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FORMIN a link between kinetochores and microtubule ends

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Abstract

The mammalian diaphanous-related (mDia) formin proteins are well known for their actin-nucleation and filament-elongation activities in mediating actin dynamics. They also directly bind to microtubules and regulate microtubule stabilization at the leading edge of the cell during cell migration. Recently, the formin mDia3 was shown to associate with the kinetochore and to contribute to metaphase chromosome alignment, a process in which kinetochores form stable attachments with growing and shrinking microtubules. We suggest that the formin mDia3 could contribute to the regulation of kinetochore-bound microtubule dynamics, in coordination with attachment via its own microtubule-binding activity, as well as via its interaction with the tip-tracker EB1 (end-binding protein 1).

Regulating chromosome alignment

During mitosis a pair of sister chromosomes captures microtubules from opposite poles of the mitotic spindle to become bi-oriented. This is followed by congression to the metaphase plate, and oscillatory movements linked to the continued growth and shrinkage of the kinetochore-bound microtubule fibers [1]. The kinetochore, a proteinaceous structure assembled at the centromere region of a chromosome, acts as the site of spindle microtubule binding. Numerous proteins have been implicated in connecting kinetochores with dynamic microtubules, although the precise functions of the majority of these proteins and the pathways that regulate them remain unclear [1–3]. The current model is that the KMN network (KNL1, Mis12, and Ndc80 complexes) serves as the core microtubule-binding apparatus with Ndc80 being the major binding site at the kinetochore for stabilizing end-on microtubule attachment [3,4]. Although there is much support for the Ndc80 complex as a crucial link in kinetochore–microtubule attachment, it is quite clear that other microtubule-associated proteins play important roles in maintaining stable end-on attachments at the kinetochore. In yeast, the Dam1 complex plays a crucial role along with the Ndc80 complex in mediating kinetochore–microtubule attachment and coupling chromosome movement to microtubule depolymerization [3]; however, vertebrate homologs of the Dam1 complex have not yet been identified. Depletion of the Ska1/RAMA complex [5–8] or the formin mDia3 [9,10] also results in chromosome misalignment phenotypes in mammalian cultured cells, reminiscent of depletion of the Ndc80 complex. Furthermore, in most metazoans bi-oriented sister kinetochore pairs undergo continuous oscillations [11]. An important

unresolved issue in the mitosis field is whether and how kinetochore components control kinetochore oscillations in coordination with microtubule attachment.

The mammalian mDia formin proteins constitute a subfamily of Rho GTPase-binding formin homology (FH) proteins [12,13]. The mDia formins nucleate and assemble unbranched actin structures, and therefore are implicated in numerous actin-based cellular functions, including cytokinesis, cell morphogenesis, cell polarity and cell migration. mDia formin proteins are also involved in regulating microtubule-dependent processes, in which they modulate the dynamics of microtubules. These topics have been reviewed elsewhere [14–16]. A potentially novel kinetochore function for the formin mDia3 was identified several years ago [10]; however, at that time it was not clear whether the chromosome misalignment phenotype caused by knockdown of mDia3 using a small interfering RNA (siRNA) might result from indirect defects due to mDia formin functions in actin dynamics. Recently, it was shown that actin nucleation-deficient mDia3 mutant constructs can rescue alignment defects seen in the mDia3 knockdown cells comparable to rescue seen using the wild-type mDia3 construct [9], strongly suggesting that the chromosome misalignment phenotype is independent of mDia3 actin-binding or nucleation activities. This eliminates the possibility that the formin mDia3 might have an effect on the mitotic spindles by regulating actin at cortical or other sites and supports the idea that mDia3 acts directly on micro-tubules at the kinetochore. Therefore, the mDia formin proteins can regulate microtubule dynamics, independent of their effects on actin, in both interphase and mitotic cells.

Current studies suggest that the mDia formin proteins could have at least two separable properties in regulating microtubule dynamics. First, the formin proteins can bind directly to microtubules independently of their actin-binding and nucleation activities [17]. Second, the mDia formins bind to the tip-tracker EB1 and its binding partner – the adenomatous polyposis coli (APC) tumor-suppressor protein [9,18], and this could promote microtubule polymerization. During chromosome congression and oscillation the leading and trailing kinetochores in a sister-kinetochore pair have two distinct stably attached microtubule populations: the trailing kinetochore is attached to growing microtubules while the leading kinetochore retains its attachment to shrinking microtubules. The EB1 and APC proteins accumulate only at the trailing kinetochore (in a sister-kinetochore pair) where there is net kinetochore microtubule growth (polymerization) [19]. Therefore, mDia3 could play different roles at leading and trailing kinetochores depending on its binding partners. Here, we examine current knowledge about the formin mDia3 at the kinetochore and discuss its possible roles in microtubule attachment, as well as the dynamic regulation of kinetochore-bound microtubules, based on its own intrinsic microtubule activity and its interaction with EB1–APC. Finally, we propose a new model for mDia3 control of kinetochore oscillations (Figure 1).

mDia3 microtubule-binding activity: mechanism and regulation

The effect of mDia formin proteins on microtubule dynamics is relatively unexplored; most descriptions come from the contributions of mDia1/2 to microtubule stabilization in migrating cells. In most undifferentiated mammalian cells, microtubules are highly dynamic.

By contrast, in migrating wound-edge cells, a subset of microtubules become stabilized with a long half-life (>1 h) near the leading edge, which enables reorientation of the MTOC (microtubule organization center) and produces a polarized micro-tubule array to facilitate cell migration [20,21]. These microtubules are capped at their plus ends [22]. The mDia formins clearly contribute to the capping process [18,21]; however, the exact role of the formin proteins remains unresolved, possibly due to the lack of biochemical characterization of mDia formin proteins in microtubule dynamics. The most informative characterization of mDia formin effects on microtubules comes from analysis of the dynamics of microtubules grown off axonemal seeds, which reveals that the mDia2 protein reduces the rates of both polymerization and depolymerization of microtubules [17]. mDia2 strongly slows microtubule shrinkage rates, an effect that is distinct from traditional microtubule-associated proteins which promote microtubule assembly rates without significantly interfering with shrinkage rates under similar experimental conditions [23–25]. The ability of formin protein to stabilize microtubules *in vitro* is consistent with a role in generating stable microtubules in migrating cells. Furthermore, mitotic cells depleted of mDia3 have an impaired ability to stabilize kinetochore-bound microtubule fibers in response to cold treatment [9].

In cells expressing an mDia3 mutant with reduced microtubule-binding activity there is a decrease in inter-kinetochore stretch (tension) between a pair of sister kinetochores [9]. This result suggests that mDia3 microtubule-binding activity could play a role in force generation at the kinetochore to stabilize attachment with microtubule bundles. Because microtubules appear to embed in kinetochores in many electron-microscopic images, it is widely accepted that kinetochores capture microtubules through end-on attachments. The Ndc80 complex would be expected to bind to the microtubule side (but very close to microtubule ends), however, because structural analysis [26], *in vitro* single-molecule assays [27], and super-resolution microscopy analysis in cells [28], all support a biased-diffusion mechanism for force generation by Ndc80 molecules acting along the microtubule axis. The formin mDia3 [9], as well as other kinetochore-associated microtubule binding proteins (e.g. the Skl/ RAMA complex [8]) are also expected to bind to the microtubule lattice. All of these microtubule-associated proteins bind to microtubules with relatively low affinity [8,9,29]. This property could be important for their roles in stabilizing kinetochore attachments to dynamic microtubules. On the other hand, the kinetochores could use alternative molecular components to attach to growing and shrinking microtubules. In the future, it will be important to address whether and how these microtubule interactors work together or independently in maintaining end-on attachments.

How the microtubule-binding activity of mDia3 contributes to the stability of kinetochore microtubule bundles remains to be defined. However, this activity is directly regulated by the Aurora B kinase [9], similar to what is seen for the KMN network [29–31]. Accurate chromosome segregation during mitosis requires that each pair of sister kinetochores captures microtubules from opposite spindle poles; however, errors frequently occur (e.g. syntelic attachments – both sister kinetochores attach to spindle microtubules from the same pole) [32]. Aurora B, a kinase that localizes to the inner centromere region between sister kinetochores, plays an essential role in attachment error correction through a ‘spatial separation’ mechanism to distinguish between correct and incorrect attachments (reviewed

in [33]). Inter-kinetochore stretch (tension) generated between a pair of bi-oriented sister kinetochores separates the Aurora B kinase at the inner centromere from its outer kinetochore substrates. Indirect immunofluorescence analysis using a phosphospecific antibody against one of the Aurora B phosphorylation sites on mDia3, Ser196, detects kinetochore staining that is substantially reduced on kinetochores aligned at the meta-phase plate upon bi-orientation [9], providing *in vivo* evidence for Aurora B phosphorylation of mDia3. Cells expressing a non-phosphorylatable mDia3 mutant cannot position chromosomes at the metaphase plate [9], although the nature of the kinetochore–microtubule attachment in these cells remains to be characterized.

Finally, cells expressing an mDia3 mutant that has reduced microtubule-binding activity, but retains its ability to bind to EB1–APC, can congress almost all chromosomes to the metaphase plate, a phenotype that is much less severe than that observed in mDia3 knockdown cells using siRNA [9]. This result suggests that, in addition to its own microtubule-binding activity, the mDia3 interaction with EB1–APC is also important for its role at the kinetochore.

mDia3–EB1–APC: a link between kinetochores and microtubule growing ends

Microtubule capture at bud sites in budding yeast is regulated by the formin Bni1, the yeast ortholog of mDia formin proteins [34,35]. Using genetic and biochemical studies, the microtubule tip-binding protein Bim1 (the yeast ortho-log of EB1) and Kar9 (speculated to represent a possible APC homolog) have been implicated in this process [36–38]. This pathway has been described as an evolutionarily conserved pathway for microtubule capture at the leading edge of migrating fibroblasts. The mDia formin proteins are probably anchored in the actin network at the cell cortex, functioning as scaffold proteins for EB1 and APC and forming a complex with these proteins at micro-tubule plus ends, thereby contributing to microtubule capping and stabilization [18]. The formin mDia3 seems to play a similar role in kinetochore–microtubule attachment during mitosis in which the mDia3 protein serves as an anchor at the kinetochore for EB1 and APC through direct interaction. A mutation in a conserved region adjacent to the FH2 microtubule-binding domain blocks the ability of both mDia2 and mDia3 to bind to EB1 [9,18]. Replacing endogenous proteins with EB1-binding-deficient mDia2 or mDia3 mutants results in inhibition of stable microtubule formation in migrating cells [18], and meta-phase chromosome misalignment in mitotic cells [9], respectively.

The microtubule tip-tracking EB1 protein, possibly along with its binding partner APC, binds to growing plus ends and promotes microtubule polymerization ([39] for review). The EB family members do not track microtubule plus ends processively and instead are exchanged rapidly at microtubule tips [40,41]. Therefore, the mDia3–EB1 interaction may represent a mechanism for EB1 accumulation at the trailing kinetochore of a sister-kinetochore pair with growing microtubules, which has long been described by indirect immunofluorescence and live-cell imaging [19]. The exact role of the stable association of EB1–APC at kinetochores is not understood; however, chromosome misalignment and mis-

segregation upon loss of EB1 and APC function have been reported in *Xenopus* egg extracts [42] and in mammalian cultured cells [43–45].

One important unresolved question in the mitosis field is how kinetochores track dynamic microtubule plus ends. Although tip-tracking behavior of Ndc80-coated microbeads has been reported [27], purified recombinant Ndc80-GFP protein has significantly impaired intrinsic microtubule-binding activity under physiologically relevant salt concentrations (100 mM NaCl) and, despite being structurally related to the tip-tracker EB1 [26], is unable to recognize growing microtubule ends efficiently [46]. By contrast, the yeast Dam1 complex can track microtubule plus ends *in vitro* and mediate the continuous association of Ndc80 with dynamic microtubule plus ends [46]. So far a Dam1 homolog has not been identified in mammals; however, other microtubule interactors at the kinetochore might have a similar effect to mediate continuous kinetochore association with dynamic microtubule plus ends. The end-binding (EB) family members can target motors or other microtubule-binding proteins to growing microtubule ends through direct interactions [40,47]. Therefore, EB1 could transmit its plus-end tracking activity to the rest of the kinetochore through its interaction with the kinetochore-associated mDia3, and this would represent a mechanism for kinetochores to track newly added tubulin subunits at the tips of growing microtubules.

Modeling the role of mDia3 in kinetochore oscillations

Metaphase chromosomes in the majority of metazoan cells make oscillatory movements around the spindle equator. Kinetochores bound to a complete kinetochore fiber associate with several microtubules (up to 25–30 bundled microtubules in mammalian cells), therefore, modeling studies predict that the dynamics of kinetochore–microtubule ends must be at least partially synchronized for chromosome oscillations to occur [48,49]. This is probably achieved by a much slower tubulin turnover rate for the individual microtubules within the kinetochore-bound microtubule bundle than is seen for free non-kinetochore microtubules [50,51]. A slower turnover rate of kinetochore microtubules could stabilize microtubules, therefore, to facilitate the attachment between kinetochores and microtubules. The molecular basis for how kinetochore components control these oscillations is not resolved. The CENP-A nucleosome-associated and CENP-A distal complex (CENP-A NAC/CAD) is proposed to control kinetochore–microtubule dynamics and chromosome oscillations, and one component, CENP-Q, directly interacts with microtubules *in vitro* [52]; however, whether this complex can directly influence microtubule dynamics remains untested. The microtubule depolymerase Kif18A has also been implicated in chromosome oscillations. Kif18A accumulates on kinetochore-bound microtubules and, depending on its motor activity, can attenuate the magnitude of chromosome oscillations [53]. A complete description of how the cell controls kinetochore oscillations clearly involves kinetochore-associated components controlling microtubule instability. A reduction, but not a complete block, of kinetochore-bound microtubule polymerization and depolymerization by the formin mDia3 at the kinetochore could play such a role. In other words, the binding of mDia3 on microtubule sides to reduce their rates of growth and shrinkage could coordinate the dynamics of these individual microtubules within the kinetochore bundles to generate directional movement. This function of the mDia3 protein could be independent of its

interaction with EB1 and APC, because the leading kinetochore, undergoing microtubule depolymerization, has been suggested to be the primary energy source for kinetochore oscillations [54].

Concluding remarks

Indirect immunofluorescence studies coupled with functional data strongly suggest that the formin mDia3 is a novel kinetochore protein that contributes to stable microtubule attachment and metaphase chromosome alignment [9,10]. The mDia3 protein has been clearly localized at the kinetochore [9,10] and does not appear to be important for the structural integrity of the kinetochore itself [9]. However, the key question of how mDia3 localizes to the kinetochore has not been addressed. There are at least three related questions that are important for understanding the functional roles of mDia3 at the kinetochore: (i) where within the kinetochore is mDia3 localized, (ii) how is mDia3 targeted to the kinetochore, and (iii) which region of the mDia3 protein is involved in kinetochore localization? In addition to phenotypic studies in cells, it will be equally important to define the biochemical properties of mDia3 itself on microtubule dynamics, its functional interplay with its binding partner EB1, as well as its possible coordination with other microtubule-binding proteins, such as the Ndc80 complex, upon their binding at microtubule sides.

A complete description of kinetochore function should involve both stable microtubule attachments and the coordination of microtubule plus-end polymerization–depolymerization. Understanding the role of mDia3 at the kinetochore, with its unique stabilization activity on both growing and shrinking microtubules, could change the current view of kinetochore–microtubule attachment and help lead to an integrated model for understanding the complicated interface between kinetochores and dynamic microtubule polymers, an issue which has great significance for understanding and controlling abnormal chromosome segregation in human genetic diseases and in cancer progression.

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Figure 1. Kinetochore–microtubule attachment and kinetochore oscillations

During metaphase kinetochore oscillations, sister kinetochores are stably attached to two distinct microtubule populations: the trailing kinetochore captures growing microtubules, while the leading kinetochore retains its attachment to shrinking microtubules. The Ndc80 complex is a central component in stabilizing end-on kinetochore–microtubule attachments. Microtubule plus-end tracking proteins, EB1 and APC, track plus ends of polymerization microtubules. The interaction between the formin mDia3 and EB1–APC may represent a mechanism for kinetochores to track newly added tubulin subunits at the trailing kinetochore with growing microtubules. The Ska1 complex also contributes to the stability of the connection between kinetochores and microtubule ends. Ska1 complex-coated beads can track depolymerizing microtubule ends. The kinesin motor Kif18A accumulates in a length-dependent manner on lagging kinetochores to control oscillation amplitude. On the leading kinetochore with depolymerizing microtubules, the formin mDia3 can directly bind to the microtubule lattice to reduce the rate of microtubule shrinkage and partially synchronize the dynamics of individual microtubules within the kinetochore bundles to control kinetochore oscillatory movements.