molecules in a folded conformation in which the extended stalk domain was looped and the head and tail appeared to be bound (Figure 1A, bottom). The low incidence of folded molecules may reflect the transient nature of this configuration, since binding between purified head and tail domains is weak (Espeut et al., 2008).

To determine whether the flexible elongated stalk of CENP-E is essential for its function, we characterized the activity of a “Bonsai” CENP-E in which 1475 amino acids (aa) of the ~1700 aa CENP-E coiled-coil domain were removed, thus shortening the stalk by 85% (Figure 1B). This shorter stalk contains the minimal segment that is sufficient for CENP-E dimerization and is predicted to form short discontinuous coiled coil (Supplemental Figure S1A). Bonsai CENP-E was expressed in insect cells, leading to production of the expected 197-kDa product (Supplemental Figure S1B). First, we tested whether Bonsai CENP-E could power the movement of microtubules in a traditional gliding assay in vitro. Bonsai CENP-E was attached to a coverslip using an antibody to its C-terminal green fluorescent protein (GFP) tag, and motions of stabilized fluorescent microtubules were recorded in the presence of ATP (Supplemental Figure S1C). This mutant retained robust motor activity, although the gliding rate and the percentage of moving microtubules were reduced compared with truncated CENP-E that lacked the entire stalk and tail (Figure 1, C and D). We next used the same conjugation strategy to attach Bonsai protein to microbeads. Laser tweezers were used to bring the beads in contact with coverslip-attached microtubules, and their mobility was assessed via differential interference contrast (DIC) microscopy (Figure 1E and Supplemental Figure S1D). This approach confirmed that Bonsai CENP-E transported cargo along microtubule tracks with a reduced velocity relative to either truncated or full-length CENP-E, producing microbead transport velocities of 5.9 ± 0.5, 17.1 ± 1.6, and 19.2 ± 2.1 μm/min, respectively (Figure 1E). We conclude that cargo-conjugated Bonsai CENP-E could support microtubule motility, but with a reduced velocity.

Strikingly different results were seen when we used total internal reflection fluorescence (TIRF) microscopy to visualize how cargo-free molecules of Bonsai CENP-E interacted with microtubules (Supplemental Figure S1E). Previous work with truncated CENP-E established that soluble molecules of this dimeric motor readily bind and walk on microtubules, whereas the full-length...
molecules either diffuse or walk processively (Kim et al., 2008; Gudimchuk et al., 2013). In contrast to both of these constructs, we observed no binding between Bonsai CENP-E molecules and microtubules; consequently, there was no detectable motion of Bonsai CENP-E along a microtubule wall or the tip tracking at the microtubule end that is characteristic of full-length CENP-E. Occasionally we observed processive motion of fluorescent complexes, which, based on their brightness, contained multiple molecules of Bonsai CENP-E, confirming that this protein was capable of moving on microtubules under these assay conditions (Figure 1F, pink arrows, and Supplemental Figure S1F). The lack of single-molecule walking on microtubules appeared to result from a reduced association rate between Bonsai and microtubules, as numerous motor molecules were present in the solution and could be seen attached nonspecifically to the coverslip (Supplemental Figure S1G). The initial brightness of these dots and the number of photobleaching steps were highly similar to those seen for dimeric kinesin 1 motor but not for the monomers of Ndc80-GFP protein (Supplemental Figure S1, H–J).

Together these results demonstrate that Bonsai CENP-E dimerizes but fails to bind to microtubules.

Of importance, the inhibition of microtubule binding by soluble, cargo-free Bonsai CENP-E dimers was stronger than that seen with full-length CENP-E, which differs from Bonsai only in the length of the flexible stalk. When we carried out a microtubule-pelleting assay in the presence of AMPPNP, a nonhydrolyzable analogue of ATP known to induce rigor binding of motor heads (Liao et al., 1994), both full-length and truncated CENP-E proteins bound strongly to microtubules (Kd = 24 ± 10 and 23 ± 7 nM, respectively). However, Bonsai CENP-E showed almost no binding (Figure 1G), confirming that in the absence of a cargo, this mutant protein has a highly reduced affinity to microtubules. Together these assays revealed that elongated flexible stalk of CENP-E plays an important role in regulating its motor activity in vitro.

Elongated stalk is essential for efficient chromosome congression via an CENP-E-dependent mechanism

Previous work using Xenopus egg extracts and cells revealed that motor activity of CENP-E is essential for chromosome congression (Kim et al., 2008, 2010). Indeed, a CENP-E “rigor” mutant, which stably binds to the microtubules and cannot walk, is not capable of mediating congression (Kim et al., 2008). In addition, it was proposed that CENP-E could promote congression by facilitating capture of already established bundles of kinetochore microtubules due to its highly elongated and flexible coiled-coil stalk (Kim et al., 2008). To examine the in vivo function of the CENP-E stalk during congression in human cells, we created several cell lines expressing different versions of Bonsai CENP-E (Figure 2A and Supplemental Figure S2A).

A gene encoding each of these mutants (which differed in the length of the CENP-E stalk), as well as full-length CENP-E (Kim et al., 2010), was integrated at a specific genomic locus in human DLD-1 cells using the FRT/Flp-mediated recombination Flip-In system. Next endogenous CENP-E, which undergoes cell cycle–dependent accumulation and loss that mimics that of cyclin B (Brown et al., 1994), was depleted using small interfering RNA (siRNA) directed...
against the 3’ untranslated region of the endogenous CENP-E mRNA (Kim et al., 2010), and the cells were “rescued” by inducing either full-length or Bonsai myc-tagged-GFP CENP-E proteins by addition of doxycycline for 8 h (Figure 2B). Induction by addition of doxycycline led to comparable accumulation level of each variant (Supplemental Figure S2B). Both long and medium Bonsai CENP-E displayed normal kinetochore localization, and each was enriched at kinetochores of unaligned chromosomes. Short Bonsai CENP-E showed highly reduced kinetochore signal (Supplemental Figure S2C). To examine the growth of these Bonsai CENP-E cell lines, we performed a clonogenic assay in cells incubated in doxycycline for >15 d. Expression of medium Bonsai CENP-E resulted in fewer colonies, consistent with a stronger perturbation of mitotic cell division (Supplemental Figure S2D), and so this construct was used for more-detailed characterization.

Because our in vitro data indicated that shortening its stalk disrupted CENP-E binding to microtubules, we first tested whether the kinetochore-bound shortened CENP-E was capable of interacting with microtubules directly. Cells were treated with GSK923295, a small-molecule inhibitor of CENP-E, which locks its kinesin motor in a “rigor” state, strongly bound to microtubules (Wood et al., 2010). In cells rescued with full-length CENP-E, this treatment caused a loss of kinetochore-bound CENP-E and its accumulation at the poles of the mitotic spindle after passive translocation via poleward microtubule flux (Gudimchuk et al., 2013). After treatment with the CENP-E inhibitor, Bonsai CENP-E also accumulated at the spindle poles, revealing that the kinetochore-bound Bonsai was still able to bind to microtubules and adopt a rigor-like state (Figure 2C).

To evaluate whether Bonsai CENP-E could support normal congression of polar chromosomes, we quantified the proportion of cells with an elevated number of polar chromosomes (more than five). A threefold increase in the number of cells with polar chromosomes was seen with Bonsai CENP-E relative to rescue with full-length CENP-E (52 ± 5 vs. 18 ± 5%; Figure 2D), indicating significant loss of CENP-E-dependent congression. The elevated number of polar chromosomes in cells rescued with Bonsai CENP-E triggered a strong activation of the mitotic checkpoint, as the average mitotic duration increased twofold compared with cells depleted of CENP-E (300 ± 29 vs. 163 ± 31 min, Supplemental Figure S2E). This delay suggested that Bonsai CENP-E could still preserve functionality of the mitotic checkpoint (Abrieu et al., 2000; Mao et al., 2003).

To test this further, we measured the recruitment of BubR1 at the kinetochores of polar chromosomes in cells rescued with Bonsai CENP-E, since wild-type CENP-E has been shown to contribute to BubR1 targeting (Weaver et al., 2003). Quantitative immunofluorescence measurements revealed comparable accumulation of BubR1 at the kinetochores of unaligned chromosomes in full-length and Bonsai-rescued cells, whereas cells depleted of CENP-E displayed 50% reduction in BubR1 signal at kinetochores of polar chromosomes (Figure 2E). Thus, these assays revealed that in a cellular context, Bonsai CENP-E retained ability to 1) localize to kinetochores (thus attaching to its cargo), 2) bind to microtubules and produce a rigor phenotype in the presence of its specific inhibitor, including passive accumulation at poles, and 3) support normal mitotic checkpoint activity. Nevertheless, Bonsai CENP-E missing the extended coiled-coil was unable to support efficient congression of polar chromosomes.

**CENP-E motor function is required for stabilization of kinetochore–microtubule attachment in metaphase**

In addition to its role in chromosome congression, CENP-E potentiates stabilization of kinetochore–microtubule attachment. The initial evidence for this was that depletion of CENP-E resulted in 50% reduction of kinetochore microtubule fibers of fully congressed, metaphase chromosomes (McEwen et al., 2001; Putkey et al., 2002). Further, genomic deletion of CENP-E led to
lagging chromosomes in mouse embryonic fibroblast and cells of regenerating liver (Putkey et al., 2002; Weaver et al., 2003). Finally, in a recent effort, two different approaches (depletion of CENP-E by RNA interference and drug-induced inhibition of CENP-E to inactivate motor activity and displace it from kinetochores) were used to demonstrate that the CENP-E motor facilitates kinetochore–microtubule attachment in metaphase and during induced microtubule depolymerization (Gudimchuk et al., 2013). Reduced kinetochore–microtubule stability after low temperature–induced microtubule depolymerization has also been found for other kinetochore microtubule-binding proteins, including the NDC80 complex (DeLuca et al., 2006), suggesting that CENP-E functions in protecting kinetochore microtubule ends from cold-induced disassembly and detachment.

We tested whether kinetochore-bound Bonsai CENP-E retained this ability. Cells rescued with Bonsai or full-length CENP-E were arrested in metaphase using MG132 (to impose mitotic arrest, whether or not the mitotic checkpoint signal was active) and subjected to cold treatment for 20 min. Compared to the full-length CENP-E control, cells rescued with Bonsai CENP-E displayed a 30% reduction of cold-stable microtubules, a level comparable to cells depleted of CENP-E (Figure 3A). This result indicated that Bonsai CENP-E was deficient in protecting microtubule ends from cold-induced disassembly. Consistent with the reduced density of cold-stable kinetochore–microtubules, measurement of interkinetochore distances in unperturbed metaphase cells revealed that they were significantly shorter in cells rescued with the Bonsai motor (Figure 3B).

Next we assessed whether the presence of Bonsai CENP-E affected the maintenance of chromosome biorientation at metaphase. Cells were arrested with MG132, as before, and chromosome alignment was monitored by live-cell imaging (Figure 3, C and D, and Supplemental Videos S1 and S2). Cells rescued with full-length CENP-E maintained proper chromosome biorientation for ~300 min before losing metaphase chromosome alignment, with multiple DNA masses separating simultaneously, a phenotype known as chromosome scattering due to loss of cohesion (Daum et al., 2011; Stevens et al., 2011). In cells rescued with Bonsai CENP-E, however, loss of chromosome alignment occurred much more quickly, with timing comparable to that in cells with no CENP-E rescue (150 and 190 min, respectively). The premature loss of chromosome alignment in Bonsai CENP-E cells affected fewer chromosomes and increased slowly over time, indicative of defective kinetochore microtubule attachment rather than a cohesion defect. These findings indicate that Bonsai CENP-E cannot properly stabilize chromosome alignment, supporting the idea that presence of a wild-type stalk is important for CENP-E-dependent stability of kinetochore–microtubule attachments in metaphase.

CENP-E motor function is required for stabilization of kinetochore–microtubule attachment in anaphase

To assess whether regulation of CENP-E motor function by its stalk influenced the ability of kinetochores to track and maintain attachment with depolymerizing microtubules ends, we arrested cells rescued with full-length or Bonsai CENP-E in mitosis in the presence of an inhibitor (S-trityl-L-cysteine [STLC]) of the kinesin motor Eg5 (Figure 4A). This treatment induces monopolar spindles with chromosome rosettes and favors establishment of attachment of their kinetochores to the ends of microtubules (Figure 4, B and C, no depolymerization). The cells were then cooled for 20 min to induce depolymerization of nonstabilized microtubules. In monopolar cells, however, this treatment also induced shortening of kinetochore...
FIGURE 4: Reduced stability of kinetochore–microtubule attachment in anaphase in cells expressing Bonsai CENP-E. (A) Schematic of the cell plating, RNA interference treatment, and drug treatment for the microtubule destabilization assay. (B) Schematic of the microtubule destabilization assay to test kinetochore attachments. The schematic also presents the outcome of the experiment. (C) Representative image of cells rescued with full-length or Bonsai CENP-E, then tested for stability of kinetochore attachments to depolymerizing microtubule ends as in B. Scale bar, 5 μm. Right, percentage of cells with attached chromosomes without microtubule depolymerization and percentage of cells with unattached outlying chromosomes after 20 min of microtubule depolymerization. Error bars, SEM. **p < 0.01, p value of a unpaired Student’s t test realized on the mean of three independent experiments. For the 20 min depolymerization condition, N = 98 cells were analyzed for full-length, 77 for Bonsai, and 97 for Empty conditions. Anti–centromere antibody (ACA) was used to identify centromeres/kinetochores. (D) Representative images from the time-lapse sequence of a cell with a lagging chromosome (yellow arrowhead). Scale bar, 10 μm. Right, percentage of cells with attached chromosomes without microtubule depolymerization and percentage of cells with unattached outlying chromosomes after 20 min of microtubule depolymerization. Error bars, SEM. **p < 0.01, p value of a unpaired Student’s t test realized on the mean of three independent experiments. N = 130 cells were analyzed for full-length, 58 for Bonsai, and 97 for Empty conditions.

The absence of motility of soluble molecules of Bonsai CENP-E in vitro, despite supporting robust motility in the gliding and bead assays, could in principle result from reduced dimerization of the shortened stalk of CENP-E. We do not favor this possibility because even our shortest Bonsai constructs have a fairly long region that is predicted to form a coiled coil, including a fragment that has been shown to be sufficient for dimerization (Kim et al., 2007). Indeed, in contrast to both full-length and truncated CENP-E motors, soluble molecules of Bonsai CENP-E are strongly defective in microtubule binding in vitro. This result implies that in the context of a full-length molecule the stalk acts as a positive regulator of interactions between microtubules and both of the CENP-E's microtubule-binding sites, one in its motor and a second in its tail.

DISCUSSION

The elongated flexible stalk of CENP-E regulates its microtubule binding and motility

Analogous to what was proposed for kinesin 1 (Verhey and Hammond, 2009), previous work with CENP-E established that binding between the head and tail domains of soluble CENP-E can inhibit its ATPase activity, with inhibition relieved by phosphorylation of the CENP-E tail by mitotic kinases (Espeut et al., 2008). Our work identifies two additional mechanisms that are likely to contribute to the regulation of CENP-E activity in cells during mitotic progression. First, similar to kinesins 1 and 3 (Blasius et al., 2007; Yamada et al., 2007), the activity of CENP-E, full length (Gudimchuk et al., 2013) and Bonsai (this work), increases in vitro when its tail is bound to a cargo. Thus, CENP-E binding to kinetochores is likely to be required for activating this motor, ensuring that only the kinetochore-bound CENP-E molecules actively interact with and move along microtubules. Second, our study implicated the CENP-E stalk in regulating microtubule binding and motility of CENP-E. Indeed, in contrast to both full-length and truncated CENP-E motors, soluble molecules of Bonsai CENP-E are strongly defective in microtubule binding in vitro. This result implies that in the context of a full-length molecule the stalk acts as a positive regulator of interactions between microtubules and both of the CENP-E's microtubule-binding sites, one in its motor and a second in its tail.

The elongated flexible stalk of CENP-E in vitro, despite supporting robust motility in the gliding and bead assays, could in principle result from reduced dimerization of the shortened stalk of CENP-E. We do not favor this possibility because even our shortest Bonsai constructs have a fairly long region that is predicted to form a coiled coil, including a fragment that has been shown to be sufficient for dimerization (Kim et al., 2008). In addition, Bonsai CENP-E failed to bind microtubules in the presence of AMP-PPN, whereas this nucleotide induces strong binding of both dimeric and monomeric kinesins (Huang and Hackney, 1994; Huang et al., 1994). Finally, our fluorescence analysis of Bonsai CENP-E molecules demonstrates directly that soluble Bonsai can form dimers, so the absence of either directed or diffusive motions of Bonsai CENP-E on microtubules appears to result from strong intramolecular inhibition.

The presence of a fully elongated CENP-E stalk is likely to be required for activating this motor, ensuring that only the kinetochore-bound CENP-E molecules actively interact with and move along microtubules. Second, our study implicated the CENP-E stalk in regulating microtubule binding and motility of CENP-E. Indeed, in contrast to both full-length and truncated CENP-E motors, soluble molecules of Bonsai CENP-E are strongly defective in microtubule binding in vitro. This result implies that in the context of a full-length molecule the stalk acts as a positive regulator of interactions between microtubules and both of the CENP-E's microtubule-binding sites, one in its motor and a second in its tail.

The elongated flexible stalk of CENP-E in vitro, despite supporting robust motility in the gliding and bead assays, could in principle result from reduced dimerization of the shortened stalk of CENP-E. We do not favor this possibility because even our shortest Bonsai constructs have a fairly long region that is predicted to form a coiled coil, including a fragment that has been shown to be sufficient for dimerization (Kim et al., 2008). In addition, Bonsai CENP-E failed to bind microtubules in the presence of AMP-PPN, whereas this nucleotide induces strong binding of both dimeric and monomeric kinesins (Huang and Hackney, 1994; Huang et al., 1994). Finally, our fluorescence analysis of Bonsai CENP-E molecules demonstrates directly that soluble Bonsai can form dimers, so the absence of either directed or diffusive motions of Bonsai CENP-E on microtubules appears to result from strong intramolecular inhibition.
The molecular mechanism of the stalk-mediated regulation of CENP-E microtubule-dependent activity is not yet known, but this effect is likely to result from the unusual structural and biomechanical properties of this coiled-coil domain. The motor domains of all kinesins share a significant similarity, but there are some interesting differences in the properties of their dimerizing stalks (Verhey and Hammond, 2009). The stalk of kinesin 1, for example, is approximately three times shorter than that of CENP-E; perhaps more importantly, the central portion of kinesin 1’s stalk is represented by a rigid coiled coil with a centrally located hinge (Coy et al., 1999; Jeppesen and Hoerber, 2012). This hinge is highly flexible and allows the stalk to fold completely; however, the tail domains bind back on to the heads, promoting autoinhibition, which in turn can be down-regulated by kinesin’s light chain (Wong and Rice, 2010; Kaan et al., 2011). In contrast, the elongated stalk of CENP-E is composed of a discontinuous coiled coil, which appears to be highly flexible (Kim et al., 2008). The stalk may directly impinge on the interactions between head and tail of CENP-E, causing repression of autoinhibition akin to the action of the light chain of kinesin 1. In wild-type CENP-E the stalk appears to partially suppress the intramolecular binding between the head and tail, thereby allowing each of these domains to engage in binding to microtubules. We propose that shortening of the stalk reinforces the head-to-tail interaction in CENP-E and strengthens autoinhibition of the motor’s activity (Figure 5).

Our hypothesis of a negative role of the CENP-E stalk in autoinhibition explains why the binding of soluble Bonsai molecules to microtubules is inhibited more significantly than seen for the full-length CENP-E molecules and why cargo-free Bonsai CENP-E supports neither directed motility (motor head mediated) nor diffusive motion (tail mediated). The role of CENP-E stalk in mediating the intramolecular interactions is also supported by the lack of microtubule-binding activity of Bonsai CENP-E even in the presence of AMPPNP, implying that the ATP-binding site of soluble Bonsai CENP-E is blocked. Full-length CENP-E, however, is induced to bind strongly to microtubules by this nonhydrolyzable ATP analogue (Figure 1G). Of interest, when the C-terminal GFP at the tail of CENP-E is conjugated via antibodies to the surface of either a bead or a coverslip, Bonsai CENP-E can bind and walk on microtubules, but still with reduced capabilities. This result suggests that effect of the stalk configuration is less pronounced with cargo-bound versus free CENP-E tail. Finally, the reduced speed of motility with Bonsai CENP-E in multiple motor assays (such as microtubule gliding) can also be attributed to a fraction of inhibited molecules present under these experimental conditions.

Mechanisms of CENP-E–mediated stabilization of kinetochore–microtubule attachments

Our experiments in cells demonstrate that shortening the CENP-E stalk causes a severe reduction in its cellular functions. Although Bonsai CENP-E is recruited robustly to the mitotic kinetochores and exhibits normal enrichment at the kinetochores of pole-proximal chromosomes, in virtually all functional assays this shortened motor produces phenotypes that are similar to or even stronger than those seen with full CENP-E depletion. Indeed, lack of the congression of pole-proximal chromosomes during prometaphase (Figure 2D), reduced stability of kinetochore–microtubule attachments in metaphase (Figure 3), and presence of lagging chromosomes in anaphase (Figure 4) strongly suggest that CENP-E stalk is required for normal CENP-E functioning. These results emphasize the important roles that CENP-E kinesin plays during different mitotic stages, but they also raise a question about why these effects appear to be more severe than in the motility assays in vitro. Indeed, whereas purified Bonsai motor maintains a fairly robust velocity of microtubule gliding and microbead motions in vitro (Figure 1, C–E), it fails to support congression of the poleward chromosomes in cells. Of importance, using the CENP-E inhibitor GSK 923295, we confirmed that the kinetochore-bound Bonsai CENP-E was able to engage in microtubule binding similar to that of full-length CENP-E, demonstrating that the lack of normal mitotic functions of Bonsai CENP-E was not due to a complete inhibition of its microtubule-binding activity.

The rate of bead motions by Bonsai CENP-E in vitro is slower than that of full-length CENP-E, but it seems unlikely that this alone could explain the strong perturbation of kinetochore–microtubule interactions in cellular assays. Bonsai CENP-E motor may have some other important biomechanical defect, which has not yet been revealed by our motility assays in vitro, including reduced processivity of Bonsai CENP-E under a load. We also note that the in vitro and in vivo situations differ in the location of GFP tags and the exact mode of cargo binding: in vitro it takes place at a C-terminal tag, whereas Bonsai CENP-E is bound to its chromosomal cargo via the kinetochore-binding site, which is located upstream from the microtubule-binding site (Figure 1B). It has also been suggested that CENP-E function in vivo requires the long reach that is provided by the extended CENP-E coiled coil (Kim et al., 2008). This length may be important for the capture of already established kinetochore microtubule bundles and efficient congression of polar chromosomes in prometaphase, because a longer stalk could expand the range of microtubule search and capture at each kinetochore early in mitosis (Figure 5, polar chromosomes). The elongated shape of CENP-E may also be important later in mitosis when CENP-E facilitates maintenance and/or capture of new microtubules at the kinetochores of already congressed chromosomes (Figure 5, aligned chromosomes). Thus, a relatively active but short CENP-E motor may not be able to carry out its cellular functions well.
A lack of normal motor activity is expected to reduce the ability of CENP-E to track with dynamic microtubule ends and stabilize end-on attachments, since microtubule tip tracking by CENP-E relies in part on its active motor (Gudimchuk et al., 2013). Indeed, we observed unstable chromosome alignment in metaphase and segregation defects in anaphase in cells rescued with Bonsai CENP-E. We note, however, that destabilization of the end-on kinetochore–microtubule attachments could also result from impaired interaction between CENP-E and some other kinetochrome protein(s), including PP1 and Ndc80 (Kim et al., 2010). If in Bonsai CENP-E cells the BubR1 inhibition of Aurora B is reduced (Guo et al., 2010), 100 U/ml penicillin, 100 U/ml streptomycin, and 2 mM phosphate-buffered saline, followed by 1 h antibody incubation. CENP-E antibody HpX-1, anti-tubulin antibody DM1A (T9026; Sigma-Aldrich, St. Louis, MO), and anti–CENP-A antibody (13939; Abcam, Cambridge, MA) were used at 1:1000 and anti–centromere antibody ACA (15-234-0001; Antibodies Incorporated, Davis, CA) at 1:500. Cells were mounted in ProLong Gold (Invitrogen). Monopolar spindles were formed with 5 μM STLC for 2 h and 20 μM MG132 to prevent mitotic exit; GSX-923295 was used at 200 nM. To induce microtubule disassembly in cells with monopolar spindle, cells were treated for 20 min with a combination of 3.3 μM nocodazole and cold by incubating on a slurry of ice and water.

Microtubule signal was quantified by measuring the total pixel intensity of a 20-pixel-wide area along the spindle axis using FIJI, and local background was subtracted.

**Live-cell imaging**

DLD-1 cells expressing histone H2B-monomeric red fluorescent protein were seeded 24 h before experiment on Nunc LabTek II chambered coverglasses (Thermo Scientific, Waltham, MA). CO2-independent medium (Life Technologies) was added, and the cells were imaged at 37°C for up to 2 h at 5 min intervals using a DeltaVision Core system.

**Protein purification**

Tubulin was purified from cow brains by thermal cycling and chromatography, then labeled with rhodamine or Hilyte647. Kinesin 1 GFP construct K560 was purified as in Case et al. (1997). Xenopus laevis Truncated CENP-E labeled with GFP was expressed and purified from Escherichia coli as in Kim et al. (2008). X. laevis full-length CENP-E was expressed and purified from High Five cells (Invitrogen; Abreu et al., 2000). X. laevis Bonsai CENP-E was expressed in High Five cells. The cells were lysed using sonication in PK100 buffer (80 mM KPIPES, pH 6.8, 200 mM KC1, 20 mM imidazole, 0.5 mM ethylene glycol tetraacetic acid [EGTA], 1 mM MgCl2, 0.1 mM MgATP, 1 mM dithiothreitol [DTT], protease inhibitors). The latter were prepared from one Complete EDTA-free protease inhibitor cocktail tablet (11 873 580 001; Roche, Basel, Switzerland) and phe- nylmethylsulfonyl fluoride, 0.2 mM. Cell lysate was spun 30 min at 15,000 rpm (Sorvall SA-600; Thermo Scientific). The supernatant was incubated for 1 h at 4°C with nickel-nitroacetic acid beads and eluted using elution buffer (40 mM KPIPES, pH 6.8, 80 mM KC1, 300 mM imidazole, 0.5 mM EGTA, 1 mM MgCl2, 1 mM DTT, 0.1 mM MgATP). Peak fractions were collected, diluted 1:4 with HiTrap Q stabilizing buffer (20 mM KPIPES, pH 6.8, 40 mM KC1, 1 mM MgCl2, 0.5 mM EGTA, 1 mM DTT, 0.1 mM MgATP), and loaded into a HiTrap Q 1 ml column. Proteins were eluted using a 0.1–1 M KC1 gradient in HiTrap Q elution buffer (25 mM KPIPES, pH 6.8, 5 mM MgCl2, 0.5 mM EGTA, 1 mM DTT, 0.1 mM ATP). Peak fractions were snap frozen after addition of 20% sucrose.

**Work with CENP-E proteins in vitro**

Microtubule gliding and single-molecule motility assays were carried out with a Nikon Eclipse Ti-E inverted microscope equipped with 100×/1.49 NA TIRF oil objective, Perfect Focus system, and TIRF Quad cube with emission wheels run with NIS-Elements Software. The temperature of the objective was kept at 32°C with a heater (Bioptechs, Butler, PA). The epifluorescence was excited with a 488 nm laser to visualize GFP and rhodamine and a 640 nm laser to visualize microtubules labeled with Hilyte647. Motility chambers were prepared with a glass slide, double-stick tape, and silanized 22 × 22 mm coverslips, as in Volkov et al. (2014). For experiments with solubile CENP-E, Taxol-stabilized, rhodamine-labeled
microtubules were attached to coverslips with anti-tubulin antibodies (Serotec), the surface was blocked with Pluronic-F127, and GFP-labeled proteins (0.5–3 nM) were imaged in the motility buffer composed of BRB80 (80 mM K-PIPES, 1 mM MgCl₂, 1 mM EGTA, pH 6.9) with 4 mg/ml bovine serum albumin (BSA), 2 mM DTT, 2 mM Mg-ATP, 7.5–10 μM Taxol, 6 mg/ml glucose, 68 μg/ml catalase, 0.1 mg/ml glucose oxidase, and 0.5% β-mercaptoethanol. Images were acquired with an Andor EMCCD at 100 ms/frame for each channel, and rhodamine and GFP signals were separated with emission filters. The number of GFP molecules in fluorescent dots for data in Supplemental Figure S1G was determined as in Gudimchuk et al. (2013). To visualize photobleaching steps, fluorescence curves were filtered with a Chung–Kennedy filter that preserves stepwise transitions (Chung and Kennedy, 1991), and the number of visual steps was counted (Supplemental Figure S1, H and I). Bonsai Ndc80-GFP protein, which is monomeric (Ciferri et al., 2005), and processive kinesin 1 proteins were used as controls.

For microtubule-gliding assay, coverslips were coated with layers of biotin-BSA (22.5 μM), NeutrAvidin (25 μM), and biotinylated anti-GFP antibody (100–100 nM; Abcam) and then blocked with Pluronic F127 (1%) and another layer of biotin-BSA (22.5 μM) or biotin-PEG (100 μM; Quant BioTech, Plain City, OH). CENP-E proteins were added at 15–600 nM for 30 min to create coatings that were similar in density for different protein constructs, as judged by GFP brightness. After removal of the unbound protein, Taxol-stabilized, HiLyte647-labeled microtubules were imaged in the same motility buffer as before, except that β-mercaptoethanol was replaced with 10 mM DTT. Images were acquired once per second with 100– to 300 ms exposure and analyzed using ImageJ. Motility of CENP-E-coated beads was examined using instruments and procedures described in Gudimchuk et al. (2013). Fluorescence-based microtubule-pelleting assay was carried out as in Gudimchuk et al. (2013). Briefly, CENP-E proteins (6 nM) were incubated with increasing concentration of microtubules and 2 mM AMPPNP for 15 min at 32°C, microtubules were pelleted, and the concentration of unbound CENP-E was quantified by GFP fluorescence of the supernatants.

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