Dynein light intermediate chains maintain spindle bipolarity by functioning in centriole cohesion

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Cytoplasmic dynein 1 (dynein) is a minus end-directed microtubule motor protein with many cellular functions, including during cell division. The role of the light intermediate chains (LICs; DYNC1LI1 and 2) within the complex is poorly understood. In this paper, we have used small interfering RNAs or morpholino oligonucleotides to deplete the LICs in human cell lines and Xenopus laevis early embryos to dissect the LICs’ role in cell division. We show that although dynein lacking LICs drives microtubule gliding at normal rates, the LICs are required for the formation and maintenance of a bipolar spindle. Multipolar spindles with poles that contain single centrioles were formed in cells lacking LICs, indicating that they are needed for maintaining centrosome integrity. The formation of multipolar spindles via centrosome splitting after LIC depletion could be rescued by inhibiting Eg5. This suggests a novel role for the dynein complex, counteracted by Eg5, in the maintenance of centriole cohesion during mitosis.

Introduction

Cytoplasmic dynein 1 (dynein) is a motor protein that carries many cargoes toward microtubule minus ends (Allan, 2011). Dynein is essential for cell division in metazoans, where it plays multiple roles. Bipolar spindle assembly requires the separation of the duplicated centrosomes in late G2/prophase, and this is driven by dynein on the nuclear envelope (NE) and at the cell cortex and by the plus end-directed motor Eg5 (a kinesin-5), which generates antiparallel sliding of overlapping microtubules (Robinson et al., 1999; Tanenbaum et al., 2008; Tanenbaum and Medema, 2010; Raaijmakers et al., 2012, 2013). At the end of prophase, dynein assists NE breakdown by pulling on the nuclear membrane (Beaudouin et al., 2002; Salina et al., 2002). In the spindle, Eg5 pushes the centrosomes apart, aided by kinesin-12, chromosome-associated kinesins, and forces provided by the polymerization of kinetochore fibers (K fibers; Tanenbaum and Medema, 2010; Maiato and Logarinho, 2014). Dynein counteracts these outward forces (Gagliò et al., 1996; Mitchison et al., 2005; Tanenbaum et al., 2008; Ferenz et al., 2009; Florian and Mayer, 2012), perhaps by driving antiparallel microtubule sliding (Tanenbaum et al., 2013).

Dynein keeps microtubules tightly focused at the spindle poles by working together with nuclear mitotic apparatus (NuMA; Gagliò et al., 1996; Merdes et al., 2000; Silk et al., 2009; Raaijmakers and Medema, 2014). Dynein at the kinetochore generates the initial lateral attachment to and translocation of chromosomes along microtubules (Yang et al., 2007; Elting et al., 2014; Sikirzhatski et al., 2014), working in opposition to the kinetochore-associated kinesin CENP-E (Kapoor et al., 2006). After end-on interactions form between the kinetochore and the K-fiber microtubules, dynein contributes to the polewards movement of sister chromatids by causing sliding of K fibers toward the poles (Elting et al., 2014; Sikirzhatski et al., 2014). Kinetochore dynein may also participate in cell cycle control by removing spindle assembly checkpoint (SAC) components from correctly aligned kinetochores (Hoffman et al., 2001; Howell et al., 2001; Wojcik et al., 2001; Mische et al., 2008; Sivaram et al., 2009), although this is controversial (Raaijmakers et al., 2013). Finally, cortical...
dynein controls the orientation of the spindle (Raaijmakers and Medema, 2014).

When dynein activity is compromised, spindles become multipolar and disorganized, chromosomes fail to attach properly to the spindle, and cell cycle progression is slowed (Robinson et al., 1999; Wojcik et al., 2001; Maiato et al., 2004; Misches et al., 2008; Tanenbaum et al., 2008; Firestone et al., 2012; Iwakiri et al., 2013; Raaijmakers et al., 2013). Impaired pole focusing and a lack of opposition to Eg5-driven forces undoubtedly contribute to aberrant spindle assembly. However, centrosomes themselves may not function normally because dynein is implicated in the accumulation of several pericentriolar material (PCM) components (Doxsey et al., 2005), including PCM-1 (Kubo et al., 1999; Dammermann and Merdes, 2002) and pericentrin (PC; Purohit et al., 1999; Tynan et al., 2000b). Dynein may also contribute directly to microtubule attachment to the centrosome (Heald et al., 1997; Burakov et al., 2008).

Each dynein complex contains two motor subunits, dynein heavy chain (DHC; DYNC1H1), along with two intermediate chains (ICs; DYNC1I1 and 2), two light ICs (LICs; DYNC1LI1 and 2), and several light chains (Allan, 2011). Although the LICs are essential for proper dynein function (Yoder and Han, 2001; Lee et al., 2005; Misches et al., 2008; Palmer et al., 2009; Sivaram et al., 2009; Horgan et al., 2010a,b; Tan et al., 2011; Raaijmakers et al., 2013), their role within the complex is not well understood. Only vertebrates have two isoforms, and, unlike other dynein subunits, LIC is not well conserved outside metazoans (Lee et al., 2005; Pfister et al., 2006; Zhang et al., 2009). Vertebrate LIC1 is phosphorylated by Cdk1 in mitosis, which leads to dynein’s release from membranes (Niclas et al., 1996; Dell et al., 2000; Addinall et al., 2001) and promotes its association with the SAC components Mad1/2 and ZW10 (Sivaram et al., 2009).

Because vertebrate dynein complexes contain either LIC1 or LIC2, but not both (Tynan et al., 2000a), an attractive idea is that they recruit specific cargoes. Indeed, LIC1, but not LIC2, binds to PC (Tynan et al., 2000b), whereas only LIC2 interacts with Par3 (Schmaranzer et al., 2009). However, both LICs can bind to the recycling endosome (RE) component FIP3 (Horgan et al., 2010a,b) and Rab-interacting lysosomal protein (Scherer et al., 2014), and the data for specific roles for LIC1 or 2 in membrane traffic are contradictory (Palmer et al., 2009; Tan et al., 2011; Hunt et al., 2013; Scherer et al., 2014). Furthermore, LICs act redundantly in centrosome anchoring to the NE in late G2 and in mitotic chromosome alignment, mitotic progression, and bipolar spindle maintenance (Raaijmakers et al., 2013).

Here, we investigate the role of LICs in cell division in cultured cells and in an organismal context using early embryos of *Xenopus laevis*. We show that although LICs are not needed for dynein’s motor activity in vitro, they are important for mitotic progression and the formation and maintenance of bipolar spindles. Upon depletion of LICs, spindle poles split apart in a process requiring the activity of Eg5, giving poles that often contain a single centriole. Because mother and daughter centrioles normally remain closely associated throughout mitosis, and only disengage during G1 (Mardin and Schiebel, 2012), this work reveals a novel role for dynein in maintaining centrosome integrity during mitosis.

**Results**

**LICs are dispensable for motor activity in vitro**

We depleted LIC1, LIC2, or both LICs from HeLaM cells (Fig. 1 A) using LIC1- or LIC2-specific siRNAs (Palmer et al., 2009). Comparable results were obtained using SMARTpools for LICs 1 and 2 (unpublished data). As previously reported (Tan et al., 2011), we found that depletion of both LICs did not affect human dynein complex integrity, because IC was stable (Fig. 1 B) and dynein migration on sucrose density gradients was unaffected (Fig. 1 C). In addition, dynein was similarly sensitive to the chaotropic agent KI, which disrupts interactions between dynein subunits (Fig. 1 C; King et al., 2002; Ori-McKenney et al., 2010).

We tested whether lack of LICs led to a loss of motor function in vitro. Dynein was purified from cells depleted of...
LIC depletion causes multipolar spindle formation and slows cell cycle progression in HeLaM cells

Although dynein lacking LICs drives microtubule gliding, previous studies have shown that LIC depletion inhibits many dynein functions in vivo, as described in the Introduction. We investigated the effect of LIC knockdown (KnD) on mitotic spindle assembly and stability in detail. Loss of both LICs led to ~70% of mitotic cells having multipolar spindles (Fig. 2, A and B). These fell into two categories: cells with three or more poles with fairly similar densities of microtubules (major multipolar; Fig. S2 A), and those with a primarily bipolar spindle that had auxiliary poles (minor multipolar; Figs. 2 A and S2 A). Similar defects were seen in human embryonic kidney (HEK; Fig. S2 B) and U2OS cells (Fig. S4 A). Depletion of DHC in HeLaMs had more severe effects (P < 0.05, compared with LICs), generating multipolar spindles with often unfocused poles and highly disorganized chromosomes (Fig. 2, A and B).

Because each dynein complex contains either LIC1 or LIC2 (Tynan et al., 2000b), we tested the effect of removing both LICs using microtubule binding and release followed by sucrose gradient sedimentation to separate dynein from kinesins (Fig. S1, A and B). Dynein purified from LIC-depleted or control cells appeared identical, save for the loss of the LICs (Fig. 1 D and Fig. S1 A). Dynein motor activity was assessed using in vitro microtubule gliding assays, in which multiple dyneins work together in a system with minimal drag. Control and LIC-free dynein both supported microtubule gliding (Video 1), and the frequency of movements was equally sensitive to dilution of the motor preparations (not depicted). Hence, their concentrations of active motor were approximately equivalent. Moreover, the mean speed of microtubule gliding was 0.57 µm/s ± 0.012 (SEM) for control and 0.56 µm/s ± 0.014 for LIC-free dynein (P = 0.632; Fig. S1 C). Microtubule gliding was completely inhibited by 50 µM sodium orthovanadate, which preferentially inhibits dynein (Cohn et al., 1989; Shimizu et al., 1995), whereas kinesin fractions were unaffected (unpublished data). Hence, LICs do not obviously influence dynein motility in vitro when multiple motors work together in the absence of cargo.
LICs individually. Loss of either LIC greatly increased the incidence of multipolar spindles in HeLaM cells but to a lesser extent than for the double depletion (Fig. 2 C, P ≤ 0.05 for LIC1 vs. LIC1&2 KnD and P ≤ 0.001 for LIC2 vs. LIC1&2 KnD). Importantly, the spindle defects in cells depleted of both LICs were rescued equally well by expression of RNAi-resistant LIC1-mKate or LIC2-mKate (Fig. 2 D), revealing that the two LICs act redundantly in spindle assembly in HeLaM cells.

To test the effects of depleting LICs on mitotic progression in HeLaM cells, we determined the mitotic index and duration of mitosis. The mitotic index increased 2–2.5-fold after depletion of both LICs, or LIC1 alone, whereas removal of LIC2 had a small but statistically insignificant effect (Fig. 3 A), as recently reported (Raaijmakers et al., 2013). Moreover, depletion of LIC1 prolonged the duration of mitosis (P = 0.048), as previously seen (Sivaram et al., 2009), whereas loss of LIC2 had no effect (P = 0.815; Fig. 3 B), even though it caused spindle multipolarity (Fig. 2 C). Strikingly, depletion of both LICs slowed mitosis far more (P < 0.001) than LIC1 KnD, and 13% of cells failed to complete mitosis within 4 h (Fig. 3 B). However, DHC-depleted cells were more profoundly retarded (P < 0.001). In keeping with delayed mitotic progression, unaligned chromosomes were common after depletion of both LICs (Figs. S2 A and S3), and they displayed high levels of the SAC proteins Bub1 and BubR1 (Fig. S2 C). The chromosome misalignment was not caused by a loss of dynein from kinetochores (Fig. S2 D). Furthermore, the kinetochores of chromosomes at the metaphase plate were under tension, as judged by the extended centromeres and reduced Bub1 and BubR1 labeling (Fig. S2 C). There was also no evidence of premature loss of chromosome cohesion (cohesion fatigue), which can occur after prolonged metaphase arrest (27 cells from three experiments; Daum et al., 2011; Stevens et al., 2011). These results suggest that depletion of both LICs affects initial chromosome alignment but not the end-on K fiber–kinetochore attachments and associated SAC silencing, as previously reported (Raaijmakers et al., 2013).

Multipolar spindles form in *Xenopus* embryos upon depletion of LICs

To test the role of LICs in an organismal context, we used morpholino (MO) oligonucleotides to knock down the LICs in *Xenopus* early embryos. Depletion of each LIC individually led to an increased mitotic index in gastrula stage embryos, but loss of both LICs had a greater effect (Fig. 3 C). Time-lapse confocal imaging of embryos expressing GFP–α-tubulin and mCherry-H2B (mCherry-histone2B) revealed a slight increase in mitosis duration after depletion of both LICs (Fig. 3 D). The mean time taken to complete mitosis was 26 min in LIC morphants compared with 15 min in controls (48 and 43 cells, respectively, from five independent experiments). However, some LIC morphant cells were particularly strongly affected, with 14% of cells failing to complete mitosis within 50 min (Fig. 3 D).

Depletion of LICs in *Xenopus* embryos disrupted spindle morphology, with multipolar spindles found in 44% of mitotic cells (Fig. 4 A and B) by developmental stage 10.5 (gastrulation), whereas no multipolar spindles were seen in controls. Unlike the multipolar spindles seen in the HeLaM cells, where the minor poles often had far fewer microtubules than the main poles and frequently overlapped the bipolar spindle, all auxiliary poles in embryo spindles were more easily distinguished (Fig. 4 A).
Depletion of LICs leads to multipolar spindles in Xenopus embryos. (A) Confocal images of spindles in embryos injected with control (Ctrl MO) or LIC 1 and 2 MOs (LIC1&2 MO) labeled with anti-α-tubulin and DAPI. Bars, 10 µm. (B) Quantification of spindle morphology in Xenopus embryos after depletion of LIC1, LIC2, or both LICs. Spindles categories: two poles = bipolar; three to four poles = minor multipolar; more than four poles = major multipolar. \( n = 4 \) independent experiments, ≥27 embryos scored in total, 240–400 spindles categorized for each condition, means ± SEM. (C) Embryos injected with control or LIC 1 and 2 MOs were rescued with a GFP plasmid or a mixture of plasmids encoding LICs 1 and 2 and scored as in B (a total of 98–159 mitotic cells from 13–18 embryos scored per condition, means ± SEM). Two-way ANOVA analysis versus controls: **, \( P < 0.01 \); ***, \( P < 0.001 \); ****, \( P < 0.0001 \). For other comparisons, see Results. C, control.

Therefore categorized the multipolar phenotype in embryos by counting the number of auxiliary poles. Around half of the aberrant spindles had one or two extra poles (minor multipolar), whereas the rest had three or more additional poles (major multipolar; Fig. 4 B). Although depletion of LIC1 alone had only a slight effect on spindle morphology compared with LIC2 loss, depletion of both LICs gave a stronger phenotype than depletion of either LIC alone (Fig. 4 B), as seen in HeLaM cells.
Importantly, the multipolar spindle phenotype was rescued by expression of GFP-tagged LICs (Fig. 4 C). Collectively, these data show that the LICs have overlapping activities during mitosis in two very different vertebrate cell systems but that the relative importance of LIC1 versus LIC2 differs.

Figure 5. Multipolar spindles form by two mechanisms in LIC1 and 2 morphant embryos. Mitotic spindles were imaged live in Xenopus embryos using GFP-α-tubulin (green) and mCherry-H2B (magenta). [A] Bipolar spindles assembled and proceeded normally through mitosis and cytokinesis in controls (Ctrl). [B] An LIC morphant that formed a multipolar spindle at the onset of mitosis and subsequently failed cytokinesis (arrows). [C] An LIC morphant that assembled a bipolar spindle, which became multipolar when spindle poles fragmented (arrows). [D] Pole fragmentation was also seen in multipolar LIC MO spindles (arrows). Bars, 10 μm; time stamps display minutes and seconds.

Two routes for forming multipolar spindles in Xenopus cells lacking LICs

To investigate how the multipolar spindles arise, we imaged the mitotic spindle in living Xenopus embryos. Embryos were injected with mRNA encoding GFP-α-tubulin and mCherry-H2B along with standard control (Fig. 5 A and Video 2) or LIC1- and 2-targeted MO oligos (Fig. 5, B–D; and Videos 3–5). Live imaging of LIC morphants revealed that multipolar spindles arose by two different routes. We found that 40% of multipolar spindles in LIC morphants (8/20 from five independent experiments) had additional poles from the very start of mitosis, forming as the spindle assembled (Fig. 5, B and D; and Videos 3 and 5). However, the other 60% of multipolar spindles assembled first as bipolar spindles and then underwent a pole fragmentation event, in which an additional pole formed by breaking away from the bona fide spindle pole (Fig. 5 C and Video 4). We also saw instances where spindles assembled as multipolar spindles but then underwent pole fragmentation to form spindles with five or more poles (Fig. 5 D and Video 5). The presence of multiple poles affected the ability of cells to complete cytokinesis, which failed entirely or showed regression of one or more cytokinetic furrows in 10 out of 20 multipolar cells, leading to multinucleate cells (e.g., Fig. 5 B, arrows).

The pole fragmentation events were particularly intriguing, as they suggested a role for the LICs in the maintenance of centrosome integrity. Importantly, the fragmentation was not simply caused by a prolonged prometaphase arrest (e.g., Sluder and Rieder, 1985; Hinchcliffe et al., 1998; Hut et al., 2003) because it occurred on average only 17 min (±2 min, SEM; n = 12 spindles, in five independent experiments) after the start of mitosis.

Depletion of LICs results in premature centriole disengagement

Spindle pole fragmentation can result from PCM fragmentation or premature disengagement of mother and daughter centrioles (Maiato and Logarinho, 2014). We therefore investigated whether the poles of multipolar spindles contained centrioles and, if so, whether these were pairs or single centrioles. We used 3View EM to assess cellular ultrastructure over a large z volume with sufficient resolution to distinguish paired centrioles in the Xenopus embryo. Although single microtubules could not be resolved, spindle poles were easily identified by following the trajectory of spindle-associated membranes (Tsai et al., 2006; Ma et al., 2009), which were unaffected by LIC depletion. In control cells, centriole pairs could be clearly seen at both poles of the bipolar spindle (Fig. 6, A and Ai; and Video 6). However, in LIC morphants the poles of multipolar spindles contained single centrioles (22 single centrioles and 12 pairs in 34 poles counted; Fig. 6, Bi–Biv; and Video 7). The presence of single centrioles suggested that premature centriole disengagement was occurring. In keeping with this, we found that in LIC morphant cells with bipolar spindles (Fig. 6, C–Cii), the distance between mother and daughter centrioles was significantly increased from 440 ± 35 nm (SEM; two cells and three centriole pairs) in controls to 696 ± 100 nm (SEM; 7 cells and 11 centriole pairs: P < 0.05) in LIC morphants.
To compare the fate of centrioles after LIC depletion in human cells, we used a HeLa cell line stably expressing GFP-tagged centrin-1 as a marker for mother and daughter centrioles (Piel et al., 2000). Analysis of mitotic cells with minor multipolar spindles revealed premature centriole disengagement (Fig. 7 A), with 58% of poles having a single centriole (304 poles scored in...
Premature centriole disengagement and PCM fragmentation occurs after LIC depletion in HeLa cells. (A, B, and D) Centrin-1–GFP HeLa cells treated with scrambled (control) or LIC 1 and 2 siRNAs were labeled with anti-α-tubulin, anti-PC, and DAPI. (A) Minor multipolar spindles in centrin-1–GFP HeLa cells. White arrowheads, mother centrioles; red arrowheads, daughter centrioles. PC spots without associated centrioles are common at minor poles (cyan arrows) and rare at major poles (yellow arrow). Bars, 5 µm. (B) Centriole number in interphase centrin-1–GFP HeLa cells was scored (>200 cells scored per condition in each of three to four independent experiments, means ± SEM). (C) HeLaM KnD cells were stained with anti-CETN3 to detect centrioles. Centriole number was counted per pole in 100 mitotic cells, in three independent experiments (means ± SEM). (D) Centriole and PC distribution at each major and minor spindle pole in LIC1- and 2-depleted centrin-1–GFP HeLa cells was scored (69 cells, from two independent experiments). ***, P < 0.001, Student’s t test.

69 cells from two independent experiments). We could also rule out that centrioles were being overduplicated in interphase after LIC depletion (Fig. 7 B). The small increase in cells with more than four centrioles likely reflects cells that had previously failed cytokinesis. Indeed, LIC2 depletion leads to failed abscission (Palmer et al., 2009), and LIC1 is found at the midbody (Horgan et al., 2011). Labeling of HeLaM cells with antibodies to the centriole component CETN3 revealed that premature centriole disengagement occurred in cells depleted of LIC1 or 2 individually, as well as in combination (Fig. 7 C). The LIC2 KnDs confirm that
cohesion fatigue plays no part in the pole fragmentation, as this treatment does not affect cell cycle progression (Fig. 3 B). We conclude that the LICs play a key role in vertebrate centriole cohesion because knockdown of the LICs leads to premature centriole disengagement and the formation of multipolar spindles.

**Fragmentation of PCM contributes to multipolar spindle formation**

One possible explanation for the effect of LIC KnD on spindle morphology is that dynein without LICs fails to recruit cargoes needed for spindle pole organization. Because PC is required for centriole cohesion (Matsuo et al., 2012) and interacts with LIC1 (Tyan et al., 2000b), we tested whether depletion of LICs led to loss of PC from the centrosome, so making the centriole–centriole linkage fragile. Surprisingly, we found that depletion of both LICs had little effect on PC localization in multipolar spindles in HeLaM (Fig. S3) or centrin-GFP HeLa cells (Fig. 7 A). Quantitation of PC levels showed no change in the total amount of PC at spindle poles versus controls (P = 0.15, n ≥ 41 HeLaM cells from four independent experiments).

We analyzed the distribution of PC in LIC-depleted centrin-GFP HeLa cells to determine whether each PC spot was associated with a centriole because the fragmentation of PCM can contribute to multipolar spindle formation (Maiato et al., 2004). Cells with the minor multipolar phenotype have a bipolar spindle with two major poles and a variable number of minor extra poles. PC was found at all major poles, regardless of whether they contained a single centriole or centriole pair, but only rarely did a major pole possess PC but no centriole (Fig. 7, A [yellow arrows] and D). Minor poles with single or paired centrioles always possessed PC. However, it was also common for minor poles to be PC positive but lack centrioles (Fig. 7, A [blue arrows] and D): PCM fragmentation was therefore taking place in addition to premature centriole disengagement. Notably, PC spots were almost always a focus for microtubules (Figs. 7 A and S3), suggesting that they act as nucleation/anchoring sites. Similar centriole-free PC spots with associated microtubules have been observed after depletion of a kinesin-3 (STARD9) and protein phosphatase 2 (Torres et al., 2010, 2011). Both major poles were positive for PC in 98% of HEK cells and 93% of HeLaM cells (Fig. S4 B). In contrast, this was true in only 20% of U2OS cells (Fig. S4, A and B), suggesting a third route for multipolar spindle formation in this cell line, which generates acentrosomal poles. The centrosomes in LIC-depleted U2OS cells were often mislocalized to the very cell periphery in late G2/prophase (Fig. S4, C and D), as previously reported (Raaijmakers et al., 2013), but this was much less common in HeLaM cells (Fig. S4 E). Such peripheral centrosomes are likely not to contribute to bipolar spindle formation (Feren et al., 2009).

**Localization of spindle assembly factors in LIC-depleted spindles**

We next investigated whether dynein and dynactin were normally localized in spindles after LIC depletion. We could not visualize dynein in HeLaM spindles with available antibodies, but dynein localization in Xenopus embryo cells was unchanged in LIC morphants (Fig. S5 A). Dynactin (p150-glued) distribution was also unaffected in most LIC-depleted Xenopus (Fig. S5 B) and HeLaM cells (Fig. 8 A, P = 0.51 for individual pole intensity in control vs. LIC KnD, n ≥ 28 cells and three independent experiments). Dynactin was prominent at kinetochores in some LIC-depleted cells (Fig. 8 A), in keeping with defective chromosome alignment.

Dynein and dynactin are involved in recruiting both NuMA and TPX2 (targeting protein for Xklp2) to spindles, which in turn contribute to correct spindle assembly (Merdes et al., 2000; Radulescu and Cleveland, 2010; Neumayer et al., 2014). However, loss of LICs in HeLaM cells or Xenopus embryos did not alter the localization of either TPX2 or NuMA to spindle poles and microtubules (Fig. 8, B and C; and Fig. S5, C and D). In addition, γ-tubulin, which is part of the microtubule nucleating γ-TuRC complex (Mardin and Schiebel, 2012), was tightly focused at all poles (Fig. 8 D and Fig. S5 E), in keeping with the ability of the additional poles to nucleate and anchor microtubules. Finally, Eg5 accumulated at normal levels at all spindle poles after LIC depletion (Fig. 8 E). Importantly, these observations were reflected in quantitation of HeLaM staining: for NuMA, γ-tubulin, and Eg5, intensity at individual poles was not significantly different between control and LIC KnD (P = 0.86, 0.31, and 0.89, respectively, n ≥ 25 cells, three independent experiments). TPX2 was slightly different, in that intensity was reduced when measured at individual poles (P < 0.0001, n ≥ 27 cells, three independent experiments) but not if the sum intensity of all poles in each cell was assessed, indicating that intensity is only reduced as a result of pole fragmentation.

The clustering of Rab11-positive REs has been reported to contribute to spindle and pole integrity (Hehnly and Doxsey, 2014), and because REs in interphase cells redistribute after LIC depletion (Palmer et al., 2009), alterations in RE positioning might contribute to the spindle phenotype we see. However, we found that only 6/236 control mitotic HeLaM cells had RE clustered at spindle poles, and furthermore, the mitotic phenotypes seen after loss of REs from spindle poles (Hehnly and Doxsey, 2014) are clearly distinct from those we observe for LIC depletion. The premature centriole splitting seen upon LIC KnD in HeLaM cells and Xenopus embryos is therefore unlikely to be caused by the loss of PC, TPX2, NuMA, γ-tubulin, Eg5, dynein, or dynactin from the spindle pole or to redistribution of REs.

**Source of the forces driving centriole separation after LIC depletion**

Because key spindle pole proteins were localized normally, we next examined whether the LIC KnD phenotype was caused by an alteration of forces within the spindle. For example, the forces exerted on spindle poles via K fibers play a part in spindle organization and function. Dynein pulls K fibers toward the poles (Goshima et al., 2005; Elting et al., 2014; Sikirzhytski et al., 2014), so generating inward tension, while kinesin-12, CENP-E, and chromokinesins push poles apart (Tanenbaum and Medema, 2010; Maiato and Logarinho, 2014). To test the role of K–fiber–mediated forces in spindle assembly after LIC depletion, we codepleted the kinetochore component NuF2 to prevent K-fiber formation (Manning and Compton, 2007; O’Connell et al., 2009; Tosso et al., 2009; Logarinho et al., 2012).
centrosomes (Tanenbaum et al., 2008; Ferenz et al., 2009; Florian and Mayer, 2012; Raaijmakers et al., 2013). We examined spindle morphology in embryos treated with STLC or DMSO as a control. Embryos injected with control MO and treated with STLC developed monopolar spindles in >90% of mitotic cells (Fig. 9, C and D; and Tables S1 and S2). In contrast, LIC morphants formed few monopolar spindles after STLC treatment. Monopolar spindle formation upon Eg5 inhibition was also reduced in LIC-depleted HeLaM cells compared with controls (Fig. 9 F). Together, these results indicate that dynein without LICs is less able to generate the force necessary to collapse the spindle to a monopolar structure when Eg5 is inhibited.

Strikingly, treatment with STLC also significantly reduced the formation of spindles with one or two additional poles in LIC morphants and concomitantly increased the proportion of bipolar spindles (Fig. 9, C and D; and Tables S1 and S2). This was not caused by a change in the number of mitotic cells because STLC treatment did not significantly alter the mitotic index in LIC morphants (Fig. 9 E). inhibition of Eg5 in HeLaM cells also rescued both the minor and major multipolar phenotypes in LIC-depleted cells (Fig. 9 F), although

Nut2 KnD alone generated multipolar spindles (Fig. 9 A and Tables S1 and S2 for statistical analysis; Logarinho et al., 2012) and in combination with LIC depletion led to >80% of cells having multipolar spindles. Because CENP-E inactivation also suppresses premature centriole splitting in some cases (Mattuuzzo et al., 2011; Logarinho et al., 2012), we inhibited CENP-E using GSK-923295 (Wood et al., 2010). This generated minor multipolar spindles in scrambled-treated cells, as previously seen (Thein et al., 2007), and also worsened the spindle defect in LIC-depleted cells (Fig. 9 B). Altogether, these results suggest that forces acting on poles via kinetochores and K fibers do not contribute to pole fragmentation after LIC depletion, but instead, their loss exacerbates the spindle defects.

We next tested the role of Eg5, which drives centrosome separation during bipolar spindle assembly. Inhibiting Eg5 with monastrol or S-trityl-l-cysteine (STLC) generates monopolar spindles (Mayer et al., 1999; Skoufias et al., 2006). Depletion or inhibition of dynein prevents the formation of monopolar spindles after Eg5 inhibition, indicating that Eg5 and dynein-driven forces normally function antagonistically to separate the centrosomes (Tanenbaum et al., 2008; Ferenz et al., 2009; Florian and Mayer, 2012; Raaijmakers et al., 2013). We examined spindle morphology in embryos treated with STLC or DMSO as a control. Embryos injected with control MO and treated with STLC developed monopolar spindles in >90% of mitotic cells (Fig. 9, C and D; and Tables S1 and S2). In contrast, LIC morphants formed few monopolar spindles after STLC treatment. Monopolar spindle formation upon Eg5 inhibition was also reduced in LIC-depleted HeLaM cells compared with controls (Fig. 9 F). Together, these results indicate that dynein without LICs is less able to generate the force necessary to collapse the spindle to a monopolar structure when Eg5 is inhibited.
A clear consequence of LIC loss is the formation of multipolar spindles, which we show occurs via two distinct major routes. One of these—the assembly of multipolar spindles in cells containing extra centrosomes—is commonly seen after reducing dynein activity using a range of approaches (Robinson et al., 1999; Wojcik et al., 2001; Maiato et al., 2004; Goshima et al., 2005; Morales-Mulia and Scholey, 2005; Quintyne et al., 2005; Tanenbaum et al., 2008; Firestone et al., 2012; Raaijmakers et al., 2017). The rescued cells were more likely to have monopolar than bipolar spindles. Eg5-driven forces are therefore required to split the centrioles prematurely when LICs are depleted.

**Discussion**

Dynein plays crucial roles in the spindle in higher eukaryotes, and we find that many of these are affected by LIC depletion. A clear consequence of LIC loss is the formation of multipolar spindles, which we show occurs via two distinct major routes. One of these—the assembly of multipolar spindles in cells containing extra centrosomes—is commonly seen after reducing dynein activity using a range of approaches (Robinson et al., 1999; Wojcik et al., 2001; Maiato et al., 2004; Goshima et al., 2005; Morales-Mulia and Scholey, 2005; Quintyne et al., 2005; Tanenbaum et al., 2008; Firestone et al., 2012; Raaijmakers et al., 2017).

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**Figure 9. Inhibition of Eg5, but not loss of K fibers, prevents the formation of minor multipolar spindles.** (A, B, and F) HeLaM cells were treated as indicated, labeled, and scored for mitotic phenotype (>100 cells in each of four independent experiments, means ± SEM). [A] Scrambled (SC) or LIC siRNA-treated HeLaM cells were incubated for 48 h and then transfected with scrambled or NuF2 siRNAs and fixed after a further 24-h incubation. [B] Depleted cells were treated with DMSO or 100 nM CENP-E inhibitor (GSK-923295; CI) for 90 min before fixation. (C) Confocal images of mitotic spindles in control (Ctrl) or LIC1 and 2Xenopus morphants treated with DMSO or STLC to inhibit Eg5. Spindles were stained with anti-α-tubulin, DAPI, and anti-γ-tubulin. Insets show zoom in of a monopolar spindle: two adjacent γ-tubulin foci indicate a single pole. Bar, 20 µm. (D) Quantitation of spindle morphology in control and LIC1 and 2 morphants treated with DMSO or STLC (means ± SEM, six independent experiments). (E) The mitotic index of control (C) and LIC morphants was determined ± STLC treatment (ns = not significant, Student’s t test, means ± SEM, n = 6 independent experiments). (F) Scrambled or LIC siRNA-treated HeLaM cells were treated with DMSO or 2 µM STLC for 2 h. See Tables S1 and S2 for two-way ANOVA results for A, B, D, and E.
et al., 2013). The second main route involves the active fragmentation of poles after spindles have assembled, by a combination of premature centriole disengagement and PCM fragmentation. This phenotype, not previously described for dynein disruption, identifies a novel role for dynein in maintaining centrosomal structure during mitosis.

We find that Eg5 is the major driving force behind this pole splitting, because inhibiting Eg5 profoundly reduced the formation of minor additional poles. Although forces exerted via K fibers contribute to pole splitting in some situations (Maiato and Logarinho, 2014), this is not the case for LIC-depleted cells, because inhibition of CENP-E or the removal of K fibers by Nuf2 depletion both worsened spindle pole fragmentation, rather than rescuing the defect. Indeed, it is likely that K fibers normally contribute to spindle stability because dynein can mediate K-fiber sliding toward the poles (Goshima et al., 2005; Elting et al., 2014; Sikirzhytski et al., 2014), which would generate inwards pulling forces (Tanenbaum and Medema, 2010). Our data also suggest that the Eg5 forces that lead to fragmentation are exerted via the interpolar microtubules rather than the K fibers. Moreover, this excludes a role for kinesin-12 (and its interactor TPX2) in pole fragmentation because it is lost from the spindle after Nuf2 depletion, whereas Eg5 is not (Sturgill and Ohi, 2013).

Importantly, both Eg5 and TPX2 are normally localized after LIC depletion, and the kinetochores of aligned chromosomes are under tension.

Exertion of a force sufficient to cause premature centriole disengagement requires that both centrioles have firmly attached microtubules. Although the mother centriole is the primary site of microtubule nucleation during interphase and mitosis (Wang et al., 2011), single daughter centrioles can clearly recruit PCM components and nucleate microtubules during late G2/prophase (Sluder and Rieder, 1985; Hut et al., 2003; Thein et al., 2007; Stevens et al., 2011; Kleylein-Sohn et al., 2012; Logarinho et al., 2012). Because dynein activity is needed for microtubule anchoring at the centrosome (Heald et al., 1997; Burakov et al., 2008), this might explain why DHC depletion does not lead to premature centriole separation but rather gives multipolar spindles with broad, disorganized poles that often consist of small microtubule bundles that lack associated γ-tubulin (Robinson et al., 1999; Wojcik et al., 2001; Maiato et al., 2004; Morales-Mulia and Scholey, 2005; Tanenbaum et al., 2008; Raaijmakers et al., 2013). Such spindles typically possess only two centrosomes, which are often dissociated from the spindle itself. In contrast, microtubules are clearly anchored to both mother and daughter centrioles in LIC-depleted cells and to the PC spots that are not associated with centrioles, which would allow Eg5 to drive pole fragmentation. The LICs are therefore not required for dynein’s microtubule anchoring activity. The microtubules are also well focused, suggesting that components such as NuMA and TPX2 are not only localized normally but are fully functional.

A key question is why LIC loss should allow Eg5 to drive apart centrioles and fragment the PCM. Centrosomes normally resist the forces exerted on them during spindle assembly, and the centrioles only come apart in late mitosis or early G1. In vertebrates, this involves separate-mediated cleavage of centrosomal cohesin (Tsou and Stearns, 2006; Ban et al., 2007; Wang et al., 2008; Nakamura et al., 2009; Tsou et al., 2009). One possibility is that centriole disengagement is triggered too early after LIC depletion, perhaps as a result of reduced recruitment of cohesin or proteins, such as Emi, that block cohesin cleavage (Ban et al., 2007; Wang et al., 2008; Nakamura et al., 2009). Indeed, cohesin and Emi interact with NuMA (Ban et al., 2007; Kong et al., 2009), whereas Emi, NuMA, and dynactin rely on each other for centrosomal localization (Ban et al., 2007). However, because LIC depletion does not affect NuMA, dynactin, or dynein localization, the centriole disengagement we see is unlikely to be caused by loss of Emi. Astrin and kinastrin also protect cohesin from premature separase cleavage, and their depletion leads to multipolar spindles with single centrioles at poles (Thein et al., 2007; Dunsch et al., 2011). Although both proteins are part of a complex that includes the dynein light chain DYNLL1 (Schmidt et al., 2010; Dunsch et al., 2011), this complex is needed for spindle positioning rather than centriole cohesion (Dunsch et al., 2012). Importantly, the centriole disengagement seen after manipulation of cohesin cleavage usually occurs after a long prometaphase arrest and premature sister chromatid separation (Maiato and Logarinho, 2014), which we do not see.

Cohesin and separase are not the only components that maintain centriole cohesion (Daum et al., 2011; Stevens et al., 2011; Oliveira and Nasmyth, 2013): structural PCM components may also play an important role (Cabral et al., 2013), some of which are transported by dynein (Doxsey et al., 2005). Of these, PC is particularly interesting, as it interacts with LIC1 but not LIC2 (Tyan et al., 2000b) and is cleaved by separase (Matsuo et al., 2012). However, although depleting LICs led to PC being redistributed to extra poles, the total amount of PC at poles was not affected. Furthermore, depletion of LIC2 alone led to spindle pole fragmentation.

Alternatively, pole fragmentation may result from an imbalance of forces within the spindle poles, primarily between dynein and Eg5. This would be analogous to the well-known opposing roles these motors play in setting up the bipolar spindle, where both motors exert force on antiparallel microtubules nucleated by the two centrosomes. This would occur primarily on interpolar microtubules, as discussed earlier. We propose that a similar situation exists between microtubules nucleated by mother and daughter centrioles during mitosis and within the PCM surrounding each centrosome, with dynein pulling the structures closer together and Eg5 pushing/pulling them apart. After LIC depletion, Eg5 forces likely outweigh those generated by dynein. This is not because of dynein or dynactin mislocalization, however. Instead, we suggest that the loss of LICs reduces dynein activity in a crucial way in a cellular context. Perhaps it generates less force, is less processive, or is less able to interact with regulatory molecules. In all these scenarios, the ability of dynein to drive cargoes under high loads, as in the spindle, will be reduced.

An observation that supports our proposal that LIC depletion reduces dynein activity in vivo comes from cells that enter mitosis with additional centrosomes. Many cancer cell lines with such extra centrosomes can nevertheless form bipolar spindles as a result of dynein and KIFC1 cooperating to cluster the centrosomes into two spindle poles, counteracting the Eg5 and
K fiber–dependent forces that push centrosomes apart (Quintyne et al., 2005; Kwon et al., 2008; Leber et al., 2010; Drosopoulos et al., 2014). However, when dynein or KIFC1 is inactivated, such cells form multipolar spindles. We see the same phenotype in LIC-depleted cells with extra centrosomes, demonstrating that dynein’s ability to counter Eg5-generated forces is compromised.

Reduced force generation in cells with normal centrosome number is also revealed when we inhibit Eg5. This normally leads to the formation of monopolar spindles, caused in part by dynein’s ability to cause antiparallel microtubule sliding (Ferenz et al., 2009; Tanenbaum et al., 2013), but when dynein is also inactivated, bipolar spindles can form (Tanenbaum et al., 2008; Ferenz et al., 2009; Florian and Mayer, 2012; Raaijmakers et al., 2013). The fact that LIC depletion rescues bipolarity in the presence of STLC (Fig. 9; Raaijmakers et al., 2013) strongly suggests that loss of LICs compromises dynein function. Furthermore, bipolar chromosome alignment on the spindle is delayed, despite dynein and dynactin being present at kinetochores. In addition, the detachment of centrosomes from the nucleus in prophase (this work; Raaijmakers et al., 2013) suggests that the dynein at the NE has reduced activity.

How does this proposal fit with our finding that purified dynein can drive microtubule gliding in vitro normally? Recent work has shown that although purified dynein is active in gliding assays in which many motors act together, single dynein molecules are not able to move along microtubules unless they are in a complex with dynactin and one of several adaptor molecules (McKenney et al., 2014; Schlager et al., 2014). The LIC1 and 2 interactor FIP3 is one such adaptor molecule. Perhaps the lack of LICs hinders the formation of a subset of these complexes, impairing certain dynein functions but leaving others intact, such as microtubule anchoring and focusing of spindle poles. Interestingly, although LIC1 and 2 have some distinct interactors and functions, we find that the LICs act redundantly in spindle formation because depletion of either LIC causes premature centriole disengagement, and overexpression of either LIC will rescue spindle bipolarity. Redundant roles for LICs in cell cycle progression, chromosome alignment, and centrosome–NE anchoring have also been reported (Raaijmakers et al., 2013). Given that invertebrates have only a single LIC gene, the redundancy in mitotic LIC functions in vertebrates suggests that these are key ancestral roles for metazoan dynein.

Altogether, our results indicate that, as well as counteracting each other in the spindle to maintain centrosome separation, Eg5 and dynein also function antagonistically within the centrosome to maintain centriole cohesion and that the LICs are key for dynein’s role in this process. This novel role has become apparent because LIC depletion does not disrupt the attachment of microtubules to the centrioles, unlike DHC depletion. Our findings are consistent with a model whereby Eg5 produces an outward force that drives the centrioles apart, which is counteracted by an inward LIC-dependent dynein-driven force. This balance of forces could help hold mother and daughter centrioles together during mitosis: a slight change in the balance could then assist centriole disengagement once mitosis is complete.

Materials and methods

Reagents

This project used mouse antibodies to dynactin p150 (BD), dynactin IC (JC74; EMD Millipore), GM130 mouse (BD), NuMA (Oncogene), phospho-histone H3 (EMD Millipore), TAT1 (K. Gull, University of Oxford, Oxford, England, UK), or DM1A (Sigma-Aldrich). The following rabbit antibodies were used: DHC (Sigma-Aldrich), KIF5B (R. Vale, University of California, San Francisco, San Francisco, CA), PC (Abcam), KIF1Bα (Bethyl Laboratories, Inc.), KIF1C (Abcam), KIF11 (Eg5; Sigma-Aldrich), LIC2 (R. Vallee, Columbia University Medical Center, New York, NY; Tan et al., 2011); NuMA (A. Merdes, Paul Sabatier University, Toulouse, France), TPX2 (C. Wiese, University of Wisconsin-Madison, Madison, WI; O’Brien and Wiese, 2006), and γ-tubulin (Keating and Borisy, 2000). The following sheep antibodies were used: Sub1, Sub1, and CENPF (S. Taylor, University of Manchester, Manchester, England, UK; Taylor et al., 2001; Hussein and Taylor, 2002), dynein N96 anti-Xenopus IC (Lane and Allan, 1999), and lamin A/C (Santa Cruz Biotechnology, Inc.). In addition, rat anti–γ-tubulin, YOL1-1 (J. Murray, University of Pennsylvania, Philadelphia, PA), human antitubulcinotermere antibodies (S. Taylor), and chicken UC1 (R. Vallee; Tan et al., 2011) were used. Fluorescently labeled secondary antibodies were obtained from Jackson ImmunoResearch Laboratories, Inc. or Invitrogen. IRDye 700CW– and 800CW–labeled secondary antibodies were obtained from LICOR Biosciences. HRP-conjugated secondary antibodies were purchased from Dako.

Cell culture and transfection

HeLa and HEK cells were grown in DMEM and 10% FCS at 37°C and 8% CO2. HeLa cells stably expressing centrin-1–GFP (Piel et al., 2000) were grown in the same medium but with the addition of G418. U2OS [human osteosarcoma cells] were grown in McCoy’s 5A and 10% FCS at 37°C with 5% CO2, siRNA transfections used INTERFERin (MP Biomedicals). siRNAs were obtained from Eurofins MWG Operon, except siGENOME lamin A/C, scrambled control, and NuF2 (Thermo Fisher Scientific). For LICs, cells were analyzed 48 h after transfection with 20 nM (mitosis experiments) or 72 h after transfection with 5–20 nM alginolucitide (other experiments). For DHC, three siRNAs (6.67 nM each) were used, and cells were analyzed after 72 h. The following sequences were used: LIC1, 5′-AGAUGACAGUGAGUAGUGUAA-3′ (Palmer et al., 2009); LIC2, 5′-ACCCUGCAGUUUGUAAUA-3′ (Palmer et al., 2009); LIC2, 5′-CAACCAACAUAGACUUACA-3′; DHC 1b, 5′-GAGAGGGGUAAUULUAA-3′; and DHC 1c, 5′-GCAAGAAAUGUGCCUAUUUA-3′. For NuF2 codeligation, cells were treated with scrambled or LIC1 and 2 siRNAs and then 48 h later were treated with either scrambled or a NuF2 SMARTpool and fixed after a further 24 h.

Human LIC1 and LIC2 (GenBank accession nos. AF078849 and AF033812) were extracted from a HeLa cDNA library and cloned into pTurboFp635-C (mKate; Evrogen). Noncoding mutations resulting in siRNA-resistant LIC1 and LIC2 were generated by PCR-based mutagenesis using the following primers: LIC1, 5′-CAGAAGATGACAGTTCGTCGTCCTCCTCGGGTCCG-3′; and LIC2, 5′-GAGAAAAACCTCGACTTGCTATACAAGTTATGGTTCC-3′. For rescues, cells were transfected with scrambled or LIC1 and 2 siRNAs for 48 h and then transfected in fresh media with siRNA-resistant LIC1-mKate or LIC2-mKate using FuGENE 6 (Promega) and fixed 24 h later.

Xenopus microinjection and MOs

Female Xenopus frogs were preprimed 4–7 d in advance with 50 U of pregnant mare serum gonadotropin (Intervet UK) and then primed with 500 U of human chorionic gonadotropin (Intervet UK) 18 h before use. Frogs were transferred to Marc’s modified Ringer’s (MMR; 100 mM NaCl, 2 mM KCl, 1 mM MgCl2, and 5 mM Hepes, pH 7.4) for egg collection. In vitro fertilization was performed by adding macerated testis to the eggs. After 30 min, embryos were dejellied in 2% cysteine (0.1x MMR) and rinsed five times in 0.1x MMR.

Embryos were microinjected into each cell at the two- or four-cell stage in 0.1x MMR plus 5% Ficoll using a Ficspirator III (Parker Instrumentation). The solution conditions were used throughout this work. The total volume was set to 5 or 2.5 nl, respectively. MOs to LIC1 and LIC2, stored as a stock solution of 1 mM diluted in water, were heated for 5 min at 65°C before being further diluted in standard control (MO sequence, 5′-CCCTTACCT-CAGTCAAATTTA-3′; Gene Tools, LLC) to the desired concentration. Translation-blocking MOs were designed against Xenopus LICs as follows: LIC1 (MO sequence, 5′-CCCTTACCT-CAGTCAAATTTA-3′; Gene Tools, LLC) and LIC2 (MO sequence, 5′-GGAGGAGGGGUAAUULUAA-3′; Dharmacon)

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Gene Tools, LLC) and were injected at a needle concentration of 0.25 mM. After microinjection, embryos were incubated at 16°C.

For rescue experiments, Xenopus LC1 and LC2 were cloned from IMAGE (Integrated Molecular Analysis of Genomes and their Expression) cDNA clones (7011679 and 8074560, respectively) and N-terminally tagged with EGFP by insertion into a custom pCS2+ N-EGFP vector. Full-length LC1 was used, but LC2 was slightly truncated at the N terminus to remove the first five amino acids, which correspond to the LC2 MO sequence, and was also truncated by 109 amino acids at the C terminus (LC2<sup>nm</sup>–<sup>377</sup>). Rescue experiments were performed by microinjecting both LC1 and LC2 translation-blocking MoIs into each cell at the two-cell stage and then injecting capped mRNA encoding LC1 and LC2<sup>nm</sup>–<sup>377</sup> into all cells of the four-cell stage embryo.

Drug treatment
For Xenopus experiments, STLC (Sigma-Aldrich; 50 mM stock in DMSO) was diluted to 1 mM in PBS and injected into the blastocoele of MO-treated embryos 21.5 h after fertilization (16°C). The needle volume was set to 18 nl, and each embryo was injected twice, into each side of the blastocoel. Control embryos were injected with the same volumes of DMSO diluted 1:50 in PBS. Embryos were incubated at RT for 2 h before fixation.

For HeLaM drug treatments, cells were transfected with scrambled or LC1 and 2 siRNAs and incubated for 70–72 h. Drug treatments were performed in DMEM + 10% FCS at 37°C followed by fixation and immunofluorescence analysis. Eg5 was inhibited with 2 µM STLC for 2 h. CENP-E was inhibited with 100 nM GSK-923295 (10 mM stock in DMSO; Medchem Express; Wood et al., 2010) for 90 min.

Immunofluorescence
Typically, Xenopus embryos were fixed for immunofluorescence 21.5 h after fertilization (16°C, stage 10.5) and processed as described previously (Danilchik et al., 1998), omitting the methanol postfix step: embryos were fixed by overnight incubation at RT in microtubule fix (3.7% paraformaldehyde, 0.25% glutaraldehyde, and 0.2% Triton X-100 in microtubule assembly buffer [80 mM Pipes, 1 mM MgCl<sub>2</sub>, 5 mM EGTA, 0.05 mM NaCl]) and each embryo was injected twice, into each side of the blastocoel. Control embryos were injected with the same volumes of DMSO diluted 1:50 in PBS. Embryos were incubated at RT for 2 h before fixation.

For HeLaM drug treatments, cells were transfected with scrambled or LC1 and 2 siRNAs and incubated for 70–72 h. Drug treatments were performed in DMEM + 10% FCS at 37°C followed by fixation and immunofluorescence analysis. Eg5 was inhibited with 2 µM STLC for 2 h. CENP-E was inhibited with 100 nM GSK-923295 (10 mM stock in DMSO; Medchem Express; Wood et al., 2010) for 90 min.

Live-cell imaging
To score mitotic index, HeLaM cells grown on coverslips were imaged on a simple phase-contrast microscope without fixation, and cells in mitosis and interphase were counted in several fields. To image the duration of mitosis in HeLaM cells, six cells were grown in glass-bottomed 24-well plates (VWR International), transfected with siRNA duplexes, and imaged 42 h later at 37°C in Ham's F12 media with 10% FCS on a microscope (IX81; Olympus) fitted with a H117 stage (ProScan; Prior Scientific). Phase-contrast images were acquired, using point viewing, every 5 min for 17 h, using a 20×, 0.50 NA Plan Fluor phase objective, a long-path diode (λ = 530 nm, Cairn Research), and an electron-multiplying charge-coupled device camera (Cascade, Photometrics). Mitosis duration was defined as the interval between the first sign of cell rounding to the first sign of cytokinesis.

For live imaging of mitotic spindles in Xenopus embryos, both cells of two-cell embryos were injected with MO before being microinjected with 2.5 nl of mRNA for EGFP–α-tubulin (nucleotide concentration of 0.5 mg/ml) or mRNA–p28 (0.1 mg/ml) into each cell of the four-cell stage. Embryos were incubated for 20 h (postfertilization) at 16°C and then mounted for live imaging in 0.1× MMR, using a ring of vacuum grease to contain the embryos and support a glass coverslip (Woolner et al., 2009). Imaging took place at developmental stages 10–11, so covering the 10.5 gastrula stage used in our fixed analysis. Single focal plane live-cell images of spindles were collected at RT (~21°C) using a confocal microscope (FluoView FV1000; Olympus) with FluoView acquisition software (Olympus) and a 60×, 1.35 NA Plan Apo S Apochromat objective. Time-lapse videos were constructed from the single focal plane images using ImageJ.

Scanning EM
Embryos were injected with standard control or LC1 and LC2 MOs and fixed in 3.7% paraformaldehyde and 0.25% glutaraldehyde in BRBB0 (BRBB0 = 50 mM stock in 1 mM MgCl<sub>2</sub>, pH 8.0) for 1 h followed by 1 h of fixation (incubated at 16°C). Samples were then processed using a high density staining method suitable for block face imaging (see supplementary protocol in Williams et al., 2011). In brief, the samples were fixed for 1 h in 2% (wt/vol) osmium tetroxide and 1.5% (wt/vol) potassium ferrocyanide in cacodylate buffer followed by 20 min in freshly prepared 1% (wt/vol) thiocarbohydrazide and then 30 min in 2% (wt/vol) osmium tetroxide and 1.5% (wt/vol) potassium ferrocyanide in 2% (wt/vol) osmium tetroxide and 1.5% (wt/vol) potassium ferrocyanide. The samples were then stained with freshly prepared Walden’s lead aspartate (0.02 M lead nitrate and 0.03 M aspartic acid, adjusted to pH 5.2) for 30 min followed by dehydration, embedding in Epon 812 (hard formulation), and trimming on a standard microtome. The samples were examined using a microscope (3View; Gatan) within a scanning electron microscope (Quanta 250 FEI; FEI). The imaging conditions were as follows: indicated quadrant magnification of 1,600×, accelerating voltage of 3.8 kV, pressure at 0.33 Torr, image at 4,000 × 5,000 pixels, and dwell time of 10 µs. Raw data was submitted to the Yasbin database at the FEI. The samples were then imaged with a Hitachi HD-2000 at the University of California, Davis (UCD), equipped with a secondary electron detector (SE) and an energy dispersive X-ray spectrometer (EDX). The data was then analyzed using the in-house quantitative analysis program (Yasbin).
were converted to an MRC file stack using IMOD (Kremer et al., 1996; procedures discussed in detail in Starborg et al., 2013). Imaging noise was removed using 2D Gaussian smoothing with a 3 x 3 kernel to aid manual segmentation. Regions of interest were cropped out using IMOD for examination and segmentation with Imaris (Bitplane). Chromosomes and centrioles were reconstructed with Imaris using the Surfaces tool. To measure the distance between centriole pairs, the Measurement points tool in Imaris was used at the center of the reconstructed surface for each centriole, and the distance between pairs was measured.

**Biochemical analysis**

Total HeLaM cell extracts for immunoblotting analysis were prepared by lysis in hot SDS sample buffer. For sucrose gradient analysis after K1 treatment, a 10-cm dish of HeLaM cells was used per condition. Cells were trypsinized, washed in complete medium, and then washed twice in BRBB. Cells were lysed for 15 min on ice in 0.5 ml BRBB plus 0.5% Triton X-100 containing Protease Inhibitor Cocktail III (Sigma-Aldrich). Lysates were centrifuged at 14,000 rpm for 10 min at 4°C and then incubated on ice for 1 h after the addition of 0, 100, or 150 mM KI (Ori-McKenney et al., 2010). Supernatants [45 µl] were applied to 5–20% (wt/vol) continuous sucrose gradients in BRBB plus 100 or 150 mM KI, as appropriate, prepared in 0.5 ml centrifuge tubes (Beckman Coulter). Samples were centrifuged in a rotor (SW 55; Beckman Coulter) with adapters, for 5 h at 38,000 rpm, 4°C, and 75 µl fractions were collected.

For dynein purification, four 15-cm dishes of HeLaM cells were transfected with LIC1/2 siRNAs, cultured overnight, and then split onto 12 dishes. Control dynein was prepared from eight 15-cm dishes of untransfected cells. Cells were trypsinized, washed in complete medium, and then washed twice in ice-cold 2% DMSO. Cells were lysed for 15 min on ice in 2 vol BRBB with 1% Triton X-100, 1 mM DTT, 0.2 mM PMSF, 10 µg/ml protease inhibitors (leupeptin, aprotinin, pepstatin, and chymostatin), and 10 µg/ml cytochalasin D. Lysates were centrifuged at 2,500 rpm for 15 min at 4°C, and then, the supernatants were spun at 110,000 g for 30 min at 4°C. The resulting supernatants were supplemented with 10 U/ml hexokinase, 20 mM glucose, 100 mM AMP-PNP (adenosine 5′-(β,γ-imino)triphosphate), 20 µM taxol, 1 mM DTT, 0.5 µg/ml cytochalasin D, and 100 µg/ml taxol-stabilized microtubules prepared from bovine brain tubulin (Cytoskeleton, Inc.). After 20 min at 30°C, microtubules were pelleted at 70,000 g, in a rotor (TLS-55; Beckman Coulter) at 25°C for 10 min through a 20% (wt/vol) sucrose cushion in BRBB, 1 mM DTT, 4 µM taxol, 1 µg/ml cytochalasin D, and 2.5 µg/ml protease inhibitors. Pellets were washed in BRBB, 1 mM DTT, 20 µM taxol, 10 µg/ml cytochalasin D, and 10 µg/ml protease inhibitors and spun as in the previous step. Pellets were resuspended in 50 µl of the same buffer containing 5 mM MgATP and incubated for 25 min at 30°C to release motors. Samples were then spun for 10 min at 70,000 g, at 22°C (TLS55). Supernatants were collected and loaded onto 600 µl 8–20% (vol/vol) sucrose continuous density gradients in BRBB containing 2.5 µg/ml protease inhibitors and 1 mM DTT. Gradients were centrifuged at 38,000 rpm for 6 h at 4°C in a rotor (MLS-50; Beckman Coulter) with adapters, and then, 50 µl fractions were collected and analyzed by SDS-PAGE on 4–15% or 5–15% gels followed by silver staining or immunoblotting using antibodies against kinesins and used for motility assays.

**In vitro motility assays**

Microtubule flow chambers were made (Allan, 1993) using coverslips cleaned by sonication for 10 min in 5% Lipsol detergent at 70°C followed by at least four washes in deionized water at 70°C with sonication. After air drying, coverslips were plasma cleaned for 30 s at 50 mTorr in a glow discharge (Emitech K100X; Quorum Technologies). Flow cells were coated for 5 min with dynein motility buffer (DMB; 10 mM K-Pipes, 50 mM K-Acetate, 2 mM MgSO4, 0.5 mM EGTA, and 10% glycerol, pH 6.9, plus 1 mM DTT and 0.2% casein) and then incubated for 5 min with 4–6 µl of motor fraction diluted to 12 µl with DMB. After flowing through 40 µl DMB containing 1 mM ATP, 10 µM taxol, and 8–10 µg/ml taxol-stabilized microtubules, microtubule gliding was visualized by video-enhanced differential interference contrast microscopy. This was performed on a microscope (BX60) equipped with a 60x, 1.40 NA Plan Achromat objective and a 1.40 NA oplanaphilam immersion universal condenser, with low shear Wallaston prisms (UDICV). Illumination was provided by a 100-W Hg lamp coupled to the microscope by a 1-mm fiber optic scanner (Technical Video), passed through 546-nm (20-nm bandwidth), heat, and UV filters. Images were passed through a 5x photo projection lens and captured by a video camera (Newvicon; Hamamatsu Photonics), and background subtraction and two-frame rolling averaging were performed by an image processor (Argus-10; Hamamatsu Photonics). Sequences were recorded onto DVD and then digitized using a Flashlab Spectrin-Pro card (Aegis Electronic Group) using SteamFixd software (Naripix), and rates of gliding were obtained using ImageX. Videos were made from cropped sequences by taking every fourth image and then performing a two-frame rolling averaging using the ImageX RunningZProtractor plugin. QuickTime videos were made using QuickTime 7 Pro with H.264 compression (Apple).

**Statistical analysis**

Statistical tests were performed using SPSS software (SPSS, Inc., Excel [Microsoft], and Prism (GraphPad Software). For experiments assessing the duration of mitosis, the percentage of cells completing mitosis in a given time was plotted as a cumulative frequency plot and analyzed using the Kaplan–Meier survival analysis test. For microtubule gliding, the mean speed per assay (12–24 microtubules, three independent experiments, 58–67 microtubules per condition in total) was determined from digitized video sequences using ImageJ. The means of these averages were calculated with SEM, and one-way analysis of variance (ANOVA) was performed. For analysis of fixed cultured cells, >100 cells per condition were scored per experiment, and the means of three independent experiments are shown, unless otherwise stated. Analysis was performed using two-way ANOVA with Tukey’s test.

To compare the mitotic index in control MO and LIC MO embryos, a one-way ANOVA with Dunnett’s multiple comparison test was performed; n = 3 independent experiments, and 14–16 embryos were analyzed per condition, with total cell counts between 1,097 and 1,992 cells. To test for significance after STLC treatment, Student’s t-tests (unpaired, two-tailed, samples of unequal variance) or two-way ANOVA with Tukey’s test were performed; n = 6 independent experiments, and 39–41 embryos were analyzed for each condition, assessing a total of between 375 and 1,175 spindles for spindle morphology and between 3,409 and 7,338 cells for mitotic index.

**Online supplemental material**

Fig. S1 shows biochemical analysis of microtubule motors prepared from HeLaM cells. Fig. S2 shows the effect of LIC loss on spindle morphology and kinetochores in human cells. Fig. S3 shows PC localization and spindle morphology in single and double LIC-depleted HeLaM cells. Fig. S4 shows the effect of LIC depletion on spindle assembly, pole composition, and prophase centromere position in U2OS and HEK cells. Fig. S5 shows dynein, dynactin, and spindle assembly factors localize normally to LIC1- and 2-depleted spindles in Xenopus cells. Tables S1 and S2 provide the statistical analysis of the data in Fig. 9. Video 1 shows that loss of LIC does not affect dynein motor function in vitro. Videos 2–5 show mitotic spindle assembly in Xenopus embryos injected with control (Video 2) or LIC1 and 2 (Videos 3–5) MOs. Multipolar spindle assembly is shown in Video 3, with pole fragmentation shown in a bipolar (Video 4) or multipolar spindle (Video 5). Videos 6–8 show 3View EM of a control spindle (Video 6), an LIC KD multipolar spindle (Video 7), and an LIC KD bipolar spindle (Video 8). Online supplemental material is available at http://www.jcb.org/cgi/content/full/jcb.201408025/DC1.

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**References**


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Figure S1.  **Biochemical analysis of microtubule motors.** (A) Microtubule motors isolated from control and LIC-depleted HeLaM cells were separated on sucrose density gradients and analyzed by SDS-PAGE and silver staining. Asterisks indicate kinesin-1 heavy chain; mm indicates the molecular mass marker lane, and numbers correspond to gradient fractions (1 = top; 12 = bottom). A side-by-side comparison of the peak dynein fraction from control and LIC-depleted cells is shown in Fig. 1. The dynein with and without LICs migrates at the same position on the gradient and contains similar proportions of IC and light chains (LC), indicating that its structure is unaffected by LIC loss. Dynactin is also present in similar amounts and migrates at the same density in both samples. The remaining band at ~60 kDa in the LIC-depleted dynein fractions is likely to be the dynactin p62 subunit. The molecular mass maker sizes are shown in kilodaltons. (B) Immunoblotting of a control microtubule motor preparation using the indicated antibodies to kinesin family members shows that sucrose density gradient centrifugation effectively separates dynein from kinesins. (C) Histogram of microtubule gliding rates in vitro generated by purified dynein with and without LICs (58 and 67 microtubules, respectively, from three independent experiments).
Figure S2. Effect of LIC loss on spindle morphology and kinetochores. (A–D) HeLaM (A, C, and D) or HEK (B) cells were transfected with scrambled siRNAs or a combination of LIC1 and LIC2 siRNAs and then fixed 72 h later, labeled, and imaged. (A) A DeltaVision deconvolved z series projection showing a major multipolar (top cell) and minor multipolar (bottom cell) phenotype in HeLaMs labeled with anti-α-tubulin and DAPI. (B) The phenotype of mitotic HEK cells was scored (>650 cells in total from three independent experiments; means ± SEM). P-values from two-way ANOVA with Sidak’s multiple comparison test for control versus LIC depletion: ***, P < 0.001; ****, P < 0.0001. SC, scrambled. (C) LIC-depleted HeLaM cells were labeled with antibodies to the centromere (anticentromere antibodies [ACA]), Bub1, or BubR1 to visualize the kinetochores. Deconvolved z-series projections are shown, with enlargements of the boxed regions. (D) HeLaM cells depleted of both LCs, or untreated cells, were treated with 10 μM nocodazole for 60 min to depolymerize microtubules, fixed, and labeled with antibodies to IC and CENPF, which localizes to kinetochores. Images are projections of a z series of deconvolved images. Bars: (A and C, main images) 5 μm; (C, enlarged images) 2 μm; (D) 10 μm.
Figure S3. PC localizes to the centrosome in LIC1, LIC2, and LIC1 and 2 siRNA-treated HeLaM cells. (A–E) HeLaM cells depleted of LIC1 (A), LIC2 (B), both LIC1 and 2 (C, minor multipolar spindle; D, major multipolar spindle), or untreated cells (E) were labeled with DAPI (blue), anti-α-tubulin (green), and anti-PC (red) and imaged on a DeltaVision microscope. Maximum projections of deconvolved z-series stacks are shown. Bar, 5 μm.
Figure S4. Effect of LIC depletion on spindle assembly, pole composition, and prophase centrosome position in U2OS and HEK cells. (A) U2OS cells depleted of both LICs were labeled with anti-α-tubulin (green), anti-PC (red), and DAPI (blue) and imaged on a DeltaVision microscope. A maximum projection of a deconvolved z-series stack is shown. (B) Cell line–specific differences in PC composition of spindle poles in cells with minor multipolar spindles. The two major poles of each minor multipolar spindle were scored for whether they possessed PC. As an example, the cell in A has one major pole that lacks PC (magenta arrows). A total of 433 (HeLaM), 294 (U2OS), and 92 HEK cells were scored from three to four independent experiments (means ± SEM). (C) LIC-depleted interphase U2OS cells with mispositioned centrosomes (arrows). Cells were labeled and imaged as in A except that a projection was made using the ImageJ extended focus plugin. (D and E) The position of each centrosome in prophase U2OS (D) and HeLaM (E) cells was scored: N, adjacent to the nucleus; C, cytoplasmic (see middle cell, image in C); PM, next to the plasma membrane (left cell, image in C). A total of ≥266 HeLaM cells from four independent experiments and ≥240 U2OS cells from three independent experiments were scored (means ± SEM). Bars, 5 µm.
Figure S5. Dynein, dynactin, and spindle assembly factors localize normally to LIC1- and 2-depleted spindles. (A–E) Immunofluorescence was used to determine the localization of dynein (A, anti-Xenopus IC), dynactin (B, p150-glued), TPX2 (C), NuMA (D), and γ-tubulin (E) in control (Ctrl MO) and LIC1 and 2 (LIC1&2 MO) MO-injected Xenopus embryos. All were found to localize normally in LIC1 and 2 morphant spindles irrespective of whether they were bipolar (two poles) or multipolar (three to four or greater than five poles) spindles. Bars, 20 µm.
Table S1.  **Statistical analysis of the effects of inhibition of Eg5 on spindle morphology, in combination with LIC depletion**

<table>
<thead>
<tr>
<th>Condition</th>
<th>Spindle morphology</th>
<th>Xenopus Eg5 inhibition p-value</th>
<th>HeLaM Eg5 inhibition p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>C + DMSO</td>
<td>C + STLC</td>
<td>LIC + DMSO</td>
</tr>
<tr>
<td>Control KnD + STLC</td>
<td>Monopolar</td>
<td>≤0.0001</td>
<td>≤0.0001</td>
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<tr>
<td></td>
<td>Bipolar</td>
<td>≤0.0001</td>
<td>≤0.0001</td>
</tr>
<tr>
<td></td>
<td>Minor multiple</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td></td>
<td>Major multiple</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>LIC KnD + DMSO</td>
<td>Monopolar</td>
<td>≤0.0001</td>
<td>≤0.0001</td>
</tr>
<tr>
<td></td>
<td>Bipolar</td>
<td>≤0.0001</td>
<td>≤0.0001</td>
</tr>
<tr>
<td></td>
<td>Minor multiple</td>
<td>≤0.0001</td>
<td>≤0.0001</td>
</tr>
<tr>
<td></td>
<td>Major multiple</td>
<td>≤0.0001</td>
<td>≤0.01</td>
</tr>
<tr>
<td>LIC KnD + STLC</td>
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<td>≤0.0001</td>
</tr>
<tr>
<td></td>
<td>Bipolar</td>
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<td>≤0.0001</td>
</tr>
<tr>
<td></td>
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<td>≤0.0001</td>
</tr>
<tr>
<td></td>
<td>Major multiple</td>
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<td>≤0.01</td>
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</table>

The data presented graphically in Fig. 9 (A, B, D and E) were analyzed by two-way ANOVA with Tukey’s test. For Eg5 inhibition in Xenopus embryos, 39–41 embryos were analyzed for each condition, assessing a total of between 375 and 1,175 spindles for spindle morphology, n = 6 independent experiments. For HeLaM Eg5 inhibition, a total of 427–485 cells were scored from four independent experiments (>100 per condition). Minus signs indicate comparisons that are made elsewhere in the table. C, control; SC, scrambled.

Table S2.  **Statistical analysis of the effects of Nuf2 depletion and inhibition CENP-E on spindle morphology, in combination with LIC depletion**

<table>
<thead>
<tr>
<th>Nuf2 and CENP-E inhibition</th>
<th>HeLaM Nuf2 depletion p-value</th>
<th>HeLaM CENP-E inhibition p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>SC + SC KnD</td>
<td>SC + Nuf2 KnD</td>
</tr>
<tr>
<td>SC KnD + treatment</td>
<td>Monopolar</td>
<td>NS</td>
</tr>
<tr>
<td></td>
<td>Bipolar</td>
<td>≤0.0001</td>
</tr>
<tr>
<td></td>
<td>Minor multiple</td>
<td>≤0.0001</td>
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<tr>
<td></td>
<td>Major multiple</td>
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<tr>
<td>LIC KnD + control treatment</td>
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<td>NS</td>
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<td>Bipolar</td>
<td>≤0.0001</td>
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<td>LIC KnD + treatment</td>
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<tr>
<td></td>
<td>Major multiple</td>
<td>≤0.0001</td>
</tr>
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</table>

The data presented graphically in Fig. 9 (A, B, D and E) were analyzed by two-way ANOVA with Tukey’s test. For HeLaM Nuf2 depletion experiments, a total of 854–872 cells were scored from four independent experiments (>200 per condition). For HeLaM CENP-E inhibition, a total of 412–438 cells were scored from four independent experiments (>100 per condition). Minus signs indicate comparisons that are made elsewhere in the table. SC, scrambled; CI, CENP-E inhibitor.

Video 1. **Loss of LIC does not affect dynein motor function in vitro.** Microtubule gliding driven by dynein purified from control (left) or LIC KnD HeLaM cells (right). Imaging was performed by video-enhanced differential interference contrast microscopy (BX60; Olympus) at RT at 25 frames per second using a video camera (Newvicon) and image processor (Argus-10) and is played back at 4× real time. Each panel is 13.5-µm across. Videos are viewed best using QuickTime 7.

Video 2. **Mitotic spindle in a control morphant.** Live confocal imaging of a mitotic spindle in a Xenopus gastrula stage embryo injected with a standard control MO. GFP-α-tubulin (green) was used to label microtubules and chromosomes were visualized using mCherry-Histone2B (magenta). Single focal plane images were collected every 6 s using a confocal microscope (FluoView FV1000; Olympus) with a 60x, 1.35 NA U Plan S Apochromat objective. Time-lapse videos were constructed in ImageJ and play back at a speed of 7 frames per second. The time stamp indicates time in minutes and seconds.
Video 3. **Multipolar spindle assembly in a LIC1 and 2 morphant.** Live confocal imaging of a mitotic spindle in a Xenopus gastrula stage embryo injected with both LIC1 and LIC2 MOs. GFP–α-tubulin (green) was used to label microtubules, and chromosomes were visualized using mCherry-Histone2B (magenta). The spindle assembles with three poles. Single focal plane images were collected every 10 s using a confocal microscope (FluoView FV1000; Olympus) with a 60×, 1.35 NA U Plan S Apochromat objective. Time-lapse videos were constructed in ImageJ and play back at a speed of 12 frames per second. The time stamp indicates time in minutes and seconds.

Video 4. **Pole fragmentation of a bipolar spindle in a LIC1 and 2 morphant.** Live confocal imaging of a mitotic spindle in a Xenopus gastrula stage embryo injected with both LIC1 and LIC2 MOs. GFP–α-tubulin (green) was used to label microtubules and chromosomes were visualized using mCherry-H2B (magenta). A bipolar spindle assembles but then undergoes spindle pole fragmentation: the first pole begins to fragment at ~24 min and 20 s, and the second fragments at 36 min. Single focal plane images were collected every 10 s using a confocal microscope (FluoView FV1000; Olympus) with a 60×/1.35 NA U Plan S Apochromat objective. Time-lapse videos were constructed in ImageJ and play back at a speed of 12 frames per second. The time stamp indicates time in minutes and seconds.

Video 5. **A multipolar spindle assembles in a LIC1 and 2 morphant and then undergoes pole fragmentation.** Live confocal imaging of a mitotic spindle in a Xenopus gastrula stage embryo injected with both LIC1 and LIC2 MOs. GFP–α-tubulin (green) was used to label microtubules and chromosomes were visualized using mCherry-H2B (magenta). The spindle assembles with three poles, but at ~20 min and 20 s, one of these poles fragments. Single focal plane images were collected every 10 s using a confocal microscope (FluoView FV1000; Olympus) with a 60×, 1.35 NA U Plan S Apochromat objective. Time-lapse videos were constructed in ImageJ and play back at a speed of 12 frames per second. The time stamp indicates time in minutes and seconds. To recenter the spindle in the field of view, the video position was adjusted at 17 min and 30 s.

Video 6. **3View EM of a control spindle.** A 3D reconstruction of a mitotic spindle in the epithelium of a control Xenopus morphant gastrula stage embryo. Images were collected using a microtome (3View; Gatan) within a scanning electron microscope (Quanta 250 FEG; FEI) with sections taken 100 nm apart. The imaging conditions were as follows: indicated quadrant magnification of 1,600×, accelerating voltage of 3.8 kV, pressure at 0.33 Torr, images of 4,000 × 5,000 pixels, and dwell time of 10 µs. Images were reconstructed using Imaris image analysis software (Bitplane). Chromosomes (blue) and centrioles (red) were reconstructed in Imaris using the Surfaces tool. Note that each pole of the bipolar spindle contains a pair of centrioles.

Video 7. **3View EM of a LIC KD multipolar spindle.** A 3D reconstruction of a mitotic spindle in the epithelium of a Xenopus morphant injected with both LIC1 and 2 MOs. Images were collected using a microtome (3View; Gatan) within a scanning electron microscope (Quanta 250 FEG; FEI) with sections taken 100 nm apart. The imaging conditions were as follows: indicated quadrant magnification of 1,600×, accelerating voltage of 3.8 kV, pressure at 0.33 Torr, images of 4,000 × 5,000 pixels, and dwell time of 10 µs. Images were reconstructed using Imaris image analysis software (Bitplane). Chromosomes (blue) and centrioles (red) were reconstructed in Imaris using the Surfaces tool. Note that all poles of the multipolar spindle contain only a single centriole.

Video 8. **3View EM of a LIC KD bipolar spindle.** A 3D reconstruction of a mitotic spindle in the epithelium of a Xenopus morphant injected with both LIC1 and 2 MOs. Images were collected using a microtome (3View; Gatan) within a scanning electron microscope (Quanta 250 FEG; FEI) with sections taken 100 nm apart. The imaging conditions were as follows: indicated quadrant magnification of 1,600×, accelerating voltage of 3.8 kV, pressure at 0.33 Torr, images of 4,000 × 5,000 pixels, and dwell time of 10 µs. Images were reconstructed using Imaris image analysis software (Bitplane). Chromosomes (blue) and centrioles (red) were reconstructed in Imaris using the Surfaces tool. Note that although each pole contains two centrioles, these centrioles are further apart than is seen in the control bipolar spindle (Video 6).