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A kinesin-mediated mechanism that couples centrosomes to nuclei

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Abstract

The M-type kinesin isoform, Kif9, has recently been implicated in maintaining a physical connection between the centrosome and nucleus in *Dictyostelium discoideum*. However, the mechanism by which Kif9 functions to link these two organelles remains obscure. Here we demonstrate that the Kif9 protein is localized to the nuclear envelope and is concentrated in the region underlying the centrosome point of attachment. Nuclear anchorage appears mediated through a specialized trans-membrane domain located in the carboxyl terminus. Kif9 interacts with microtubules in in vitro binding assays and effects an endwise depolymerization of the polymer. These results suggest a model whereby Kif9 is anchored to the nucleus and generates a pulling force that reels the centrosome up against the nucleus. This is a novel activity for a kinesin motor, one important for progression of cells into mitosis and to ensure centrosome-nuclear parity in a multinuclear environment.

Keywords

Centrosome; Kinesin; Microtubule; *Dictyostelium*

Introduction

Interactions between centrosomes and nuclei are well known to play fundamental roles in eukaryotic cell function, particularly during motility and division processes. During

interphase, centrosomes nucleate microtubules that connect nuclei to force-generating machinery. These interactions are important because they coordinate nuclear transport, maintain nuclear position during cell movement, and perhaps even couple extracellular signals to nuclear alterations that influence gene expression. Upon entering mitosis, a close association between centrosomes and nuclei facilitates the attachment of spindle microtubules to chromosomes, which is necessary to ensure the accurate segregation of genetic material. Primarily acting through microtubules, genetic and molecular analyses have begun to reveal a complex framework of proteins that interconnect centrosomes and nuclei, including the KASH/SUN families that span the nuclear envelope (NE) and serve to establish linkages with dynein and kinesin motors (reviewed in [1]). Yet despite this work, the full range of molecular connections, particularly in nonvertebrate eukaryotes with structurally diverse centro-some-nuclear relationships remains poorly understood.

In vertebrate cells, centrosomes are located in the cytoplasm during interphase and their interactions with nuclei are sufficiently dynamic to permit independent movements of these two organelles. Interphase centrosomes in the social amoebae *Dictyostelium* are also located in the cytoplasm. In contrast to vertebrate models, these centrosomes remain adjacent to (and tightly connected with) nuclei, and cycle into and out of the nuclear envelope for a closed mitosis. Early electron microscopy studies revealed that *Dictyostelium* centrosomes are physically attached to interphase nuclei through visible linkages, leading to their identification as nuclear-associated bodies [2–5]. This strategy of cytoplasmic but nuclear-attached centrosomes can be found in a number of other organisms [6–9], but the underlying mechanism(s) of how these attachments are made and their functional significance are not known.

We previously demonstrated that disruption of the M-type kinesin isoform, Kif9, allows *Dictyostelium* centrosomes to separate from nuclei during interphase, suggesting that a microtubule-based mechanism forms a major component to the linkage machinery [10, 11]. This disruption also leads to the generation of supernumerary centrosomes and ultimately produces defective spindle arrangements during division [11]. By comparing functional differences between vertebrate and *Dictyostelium* models, we seek to better understand the mechanisms and regulation of how eukaryotes manage interactions between centrosomes and nuclei. In the current study, we characterize the biochemical properties and cellular distribution of the Kif9 kinesin. Our work leads to a model whereby Kif9 is directly anchored into the nuclear envelope and engages microtubules to maintain centrosome proximity during interphase. This is a new activity for a kinesin motor and one that underscores the evolutionary adaptability of kinesins to perform specific tasks.

Results

Kif9 participates in the centrosome-nuclear linkage

To quantify the separation between nuclei and centrosomes, we imaged cells and isolated nuclei with an antibody to DdCP224, a well-known component of the centrosomal corona [12, 13] (Fig. 1). In the wild type, 98 % of cells and 65 % of isolated nuclei contained a centrosome-nuclei pair within 1 μm of each other (Table 1). In the absence of Kif9, 50 % of cells but only 12 % of isolated nuclei contain such closely apposed structures. This result indicates that even though the centrosome may appear adjacent to the nucleus in many of the *kif9* null cells, the linkage components that firmly connect these two organelles are disrupted. EM analysis further illustrates the centrosome disjunction in *kif9* null cells (Fig. 1). Plasmid expression of Kif9 in the *kif9* null background restores the physical connection between centrosomes and nuclei (Table 1), indicating that the Kif9 kinesin is sufficient to complement the mutant disconnect phenotype.

The separation between these two organelles could be due to an underlying structural defect in the centrosome itself or to removal of a linker component acting indirectly on the microtubule array. To examine whether there were any obvious defects to the centrosome core, we compared their ultrastructure in wild-type and *kif9* null cells (Fig. 1). Unlike the centriolar pair found in many other eukaryotes, the core structure in Dictyostelids is a single, multilayered disc-shaped object [5, 14]. Central discs are surrounded by an electron dense cloud or corona that nucleates microtubules and is considered functionally equivalent to pericentriolar material.

By EM, the vast majority of centrosomes appear identical in wild-type and *kif9* null cells ($n > 50$), indicating that Kif9 does not play a direct role in centrosome ultra-structure. However, LM imaging of centrosomes does reveal an interesting perturbation. While 95 % of wild-type (221/233) and 77 % (175/226) of *kif9*⁻ centrosomes show a similar compact geometry, 23 % (51/226) of the *kif9*⁻ centrosomes appear unusually elongated. By EM, the elongation only occurs in the *X* and *Y* dimensions of the centrosome core, whereas its thickness and appearance of the surrounding corona are identical to the wild type (Fig. 1). Similar core elongation is seen in 5 % of the wild-type centrosomes, and has been reported in dynein-mutant cells [15]. We postulate that these alterations are related to aberrant duplication of centrosomes, perhaps as a consequence of failing to integrate into the nuclear envelope during division. Co-labeling *kif9*⁻ cells with DdCP224, γ -tubulin, and microtubule markers suggests that all of the visually distinct centrosomes are competent to nucleate microtubules (not shown). Thus, despite morphological variation and nuclear displacement, the basic interphase function of these centrosomes is preserved. Together, these results indicate that Kif9 connects centrosomes and nuclei through a linkage outside of the centrosome proper.

Kif9 contains multiple functional domains

Kif9 is a 1,222-residue polypeptide with a predicted molecular mass of 136 kDa. The conserved kinesin motor domain is centrally located at residues 356–720, followed immediately downstream by a heptad repeat pattern predicted to form a coiled coil. To broadly map functional domains, we made a series of motor constructs for analysis in *Dictyostelium* (Fig. 2). Despite using a promoter construct that produces highly overexpressed fragments of the dynein motor [16], all of the Kif9 constructs were weakly expressed, detectable only by immunoblot analysis. And instead of a single distinct polypeptide at the predicted molecular mass, immunoblots revealed a multibanded appearance to the Kif9 polypeptide (Fig. 2). Treatment of lysates with lambda phosphatase reduces this pattern to largely a single band, suggesting that mass variability may result from phosphorylation. Comparison of truncated fragments demonstrate that the variability occurs through modifications upstream of the motor domain (Fig. 2c).

Immunoblots also reveal that the full-length polypeptide readily sediments at low speed centrifugation ($30,000\times g$), indicating that Kif9 is bound to some high mass cellular compartment (Fig. 2b). Previous analyses highlighted a short 23-residue hydrophobic region located 18 residues from the Kif9 carboxyl terminus (aa1182–1204) that could serve as a transmembrane domain [17]. To test whether this region anchors the Kif9 protein, we expressed a truncated version of Kif9 construct lacking the final 43 residues (Kif9₁₁₇₉). The truncated version remained in the supernatant even after centrifugation at $150,000 \times g$ for 60 min, demonstrating that the C-terminus does act in some way to anchor Kif9.

Kif9 further contains a SxIP sequence in the amino-terminal region (aa100–103). This motif is known to target the kinesin-13 isoform MCAK, to the microtubule +tip binding protein, EB1 [18]. To test whether a similar interaction occurs with Kif9, we immunoprecipitated EB1 from cells expressing the near full-length, cytosolic version, Kif9₁₁₇₉. Immunoblots

reveal that Kif9 does co-precipitate with EB1 (Fig. 2d), consistent with an interaction in the cytosol.

Motor domain fragments of Kif9 containing either the amino or carboxyl termini sequences interact differently with microtubules in simple pelleting and ATP-extraction assays (Fig. 2e). The motor plus amino-terminal domain weakly interacts with microtubules and readily extracts with ATP; the motor plus carboxy-terminal domain tightly binds microtubules and only weakly extracts with ATP; and the near full-length polypeptide binds well but only partially extracts from microtubules with ATP. These results are similar to other kinesin isoforms (e.g., kinesin 5, 7, 8, and 10 families) that contain additional ATP-insensitive microtubule-binding activities in their tail domains [19–22]. These cursory analyses hint at a complex motor activity that includes multiple regulatory and anchorage domains.

Kif9 localization suggests a tethering mechanism to the nucleus

To determine the cellular distribution of Kif9, we placed GFP tags at either the 5' or 3' ends of the *kif9* coding sequence and expressed these polypeptides in mutant and wild-type cells. Both tagged versions of the full-length polypeptide showed similar distribution, with a striking enrichment in the region near the centrosome-nuclear connection (Fig. 3). In some cells, the 3' tagged version was also seen to decorate a subset of interphase micro-tubules and form spot-like aggregates in the cytoplasm; this additional labeling was frequently associated with higher levels of expression and could very well result from steric interference between the GFP tag and the carboxy terminal anchorage domain. Consistent with the in vitro analysis, deletion of the carboxyl terminal trans-membrane and tail domains (Kif9₁₁₇₉) redistributes the polypeptide into the cytoplasm and onto microtubules. At higher levels of expression, some of the cytoplasmic microtubules also appear to be bundled (Fig. 3c, right two frames). Localizing the C-terminal fragment containing only the transmembrane and tail domains (Kif9_{1181–1222}), in a wild-type background shows a GFP distribution at the nucleus similar to the full-length Kif9 polypeptide (Fig. 3). Expression of the same sequence in a Kif9 null cell background shows a dramatically different result, with a general nuclear envelope labeling and what appears to be endoplasmic reticulum. This result indicates that wild-type Kif9 activity is required (directly or indirectly) for proper polypeptide positioning, and further indicates that the carboxyl terminus plays a critical role in Kif9 anchorage.

To address Kif9 anchorage in greater detail, we examined labeling patterns in isolated nuclei (Fig. 4). Here, the Kif9 polypeptide is largely positioned at the point of centrosome-nuclear attachment. In nuclei where centrosomes had detached during pelleting onto coverslips, Kif9 labeling remains associated with the nucleus. Free centrosomes that are spatially separated from nuclei do not contain detectable amounts of Kif9 indicating that the motor protein is firmly anchored at the nuclear envelope.

A transmembrane domain followed by a short tail sequence to the C-terminus in a protein targeted to the nuclear envelope is an architecture characteristic of KASH-domain proteins [1]. KASH-domain proteins typically span the outer nuclear envelope, and interact in the perinuclear space with SUN proteins. To examine the possibility that Kif9 functions as a KASH domain protein, we localized Kif9 and Sun1 polypeptides (Fig. 5) [23]. Consistent with forming an integral complex, both proteins show a similar distribution on the nuclear envelope. In wild-type cells, Sun1 staining forms patches that are asymmetrically distributed to one side of the nuclear surface, with their greatest density underlying the point of centrosome attachment [23] (Fig. 5). In the absence of Kif9, Sun1 remains clustered in discrete patches, but these patches are evenly spread around the nuclear envelope. Sun1 is also required for a close clustering of centromeres in the nucleus underlying the point of centrosome attachment [23]. While centromeres remain clustered as a group, the loss of Kif9

promotes their random distribution in the nuclear volume (Fig. 6). These results indicate that Kif9 and Sun1 at least interact in the same pathway, if not form a direct connection.

Kif 9 is a motor protein that depolymerizes microtubules

To address whether Kif9 functions as a microtubule motor, we utilized a yeast TAP-tag/microtubule affinity strategy to purify near full-length Kif9 (Kif9₁₁₇₉) from *Dictyostelium* (Fig. 7), and set up simple perfusion chambers to test for motor activity. Rhodamine-labeled, Taxol-stabilized microtubules readily bound to Kif9-coated coverslips, but under these assay conditions, did not show any gliding activity. Instead, the addition of Kif9 protein in the ATP wash promoted slow endwise depolymerization of the microtubule polymer. In our assay buffer (50 % PHEM), this depolymerization occurred at a rate of 1.9 ± 1.0 nm/s (SD, $n = 37$). When the entire microtubule is visible, depolymerization occurs at both ends, although one end of the polymer appears to be favored. In addition, Kif9₁₁₇₉ appears to generate ATP-insensitive microtubule bundles (Fig. 7), a result consistent with the in vivo analyses presented in Fig. 3c. This activity, coupled with sedimentation analyses that indicate a significant fraction of polypeptide remains associated with the microtubule pellet after ATP extraction (Figs. 2, 7) is further consistent with Kif9 containing a second ATP-insensitive microtubule binding domain.

Discussion

Dictyostelium exhibits an interesting housekeeping strategy of firmly coupling a cytoplasmic centrosome to the nucleus. In addition to linking nuclei to force generating machinery for movement, such coupling has two obvious benefits: it maintains the centrosome close to the nucleus allowing for efficient NE integration during mitosis, and it reduces the potential for mis-integration in multinucleated cells that frequently occur in the population [11]. Similar strategies are utilized by a number of other lower eukaryotes, and while linkages that connect these organelles can be seen by EM, their underlying biochemical mechanisms are largely unknown. In *Dictyostelium*, a kinesin motor protein provides a critical component of the centrosome-nucleus linkage. In this paper, we have broadly mapped the functional domains of this kinesin and constructed a testable model for its mechanism of operation.

Several proteins have been demonstrated to play a role in connecting centrosomes to nuclei in *Dictyostelium*, including the Sun1 homolog [23, 24], a centrin B homolog [25], and the centrosomal protein, CP148 [26]. Together they play structural or regulatory roles in anchoring microtubule minus ends into the centrosomal corona or in stabilizing components at the nuclear envelope. Given the close apposition between centrosomes and the outer nuclear envelope, it is also probable that some of these proteins directly bridge the two structures and form primary contacts. However, centrosomes in *Dictyostelium* are subject to significant cortical and cytosol-mediated pulling forces that act through attached microtubules. Centrosomes can be seen to move through the cytoplasm at rates up to 1–2 μ m/s, producing sufficient force to distort and drag the attached nuclei [27, 28]. Therefore we postulate that *Dictyostelium* requires an additional, microtubule-based support mechanism to counteract these forces and maintain contact between centrosomes and nuclei.

Microtubules and motor proteins are known to engage and move nuclei in a number of different contexts. Dynein is well known in nuclear-centrosomal associations in other organisms (e.g., [29–39]), but its role in *Dictyostelium* is not so clear. While disruption of the dynein regulatory protein Lis1 leads to the dislocation and circulation of interphase centrosomes in the cytoplasm [28], disruption of dynein cargo-binding activity [15] or dominant negative expression of dynein motor domain fragments [27] does not dramatically affect centrosome nuclear connections. Regardless of its activities, dynein alone is not

sufficiently robust to maintain the centrosome-nuclear linkage in the absence of Kif9, and thus may play only a minor role here at the NE.

Kinesin-1 and -3 motors also bridge centrosomes and nuclei in other systems. However, here they are primarily active in promoting nuclear movement toward the micro-tubule plus ends [37–41]. In contrast to these systems, *Dictyostelium* appears to utilize a so-far-undescribed kinesin-based motor configuration for minus-end directed movement of nuclei, one that combines multiple functional elements.

Our results suggest the following working model for Kif9 activity (Fig. 8). We postulate that Kif9 is anchored into the NE through the hydrophobic motif located near the protein's carboxyl-terminus [10, 17]. The extreme C-terminal tail would then be positioned in the perinuclear space and available to interact with Sun1 to strengthen and support this anchorage. In this regard, Kif9 may fulfill the function of a KASH-domain protein. Although kinesin-1 isoforms in other organisms have been shown to interact with KASH-domain proteins and form indirect connections between microtubules and nuclei, a direct linkage to the nuclear envelope would be a new and novel property for a kinesin motor. *Dictyostelium* contains two isoforms of SUN proteins; Sun1 is clearly involved in centrosome anchorage [23, 24], whereas SunB appears to have a signaling role for cell differentiation during development [42]. Based on sequence homology alone, there are no readily identifiable members of the KASH-domain families in *Dictyostelium*. Interaptin has been considered a *Dictyostelium* KASH protein but the primary isoform seems to be developmentally regulated and its disruption does not alter Sun1 distribution [24, 43]. The redistribution of Sun1 in the absence of Kif9 clearly indicates that these two proteins participate in the same functional pathway, and suggests the interesting possibility that Kif9 directly binds to Sun1.

With the carboxy terminal end of Kif9 embedded in the NE, the kinesin motor domain is available in the cytosol to engage cytoplasmic microtubules. Our in vitro analyses indicate that Kif9 binds to microtubules and effects an end-on depolymerization. We cannot yet exclude other motor activities for Kif9, but a depolymerase action would create a pulling force between nuclei and centrosomes, and produce two visible effects. First, the motor would create a microtubule-minus end directed force that reels the centrosome up against the nuclear envelope. Second, Kif9 and its underlying connected framework would travel laterally through the lipid bilayers of the NE and concentrate in the region where force is being produced. Both of these events are reflected in the localization data presented here. A plus-end-directed microtubule motor acting similar ought to have exactly the opposite effect, increasing the distance between the centrosome and nucleus, and redistributing its NE anchor on the nuclear surface distal from the centrosome. Interestingly, this effect was observed in a kinesin-1/nesprin 4 linkage in vertebrate cells [39]. These results suggest similar actions to move the nucleus relative to the centrosome, but highlight the functional differences between a plus-end-directed motor and a minus-end-directed microtubule depolymerase.

As a microtubule depolymerase anchored in the nuclear envelope, Kif9 provides a dynamic mechanism to couple centrosomes and nuclei during interphase. At least in *Dictyostelium*, this coupling is essential for normal cell division [11]. Microtubules are required for changes in the distance between centrosomes and nuclei [23], and if a centrosome does break free of the nucleus, this mechanism would allow it to be reeled back into place. Interestingly, the Kif9 sequence also contains an amino-terminal SxIP domain. SxIP motifs target polypeptides, including the kinesin-13 MCAK to the microtubule +tip organizing protein, EB1 [18]. Moreover, the interaction between MCAK and EB1 is regulated by phosphorylation through serine residues clustered near the SxIP motif. We see evidence for

a similar targeting mechanism in Kif9. While most visible interphase microtubules in *Dictyostelium* are relatively stable and their + tips project beyond the nuclear region, there are dynamic pools of tubulin polymer and EB1 localized at the centrosome [44, 45]. It is possible that Kif9 specifically targets this nascent pool of microtubule polymer to remain attached to the centrosome. Upon mitotic entry, there is a near complete loss of cytoplasmic microtubules that precedes centrosome integration into the nuclear envelope [11, 14]. At this point, all external microtubule-based pulling forces acting on the centrosome cease, and the Kif9-mediated mechanism would no longer be required to maintain its position.

Central-motor domain kinesins in other organisms (e.g., the kinesin-13 family) are primarily active during mitosis to modulate spindle and kinetochore microtubule activities [46], or have interphase functions to regulate microtubule, centriole, and axonemal length [47–53]. Outside of the conserved features integral to kinesin motor domains and a central motor position in the polypeptide, Kif9 bears little primary sequence homology with Kinesin 13s. Yet, Kif9 may function in a related capacity, targeted to microtubule ends and effecting their depolymerization. These results underscore the evolutionary adaptability of the kinesin family and illustrate additional roles for kinesins in microtubule length control during interphase.

Materials and methods

Light microscopy (LM)

Wild-type (WT) AX-2 and *kif9* null cells were grown and processed for immunofluorescence following methods described in refs [16, 54, 55]; α -tubulin, DdCP224, Cenp68, and Sun1 antibodies have been previously described [12, 22, 56]; DNA was labeled with Hoechst 33342. Image stacks in the Z direction were collected on a DeltaVision microscope workstation with a 100 $\times$ objective, and de-convolved using softWoRx 2.5 (Applied Precision, Issaquah, WA, USA). Maximum intensity projections were prepared with ImageJ (NIH); figures were assembled in Adobe Photoshop. Live cell recordings were performed using WT and *kif9*⁻ cells that had been transformed with a GFP-alpha tubulin expression plasmid. For imaging, cells were washed in phosphate buffer (20 mM KCl, 2.5 mM Na₂HPO₄, 2.5 mM NaH₂PO₄, 0.24 MgCl₂, pH 6.4) and placed under agarose in humidified Rose chambers [57]. Image frames (fluorescent and DIC) were collected at 10-s intervals on a Nikon TE200 microscope, using a 60 $\times$ objective and an Orca-R2 camera (Hamamatsu) controlled by IP Lab software (BD Biosciences). For long-term cell cycle analysis, cells were placed into Nunc Lab-Tek chambers in growth medium. The chambers were modified to accommodate a top glass coverslip, and cells were viewed via DIC optics on a Nikon TE2000 microscope. Images were collected with a 60 $\times$ objective at 1-min intervals over 20–24 periods.

Electron microscopy (EM)

Cells were attached to glass coverslips in phosphate buffer for 1 h, then fixed in 0.5 % glutaraldehyde, and 0.2 % Triton X-100 in 15 mM PIPES pH 7.0, 1 mM EGTA for 5 min. This solution was replaced with buffer containing 0.5 % glutar-aldehyde for an additional 25 min (no detergent). Following free aldehyde reduction with sodium borohydride and washes in PBS, cells were labeled with the DdCP224 primary antibody and a FITC-conjugated secondary antibody. LM image stacks of labeled centrosomes were collected and processed as described above, and cell positions were marked with a diamond scribe. Cells were postfixated with 1 % OsO₄ for 30 min at room temperature and then dehydrated and embedded in EPON/Araldite. LM-imaged cells were relocated and serially sectioned. Sections were stained with 2 % uranyl acetate (1 h) and Reynolds lead citrate (15 min), and then viewed in a Zeiss 910 TEM.

GFP-Kif9 expression

The coding sequence for Kif9 was obtained by PCR amplification of genomic DNA. Three overlapping fragments were isolated, each between 1.1 and 1.5 kb in length, and a full-length construct was reassembled in a *Dictyostelium* expression plasmid containing the dynein heavy chain promoter [57]. Primer sequences and detailed cloning strategies are available upon request. The entire construct was sequenced to ensure that no errors were introduced from the amplification or cloning steps. The GFP sequence was excised from pEGFP-C1 (Clontech) by PCR, and inserted via engineered *NheI* restriction enzyme sites either upstream of the initiating ATG codon (5' tag), or after the terminal Thr₁₂₂₂ coding residue (3' tag). Short linker sequences of six and eight residues were incorporated to facilitate cloning of the 5' and 3' tags, respectively. Truncated Kif9 fragments were prepared using combinations of restriction enzyme sites and PCR amplification (detailed sequences and strategies are available on request). Expression constructs were introduced into *Dictyostelium* cells and transformants were isolated as previously described [16].

Biochemistry

The preparation of cell lysates, microtubule binding, immunolocalization, and immunoblotting were performed as described in [58]. Nuclei were isolated by detergent extraction, mechanical shearing, and sedimentation onto coverslips [59]. λ phosphatase was purchased from New England Biolabs and was added directly to cell lysates. Immunoprecipitation was performed by adding DdEB1 antibody [44] to 1.5 ml of high-speed supernatant (30 min at RT), followed by the addition of Protein-G Sepharose (GE Healthcare) (30 min at RT), then 4 × 1 ml washes in Tris buffer (50 mM Tris, pH 7.5, 150 mM KCl, 1 mM MgCl₂, 0.5 mM EDTA, 1 mM MgATP). Pellets were suspended in 50 μ l of Tris buffer, and processed for PAGE/ immunoblotting. Blots were probed with an anti-c-myc antibody (clone 9E10), to recognize an epitope tag at the amino terminus of Kif9₁₁₇₉.

For protein purification, we inserted the near full-length coding sequence (Kif9₁₁₇₉) into the pDV-NTAP-CGFP *Dictyostelium* TAP tag vector [60] and then transformed wild-type cells for protein expression (sequence details available on request). High-speed supernatants were incubated with IgG-agarose beads (Sigma) (100 μ l bed volume per milliliter of cell pellet, 1 h at 4 °C). Following three washes with Tris buffer, the resin was incubated with TEV protease (Invitrogen) (10 U/ml cell pellet, 1 h at 4 °C) to release Kif9 into solution. The resin supernatant was then collected, diluted to 1.5 ml with assay buffer (50 mM Pipes, pH 7.2, 5 mM EGTA, 0.5 mM EDTA, 4 mM MgCl₂, 0.9 M glycerol), supplemented with 10 μ M paclitaxol, and purified bovine microtubules to a final concentration of 0.5 mg/ml. Following a 20-min incubation at RT, microtubules were sedimented, the pellet was extracted with 10 mM MgATP in assay buffer, and then recentrifuged. The supernatant (ATP extract) contains the purified Kif9 protein.

For motility analyses, a perfusion chamber was constructed on a glass slide with double stick tape and a coverslip. The chamber was incubated sequentially with 0.5 mg/ml casein, Kif9, rhodamine-labeled microtubules, and buffer containing Kif9, 1 mM ATP, and a O₂ scavenging system composed of catalase, glucose oxidase, and glucose (Mitchison Lab Protocols, <http://mitchison.med.harvard.edu/protocols/flowcell.html>), then sealed with VALAP and imaged in the LM as described above. For statistical analyses, data were recorded from multiple (3) independently prepared coverslips or cell preparations.

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<http://dictybase.org> to archive and annotate *Dictyostelium* sequence information, and distribute stock materials for *Dictyostelium* research. This work was supported in part by the NSF (MCB-1051612 to MPK), the NIH (GM59363 to A.K), and the Deutsche Forschungs-gemeinschaft (DFG GR1642/3-1 to R.G). The experiments presented in this manuscript submitted for publication were performed in compliance with the current laws of the United States and Germany.

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Fig. 1.

Centrosome detachment and morphologies. **a, b** LM images of CP224 antibody staining (green) in cells and isolated nuclei, respectively. The *top row* presents the wild type and the *bottom row* shows the *Kif9* null. Note the close apposition of centrosomes to nuclei (blue) in the wild type but not in the *Kif9* null. **c** EM examples of normal centrosome ultrastructure in the wild type (*top*) and *Kif9* null cells (*bottom*). **d** Side (*left*) and top-down (*right*) views of wild-type normal and elongated *Kif9* null centrosomes. In the elongated examples, the core is extended in length and width, but the thickness and surrounding corona appear the same as in the wild type. *Insets* show LM panels of the same centrosomes stained with the CP224 antibody. **e** The normal close contact between the nuclear envelope (*arrowheads*) and centrosome in a wild-type cell. **f** The separation between nucleus and centrosome in a *kif9* null cell. Serial section analyses of similar *Kif9* null centrosomes do not reveal definitive connecting structures or membranes between these two organelles. *LM bar*, 2.5 μ m, *EM bar*, 250 nm

Fig. 2.

Kif9 expression analysis. **a** Schematic of Kif9 expression constructs. The *yellow bar* indicates the position of the SxIP motif; the *light blue bar* shows position of the kinesin motor domain; the *dark green region* is predicted to form a coiled coil; the *red bar* indicates the position of the transmembrane domain. **b–e** Immunoblot panels of epitope-tagged Kif9 constructs expressed in *Dictyostelium* and probed with a c-myc antibody. **b** Full-length Kif9₁₂₂₂ is present in cell lysates (Lys), but absent in the supernatant following high-speed centrifugation (HSS). In contrast, removal of the c-terminal 43 residues (Kif9₁₁₇₉) produces a fully soluble polypeptide. **c** Kif9 appears to be phosphorylated at sites in the N-terminus. λ phosphatase treatment reduces a heterogeneous mixture of polypeptides into primarily a single band. In cell lysates without phosphatase treatment, Kif9₇₂₀ is heterogeneous in size, whereas Kif9_{356–1222} is primarily a single sharp band. These results indicate that band heterogeneity is due to domains located between residues 1–356. **d** Kif9 coprecipitates with EB1. *Left panel* shows HSS starting material in WT and cells expressing Kif9₁₁₇₉; *right panel* shows end pellets after incubation with an EB1 antibody. Kif9 readily cosediments with EB1 (*middle lane*). The *far right lane* shows a control experiment in Kif9₁₁₇₉ cells, performed in the absence of EB1 antibody. **e** Three motor domain constructs show differential microtubule-binding activity. Kif9₁₁₇₉ is roughly equally distributed between the microtubule pellet and ATP supernatant after extraction. Kif9₇₂₀ shows relatively weak affinity for microtubules and is readily extracted with ATP. The carboxy-terminal motor domain Kif9_{356–1222} binds relatively tightly to microtubules and preferentially remains in the pellet following ATP extraction.

Fig. 3.

Kif9 distribution in *Dictyostelium* cells. GFP-labeled Kif9 is displayed in *red*, microtubules in *green*, and nuclei in *blue*. Interphase centrosomes are marked with *arrowheads* in **a–d**. **a** Distribution of GFP-Kif9₁₂₂₂ (N-terminal tag) expressed in Kif9 null cell (frames 1,2) and WT cell backgrounds (frames 3,4). **b** Kif9₁₂₂₂-GFP (C-terminal tag) distributions in WT cells. **c** Distribution of the Kif9 polypeptide fragment lacking the C-terminal transmembrane domain (GFP-Kif9₁₁₇₉). Note the cytoplasmic redistribution and microtubule decoration of the polypeptide. **d** Distribution of the C-terminal transmembrane domain (GFP-Kif9_{1181–1222}), in a WT cell background (*left two frames*) and in a null cell background (*right two frames*). **e** Kif9₁₂₂₂ distribution in mitotic cells. Examples (*left to right*) include prophase (5' GFP-tag), metaphase (3' GFP), anaphase (5' GFP), and telophase cells (5' GFP), respectively. Bar 5 μ m

Fig. 4. Kif9 localization on isolated nuclei. **a** The relationship between GFP-Kif9₁₂₂₂ (*red*), centrosomes (CP224 in *green*), and nuclei (*blue*), in a wild-type background. **b** Examples of nuclei that have lost their centrosomes while images in **c** show centrosomes that have separated from nuclei. In all cases, Kif9 labeling is most closely associated with nuclei. *Bar* 2.5 μ m

Fig. 5. Kif9 and Sun1 distribution. **a** GFP-Kif9₁₂₂₂ (*green*), Sun1 (*red*), and nuclei (*blue*). Staining is performed on permeabilized cells. Note the close relationship between Kif9 and Sun1 distribution. **b, c** Sun1 distribution on isolated nuclei. **b** Examples of the asymmetric distribution seen in wild-type cells. **c** Distributions in *kif9* null cells. Note that the label is more evenly distributed on the nucleus in the absence of Kif9. *bar*, 2.5 μ m

Fig. 6.

Centromere position is dependent on Kif9 activity. **a** Cenp68 antibodies (*red*) mark the position of the centromere cluster; the centrosome is indicated with *arrowheads* as the focal point of microtubule staining (*green*). Note that in wild-type cells, the centromere cluster is located proximal to the centrosome, but in the two mutant cells, it is located on the distal side of the nucleus, *bar* 5 μm . **b** Quantification of centromere position. In 97 % of the wild-type cells (*light blue* column, 100/103 cells), the centromere is positioned on the nuclear side proximal to the centrosome. In kif9 null cells, only 46 % of the centromere clusters were similarly located (in *red*, 46/101 cells), and many were found in the nuclear mid-region and distal to the centrosome position. *Error bars* denote 95 % confidence interval

Fig. 7.

In vitro motor activity. **a** Coomassie-stained PAGE showing Kif9 purification (*arrow*). **b** Sequence demonstrating endwise depolymerization of Taxol-stabilized, rhodamine-labeled microtubules in the presence of Kif9. Reference *arrowheads* are provided to mark the initial ends and two fixed points on the longer microtubule. Note that depolymerization of the right microtubule end is substantially greater than the left end. No such microtubule activity is seen in the absence of Kif9 protein. *T* in min, bar, 2 μm . **c** Two images of rhodamine-labeled microtubules in the absence (*left*) and presence (*right*) of Kif9. Both preparations contain ATP and were recorded using identical camera settings. The fluorescent structures on the right differ in brightness, in smoothing of the speckled labeling, and sweeping together in long, curving structures, indicating polymer bundling, bar 5 μm

Fig. 8. Model of Kif9 function. **a** Overview of nucleus (*blue*) centrosome (*olive*), and microtubule (*green*) interactions. **b** Detail of Kif9's interaction with the nuclear envelope and centrosome-linked microtubules. *ONE* and *INE* indicate the outer and inner nuclear membrane, respectively

Table 1Percentage of nuclei with adjacent centrosomes ($\geq 1 \mu\text{m}$)

Construct	Intact cell	Isolated nuclei
WT	98 % (221/225)	65 % (647/998)
<i>kif9</i> ⁻	50 % (158/314)	12 % (115/976)
<i>kif9</i> ⁻ + <i>kif9</i>	92 % (305/330)	58 % (546/942)
<i>kif9</i> ⁻ + <i>kif9</i> ₁₁₇₉	60 % (82/137)	n.d.