

Microtubule-dependent path to the cell cortex for cytoplasmic dynein in mitotic spindle orientation

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During animal development, microtubules (MTs) play a major role in directing cellular and subcellular patterning, impacting cell polarization and subcellular organization, thereby affecting cell fate determination and tissue architecture. In particular, when progenitor cells divide asymmetrically along an anterior-posterior or apical-basal axis, MTs must coordinate the position of the mitotic spindle with the site of cell division to ensure normal distribution of cell fate determinants and equal sequestration of genetic material into the two daughter cells. Emerging data from diverse model systems have led to the prevailing view that, during mitotic spindle positioning, polarity cues at the cell cortex signal for the recruitment of NuMA and the minus-end directed MT motor cytoplasmic dynein.¹ The NuMA/dynein complex is believed to connect, in turn, to the mitotic spindle via astral MTs, thus aligning and tethering the spindle, but how this connection is achieved faithfully is unclear. Do astral MTs need to search for and then capture cortical NuMA/dynein? How does dynein capture the astral MTs emanating from the correct spindle pole? Recently, using the classical model of asymmetric cell division—budding yeast *S. cerevisiae*—we successfully demonstrated that astral MTs assume an active role in cortical dynein targeting, in that astral MTs utilize their distal plus ends to deliver dynein to the daughter cell cortex, the site where dynein activity is needed to perform its spindle alignment function. This observation introduced the novel idea that, during mitotic spindle orientation processes, polarity cues at the cell

cortex may actually signal to prime the cortical receptors for MT-dependent dynein delivery. This model is consistent with the observation that dynein/dynactin accumulate prominently at the astral MT plus ends during metaphase in a wide range of cultured mammalian cells.

Spindle Positioning in Budding Yeast

In budding yeast, at least two distinct but complementary mechanisms dictate the alignment and positioning of the mitotic spindle along the cell polarity axis—termed Kar9 and Dynein pathways. The Kar9 pathway uses a class-V myosin, Myo2, which guides the plus ends of astral MTs along cortical actin cables toward the apex of the bud cell cortex.^{2,3} Myo2 binds Kar9, the yeast Adenomatous Polypoidosis Coli (APC) homolog, via its tail domain. Kar9 in turn binds Bim1, the yeast EB1 homolog, which interacts with the plus end of astral MTs. As Myo2 motors along the actin cables (which are polarized toward the bud tip), it transports the MT plus end (as cargo) toward the bud cell tip. There, an attachment-coupled MT depolymerization event (or capture-shrinkage) results in the translocation of the spindle toward the bud cell. These capture-shrinkage events, which are biased toward the bud cortex,⁴ are thought to be mediated by a MT depolymerase, the identity of which is still unknown, although evidence points to Kip3 (a kinesin-8)^{5,6} and/or Kar3 (a kinesin-14),⁷⁻¹⁰ both of which exhibit plus end-specific MT depolymerase activity. The motor may induce MT depolymerization

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while keeping the MT attached to a yet unknown cortical receptor protein.^{7,11} This capture-shrinkage mechanism generates enough pulling force to move the spindle toward the bud. Based on in vitro force measurements, such depolymerization-driven events could generate at least 30–65 pN of force per MT.¹²

In contrast, during the Dynein pathway,^{4,13} dynein motors attached to the bud cell cortex cause spindle movements by walking along astral MTs toward the spindle pole body (SPB; equivalent to centrosome in animal cells). Because the motor is anchored, dynein's walking generates pulling forces at the cortex. Also, because the minus end of the astral MT is stably attached at the SPB, the astral MT that is being pulled by dynein slides along the bud cortex, dragging the spindle into the bud neck, thereby aligning the spindle along the polarized cell axis prior to anaphase onset. The events that conspire to properly position the spindle are seemingly well coordinated and tightly regulated since cell division appears to occur in a rhythmic and dependable manner. Our recent data are beginning to uncover the intricate and complex series of events that ultimately result in the targeting of dynein to the cell cortex in a timely and spatially restricted manner.

From the Cytoplasm to the MT Plus End

In a wide variety of cell types, cytoplasmic dynein is found at the distal tip, or the plus end, of astral MTs.^{14–17} Studies from budding yeast have indicated that the targeting of dynein to this site is not sufficient for Dynein pathway activity in this organism; rather, the association of dynein with the cell cortex is required for dynein function in vivo. Thus, dynein does not elicit its activity from MT plus ends. What is the role of plus end-targeting in dynein function? The first insight into this question came from genetic studies in which the gene coding for the cortical receptor protein, Num1,^{18–20} was deleted. In addition to a complete loss of cortical dynein foci and disrupted dynein pathway activity, these mutants exhibited an enhanced accumulation of

dynein at astral MT plus ends compared with wild-type cells.^{13,21} A similar phenotype was observed when genes encoding subunits of the dynactin complex were deleted (discussed below). These data led to the proposal that cortical dynein is delivered (or 'offloaded') from the plus ends of dynamic astral MTs to the cell cortex (see Fig. 1). The mechanism by which a minus end-directed motor accumulates at the plus ends of astral MTs and stays associated with the growing or shrinking plus end remains a confounding and intriguing mystery. However, the identities of numerous factors that facilitate the targeting and/or the maintenance of dynein at this site have been revealed, and to a certain extent their distinct roles have been characterized.

DHC/Dyn1. As in higher eukaryotes, the dynein complex in budding yeast consists of four different polypeptides, including the light chain (LC8/Dyn2), the light-intermediate chain (LIC/Dyn3), the intermediate chain (IC/Pac11), and the heavy chain (HC/Dyn1), the latter of which is solely responsible for the motor activity of the complex. Each dynein complex at the astral MT plus end consists of two subunits of HC/Dyn1, four of IC/Pac11, three of LIC/Dyn3, and likely eight of LC8/Dyn2.^{22,23} Dyn1 consists of an N-terminal tail domain, which is responsible for cortical targeting in budding yeast (discussed below), and a C-terminal motor domain (Fig. 2). The motor domain consists of a MT-binding domain amidst a concatenated series of six

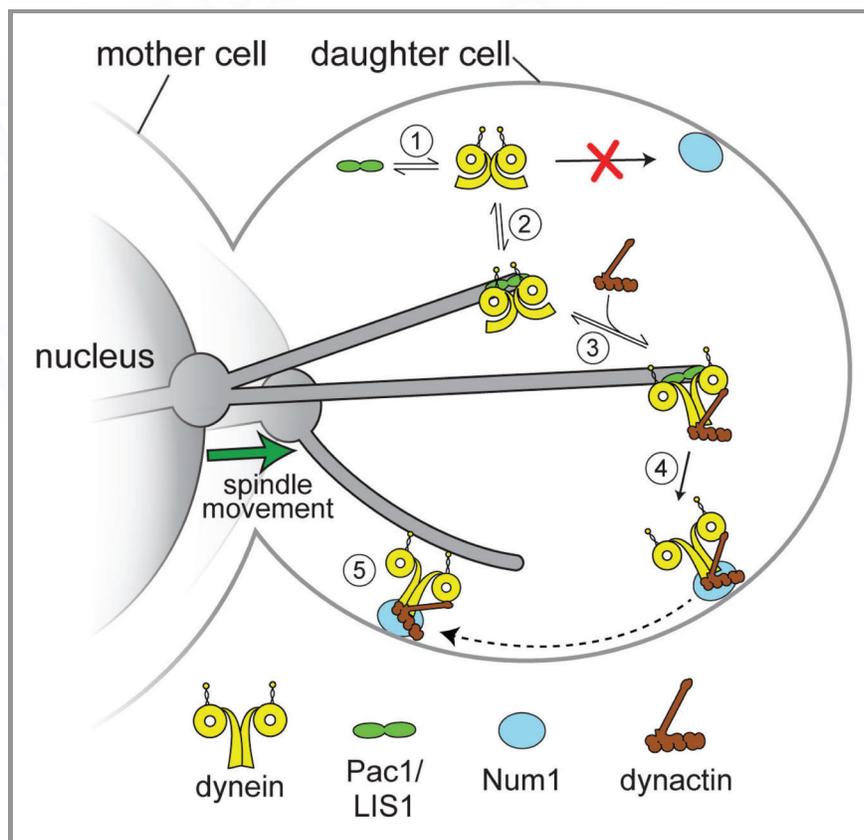


Figure 1. Offloading model for dynein function in budding yeast. Cytoplasmic dynein adopts a 'masked' configuration, thus preventing it from being directly recruited from the cytoplasm to cortical Num1 receptor sites. Dynein associates with Pac1 in the cytoplasm (step 1) forming a complex that is competent for binding to the astral MT plus end (step 2), which occurs in a Bik1-dependent manner. Plus end-associated dynein then recruits the dynactin complex (step 3), which triggers the unmasking event allowing the dynein tail domain to bind to cortically anchored Num1 (step 4; offloading). Cortical dynein-dynactin then binds to astral MTs anchored at the spindle pole body, and walks toward the minus end, thus pulling the nucleus and associated mitotic spindle into the neck connecting mother and daughter cells (step 5).

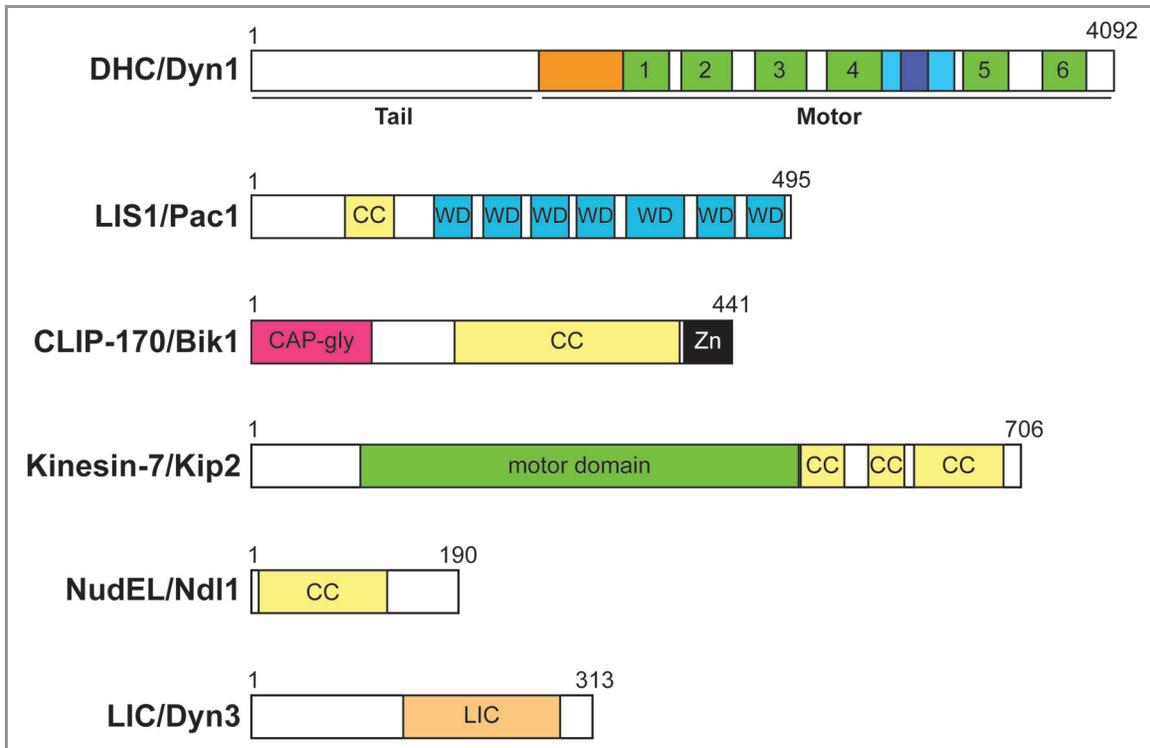


Figure 2. Domain organization of proteins involved in dynein plus end-targeting. With the exception of DHC/Dyn1, all schematics are drawn to scale. Domains in DHC/Dyn1²⁶: orange region, 'linker' domain; green regions, six AAA domains; light blue regions, anti-parallel coiled coil of the stalk; dark blue region, MT-binding domain. CC, coiled-coil domain; WD, WD40 repeat; Zn, zinc fingers or metal-binding domain.^{46,47}

AAA domains (ATPase associated with various cellular activities) located C-terminal to a linker element that is thought to function as a mechanical lever during the dynein power stroke.^{24,25} Truncation analyses of the heavy chain have found that the minimal domain that is sufficient for motor activity is also sufficient for plus end-targeting.²⁶ In fact, when expressed from its native promoter, the dynein motor domain is a more robust plus tip-tracker than the full-length protein, likely due to its enhanced association with Pac1, the yeast LIS1 homolog (see below). Association of the motor domain with the MT plus end does not require motor activity, since rigor-inducing point mutations in the Walker A and B motifs do not preclude plus end-targeting.²⁶

LIS1/Pac1. The best characterized of the dynein plus end-targeting factors is LIS1, the causative gene for human lissencephaly, a severe brain developmental disorder characterized by defective neuronal migration. The budding yeast homolog, Pac1, which contains a coiled-coil domain and seven consecutive

WD40-repeat domains (Fig. 2), is required for the targeting of dynein to MT plus ends and consequently the cell cortex. Our recent genetic, biochemical and quantitative fluorescence microscopy studies have revealed that Pac1 associates with the dynein complex in a 1:1 ratio in the cytoplasm prior to its association with MT plus ends (Fig. 1, steps 1 and 2).²² Interestingly, Pac1 is unable to associate with plus ends directly, but requires dynein for this activity. Thus, dynein and Pac1 together comprise a complex that is competent for plus end-targeting.

A precise mechanistic role for Pac1/LIS1 in affecting dynein plus end-targeting remains unknown, but studies in which dynein motility has been reconstituted in vitro have revealed possible mechanisms of action. Recent studies with yeast dynein²⁷ and vertebrate dynein^{28,29} showed that both Pac1 and LIS1 reduce the velocity of dynein motility. One such study using an optical tweezer found that LIS1 induces a persistent MT-bound state of dynein under high load conditions (although dynein would not be under load at the

plus end). These data suggest that LIS1/Pac1 may be important to maintain dynein at the plus end by keeping the motor in an 'off' state, thus preventing it from walking toward the minus end. To test this hypothesis, we believe it would be important for future studies to reconstitute dynein plus end-targeting on dynamic MTs using purified components.

CLIP-170/Bik1. The first protein found to be associated with the dynamic ends of MTs was CLIP-170 (cytoplasmic linker protein).³⁰ Since then, it has been joined by a host of numerous other such factors, including the end-binding (EB) family of tip-tracking proteins (reviewed in ref.³¹). The budding yeast CLIP-170 homolog, Bik1, has been shown to be crucial for the plus end-targeting of dynein and Pac1.^{21,22} The association of Bik1 with astral MT plus ends is mediated by the N-terminal CAP-Gly (cytoskeleton associated protein, glycine rich) domain (Fig. 2),³² a motif found in several MT-associated proteins that bind to the acidic tails (EEY motif) of α -tubulin and EB1. Studies using purified

mammalian CLIP-170 have shown that it is unable to tip-track autonomously in a reconstituted experimental system. Rather, it requires the presence of EB1 for this activity.^{33,34} CLIP-170 appears to recognize a composite binding site consisting of both tyrosinated tubulin and tyrosinated EB1 *in vitro*,³⁴ and also requires tyrosinated tubulin for tip-tracking *in vivo*.³⁵

Our recent studies have shown that Bik1 plays a role in dynein plus end-targeting that is clearly distinct from Pac1.²² Bik1 associates with astral MT plus ends in a manner that is independent of either dynein or Pac1. Furthermore, Bik1 is found at astral MT plus ends in excess with respect to the number of dynein-Pac1 complexes. Interestingly, in contrast to reconstitution experiments performed with purified mammalian CLIP-170, Bik1 possesses the ability to associate with astral MT plus ends independently of the yeast EB1 homolog, Bim1.^{22,36} Whether this discrepancy is due to evolutionary divergence (yeast vs. vertebrate tubulin, or Bik1 vs. CLIP-170), or differences between *in vitro* and *in vivo* conditions, remains to be determined. Thus, it appears as though association of dynein with astral MT plus ends requires only Pac1 and Bik1.

Kinesin-7/Kip2. This plus end-directed kinesin has been shown to transport Bik1 along astral MTs toward the distal tip.³⁶ *In vivo* studies³⁷ and *in vitro* reconstitution experiments³⁸ demonstrated that the same phenomenon also occurs with Tea2 and Tip1, the fission yeast homologs of Kip2 and Bik1, respectively. Since the dynein-Pac1 complex presumably associates with Bik1 at astral MT plus ends, it is not surprising that dynein has also been observed to be transported toward the plus end in a Kip2-dependent manner.²⁶ However, fluorescence intensity measurements of Dyn1 in wild-type and *kip2Δ* mutant cells indicate that Kip2-mediated transport accounts for only ~30% of the dynein-Pac1 complex at astral MT plus ends. Direct recruitment from the cytoplasm likely accounts for the remaining ~70% of these proteins at the plus end, although further studies will be required to test this.

NudEL/Ndl1. Ndl1, a NudE-like protein, is required for the normal wild-type frequency of dynein targeting to astral MT

plus ends.³⁹ Genetic and cell biological analyses showed that Ndl1 may stabilize or enhance the efficiency of Pac1 binding to dynein at the plus end, a role consistent with a subsequent *in vitro* study in which the effects of mammalian NudE and LIS1 on dynein motility were determined.²⁸ Intriguingly, a truncated form of yeast dynein containing only the motor domain (termed Motor; see Fig. 2) requires Pac1, but not Ndl1, for astral MT plus end-targeting,²⁶ suggesting that the dynein tail domain (termed Tail; see Fig. 2) and Ndl1 are involved in the steps prior to dynein-Pac1 interaction with the MT plus end. Both Tail and Ndl1 may cooperate to promote Pac1 binding to Motor (which then becomes competent for plus end-binding via Bik1), as proposed.²⁸ Alternatively, as suggested by our data, Tail might inhibit Pac1 binding to Motor, and Ndl1, or other factors may help relieve this inhibition.

LIC/Dyn3. Unlike higher eukaryotes in which multiple isoforms of the dynein light intermediate chain are found, budding yeast possesses only a single gene coding for this accessory chain. Although the role of Dyn3 in the Dynein pathway is still unclear, our studies have revealed that it functions in promoting a proper and stable conformation of the dynein heavy chain. Initial efforts to characterize Dyn3 led to the proposal that it was important for the offloading of dynein to the cell cortex. This was based on the observation that cortical Dyn1 foci were absent in cells lacking Dyn3, whereas motile cytoplasmic foci of Dyn1, presumed to be plus end-associated, appeared to be unaffected.⁴⁰ However, two-color imaging of Dyn1-3GFP and mCherry-labeled MTs (mCherry-Tub1) revealed that these motile cytoplasmic Dyn1 foci are not associated with MT plus ends or any other apparent cellular structure, suggesting aggregation of Dyn1 in *dyn3Δ* cells (Fig. 3A). Immunoblotting of Dyn1 revealed that loss of Dyn3 partially compromises Dyn1 protein stability (Fig. 3B). These data are consistent with findings in other organisms, in which disruption of LIC significantly reduced the protein levels of DHC.^{41,42} Thus, Dyn3 is required for the proper targeting of the dynein complex to astral MT plus ends, possibly

due to the maintenance of a proper Dyn1 conformational state.

From the Plus Ends to the Cell Cortex

As in higher eukaryotes,^{16,43} the recruitment of dynein to the cell cortex in budding yeast occurs in a spatially and temporally restrictive manner. Our studies in budding yeast have indicated that dynein must first be targeted to the plus ends of MTs prior to associating with cortical Num1 receptor sites at the daughter cell cortex (see Fig. 1). What prevents dynein from being directly recruited to Num1 in the absence of plus end-targeting? If dynein has the inherent ability to bind to cortically anchored Num1, then its inability to bind to Num1 in the absence of plus end-targeting intimates the presence of an inhibitory mechanism. Intriguingly, a truncated form of yeast dynein containing only the tail domain is capable of associating with Num1 receptor sites in the absence of plus end-targeting. In fact, association of Tail with the cell cortex is much more robust than full-length Dyn1. These data strongly suggest that the dynein tail domain has an inherent affinity for cortical Num1 that is independent of MT-binding.²⁶ Thus, we propose that, in the context of the full-length dynein molecule, this activity is negatively regulated through an intramolecular 'masking' of the tail domain by its motor head.

We recently obtained evidence supporting this hypothesis using an engineered yeast dynein mutant in which a peptide was inserted into the junction between the tail and motor domains.²⁷ This mutant possessed wild-type motor activity but exhibited targeting characteristic of a constitutively unmasked dynein, which can also be seen offloading from astral MT plus ends to the cell cortex. We observed that the majority of offloading events for both the mutant as well as wild-type dynein took place at the daughter cell cortex. Thus, delivery of dynein to the place where it is needed appears to be intrinsic to the offloading mechanism. We believe that dynein recruitment to the astral MT plus ends relieves the Motor-dependent inhibition of the Tail, thus

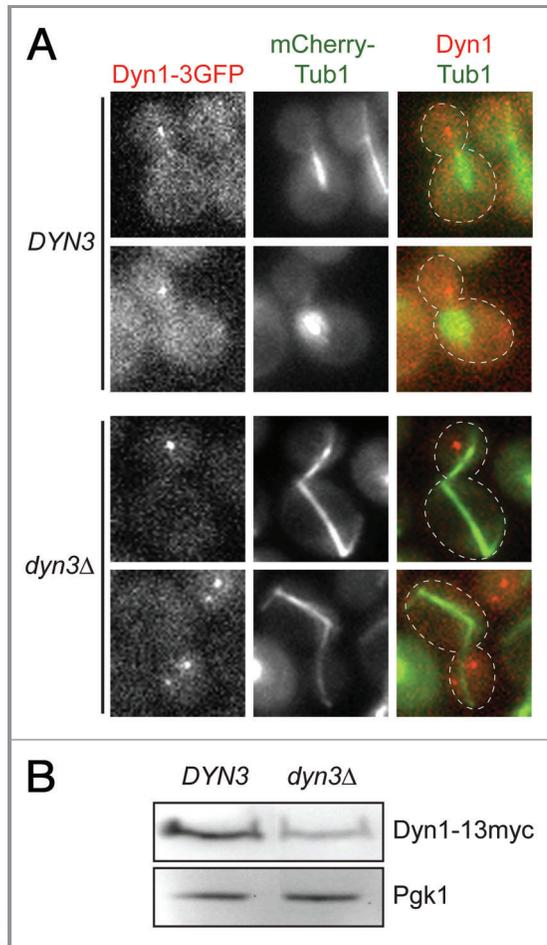


Figure 3. Role of budding yeast LIC/Dyn3 in plus end-targeting and stability of DHC/Dyn1. (A) Wild-type (top; *DYN3*) or *dyn3Δ* (bottom) cells expressing Dyn1-3GFP and mCherry-Tub1. Merge images show Dyn1-3GFP in red and mCherry-Tub1 in green. Dyn1 foci are absent from astral MT plus ends in *dyn3Δ* cells. Each image is a maximum intensity projection of a 2- μ m Z-stack of wide-field images. (B) Protein gel blot of wild-type and *dyn3Δ* strains carrying *DYN1-13myc*. Equal amount of total cell lysate was loaded in each lane, transferred to PVDF and probed with either anti-c-Myc (top) or anti-PGK1 (for loading control, bottom) antibodies.

priming the dynein molecule for offloading to cortical Num1 sites. Our data also suggested that, after astral MT plus end-targeting, subsequent association of dynein with the dynactin complex may mediate the unmasking event, thus ensuring that only intact dynein-dynactin complexes are offloaded to the cortex (see Fig. 1, steps 3 and 4). In support of this hypothesis, deletion of genes encoding the various components of the dynactin complex results in a loss of dynein from the cell cortex without affecting plus end-targeting of dynein.¹³ If true, our model proposes that dynactin would trigger a conformational change in the dynein heavy chain. Future structural studies utilizing such techniques as super-resolution fluorescence

microscopy or electron microscopy will shed light on whether dynactin can affect conformational changes within dynein and how dynactin controls dynein function at the astral MT plus ends. Reconstitution of the offloading phenomenon in vitro will provide invaluable information pertaining to the role of each of the dynein pathway components in offloading, and will shed light on the mechanism by which it occurs.

Cell cycle regulation of Dynein pathway activity. The requisite course of delivery for dynein from the cytoplasm to MT plus ends to the cell cortex provides numerous possibilities for cellular dynein pathway activity to be regulated. In fact, overexpression of either Pac1 or Dyn1 causes an increase in cortical dynein

activity as a result of the enhanced association of dynein with MT plus ends and consequently the cell cortex.²² Interestingly, Motor possesses a higher affinity for Pac1 than full-length Dyn1 and as a result exhibits enhanced plus end-targeting compared with Dyn1, based on frequency and intensity measurements at the astral MT plus ends. These observations further support the notion that the dynein tail domain plays a role in modulating Pac1 binding to the motor domain. Whether this is directly attributable to the tail domain or to a Tail-interacting factor, such as NudEL/Ndl1, is currently unknown.

Given the localization of dynein to astral MT plus ends in a wide variety of cell types, plus end-targeting is likely a key point of regulation for cortical dynein activity. In budding yeast, a regulated interaction between Dyn1 and Pac1 is likely an important means by which plus end-targeting, and thus Dynein pathway activity, is regulated. Quantitation of plus end and cortical dynein foci revealed that dynein targeting is enhanced at both sites as cells progress from G1 through mitosis.²⁶ At least two possible scenarios may account for this enhancement of dynein-Pac1 targeting to MT plus ends: (1) upregulation of Pac1 or Dyn1 expression levels as the cell approaches mitosis; or, (2) an enhanced affinity of Pac1 for Dyn1. The first possibility provides a simple, yet elegant manner to restrict dynein activity to the time it is needed most. However, such a mechanism would be slow to decrease dynein activity after the time it is needed, and would be limited by the half-life of the dynein complex stability in the cell. Combining such a mechanism with targeted proteolysis (e.g., ubiquitination or sumoylation) may provide the needed timely removal of dynein from the astral MT plus ends, and the concomitant reduction in dynein activity, however it is unclear whether such a mechanism would provide the potentially rapid response time needed for proper dynein-mediated spindle orientation. An enhanced interaction between dynein and Pac1, the second possibility, would result in an enhanced association of the dynein-Pac1 complex with astral MT plus ends, and concomitantly the cell cortex. This

potential regulatory mechanism for dynein plus end-targeting may be sufficient for the rapid delivery of dynein to sites of action. Conversely, downregulation of the dynein-Pac1 interaction could be used to prevent the delivery of dynein to the cell cortex. The means by which this interaction is regulated is unknown, although, as discussed above, our data indicate that Tail plays a role in affecting the dynein-Pac1 interaction.

While regulated plus end-targeting of dynein may be sufficient to ensure the timely delivery of dynein to sites of action, other means would be required to ensure that the activity of cortically-anchored

dynein is switched 'off' when it is no longer wanted. One such potential mechanism may be a regulated interaction between cortical dynein and its activator, dynactin. Our studies revealed that, in wild-type cells, the normal steady-state ratio of cortical dynein-dynactin is roughly 2 dynein to 1 dynactin complex.²² This is in contrast to in vitro reconstitution studies, which demonstrated that, when walking along a MT, each dynein complex is bound to one dynactin complex.⁴⁴ Intriguingly, when the gene encoding She1 (Sensitive to High Expression-1, a recently characterized Dynein pathway component⁴⁵) is deleted from the cell,

the dynein:dynactin ratio is reduced to from 2:1 to 1:1. Accordingly, *she1Δ* cells exhibit a significant augmentation of Dynein pathway activity. While She1 may affect other aspects of Dynein pathway activity, these data suggest that She1 has a role in regulating the interaction between cortically-anchored dynein with dynactin, and that this interaction poses yet another means by which dynein pathway activity may be regulated in the cell.

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