Drosophila Klp67A binds prophase kinetochores to subsequently regulate congression and spindle length

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Summary

The kinesin-8 proteins are a family of microtubule-depolymerising motor molecules, which, despite their highly conserved roles in chromosome alignment and spindle dynamics, remain poorly characterised. Here, we report that the *Drosophila* kinesin-8 protein, Klp67A, exists in two spatially and functionally separable metaphase pools: at kinetochores and along the spindle. Fixed and live-cell analyses of different Klp67A recombinant variants indicate that this kinesin-8 first collects at kinetochores during prophase and, by metaphase, localises to the kinetochore outerplate. Although the catalytic motor activity of Klp67A is required for efficient kinetochore recruitment at all times, microtubules are entirely dispensable for this process. The tail of Klp67A does not play a role in kinetochore accumulation, but is both necessary and sufficient for spindle association. Using functional assays, we reveal that chromosome position and spindle length are determined by the microtubule-depolymerising motor activity of Klp67A exclusively when located at kinetochores, but not along the spindle. These data reveal that, unlike other metazoan kinesin-8 proteins, Klp67A binds the nascent prophase and mature metaphase kinetochore. From this location, Klp67A uses its motor activity to ensure chromosome alignment and proper spindle length.

Key words: Kinesin-8, Mitosis, Microtubule, Depolymerase, Motor protein

Introduction

The founder members of the kinesin-8 family of conserved microtubule (MT)-depolymerising motor proteins were originally identified in budding yeast and fission yeast mutants (*kip3* and *klp5/6*, respectively) exhibiting elongated and hyperstable MTs and spindles (Cottingham and Hoyt, 1997; Straight et al., 1998; West et al., 2001; West et al., 2002). Similarly, reduced levels of human Kif18A or *Drosophila* Klp67A also lead to abnormally long and distorted mitotic spindles. The chromosomes in these cells fail to congress to the spindle equator or form a stable metaphase plate, often dwelling near the poles (Goshima and Vale, 2003; Gandhi et al., 2004; Goshima et al., 2005a; Zhu et al., 2005; Mayr et al., 2007; Stumpff et al., 2008). These defects might be explained, in part, by the findings that kinesin-8 proteins are plus-end-directed translocating motors, some of which exhibit plus-end-specific MT-depolymerising activity (Pereira et al., 1997; Gupta, Jr et al., 2006; Varga et al., 2006; Mayr et al., 2007; Grissom et al., 2008).

During mitosis, the metazoan kinesin-8 proteins Klp67A and Kif18A concentrate in the vicinity of the centromeres, in the region of the kinetochore and MT interface. Whereas Klp67A also spans the entire length of the associated MTs, Kif18A is largely limited to the equatorial region, where it forms a concentration gradient that diminishes towards the spindle poles (Goshima and Vale, 2005; Mayr et al., 2007; Stumpff et al., 2008). To date, only two other groups of kinesins have been identified in the vicinity of the centromeres and kinetochores. The first is the non-motile, MT-depolymerising kinesin-13 family, and includes vertebrate Kif2A, Kif2B and Kif2C (also known as MCAK). The *Drosophila* genome likewise encodes three kinesin-13 proteins, although functions have only been assigned to Klp10A and Klp59C, which are most similar to Kif2A and Kif2C, respectively. From their position at mitotic centromeres and kinetochores and, in some instances, at spindle poles, these motor proteins regulate spindle MT dynamics for proper bipolar spindle formation, chromosome alignment and subsequent anaphase segregation (Wordeman and Mitchison, 1995; Maney et al., 1998; Kline-Smith and Walczak, 2002; Ganem and Compton, 2004; Kline-Smith et al., 2004; Rogers et al., 2004; Goshima and Vale, 2005; Manning et al., 2007). The centromere targeting of both Kif2A/Klp10A and Kif2C initiates in prophase (Wordeman et al., 1999; Goshima and Vale, 2005), whereas Klp59C and Kif2B seem to collect near centromeres and kinetochores, respectively, following the degradation of the nuclear envelope at the onset of prometaphase and the initiation of spindle formation (Rogers et al., 2004; Manning et al., 2007). This late accumulation is similar to that of the kinesin-7 protein CENP-E (*Drosophila* CENP-meta), which also binds to kinetochores after the nuclear envelope begins to rupture (Cooke et al., 1997; Yao et al., 1997; Yucel et al., 2000). Following accumulation at the outerplate, this motor protein facilitates chromosome alignment by translocating towards the plus ends of pre-existing kinetochore-associated MTs and stabilising kinetochore and MT interactions (Schaar et al., 1997; Yucel et al., 2000; McEwen et al., 2001; Kapoor et al., 2006). These observations suggest that Klp67A might reside at the centromere and kinetochore, or might collect at the plus ends of the MTs that bind the kinetochore. Equally ambiguous are the contributions made by this or pools of spindle-associated Klp67A in determining spindle length and directing chromosome alignment, or when these associations occur. Defining the mechanisms responsible for the distributions of Klp67A and their functional consequences will provide insights into metaphase spindle function.

With this in mind, we have taken a genetic approach to identifying the localisation and functional properties of *Drosophila* Klp67A. Using a combination of transgenic tissue culture cells and living neuroblasts, we show that Klp67A is a core component of the

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kinetochore. Comparisons of different truncated or mutant enhanced GFP (EGFP)-tagged Klp67A variants reveal that the kinetochore- and spindle-associated Klp67A populations are separable. Although it is necessary, the motor domain of Klp67A is not sufficient for kinetochore loading. The association with kinetochores does not require MTs and begins during prophase. Although the C-terminal tail domain of the protein is not needed for kinetochore targeting, it does promote robust association with spindle MTs. Using a rescue assay, we find that it is the placement of the MT-depolymerising motor activity of Klp67A at kinetochores, but not along the spindle, that is responsible for chromosome congression and spindle length determination. Our data reveal that Klp67A is a MT depolymerase that uniquely associates with prophase and metaphase kinetochores, a distribution that ensures faithful chromosome alignment and spindle morphology.

Results

The kinetochore- and spindle-targeting domains of Klp67A are separable

A previous localisation study revealed that during mitotic metaphase, Klp67A is found as paired, chromosome-proximal foci and along the length of the spindle (Goshima and Vale, 2005). To identify the significance of these distributions, we undertook a molecular dissection of the Klp67A protein. Analysis of the primary sequence of full-length Klp67A (814 amino acids) suggests that it comprises three domains (Fig. 1): an N-proximal motor domain consisting of a catalytic motor and neck (amino acids 1-358), a centrally positioned putative coiled-coil-rich region (amino acids 359-611) and a C-terminal tail (amino acids 612-814) that contains a predicted nuclear localisation signal (NLS; amino acids 670-673). To determine which of these domains might influence the localisation of Klp67A, we created a series of transgenic *Drosophila* Dmel-2 tissue culture cell lines that used the inducible metallothionein promoter to express a series of different C-terminal EGFP-tagged protein variants. Each of these non-clonally derived lines expressed recombinant proteins of the predicted size, as confirmed by western blot (supplementary material Fig. S1). As these populations were not clones, recombinant protein levels varied among cells. We therefore concentrated our studies on those cells with mid-levels of expression, as determined by EGFP fluorescence intensity.

An examination of cells expressing EGFP-tagged wild-type Klp67A [Klp67A(WT)EGFP] revealed that the protein was sequestered into nuclei during interphase (supplementary material Fig. S2). As with previous observations using fluorescently labelled recombinant Klp67A (Goshima and Vale, 2005) and immunolocalisation studies (supplementary material Fig. S3), we observed that, during mitotic metaphase, Klp67A(WT)EGFP homogeneously coated the spindle and formed prominent punctae near the centromeres of each chromosome (Fig. 1). The consistent accumulation of Klp67A(WT)EGFP adjacent and external to centromeres (120 out of 120 centromeres scored) could reflect recruitment to kinetochores, as previously proposed (Goshima and Vale, 2005), or might be due to the concentration of the protein at MT plus ends. Several lines of evidence, described in detail below, indicated that the motor protein was associated with the kinetochore.

To identify the contribution of the catalytic motor domain in determining the subcellular distribution of Klp67A, we generated an ATPase-compromised 'rigor' form of the protein, Klp67A(RIG)EGFP. This required introducing the G116E mutation (see Materials and Methods), analogous to that described by Meluh and Rose (Meluh and Rose, 1990), which allows tubulin binding but prevents motor release, thereby preventing translocation and motility. This rigor mutation did not affect interphase nuclear sequestration (supplementary material Fig. S2). However, as

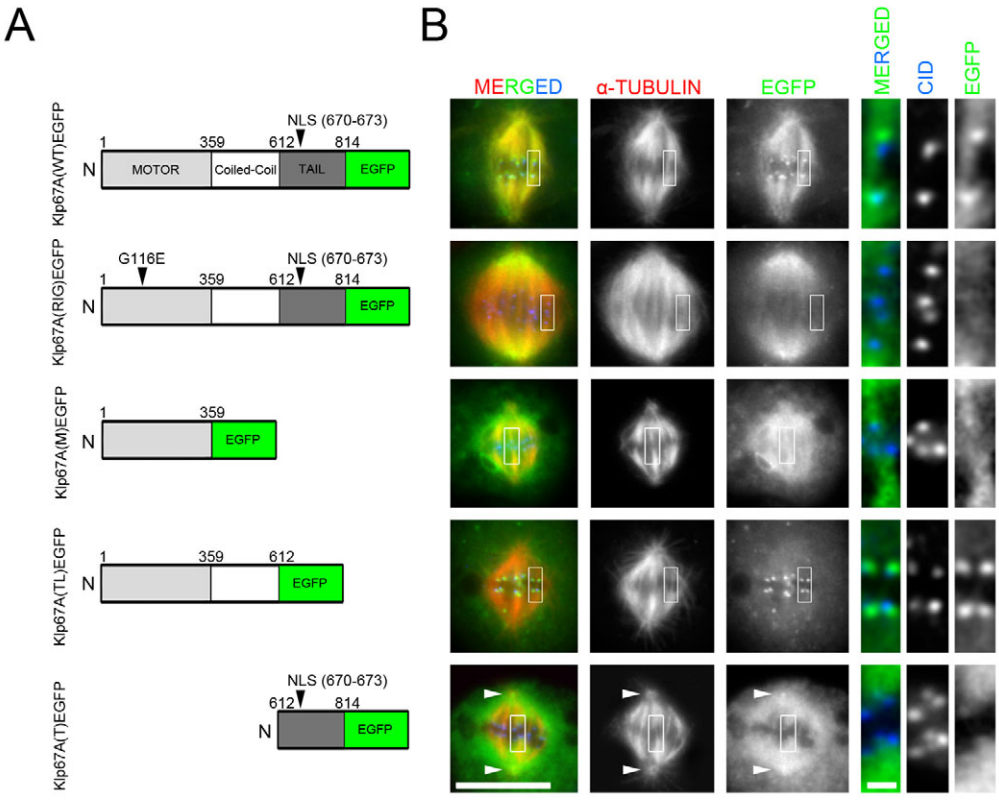

Fig. 1. Kinetochore and spindle localisation of Klp76A during metaphase is differentially determined by its domains. (**A**) Domain composition of each of the Klp67AEGFP variants used in this study: WT is wild-type, RIG denotes the rigor mutant G116E, M is motor-only, TL indicates tail-lacking; T represents tail-only. Numbers indicate amino acid residues and NLS is the nuclear localisation signal. (**B**) Metaphase distributions of each variant (EGFP; green) relative to MTs (α-tubulin; red) and centromeres (CID; blue). Only the wild-type and tail-lacking EGFP variants efficiently accumulate at centromeres. Boxed regions are magnified and contrast enhanced at right. Arrowheads in the bottom panels indicate polar-positioned Klp67A(T)EGFP foci. See text for details. Scale bar: 10 μm. Magnified region scale bar: 1 μm.

represented in Fig. 1, accumulation near metaphase centromeres was greatly compromised and only 14% of these structures were flanked by an EGFP signal (*n*=120). When present, Klp67A(RIG)EGFP could be found at varying intensities among kinetochores and, in extreme cases, was visible on only one of the two sisters. Loss of motor activity did not inhibit spindle localisation, although the mutant form appeared diminished at MT plus ends near the spindle equator and instead concentrated near the spindle poles. This accumulation might result from the pole-ward movement of stably bound motor subunits towards fluxing MTs during metaphase.

We next examined the localisation of the first 358 residues of Klp67A, corresponding to the catalytic motor and neck domain fused to EGFP. This construct lacks the predicted C-terminal NLS and this motor-only variant, Klp67A(M)EGFP, was consistently found throughout the interphase cytoplasm. In some of these cells, the MT cytoskeletons were diminished or completely absent (supplementary material Fig. S2).

As with many kinesins, the C-terminal tail of Klp67A is a putative cargo-binding domain. To determine whether the tail contributed to subcellular targeting, two variants were generated: the tail-lacking Klp67A(TL)EGFP, which contains the functional catalytic motor and centrally positioned coiled-coil domains but lacks residues 612-814; and the complementary tail-only Klp67A(T)EGFP, which consists exclusively of those terminal 204 residues. The distribution of each was markedly different throughout the cell cycle. As with the motor-only variant, tail-lacking Klp67A(TL)EGFP lacks the predicted NLS and was not imported into the nucleus. This also resulted in some cells lacking an MT cytoskeleton (supplementary material Fig. S2). Conversely, tail-only Klp67A(T)EGFP was always sequestered into interphase nuclei. There it covered the chromatin and, unlike the other variants, formed a large mass corresponding to the nucleolus, as demonstrated by colocalisation with the fibrillarin protein (supplementary material Fig. S2).

These truncation studies indicated that, when free in the interphase cytoplasm, Klp67A variants containing the motor domain are functional MT depolymerases. To determine whether this activity was due to the catalytic ATPase activity of the motor, we performed an in vivo MT-depolymerisation assay (Stumpff et al., 2007). This entailed generating a cell line expressing a motor-dead rigor form, Klp67A(RIG)EGFP, in which the predicted NLS (amino acids 670-673) had been mutated. As a positive control, we engineered the same NLS mutation in the otherwise wild-type Klp67A(WT)EGFP. The depolymerising ability of these NLS-compromised Klp67AEGFP forms was examined alongside that of Klp67A(M)EGFP and Klp67A(TL)EGFP. Only the motor-dead rigor form failed to depolymerise interphase MTs (supplementary material Fig. S4). It was unlikely that this resulted from an inability to interact with the MT, because this NLS mutant seemed to associate with the interphase cytoskeleton, as shown by a fibrous distribution that could overlap with that of the MTs. The ability of the remaining Klp67AEGFP variants to depolymerise MTs did not entirely correlate with protein concentration and, within a given EGFP fluorescence intensity range, unaffected and greatly diminished cytoskeletons were observed. Together, these data recapitulate the observation that Klp67A does not require a mitotic cytoplasm, the tail or coiled-coil domains for MT depolymerisation (Goshima and Vale, 2005). They further demonstrate that, in vivo, this activity requires ATP hydrolysis by the catalytic motor domain. These data suggest that Klp67A is normally prevented from depolymerising interphase MTs by its sequestration into the nucleus, an import function using the C-terminal NLS found at amino acids 670-673.

We next wished to examine how the motor-only, tail-lacking and tail-only Klp67AEGFP truncations were distributed during metaphase. Despite the contribution of the motor domain to kinetochore loading, established above, Klp67A(M)EGFP did not collect at any of the 120 centromeres examined (Fig. 1). Instead, the motor domain was enriched in the vicinity of the spindle. Here, it was found to varying extents within the spindle interstices, as well as along its MTs, although not to the same extent as in the wild-type form (Fig. 1). Thus, while the motor ATPase activity of Klp67A promotes efficient kinetochore loading, this domain is insufficient for that same recruitment. However, the motor is competent for spindle targeting.

As shown in Fig. 1, removal of the tail domain reduced spindle association to near background cytoplasmic levels. It did not affect recruitment to centromeric sites and Klp67A(TL)EGFP was found adjacent to 100% of these structures (n=120).

Conversely, the spindle was often found in a pool of tail-only Klp67AEGFP. Like the motor-only variant, this truncation was found between the spindle-associated MTs and, as revealed by cells with lower levels of expression, could also be seen to localise on them. Detailed examination failed to identify any centromere localisation (n=120). However, Klp67A(T)EGFP collected at above background levels to form discreet spots at the spindle poles (Fig. 1; arrowheads). Staining against the pericentriolar marker centrosomin confirmed that these sites of accumulation corresponded to centrosomes (n=50; supplementary material Fig. S5A). This association was observed throughout mitosis (M.S.S. and D.M.G., unpublished observations) and required intact MTs (n=30 centrosomes; supplementary material Fig. S5B). Thus, the C-terminal portion of Klp67A is not essential for recruitment to centromere-proximal sites, but is both necessary and sufficient for spindle association.

Klp67A binds to kinetochores in a microtubule-independent manner

To determine whether Klp67A accumulated at the kinetochore per se rather than at the plus ends of the impinging kinetochore-associated MTs, cells were incubated with colchicine overnight to depolymerise their MTs (Lemos et al., 2000). This treatment resulted in total spindle ablation, but did not prevent wild-type Klp67AEGFP from concentrating in the predicted kinetochore region (Fig. 2A; supplementary material Fig. S6). Of the 150 centromeres examined, 130 were flanked by a Klp67A(WT)EGFP signal. Thus, Klp67A is a core kinetochore component. Unlabelled centromeres were never found in cells with EGFP-tagged ones. This undecorated minority of cells (2 out of 15) might therefore represent a distinct subpopulation, for example, those in the process of 'slipping' out of mitosis. In agreement with this, Klp67A does not associate with kinetochores beyond late anaphase (Goshima and Vale, 2005) (M.S.S. and D.M.G., unpublished observations).

We then expanded this approach and examined how MT removal affected the kinetochore loading of our other variants (Fig. 2A). Incubation with colchicine did not prevent kinetochore recruitment of the tail-lacking Klp67A(TL)EGFP. However, as with the wild-type, we now observed a cell-specific 15% decrease in the number of centromeres adjacent to a pronounced Klp67A(TL)EGFP signal (150 centromeres scored). Thus, MTs are not required for Klp67A loading onto metaphase kinetochores.

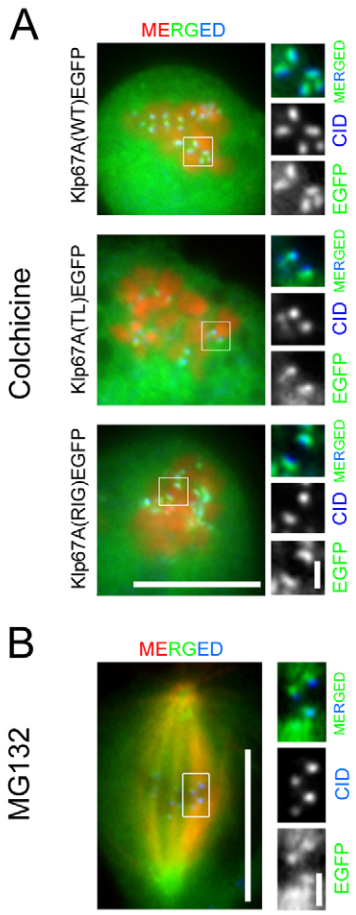

Fig. 2. Treatments that depolymerise MTs or cause mitotic arrest promote Klp67A recruitment to kinetochores. (**A**) Cells were treated with the MT-depolymerising agent colchicine and stained to show the distribution of each Klp67AEGFP variant (EGFP; green) relative to DNA (red) and centromeres (CID; blue). Kinetochore recruitment occurs for the wild-type (WT), tail-lacking (TL) and rigor mutant (RIG) forms in the absence of MTs. (**B**) Treatment with the proteosome inhibitor MG132 also restores the rigor mutant (EGFP; green) to kinetochores (centromeres are labelled CID; blue). This is not due to disruption of the spindle (α-tubulin; red), which remains intact during the prolonged mitotic arrest that accompanies MG132 exposure. Insets show magnified and contrast-enhanced views of the boxed regions. Scale bars: 10 μm. Magnified region scale bars: 2 μm.

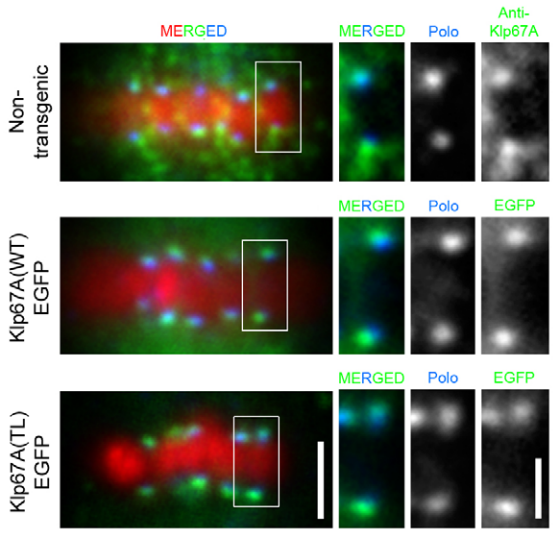

Fig. 3. Klp67A and its EGFP-tagged variants localise to the outer plate of the kinetochore. Comparison of the distribution of endogenous Klp67A (non-transgenic, anti-Klp67A), and recombinant wild-type (WT) and tail-lacking (TL) Klp67AEGFP variants (green) relative to the outer kinetochore plate protein Polo kinase (blue) and DNA (red). In all cases, Klp67A colocalises with the Polo signal. The boxed regions are magnified at right. Scale bar: 10 μm. Magnified region scale bar: 1 μm.

Remarkably, whereas the rigor motor mutant Klp67A(RIG)EGFP only associated with 14% of kinetochores in untreated cells (see above), colchicine treatment restored its accumulation to 95% of the kinetochores examined (190 out of 200 centromeres; 19 out of 20 cells with labelled kinetochores) (Fig. 2A). To determine whether this restored loading was due to either the changes in kinetochore properties that are known to occur in the absence of MTs or the sustained mitotic state accompanying drug incubation, we applied an alternative means of delaying mitotic progression. This involved treating cells with the proteosome inhibitor MG132, which prolongs mitosis without altering spindle integrity (Kallio et al., 2002). As shown in Fig. 2B, the mitotic arrest resulting from MG132 treatment was also highly effective at promoting kinetochore loading; the number of Klp67A(RIG)EGFP-labelled kinetochores rose from 13% in control DMSO solvent treatments to 93% (n=120 centromeres each). Neither treatment led to kinetochore association of Klp67A(M)EGFP or Klp67A(T)EGFP (data not shown).

Although the tail domain was not required for recruitment to kinetochores, it might influence the position of Klp67A within this trilaminar structure. Its distribution was therefore compared to that of the outer kinetochore plate protein Polo kinase (Blower and Karpen, 2001). As with male meiotic cells (Savoian et al., 2004), we found that, during mitotic metaphase, endogenous, wild-type and tail-lacking EGFP-tagged Klp67A variants colocalised with Polo (Fig. 3). From these experiments, we conclude that Klp67A is a core kinetochore component that resides in the outerplate region during metaphase. This subcellular distribution does not require the tail domain. However, when the ATPase activity of the catalytic motor domain is compromised, kinetochore accumulation is greatly reduced. This association can be restored to near wild-type levels by prolonging the duration of mitosis. This suggests that Klp67A is still able to bind to the kinetochore, but that binding is less efficient when the ATPase cycle is perturbed.

Kinetochore loading begins in prophase and requires a functional motor domain

Our fixed-cell studies of Klp67A(WT)EGFP-expressing Dmel cells indicated that kinetochore recruitment occurred early in mitosis. We noted several examples of prophase cells with paired and centromere-proximal EGFP foci. These signals partially overlapped with the centromeres and extended away from the chromatin, suggesting that wild-type Klp67A(WT)EGFP binds to kinetochores in prophase before MTs gain entry into the nuclear volume (Fig. 4A). To document the dynamics of these initial associations, we turned to the *Drosophila* neuroblast system, which provides a rich source of prophase cells that are amenable to high-resolution live cell imaging (Savoian and Rieder, 2002). Spinning disk confocal microscopy was used to follow primary cultures of neuroblasts

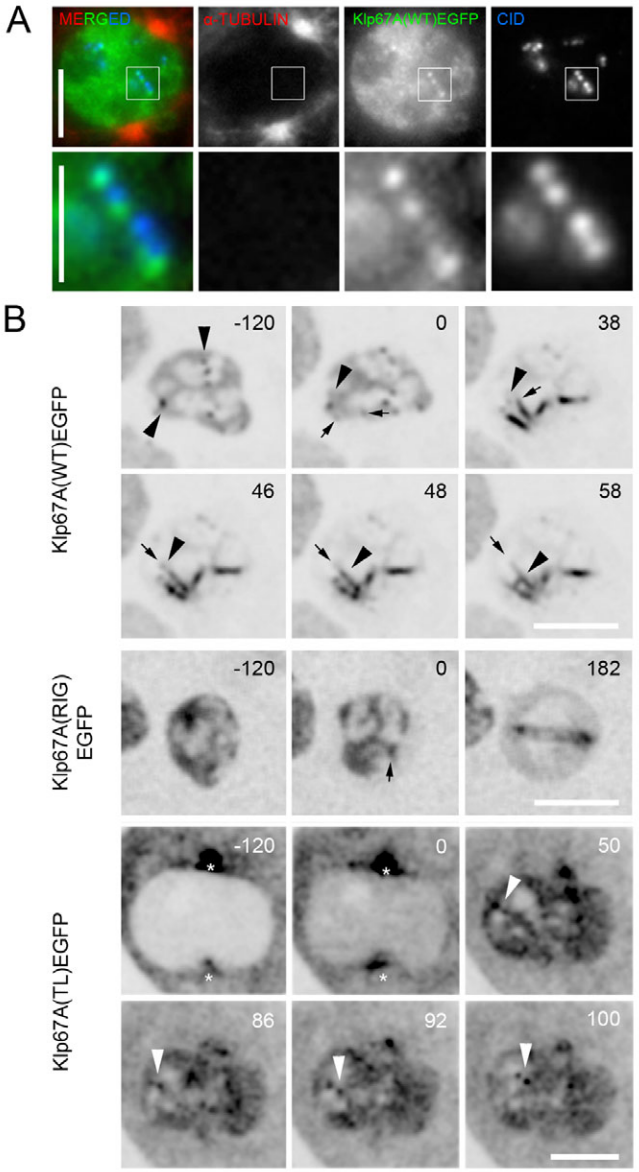

Fig. 4. Klp67A associates with prophase and prometaphase kinetochores in a manner that is dependent on motor activity, but independent of the presence of the tail domain and MTs. (**A**) A prophase Dmel cell fixed and stained to compare the distribution of wild-type Klp67A (EGFP; green), MTs (α-tubulin; red) and centromeres (CID; blue). The boxed region is magnified in the second row. Klp67A(WT)EGFP is found adjacent to the centromeres in the MT-free prophase nucleus. Scale bar: 5 µm. Magnified region scale bar: 2 µm. (**B**) Selected frames from time-lapse series of neuroblasts expressing wild-type (WT), rigor mutant (RIG) and tail-lacking (TL) Klp67AEGFP. The fluorescence signal has been inverted. In wild-type cells, Klp67AEGFP forms chromatin-associated punctae during prophase (–120; arrowheads), which contact and then travel along the invading MTs of the forming spindle (0-58; arrows) at prometaphase onset. In this cell, the centrosomes separate late and their MTs oppose one another. Prophase punctae do not form in the rigor mutants, although Klp67A(RIG)EGFP still binds to the impinging MTs at prometaphase onset (0; arrow) and metaphase (182). In Klp67A(TL)EGFP-expressing neuroblasts, the tail-lacking protein is found in the prophase cytoplasm and on putative centrosomes (–120; asterisks) until prometaphase onset (0), at which time it enters the nuclear volume and also assumes a paired-spot configuration on the chromosomes (50; arrowheads). These spots can form before chromosome movement (86-100). See text for details. Time is in seconds relative to prometaphase onset. Scale bars: 5 µm.

expressing Klp67A(WT)EGFP, Klp67A(RIG)EGFP and Klp67A(TL)EGFP, each under the control of the Gal4-UAS regulatory system. A representative Klp67A(WT)EGFP-expressing neuroblast is shown in Fig. 4B. A few minutes (–120 seconds) before prometaphase onset and the initiation of spindle formation (0 seconds), bright paired spots appeared on each chromosome (Fig. 4B, arrowheads). During nuclear-envelope fenestration, MTs from the two presumptive late separating centrosomes invaded the nuclear space and became coated with Klp67A(WT)EGFP, enabling their imaging. One such MT (Fig. 4B, arrow) grows towards one of the puncta (38-46 seconds) and then contacts it. Immediately thereafter, both spots in the pair move along the MT towards its predicted minus end (48-58 seconds; supplementary material Movie 1). Paired Klp67A(WT)EGFP signals were observed several minutes before prometaphase onset in all 12 cells filmed. At least one pair in each cell exhibited movements similar to those described above and identical to those that occur as a kinetochore captures a MT when spindle formation initiates (Hayden et al., 1990; Rieder and Alexander, 1990). Following prometaphase, Klp67A(WT)EGFP decorated the kinetochores and spindles of metaphase cells in a manner indistinguishable from tissue culture cells before exiting mitosis (data not shown). From our combined studies of Dmel cells and neuroblasts, we conclude that Klp67A begins to load onto kinetochores in prophase. Spindle association is a subsequent event that initiates with MTs being coated by the nuclear pool of Klp67A as they first penetrate beyond the disintegrating nuclear envelope.

In stark contrast to wild-type Klp67A(WT)EGFP, the Klp67A(RIG)EGFP rigor mutant did not appear as spots on the chromosomes during spindle formation (n=12) (Fig. 4B; supplementary material Movie 2) or at later stages (data not shown). The ability of the kinesin to bind to MTs seemed unaffected and it labelled the spindle from the entry of the first MT into the nuclear space (0 seconds; Fig. 4B, arrow) until this recording was terminated following the loose metaphase alignment of chromosomes (182 seconds). Thus, loss of motor activity prevents prophase loading of Klp67A onto kinetochores.

Because Klp67A(TL)EGFP lacks an NLS, it is unable to interact with kinetochores in the prophase nucleus. Nevertheless, Klp67A(TL)EGFP is found on metaphase kinetochores (Figs 1-3). We therefore filmed 19 neuroblasts to determine when tail-lacking Klp67A(TL)EGFP loads onto these structures (Fig. 4B; supplementary material Movie 3). As cells advanced into prophase, Klp67A(TL)EGFP transiently concentrated on presumptive centrosomes (–120 seconds; Fig. 4B, asterisks), where it remained until the mid-stages of spindle formation (data not shown). Shortly after prometaphase onset, as judged by the influx of the cytoplasmic fluorescence signal into the nucleus (0 seconds), the protein coalesced into paired spots on each chromosome (50 seconds; Fig. 4B, arrowhead). In some cells, the chromosomes underwent movement almost simultaneously with labelling. In this example, the chromosome was stationary for over half a minute after the kinetochores became decorated, before exhibiting the first signs of motion and ‘jerking’ towards the predicted spindle pole at the upper portion of the cell (86-100 seconds; Fig. 4B, arrowheads). Despite the temporary enrichment of Klp67A(TL)EGFP within the nucleus following membrane fenestration at prometaphase onset (e.g. Fig. 4B), we never observed EGFP-labelled MTs on any nascent or mature metaphase bipolar spindle. In summary, these live cell observations reveal that Klp67A targets to kinetochores as early as prophase, but

loading can also occur in prometaphase. Efficient kinetochore, but not spindle, association at any time requires motor ATPase activity.

Klp67A governs spindle length and chromosome alignment through kinetochore-localised motor activity

Finally, to identify the contribution of kinetochore- and spindle-associated Klp67A to metaphase spindle function, we applied an RNAi-based depletion and transgene rescue strategy. Each Dmel cell line was treated with double-stranded (ds) RNAs that specifically target the 5′ and 3′ untranslated regions (UTR RNAi) of the endogenous *klp67A* message, but that were absent from the transgenes used above. In agreement with previous studies (Goshima and Vale, 2003; Goshima et al., 2005b; Buster et al., 2007), we found that diminution of endogenous Klp67A in a non-transgenic background prevented chromosome alignment at the spindle equator; instead, the chromosomes were found along the length of abnormally elongated and often bent spindles. Following Klp67A downregulation, the average spindle length increased from 8.7±0.2 μm to 13.0±0.3 μm. We then induced the expression of each Klp67AEGFP variant after *klp67A* UTR RNAi treatment and assessed 80 or more cells to determine whether spindle length and chromosome alignment were restored relative to mock-depleted controls treated with dsRNAs against the prokaryotic *Kanamycin Resistance* gene. The *klp67A* UTR RNAi treatment provided a method to selectively deplete the endogenous Klp67A, but did not affect the production and distribution of the recombinant proteins (Fig. 5). The results of these rescue experiments are illustrated in Fig. 5B and summarised in Table 1.

As expected, expression of wild-type Klp67AEGFP fully rescued the scattered chromosome and elongated spindle phenotypes. This resulted in morphologically normal metaphase plates on spindles similar in length to mock depleted controls (average spindle lengths of 8.3±0.5 μm and 7.8±0.2 μm, respectively). By contrast, neither spindle length nor chromosome alignment were rescued following expression of the rigor mutant Klp67A(RIG)EGFP (Fig. 5B and Table 1). Even in mock depleted cells containing endogenous Klp67A, induction of the motor-compromised protein led to spindles that were abnormally long (average length 10.8±0.9 μm). Congression also appeared compromised and, whereas most chromosomes collected near the spindle equator, we commonly found others dwelling at the spindle poles. This alignment defect became more severe following endogenous Klp67A depletion and the chromosomes became strewn along spindles that were on average 11.2±0.3 μm long.

These data suggested that motor activity was vital to Klp67A activity. Indeed, we found that tail-only Klp67A(T)EGFP was unable to rescue any of the defects occurring after endogenous Klp67A knockdown (Fig. 5; Table 1). The spindles in this background became distorted in morphology and increased in length by 50%, from an average length of 8.2±0.2 μm to 12.4±0.3 μm.

Table 1. Average spindle lengths following dsRNA treatment

Variant	*Kanamycin Resistance* dsRNA $n \geq 80$ cells (μm)	*klp67A* 5′ and 3′ UTR dsRNA $n \geq 80$ cells (μm)
Non-transgenic	8.7±0.2	13.0±0.3
Klp67A(WT)EGFP	7.8±0.2	8.3±0.5
Klp67A(RIG)EGFP	10.8±0.9	11.2±0.3
Klp67A(M)EGFP	8.6±0.2	13.5±0.4
Klp67A(TL)EGFP	8.1±0.2	8.6±0.2
Klp67A(T)EGFP	8.2±0.2	12.4±0.3

Moreover, the chromosomes did not congress to form a distinct metaphase plate.

We next compared the rescue efficiency of tail-lacking Klp67A(TL)EGFP, which robustly accumulates at kinetochores but

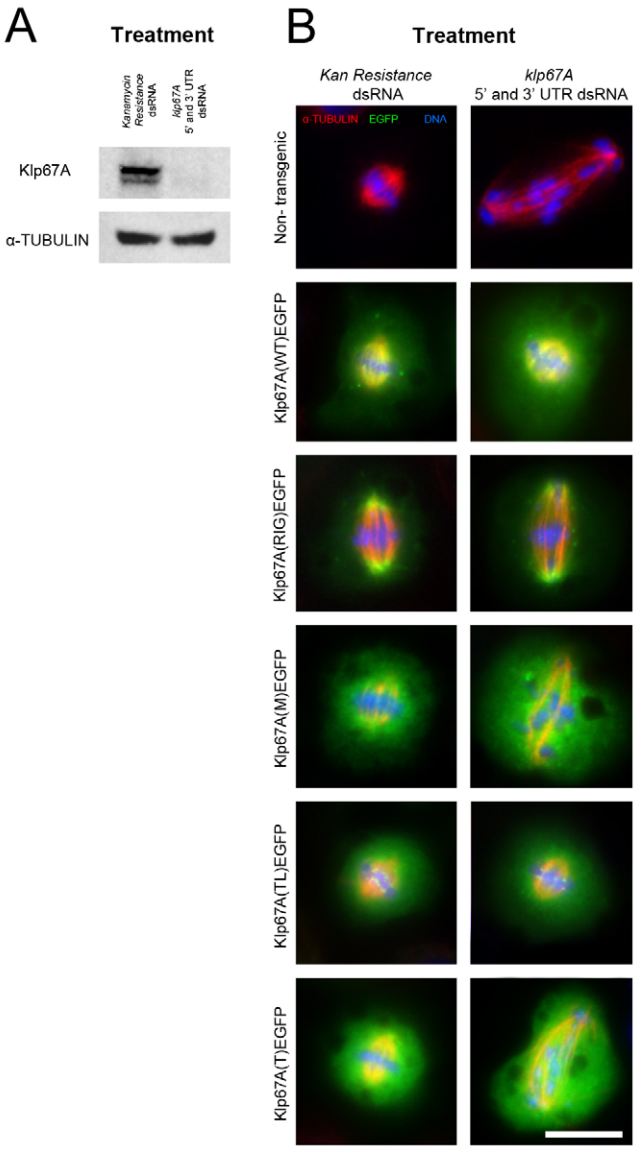

Fig. 5. Results of an RNAi-based rescue assay using different Klp67AEGFP variants. (**A**) Cells were treated with dsRNAs targeting either the *Kanamycin Resistance* gene (mock depletion) or the 5′ and 3′ UTRs of the endogenous *klp67A* mRNA. Cell extracts were probed with antibodies against Klp67A. Cells treated with the *Kanamycin Resistance* dsRNA display the Klp67A doublet characteristic of control cells, whereas virtually no signal can be detected after incubation with *klp67A* 5′ and 3′ UTR dsRNAs. α-Tubulin staining is shown as a loading control. (**B**) Gallery of representative phenotypes following dsRNA treatment and induction of the indicated transgenes. Images show the merged distributions of MTs (α-tubulin; red), each Klp67A variant (EGFP; green) and the chromosomes (DNA; blue). Endogenous Klp67A depletion does not alter the distribution of any of the recombinant forms. Only the kinetochore-localised wild-type (WT) and tail-lacking (TL) Klp67AEGFP variants can rescue the elongated spindle and scattered chromosome phenotypes that occur after *klp67A* 5′ and 3′ UTR dsRNA treatment. See text for details. Scale bar: 10 μm.

not at the spindle, with that of spindle-associated motor-only Klp67A(M)EGFP (Fig. 5; Table 1). Importantly, both of these truncations are catalytically active and can depolymerise MTs, as demonstrated above (supplementary material Figs S2 and S4). Klp67A(TL)EGFP rescued the endogenous Klp67A knockdown phenotypes as efficiently as the wild-type protein. In the presence of tail-lacking Klp67A, diminution of the endogenous protein did not perturb chromosome alignment and each chromosome again took up an equatorial position. Likewise, spindle size was fully rescued and the average spindle length after treatment with *klp67A* UTR dsRNAs was 8.6±0.2 μm (average spindle length after *Kanamycin Resistance* dsRNA treatment: 8.1±0.2 μm). Strikingly, expression of Klp67A(M)EGFP did not rescue the phenotypes resulting from *klp67A* UTR dsRNA treatment. The decoration of MTs by this depolymerase did not restore spindle morphology and these structures assumed an elongated bent form about 150% the length of their mock-depleted control counterparts (8.6±0.2 μm versus 13.5±0.4 μm). Furthermore, Klp67A(M)EGFP expression did not lead to well-defined metaphase plates; instead, the chromosomes were found at equatorial and polar locations. These data implicate the kinetochore but not spindle-associated pools of the Klp67A MT depolymerase in regulating spindle length and chromosome alignment.

Discussion

It was previously reported that EGFP-tagged Klp67A is found adjacent to mitotic metaphase centromeres and along the spindle (Goshima and Vale, 2005; Goshima et al., 2005b). Our work here extended these observations and identified the domains of the protein responsible for each of these separate localisations and their contributions to the function of Klp67A in metaphase as a key regulator of spindle length and chromosome alignment (Goshima and Vale, 2003; Goshima et al., 2005b; Buster et al., 2007) (this study).

We found that Klp67A is a core kinetochore component that is first recruited during prophase and that, by metaphase, colocalises with Polo kinase (Fig. 3) and Ndc80 (supplementary material Fig. S6) on the outerplate (Blower and Karpen, 2001; Przewloka et al., 2007). Three main lines of evidence support the identification of Klp67A as a core kinetochore component. First, immunolocalisation studies reveal centromere-proximal Klp67A punctae in regions devoid of MTs. Thus, it is unlikely that the Klp67 foci correspond to labelled MT plus ends. Second, time-lapse examinations of neuroblasts show that Klp67A collects as a pair of spots on each prophase chromosome. These foci are visible several minutes before the nuclear pool of Klp67A spills into the cytoplasm as the nuclear envelope becomes fenestrated. Only after interacting with the putative invading MTs of the forming spindle do the spots show any signs of movement. At this time, they contact and then rapidly move along the MT towards the predicted minus end in a manner identical to that previously described during spindle formation as a kinetochore captures and then glides along an astral MT (Hayden et al., 1990; Rieder and Alexander, 1990). Third, incubation of cells with the MT-depolymerising agent colchicine did not prevent Klp67AEGFP accumulation adjacent to centromeres, whereas it completely dismantled the spindle, as assayed by immunofluorescence (supplementary material Fig. S6). Furthermore, after drug treatment, Klp67A(WT)EGFP assumed an enlarged and crescent-shaped morphology (compare insets in Fig. 1B; Fig. 2A), a change characteristic of outerplate components in the absence of MTs (supplementary material Fig. S6) (Rieder, 1982; Hoffman et al., 2001). Thus, the *Drosophila* kinesin-8 protein Klp67A is a newly identified metazoan core kinetochore component.

Members of another MT-depolymerising family, the kinesin-13 proteins, also load onto prophase chromosome structures. However, these proteins either associate with the centromere (Kif2A/Klp10A) or redistribute to the kinetochore during the course of mitosis (Kif2C). Because kinesin-13 proteins lack translocating motor activity (Desai et al., 1999; Rogers et al., 2004), Klp67A is, to the best of our knowledge, the only motile kinesin that loads adjacent to the centromere before nuclear-envelope breakdown.

Given the expendable nature of MTs in the kinetochore loading of Klp67A, it is surprising that this action requires the catalytic activity of the motor. Our data indicate that the failure of the rigor form to load onto kinetochores is not due to the sequestration of the mutated motor by non-reversible MT binding. This is illustrated by the failure of Klp67A(RIG)EGFP to collect on kinetochores in the MT-free environment of the prophase nucleus. Furthermore, kinetochore recruitment is restored if cells are arrested for prolonged periods, even in the presence of a spindle. An analogous centromeric mislocalisation occurs when the motor domain of the kinesin-13 protein Kif2C is mutated into a rigor form (Wordeman et al., 1999). Thus, it might be that the conformational changes experienced by MT-depolymerising kinesin motors during the ATP hydrolysis cycle facilitate interactions with proteins other than tubulin. Further work will be required to address this interesting possibility.

Even when functional, the motor domain of Klp67A is insufficient for kinetochore recruitment, as such targeting further requires the centrally positioned coiled-coil region. Attempts to directly determine the contribution of this domain to subcellular targeting and Klp67A function were unsuccessful, with the recombinant protein consistently forming cytoplasmic aggregates (data not shown). In other motor proteins, the coiled-coil region facilitates the formation of higher order structures. Although the oligomeric state of Klp67A is currently unknown, we note that both budding (Gupta, Jr et al., 2006; Varga et al., 2006) and fission (Grissom et al., 2008; West and McIntosh, 2008) yeast kinesin-8 members are suggested to homodimerise. However, the different localisation pattern of tail-lacking Klp67AEGFP (which retains the coiled-coil domain) compared with the wild type, and the subsequent lack of changes in distribution after endogenous Klp67A depletion argue against a stringent requirement for homo-oligomeric structures in subcellular targeting. Many organisms encode two kinesin-8 proteins. In fission yeast, Klp5 and Klp6 form a heterodimer required for retention in the nucleus and subsequent kinetochore and spindle localisation (Garcia et al., 2002; West et al., 2002; Unsworth et al., 2008). A similar targeting mechanism reliant on the second identified metazoan kinesin-8, Kif19 (Wickstead and Gull, 2006), seems unlikely however. Depletion of Kif19 in both flies and vertebrate systems fails to phenocopy Klp67A/Kif18A downregulation defects or induce any mitotic abnormalities (Goshima and Vale, 2003; Zhu et al., 2005).

Our complementary studies using tail-lacking and tail-only Klp67AEGFP truncations indicated that the C-terminal portion of the protein promotes spindle association. Sequence analyses indicate that the tail is rich in basic residues (34 out of 204 residues), a feature that facilitates the ATP-independent and charge-based binding of kinesins to MTs, probably through interactions with the C terminus of tubulin (Chandra et al., 1993; Ovechkina et al., 2002; Shiroguchi et al., 2003; Straube et al., 2006). The reduced spindle association that occurs after deletion of the tail might indicate that such electrostatic interactions promote binding along the MT

lattice. Consistent with this, a tail-only variant collected at and coated the spindle. The combined affinities of the motor and tail for MTs might account for the high processivity exhibited by full-length recombinant kinesin-8 proteins (Varga et al., 2006; Mayr et al., 2007).

Our attempts to rescue the effects of depleting endogenous Klp67A identified the importance of interactions between the motor domain of this kinesin and the kinetochore for spindle length determination and equatorial chromosome alignment. This was revealed by the inability of the spindle-associated motor-only variant to restore these parameters to those seen in mock-depleted controls. By contrast, the depleted protein was fully complemented by the kinetochore-positioned tail-lacking Klp67A. Although these truncations differ by the presence of the coiled-coil domain, we do not believe that this can account for the differences in rescue efficiency. Both the motor-only and tail-lacking Klp67AEGFP variants are functional MT depolymerases (supplementary material Figs S2 and S4) (Goshima and Vale, 2005). Rather, we propose that it is the distribution of this depolymerising activity that determines Klp67A function. Kinesin-8 proteins exclusively depolymerise MT plus ends (Gupta, Jr et al., 2006; Varga et al., 2006; Mayr et al., 2007). The insertion of MTs into the Klp67A-enriched kinetochore outerplate during kinetochore fibre maturation (Rieder, 1982; Maiato et al., 2006) would position the depolymerase in contact with these target sites. Although our data indicated that motor-only Klp67A(M)EGFP extends towards the centromere, the limitations of light microscopy prohibit us from determining whether, in fact, the protein is in contact with MT plus ends. Even if present, motor-only Klp67A might fail to collect to the threshold concentration of kinesin-8 proteins needed for efficient depolymerisation (Varga et al., 2006; Mayr et al., 2007). In either case, this population is insufficient to depolymerise kinetochore-associated MTs.

Interestingly, the human homologue of Klp67A, Kif18A, has not been observed to bind to the prophase kinetochore or accumulate in the region of the kinetochore in the absence of a spindle (Mayr et al., 2007; Stumpff et al., 2008). Thus, unlike *Drosophila* Klp67A, vertebrate Kif18A does not seem to be a core kinetochore component. Kif18A is proposed to direct mitotic events through the formation of a concentration gradient exclusive to kinetochore-associated MTs. Colocalisation studies consistently indicate that Kif18A concentrates external to the outerplate protein Hec1 (also known as Ndc80), suggesting accumulation at the plus ends of MTs as they interface with the kinetochore. Kif18A collects at this site and orchestrates chromosome movement to and oscillations upon the metaphase plate (Mayr et al., 2007; Stumpff et al., 2008). Such a distribution would be functionally analogous to the rich pool of Klp67A on the kinetochore in *Drosophila*. However, chromosomes exhibit little to no oscillatory movement during *Drosophila* mitosis. Although no detailed kinetic analyses of kinetochore movement have been performed on mitotic cells depleted of Klp67A, live cell studies of *klp67A* mutant primary spermatocytes indicate an inability to assume a stable position at the spindle equator (Savoian et al., 2004). This phenomenon is also observed following RNAi-mediated depletion of Kif18A (Stumpff et al., 2008). Thus, loss of kinesin-8 function induces or exaggerates chromosome movements, leading to the scattered chromosome phenotypes seen in fixed preparations. Conversely, overexpression of Kif18A dampens chromosome oscillations (Stumpff et al., 2008). This suggests that threshold amounts of kinesin-8 proteins act at the kinetochore and the spindle-interacting MT plus ends to determine chromosome position. It will be of future interest to test this possibility by comparing the behaviour of kinetochores enriched with motor-dead or tail-lacking Klp67A relative to that of their wild-type counterparts. Such studies might provide insights into the evolutionary significance of the divergent Klp67A and Kif18A distributions, possibly by revealing additional roles for kinetochore-associated Klp67A. One such role might be in helping to ensure attachment to the spindle, possibly by providing a link to shrinking MT plus ends, a cargo-bearing function recently demonstrated for fission yeast Klp5-Klp6 (Grissom et al., 2008).

This leaves the question of what the primary function of the spindle-associated pool of Klp67A might be. We speculate that, although not required for metaphase, this population might be crucial for cytokinesis. After the transition into anaphase, Klp67A concentrates at the midzone of the central spindle (Savoian et al., 2004; data not shown). Live cell observations indicate that, during these late stages, Klp67A maintains central spindle integrity for faithful cleavage furrow positioning and ingression (Gandhi et al., 2004; Gatt et al., 2005). We are currently investigating which properties of Klp67A enable it to perform this stabilising task.

The work described here reveals that Klp67A exists in two spatially and functionally separable metaphase pools: on kinetochores and on the spindle. By identifying the domains of the protein responsible for each of these distributions, we have shown that it is the positioning of the MT-depolymerising catalytic motor activity at kinetochores that is required for faithful chromosome alignment and spindle length determination. This different localisation compared with human Kif18A suggests a previously unrecognised level of functional flexibility among MT-destabilising proteins. Identifying how this is regulated as mitosis progresses will be crucial to understanding the interactions and extent of redundancy of spindle-regulating molecules.

Materials and Methods

Domain and sequence predictions

The motor and neck region has been described (Goshima and Vale, 2005). The COILS v2.1 program (Lupas et al., 1991) was used to identify coiled-coil-rich regions. The NLS (amino acids 670-673) was identified using the PSORT II programme (Nakai and Horton, 1999).

Mutagenesis and transgenic tissue culture cell generation

Metallothionein-regulated constructs were created using the Gateway recombination system (Invitrogen). For each insert (wild type: nucleotides 1-2245; motor only: nucleotides 1-1074; tail lacking: nucleotides 1-1834; tail only: nucleotides 1835-2245), PCR was used to amplify Gateway-compatible sequences using the full-length *klp67A* cDNA as the template. The stop codons were replaced by triplets encoding glycine, all of which were in frame with the downstream *EGFP* sequence. The rigor variant G116E (Meluh and Rose, 1990) and NLS-altered R671G mutants were constructed using the QuickChange II site-directed mutagenesis kit (Stratagene). Mutagenic primer sequences are available upon request. PCR products were sequenced on both strands. Non-clonally selected cell lines were generated as described previously (D'Avino et al., 2006) and maintained in Express Five serum-free media (Invitrogen) supplemented with 30 μg/ml blasticidin, L-glutamine, penicillin and streptomycin.

RNAi treatment and western blotting

For each of two trials, 30 μg of dsRNA against either the *Kanamycin Resistance* gene [for primer sequences, see Przewloka et al., 2007 (Przewloka et al., 2007)] or a 1:1 mixture containing 15 μg each of dsRNAs targeting the *klp67A* 5′ and 3′ UTRs was used [for primer sequences, see Goshima and Vale, 2005 (Goshima and Vale, 2005)]. Cells were treated with dsRNAs for a total of 5 days. On day 3, 1 mM copper sulphate was added to control and transgenic cells. Protein depletion and Klp67A recombinant variant protein sizes were assayed by western blot according to standard protocols. Blots were probed with the following antibodies and dilutions: mouse anti-α-tubulin (DM1A, 1:5000) and anti-GFP 1:1000 and rabbit anti-Klp67A (Savoian et al., 2004) at 1:500.

Drug treatments, immunofluorescence, wide-field imaging and analysis

MTs were depolymerised by 18 hours incubation in 25 μM colchicine. Cell-cycle arrest experiments were performed by exposing cells to 25 μM MG132 in DMSO

or 0.1% DMSO as a control for 9 hours. All cells were plated on coverslips coated with 0.5 mg/ml concanavalin A for 1.5 hours before fixation with 4% paraformaldehyde. The following previously described or commercially available antibodies and dilutions were used: rabbit anti-CNN (7647) 1:200, rabbit anti-fibrillarin (Ab5821) 1:1000, rabbit anti-Ndc80 1:500, rabbit anti-Klp67A 1:100, mouse anti-Polo kinase (M294) 1:50, mouse anti-α-tubulin 1:500 and chicken anti-CID 1:3000. Epitopes were visualised with appropriate Alexa-Fluor 350, 488 and 568 tagged secondary antibodies. DNA was labelled with DAPI-containing mounting media. Z-series were acquired at 0.25 μm steps on a Zeiss Axiovert200 microscope with a 100× (N.A. 1.4) lens and 2×2 bin using a Coolsnap HQ camera running Metamorph software. Centromeres were scored as positive for the presence of EGFP if a distinct signal intensity peak formed relative to the adjacent background, as confirmed by linescan analyses. Spindle lengths were determined using the Metamorph segmented line function to measure the distance between each spindle pole following the shape of the spindle long axis in maximum intensity projections. Images were processed and figures generated in Adobe Photoshop using projections consisting of a variable number of sections from selected z-series.

In vivo microtubule depolymerisation assay

MT depolymerisation efficiency was determined for each Klp67AEGFP variant according to the method of Stumpff and co-workers (Stumpff et al., 2007). Briefly, cells were induced and plated on coverslips as described above before fixation with 1% formaldehyde in –20°C methanol for 10 minutes. Cells were stained for α-tubulin and DNA. For each variant, single sections of 60 or more cells were acquired using a 63× (N.A. 1.4) lens and 2×2 bin on a Zeiss Axiovert200 microscope outfitted with a Coolsnap HQ camera and controlled by Metamorph. All images were captured using identical acquisition parameters. Non-scaled and merged RGB colour images were imported into the public domain program Image J (http://rsb.info.nih.gov/ij) for analysis. The average EGFP and tubulin fluorescence intensities were measured for each expressing cell and an adjacent non-expressing cell of similar size and morphology. The tubulin fluorescence intensity ratio of Klp67AEGFP-expressing and non-expressing cells was determined in Microsoft Excel and plotted in the same program. We empirically determined that tubulin fluorescence intensities ≤0.6 indicated complete depolymerisation of the MT cytoskeleton. A large population of cells from all genetic backgrounds had ratios greater than the value of 1.0 expected when no net change in tubulin polymer occurs. We do not believe that this indicates MT-stabilising activity, but instead results from the experimental noise inherent when comparing similar but individual cells.

Transgenic flies and neuroblast imaging

Gal4-UAS-regulated *klp67AEGFP* variants were made using the Gateway system and recombination into a modified pUAST vector containing a downstream *EGFP* gene (pUASM; a gift of Pier Paolo D'Avino and Feng Chen, University of Cambridge, UK). These constructs were used to generate transgenic flies according to standard procedures.

Primary cultures of neuroblasts heterozygous for the driver Gal4-69B or *elav*-Gal4 and two different strains for each *klp67AEGFP* transgene were prepared and maintained at 25°C in open chambers (Savoian and Rieder, 2002; Inoue et al., 2004). Images were acquired on a Zeiss Axiovert200 microscope fitted with a PerkinElmer RSIII spinning disk confocal unit (PerkinElmer Life Sciences) and running the Ultraview Imaging Suite. Single optical sections were captured at 2 second intervals with a 100× (N.A. 1.4) lens and a 2×2 bin. Data sets were imported into Metamorph and Photoshop for movie export and figure generation, respectively.

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http://jcs.biologists.org/cgi/content/full/123/5/767/DC1

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