CLASPs function redundantly to regulate astral microtubules in the C. elegans embryo

Eugenel B. Espiritu 1, Lori E. Krueger 1, Anna Ye, Lesilee S. Rose *

Department of Molecular and Cellular Biology, University of California, Davis 95616, USA

A R T I C L E   I N F O
Article history:
Received 8 March 2012
Received in revised form 26 April 2012
Accepted 11 May 2012
Available online 19 May 2012

Keywords:
Asymmetric division
Spindle positioning
Mitosis
Microtubules

A B S T R A C T

Microtubule dynamics are thought to play an important role in regulating microtubule interactions with cortical force generating motor proteins that position the spindle during asymmetric cell division. CLASPs are microtubule-associated proteins that have a conserved role in regulating microtubule dynamics in diverse cell types. Caenorhabditis elegans has three CLASP homologs in its genome. CLS-2 is known to localize to kinetochores and is needed for chromosome segregation at meiosis and mitosis; however, CLS-1 and CLS-3 have not been reported to have any role in embryonic development. Here, we show that depletion of CLS-2 in combination with either CLS-1 or CLS-3 results in defects in nuclear rotation, maintenance of spindle length, and spindle displacement in the one-cell embryo. Polarity is normal in these embryos, but reduced numbers of astral microtubules reach all regions of the cortex at the time of spindle positioning. Analysis of the microtubule plus-end tracker EB1 also revealed a reduced number of growing microtubules reaching the cortex in CLASP depleted embryos, but the polymerization rate of astral microtubules was not slower than in wild type. These results indicate that C. elegans CLASPs act partially redundantly to regulate astral microtubules and position the spindle during asymmetric cell division. Further, we show that these spindle pole-positioning roles are independent of the CLS-2 binding proteins HCP-1 and HCP-2.

© 2012 Elsevier Inc. All rights reserved.

Introduction

CLASPs are non-motor, microtubule-associated proteins that have roles during both mitosis and interphase in multiple cell types. Studies in several systems indicate an important role for CLASP in stabilizing microtubule length. Measurements of the parameters of microtubule dynamics after CLASP depletion indicate that CLASPs can act by either preventing catastrophes or promoting rescue from catastrophe (e.g., Al-Bassam and Chang, 2011; Maiato et al., 2003, 2005; Ortiz et al., 2009; Slep, 2009; Sousa et al., 2007). Recent in vitro evidence shows that S. pombe CLASP rescues microtubules from depolymerization by associating with the microtubule lattice and promoting tubulin addition (Al-Bassam and Chang, 2011; Al-Bassam et al., 2010).

CLASPs were first identified as CLIP-associated proteins that localize to microtubule plus-ends in mammalian cells at interphase, through interactions with the microtubule plus-end tracking proteins, CLIP and EB1 (Akhmanova et al., 2001). In some mammalian tissue culture cells, CLASP acts to regulate the dynamic properties of a localized subset of microtubules within the cell or to polarize microtubule arrays. For example in non-motile Hela cells, CLASPs interact with EB1 to promote microtubule rescue specifically at the cell periphery (Mimori-Kiyosue et al., 2005), and in motile fibroblasts CLASPs respond to polarity cues through the GSK3β signaling pathway to stabilize microtubules at the leading edge (Akhmanova et al., 2001; Kumar et al., 2009). Similarly, in migrating epithelial cells CLASPs are asymmetrically localized to the leading edge of the cell, but in this case they are present along the microtubule lattice in addition to being on plus-ends (Wittmann and Waterman-Storer, 2005).

In most mitotic cell types where CLASP function has been examined, localization at microtubule plus-ends has not been seen. Rather, CLASPs localize to kinetochores and to the central spindle where they play important roles in chromosome segregation and the establishment and/or maintenance of bipolar spindle structure respectively (Cheeseman et al., 2005; Hannak and Heald, 2006; Lemos et al., 2000; Maiato et al., 2003, 2005; Mimori-Kiyosue et al., 2005; Ortiz et al., 2009; Pereira et al., 2006). Interestingly, Drosophila CLASP (called mast/orbit) is also needed for the asymmetric division of Drosophila germ-line stem cells and the subsequent oriented divisions of the developing oocyte cysts (Mathe et al., 2003). In these cells, spindles are anchored by one pole to a cytoskeletal structure called the fusome. CLASP localizes to this structure and the spindle, and loss of CLASP results in defects in fusome structure and abnormal...
or monopolar spindles that fail to orient to the fusome. However, whether mast/orbit also plays a role in spindle orientation events that are regulated by interactions of astral microtubules with polarized cortical cues has not been addressed.

In the *Caenorhabditis elegans* one-cell embryo, as in many cell types, PAR polarity proteins establish cortical domains required for cell polarity. The PAR proteins also regulate the forces that align the spindle with the polarity axis to accomplish an asymmetric division (reviewed in Galli and van den Heuvel, 2008; Gonczy, 2008; Knoblich, 2010). Spindle positioning forces are generated when astral microtubule plus-ends interact with force generators at the cell cortex, thereby translating cortical pulling forces into forces acting on centromeres or spindle poles. The PAR proteins regulate cortical forces via a conserved pathway involving Cz proteins and their partners GPR-1/2 and LIN-5 (known as LGN/PINS and NuMa/Mud in vertebrate cells and *Drosophila* respectively). GPR-1/2 and LIN-5 are asymmetrically localized at the cortex and interact with regulators of the microtubule motor dynein, suggesting that the asymmetric activation of dynein leads to spindle positioning events. In the *C. elegans* one-cell, asymmetric cortical pulling forces drive anteriorly directed nuclear centration and rotation movements at prophase, as well as posteriorly-directed spindle displacement at metaphase and anaphase.

Microtubule-associated proteins that regulate microtubule plus end dynamics can potentially determine the frequency and duration of growing microtubule plus ends and thus, interactions with dynein or other force-generators at the cortex (Kozlowski et al., 2007; Labbe et al., 2003; Srayko et al., 2005). Consistent with this view, the XMAP215 ortholog, ZYG-9 and the doublecortin ortholog ZYG-8 are required for spindle positioning in the *C. elegans* one-cell embryo. Mutations in the genes for either of these proteins result in short astral microtubules and abnormal nuclear and spindle positioning at all stages (zyg-9) or metaphase/anaphase (zyg-8), presumably due to a failure of microtubule-cortex interactions (Gonczy et al., 2001; Matthews et al., 1998; Srayko et al., 2003). However, the depletion of several other proteins shown to influence microtubule dynamics in other systems, including an ortholog of CLASP, CLS-2, the plus-end binding proteins EB1 and CLIP, and the depolymerizing kinesin MCAK, has not been reported to produce defects in asymmetric division (Srayko et al., 2005). The failure to identify a phenotype in these studies could be due to redundancy with gene family members or partially overlapping functions between different families.

Three genes in the *C. elegans* genome have homology to CLASP: cls-1 (C07H6.3), cls-2 (R107.6), and cls-3 (ZC843.4). Of the *C. elegans* CLASP homologs, CLS-2 localizes to the kinetochore-microtubule interface and is required for mitotic fidelity in embryos. Depletion of cls-2 by RNA interference leads to premature separation of spindle poles before anaphase onset, and chromosome mis segregation (Cheeseman et al., 2005). CLS-2 is also required for meiosis where it plays roles in cytoplasmic streaming and the structure of the anaphase spindle (Dumont et al., 2010; Yang et al., 2003). CLS-1 and 3 are similar to CLS-2 throughout their lengths, except for an N-terminal extension of CLS-1. This region of CLS-1 aligns with the first Tumor Overexpressed Gene domain-like domain (TOG-like) of mammalian CLASPs, suggesting that CLS-1 may have two TOG-like domains. This makes CLS-1 more similar to mammalian CLASPs than CLS-2 and CLS-3, which contain only one predicted TOG-like domain. However, knockdown of CLS-1 or CLS-3 via RNA interference does not give lethality (Sonnichsen et al., 2005; Yang et al., 2003).

In this study, we use the *C. elegans* embryo as a system to study the roles of CLASP proteins in spindle movements that are generated by astral microtubules during asymmetric division. Using RNA interference of the three genes singly and in combination, we found that the three *C. elegans* CLASP homologs function redundantly during nuclear rotation and spindle displacement. Together, these results provide evidence for a role for *C. elegans* CLASP in regulating astral microtubules during asymmetric cell division.

Materials and methods

Worm strains and growth

*C. elegans* were cultured on MYOB plates using standard methods, as previously described (Brenner, 1974; Church et al., 1995). The following strains were used: N2 (wild-type Bristol variant); AZ244, unc-119(ed3); ru57[pa2417: pie-1::β-tubulin::GFP; unc-119(+)]; FM102, lin-5[ev5771s] unc-119(ed3); ru57[pa2417: pie-1::β-tubulin::GFP; unc-119(+)]; FM125, (unc-119(ed3), ru57[pa2417: pie-1::β-tubulin::GFP; unc-119(+)), tdi37[unc-119(+)] pie-1::mCherry::H2B; JH1512, lax1137 [pRF-4, pJH7.04 pie-1::GFP-Par6, genomic DNA]

*JH2648, [PAR-6::mCherry unc-119; ax1Is1928 [pEC66]; TH66, unc-119(ed3); Is[pie-1:EBP-2::GFP]].* N2 worms were maintained at 20 °C, while transgenic strains were maintained at 23–25 °C for optimal transgene expression.

RNA interference

RNA interference (RNAi) was carried out by bacterial feeding (Timmons et al., 2001) using the following Ahringer library bacterial clones where the numbers refer to nucleotide positions in the unsupplied CDS (Kamath et al., 2003): cls-2 618-1733 bp (III-A410), cls-3 (III-4N20), cls-1 4214-5350 (III-A13), hcp-1 (V-4H10), hcp-2 (V6P23). Bacteria were used undiluted for single RNAi, or mixed 1:1 for double RNAi respectively. Feeding was conducted at 20 °C for the N2 background and 23–25 °C for GFP transgenic strains; embryos were screened for depletion phenotypes from 36–48 and 20–40 h post injection respectively, with the exception of hcp-1/2(RNAi) embryos for which 13/21 were examined at 48–72 h. To generate dsRNA for injection, the inserts of plasmids from the above clones were amplified with a T7 primer, and the product used as a template to synthesize dsRNA using the MegaScript T7 Riboprobe Kit (Roche); dsRNA was resuspended at a final concentration of 0.5 or 1 mg/mL for each dsRNA. The entire inserts from the above constructs were also cloned in tandem into the RNAi feeding vector L4440 to create pEE1 [cls-1;cls-2 ], pEG66 [cls-2; cls-3], dsRNA was generated from these new inserts and mixed with either cls-3 or cls-1 dsRNA to generate a triple RNAI mixture. L4 larvae were injected at room temperature and subsequently stored and analyzed as above depending on the background strain. No difference in phenotype was seen for double RNAi carried out at 0.5 versus 1 mg/mL concentration.

Imaging and quantification

For live imaging experiments shown in Figs. 1–3, embryos were cut from gravid hermaphrodites in egg buffer, mounted on 2% agarose pads and sealed under a coverslip. For the PAR-6::mCherry experiments, embryos mounted on agar exhibited strong autofluorescence of the egg shell. For that experiment and those involving hcp-1/2 and lin-5, embryos were mounted on polylysine coated coverslips and inverted over spacers for imaging. DIC imaging of N2 and epifluorescence imaging of worms expressing fusion proteins were carried out on an Olympus BX60 microscope, using an Olympus PlanFl 100X, 1.3 NA objective or a PlanApo N 60X, 1.42 NA objective lens respectively. Images were acquired using a Hamamatsu Orca 12-bit digital camera and OpenLab Software. For the quantification of spindle
movements shown in Figs. 1 and 2, DIC images were captured at one-second intervals, and tracked manually using the Manual Tracking plugin for ImageJ (rsbweb.nih.gov/ij/plugins/track/track.html); data was transferred to Microsoft Excel for processing. Scatter plots and statistical analysis were carried out using PrismPlot (GraphPad Software, Inc). For DIC and epifluorescence imaging experiments, brightfield timelapse images were captured at 12 frames/min. Single fluorescent images were taken at 60, 120 and 180 s post NEB to monitor the GFP::tubulin signal, or at pseudocleavage, NEB, and cytokinesis to examine PAR-6. To determine the extent of the PAR-6 domain, the free hand tool in ImageJ was used to trace the cortex from the anterior to the posterior, and the intensity values were plotted. In wild type embryos, the posterior cortex values were the same or lower than the adjacent cytoplasm. The absolute pixel intensities at the anterior cortex varied, but dropped in a gradient such that levels reached 50% maximum at 50% egg length (EL) in all embryos, further dropping to the posterior minimum by 70% EL. In the few CLASP embryos with extended domains, the gradient was shifted such that the minima were reached at 80% EL, but the posterior-most cortex still exhibited an absence of PAR-6 signal as in wild type.

For confocal imaging of GFP::tubulin or EBP-2::GFP strains, embryos were mounted on agar as above and imaged on a Zeiss 3i Hybrid Spinning Disk Confocal- TIRF-Widefield Marianas microscope with a Plan-Apo 63x, 1.3 NA objective lens (Zeiss).

Fig. 1. Spindle positioning phenotypes in CLASP depleted embryos. (A) Still images from DIC time-lapse movies of representative embryos for wild type and the CLASP RNAi treatments indicated. Anterior is to the left in these and all subsequent images. Time to NEB (0) is given for each frame; dots mark centrosomes. Scale bar is 10 μm. (B) Spindle pole traces during the cell cycle generated from tracking each embryo in A are shown directly under each corresponding embryo; anterior spindle poles shown in light blue, posterior in dark blue.
Acquisition was controlled by Slidebook 5 software (3i Incorporated). Embryos were excited with 488 and 561 wavelengths for 400 ms each sequentially incorporated into a 4 s interval between acquisitions. In Slidebook, acquisitions were photobleach corrected following a single exponential fit. For higher resolution imaging of GFP::tubulin labeled microtubules, embryos were filmed starting at NEB using the same microscope, acquisition software, and a Plan-Apo 100 x, 1.3 NA objective lens (Zeiss). Embryos were excited with 488 and 561 wavelengths for 800 ms and 100 ms respectively, incorporated into a 1.52 s interval between acquisitions. All raw images were exposed and scaled with the same parameters, and were photobleach corrected as above; however, because cls2; cls-1 and cls2; cls-3 embryos exhibited a lower signal to noise ratio, images for these were adjusted using the auto-contrast tool in Image J in order to better visualize microtubule ends. Microtubule segments that came within 1 μm of the cortex were manually tracked with the ImageJ pencil tool. Cortical contacts per time point were measured from 40 s before to 40 s after the onset of posterior spindle displacement (PD) and were then divided into eight 10-s windows. No significant differences in the average number of cortical contacts in wild-type embryos were seen over this time period, and thus the data were pooled into two groups (before PD and after PD) for presentation in Fig. 4B. The ten time-points prior to posterior spindle displacement (15.2 s total) were reanalyzed to track the number of contacts per four cortical domains defined by the location on the anterior-posterior axis, where 0% is most anterior and 100% is most posterior. The length of the cortical region measured in the anterior (0–25%) and posterior (75–100%) domains is longer than in the lateral domains due to the curvature of the embryo, and so the number of microtubule contacts was normalized relative to length for graphing in Fig. 4C.

The plus-ends of growing microtubules were visualized in EBP-2::GFP expressing embryos excited with a 488 wavelength for 300 ms, incorporated into a 500 ms interval between acquisitions over 32 s. The 64 time points prior to posterior displacement

---

Fig. 2. Quantification of defects exhibited by CLASP RNAi embryos. Scatter plots show specific measurements for all embryos in the data sets from Table 1. In each plot, the means for each genotype are indicated by horizontal lines; the values for the means and statistical significance are summarized in Table 1. (A) Rotation angle of nuclear-centrosome complex at NEB, where complete rotation onto the A/P axis = 0°; complete failure = 90°. (B) Centration point of the nuclear–centrosome complex, expressed as percent egg length (%EL) where the anterior end of the embryo = 0%, posterior = 100%. (C) Minimum spindle length after NEB. (D) Maximum position (i.e. the farthest posterior point obtained) of the anterior centrosome, expressed as percent egg length (%EL). (E) The time of onset of spindle displacement. (F) Maximum position of the posterior centrosome.
followed by fixation for 5 s in the cortex; quantifications were restricted to a half-circle radius on the outside of each cortex as shown in Fig. 6A (Srayko et al., 2005) to ensure scoring EBP-2::GFP comets on astral microtubules projected towards the cortex, rather than on interpolar microtubules. Five comets each for the anterior and posterior centrosomes were quantified for each time interval per embryo, and are presented as an average sum for five embryos per condition. For quantification of microtubule growth rates, EBP-2::GFP comets were tracked using the Manual Tracking plugin in Image J. Growth rate was quantified per comet by dividing the distance traveled by the time interval the comet could be detected; 5 comets that persisted for at least 5 time points were quantified per centrosome per embryo.

Antibodies and immunolocalization

The following regions were used as antigens for antibody production: CLS-1 (aa42-333), CLS-2 (aa689-759), CLS-3 (aa660-736). The specified regions were amplified by PCR using CDNA templates (cls-3 and cls-1 full-length cDNA clones were obtained from Open Biosystems, cls-2 cDNA from (Yang et al., 2003) and cloned into pGEX or pMAL protein purification vectors. MBP and GST fusion proteins were expressed in bacteria and purified using amylose resin or Glutathione Sepharose 4B resin respectively according to the manufacturer’s instructions (Amersham Bioscience, GE Healthcare). Purified GST-CLS-1, MBP-CLS-1 and MBP-CLS-3 were injected into rabbits and rats (Covance). Antisera were purified on affinity columns made by cross linking GST-CLS-2 and GST-CLS-3 to Affigel-15, and GST-CLS-1 to Affigel-10 (BioRad); antibodies were eluted with glycine-HCl, pH 2.5 and neutralized with 1M Tris pH 8.0. To test antibodies, purified GST-fusion proteins were separated on a 12% acrylamide gel, transferred to nitrocellulose membrane (GE Healthcare) and incubated with primary antibodies (1:1000). Secondary antibodies (Licor) were diluted 1:20,000 in PBS, and images acquired on an Odyssey Infrared Imaging System.

For western blot analysis of embryo extracts, synchronized L4 worms were collected and the embryos obtained by the alkaline feeding in a wild-type background to deplete CLASP proteins singly and in combination. Embryos were examined during the first division by DIC time-lapse microscopy. Wild-type embryos follow a stereotypical sequence of nuclear and spindle movements during the first cell cycle (reviewed in Galli and Van Den Heuvel (2008) and Gonczy (2008)). Male and female pronuclei meet at the posterior of the embryo. The pronuclear-centrosome complex then moves to the center of the embryo (centration), simultaneously rotating to align the centrosomes along the anterior-posterior axis of cell polarity (nuclear rotation) before nuclear envelope breakdown (NEB) (Fig. 1, Movie 1). The spindle forms parallel to the anterior/posterior (A/P) axis and in many embryos the entire spindle shifts slightly towards the posterior during metaphase. This movement and asymmetric elongation of the spindle at anaphase leads to unequal cleavage and are together referred to as posterior spindle displacement. During spindle elongation, the entire spindle pole making larger oscillations perpendicular to the A/P axis than the anterior pole.

Supplementary material related to this article can be found online at http://dx.doi.org/10.1016/j.ydbio.2012.05.016.

Results

CLASPs are required for spindle positioning in the one-cell embryo

To determine if C. elegans CLASPs act redundantly during spindle positioning, we used RNA interference (RNAi) by bacterial feeding in a wild-type background to deplete CLASP proteins singly and in combination. Embryos were examined during the first division by DIC time-lapse microscopy. Wild-type embryos follow a stereotypical sequence of nuclear and spindle movements during the first cell cycle (reviewed in Galli and Van Den Heuvel (2008) and Gonczy (2008)). Male and female pronuclei meet at the posterior of the embryo. The pronuclear-centrosome complex then moves to the center of the embryo (centration), simultaneously rotating to align the centrosomes along the anterior-posterior axis of cell polarity (nuclear rotation) before nuclear envelope breakdown (NEB) (Fig. 1, Movie 1). The spindle forms parallel to the anterior/posterior (A/P) axis and in many embryos the entire spindle shifts slightly towards the posterior during metaphase. This movement and asymmetric elongation of the spindle at anaphase leads to unequal cleavage and are together referred to as posterior spindle displacement. During spindle elongation, the entire spindle pole making larger oscillations perpendicular to the A/P axis than the anterior pole.

In situ immunolocalization

Gravid hermaphrodites were rinsed in ddH2O, mounted on polylysine-coated slides, and cut to release embryos. Embryos were prepared for immunolocalization in liquid nitrogen, followed by fixation for 5 s in −20 °C methanol then 30 min in 4% paraformaldehyde/24M sorbitol/PEM (PEM is 100 mM PIPES/5 mM EDTA/ 5 mM MgCl2 pH6.9) as described in Toya et al. (2010)). CLS-2, CLS-3, and DM1x, (Sigma) antibodies were diluted in PBS at 1:50, 1:10, and 1:500 respectively; fluorescently conjugated secondary, goat anti-rabbit FITC and goat anti-mouse Rhodamine, were diluted in PBS at 1:100. CLS-2 and CLS-3 were pre-absorbed with acetone powders of GST-expressing bacteria and MBP-expressing bacteria respectively and secondary were pre-absorbed with wild-type worm acetone powder prior to use. Embryos were stained with DAPI to visualize DNA and determine cell-cycle stage, and mounted in Vectashield mounting medium (Vector Laboratories, Inc.). Similar results were obtained with standard MeOH fixation conditions (Miller and Shakes, 1995). Confocal sections were acquired on an Olympus Fv1000 Fluoview Laser Scanning Confocal Microscope, using a 60X Plan-Apo NA 1.42 objective. For each embryo, Z-series of five confocal sections were taken at mid-embryo focal plane in 0.2 µm steps, with below-saturation acquisition settings. Images shown in panels are maximum intensity projections made in Image J.
variable (Table 1, Fig. 2B). Some embryos exhibited a partial failure of centration (Fig. 1A, Movie 3: cls-2; cls-1 embryo shows an example) while others showed an over-centering phenotype (Fig. 1A, Movie 4: cls-2; cls-3).

After the centration/rotation phase, many cls-2; cls-1 and cls-2; cls-3 (RNAi) embryos exhibited a spindle collapse phenotype in which the spindle poles did not maintain their normal separation after NEB and instead moved closer together (Fig. 1 cls-2; cls-1 and cls-2; cls-3 show representative embryos). Quantification of spindle pole-to-pole distance showed that the average minimum spindle length after NEB for cls-2; cls-1 and cls-2; cls-3 (RNAi) embryos was significantly different from wild type (Table 1) and many embryos exhibited a minimum spindle length smaller than the wild-type range (Fig. 2C). In the majority of embryos in this data set, spindle collapse was followed by an excessive spindle displacement phenotype in which the entire spindle moved towards the posterior (Fig. 1). The two poles of the collapsed spindle traveled as a unit, and thus the anterior spindle pole moved significantly farther to the posterior than in controls (Fig. 2D, Tables 1 and 2). The timing of this spindle displacement was within the normal range for most embryos, but delayed in some (Fig. 2E, Table 1). In contrast, the posterior spindle pole did not travel farther towards the posterior on average than in wild type (Fig. 2F, Table 1). After excessive spindle displacement, spindle poles moved apart at anaphase and revealed the weak spindle phenotype typical of cls-2 RNAi alone (Fig. 1, Table 2). The final position of both spindle poles was on the anterior-posterior axis, as in wild type (Fig. 1) and the embryos completed cytokinesis (n > 8 embryos for each type of RNAi condition).
Quantification of the same parameters in cls-2 single (RNAi) embryos revealed that some cls-2(RNAi) embryos exhibited shorter spindles and excessive spindle displacement (Tables 1 and 2; Fig. 2). For example, 6/17 cls-2(RNAi) embryos showed a minimum spindle length that was smaller than the wild type range (Fig. 2C, Table 1). The mean spindle length of these cls-2 embryos was smaller than wild type, but still longer than in cls-2; cls-1 and cls-2; cls-3 (RNAi) embryos (Table 1). In contrast, the maximum length of the spindle reached during anaphase was not significantly different in any of the CLASP depletion treatments compared to wild type (Table 1).

Depletion of CLASPs by bacterial feeding in a strain expressing GFP::tubulin, mCherry::histone (McNally et al., 2006); Fig. 3, Table 2, Movies 6–10) was used for a more careful examination of chromosome movements and spindle structure after single versus double CLASP depletion. As previously reported (Cheeseman et al., 2005), all cls-2 (RNAi) embryos showed a chromosome congression defect and either a complete absence of microtubules (4/6) or greatly reduced numbers of microtubules (2/6) in the central spindle at anaphase (Movie 7). This phenotype was often accompanied by lagging chromosomes (2/6) or an extended mass of chromosome material at anaphase that appeared stretched out on the remaining midzone microtubules (2/6). Similarly cls-2; cls-1 and cls-2; cls-3 double RNAi embryos showed chromosome congression defects (2/4 and 7/8 respectively), missing or reduced central spindle microtubules (4/4, 7/8), and lagging chromosomes or chromatin at anaphase (2/4, 2/8; Movie 8, 9). Thus, no additional spindle structure or chromosome phenotypes were seen after cls-2; cls-1 and cls-2; cls-3 double depletion compared to cls-2 RNAi alone. In some cls-2; cls-1 and cls-2; cls-3 embryos, although the chromosomes exhibited initial congression defects, chromosomes appeared aligned in late metaphase (Movie 9). Improved alignment correlated with spindle pole collapse. It was previously shown that attenuating cortical pulling forces suppressed the premature spindle pole separation defect and improved chromosome segregation in cls-2(RNAi) embryos (Cheeseman et al., 2005). Thus, the spindle collapse phenotype of cls-2; cls-1 and cls-2; cls-3 embryos, which prevents premature spindle pole separation, likely allows more time for kinetochore attachments to occur in these embryos.

Similar spindle positioning and spindle collapse phenotypes were observed after single and double RNAi of CLASPs by bacterial feeding in other backgrounds and by injection of dsRNA (Table 2).
RNAi directed against all three CLASPs did not result in a stronger phenotype. Although the penetrance of each phenotype among data sets varied, the nuclear rotation and spindle displacement defects were typically stronger in cls-2; cls-1 and cls-2; cls-3 double RNAi embryos compared to cls-2 single RNAi embryos (Table 2). In contrast, no obvious spindle positioning phenotypes were observed in cls-1; cls-3 (RNAi) experiments, which were carried out in parallel with the other double RNAi experiments (Figs. 1 and 2 and Tables 1 and 2, Movie 5). The only difference between cls-1; cls-3 (RNAi) embryos and wild type was a slightly smaller minimum spindle length (Table 1). Likewise, when cls-1 and cls-3 were depleted simultaneously in a strain expressing GFP::tubulin, mCherry::histone, chromosomes congressed on the

Together, these observations indicate that cls-1 and cls-3 partially redundantly with (smaller minimum spindle length (Table 1). Likewise, when depleted simultaneously in a strain expressing GFP::tubulin, mCherry::histone, chromosomes congressed on the metaphase plate and segregated normally, and the spindle-mid-zone microtubules appeared robust (11/11, Fig. 3; Movie 10). Together, these observations indicate that cls-1 and cls-3 act partially redundantly with cls-2 during centration, rotation and spindle displacement in one-cell embryos, as well as in the maintenance of spindle length.

**Defects in spindle positioning in CLASP depleted embryos are not due to polarity defects**

CLS-2 was previously shown to function prior to the first mitosis in *C. elegans*, during the microtubule-mediated cytoplasmic streaming that occurs just after fertilization and in spindle formation and chromosome segregation in meiosis (Dumont et al., 2010; McNally et al., 2010). After the completion of meiosis, the polarity axis for asymmetric division is established. The growth of the sperm's microtubule aster correlates with the timing of polarity establishment (Tsai and Ahringer, 2007; Wallenfang and Seydoux, 2000). Thus, the spindle positioning phenotypes of CLASP depleted embryos could result from pre-mitotic defects in microtubule function that affect polarity. To test this, we analyzed embryos expressing GFP::PAR-6 or mCherry::PAR-6 during the first cell cycle (Zonies et al., 2010). In cls-2, cls-2; cls-1 or cls-2; cls-3 (RNAi) embryos, an anterior PAR-6 domain of normal size formed by the time of pronuclear meeting in the majority of embryos, just as in controls (14/16, 7/8, 7/8, respectively; Fig. 4). Similar results were observed in cls-1; cls-2; cls-3 (RNAi) embryos (6/8 embryos exhibited a normal PAR-6 domain). In the remaining embryos for all treatments, the PAR-6 domain extended farther than 50% egg length, but PAR-6 remained absent at the very posterior cortex (see Methods for quantification). Of the 6 embryos among all treatments that showed an extended PAR-6 domain, 3 of these had no spindle positioning defects. Further, of the normally polarized embryos, 3/14 cls-2 (RNAi) embryos, 4/7 cls-2; cls-1, 3/7 cls-2; cls-3, and 5/7 cls-1; cls-2; cls-3 embryos showed either a nuclear rotation defect, excessive spindle displacement, or both. Thus, there was no correlation between the embryos with an extended PAR-6 domain and alterations in spindle positioning.

To test whether the posterior movements of the spindle observed after CLASP depletion are due to the normal cortical forces, we examined the effects of a loss of lin-5 activity on the phenotype. LIN-5 is a component of the G protein pathway that results in cortical pulling forces and asymmetric spindle displacement in wild-type embryos. Strains expressing GFP::tubulin with or without the lin-5(ev571ts) mutation were used for this analysis (Park and Rose, 2008). For cls-2; cls-1 and cls-2; cls-3 (RNAi), 67% and 72% of the embryos exhibited spindle collapse and excessive spindle displacement respectively (n=18). In contrast, none of the lin-5; cls-2; cls-1 (RNAi) and lin-5; cls-2; cls-3 (RNAi) embryos showed excessive spindle displacement or any posterior movement of the spindle during metaphase (n=19). Nonetheless, spindle collapse occurred in 61% of lin-5; cls-2; cls-3 (RNAi) embryos. In addition, in the subset of embryos for which tubulin fluorescence was examined, central spindle microtubules were absent or greatly reduced in 8/9 lin-5; cls-2; cls-1 (RNAi) and 7/8 cls-2; cls-1 (RNAi) embryos, indicating that the weak spindle phenotype is present even when forces are attenuated. We conclude that the spindle positioning defects observed in CLASP depleted embryos are not caused by abnormal polarity but do depend on asymmetric cortical forces.

**CLASPs are required for normal numbers of microtubule-to-cortex contacts**

CLASPs are known to affect microtubule dynamics and in particular can promote long microtubule length by preventing catastrophe (Al-Bassam et al., 2010; Maiato et al., 2003; Maiato et al., 2005; Ortiz et al., 2009; Slep, 2009; Sousa et al., 2007). The defects in spindle positioning observed could therefore be caused by alterations in microtubule dynamics and length that reduce interactions of astral microtubules with the cortex. To assay for microtubule interactions with the cortex, we first examined astral microtubules in GFP::tubulin expressing embryos, using higher resolution imaging. Control and RNAi embryos were imaged from NEB through anaphase, and the number of microtubules that contacted the cortex were quantified (Fig. 5; see Methods for details). In this data set, the spindle positioning phenotypes were weaker, although many embryos exhibited a collapsed spindle (Table 2). Nonetheless, cls-2 (RNAi) embryos exhibited significantly fewer microtubule-cortex contacts when compared to control embryos (Fig. 5). In addition, cls-2; cls-1 and cls-2; cls-3 double RNAi embryos had fewer microtubules contacting the cortex than cls-2 single RNAi embryos or controls. These results demonstrate that the three CLASPs act redundantly in regulating astral microtubule contact with the cortex.

We next examined whether depletion of CLASPs affects microtubule-cortex contacts globally or only at certain regions of the cortex. The number of microtubule-cortex contacts in each of four domains across the anterior/posterior axis of the embryo were calculated. Control embryos showed a similar number of cortical contacts in all domains. In cls-2 and cls-2; cls-1 embryos, microtubule contacts were significantly reduced at all cortical regions (Fig. 5C). We conclude that depletion of CLASPs reduces astral microtubule contact with the cortex throughout the embryo.

To further characterize the effects of CLASP depletion on microtubules, we examined a strain expressing EBP-2::GFP after depletion of CLASPs by RNAi. EBP-2 is a *C. elegans* ortholog of EB1, which labels the plus ends of growing microtubules only and is thus visible as fluorescent “comets” (Srayko et al., 2005). We found no difference in the number of EBP-2::GFP comets nucleated from the centrosome that grew to a distance of 10 μm in cls-2 (RNAi) embryos compared to wild type (Fig. 6), consistent with a previous study (Srayko et al., 2005). However, cls-2; cls-3 embryos had fewer growing microtubules extending to this distance, and all CLASP depleted embryos showed significantly fewer EBP-2::GFP comets growing to within 2 μm of the cortex. As with the analysis of microtubules using GFP::tubulin, the double CLASP depletion embryos exhibited fewer growing microtubules near the cortex than embryos depleted for CLS-2 alone. Measurements of the velocities of EBP-2::GFP comets, an assay for microtubule polymerization rate, showed a very small increase in cls-2 and cls-2; cls-1 embryos compared to controls; the difference in velocities between cls-2; cls-3 and control embryos, and between cls-2; cls-3 and the other cls-2 depletions, was not significant (Fig. 6). Overall, these results indicate that fewer growing microtubules reach the cortex in CLASP depleted embryos. In this data set, the excessive spindle displacement phenotype was observed in 0% of cls-2 (RNAi) embryos, 60% of cls-2; cls-1 embryos and 37.5% of cls-2; cls-3 embryos. Thus, the strength of the spindle positioning phenotypes exhibited by
CLASP depleted embryos correlates with defects in the number of microtubules that grow to and contact the cortex.

CLS-2 localize to the cytoplasm and mitotic spindle throughout early development

To further investigate the function of *C. elegans* CLASPs, we raised antibodies to all three proteins. The region from amino acids 680–780 of CLS-2 showed the lowest homology among all three CLASPs and this was used as an antigen for raising antibodies against CLS-2 and CLS-3. The 330 amino acid N-terminal region of CLS-1, which is not present in CLS-2 or CLS-3, was used as antigen for CLS-1. Western blotting of embryo extracts made from wild type, which was greatly diminished in extracts made from *cls-2* RNAi treated worms; similarly, immunolocalization in embryos showed staining that was abolished by RNAi (described below, Fig. 7). The anti-CLS-1 and anti-CLS-3 antisera or purified antibodies recognized their respective fusion proteins on westerns. However, a specific signal that could be detected in wild type but not clasp depletion embryos was not observed with immunostaining or western blotting using these reagents (unpublished results).

Previous studies in *C. elegans* showed that CLS-2 localizes weakly at centrosomes and strongly to kinetochores in the one-cell mitotic embryo, but the localization pattern throughout early embryo divisions was not reported (Cheeseman et al., 2005). From analyzing staining throughout the cell cycle (Fig. 7), we found that CLS-2 first appeared on the centrosomes at prophase in some embryos (3/7). By prometaphase, centrosome staining was evident in most embryos and the signal also entered the nucleus (8/9). CLS-2 localized to the kinetochore interfaces and on the spindle at metaphase in one-cell embryos, in addition to the centrosomes (n = 12). Spindle staining persisted into anaphase (17/19), especially in the mid spindle region (Fig. 7); anaphase spindle staining was not reported in *C. elegans* mitotic embryos previously, but was shown for meiotic spindles (Cheeseman et al., 2005; Dumont et al., 2010). Centrosome staining also persisted through anaphase and into telophase (19/19 and 17/18 respectively).

All embryos also showed cytoplasmic staining which was absent after RNAi depletion. In multicellular embryos, interphase cells showed strong cytoplasmic staining that appeared enriched at cell contacts but did not appear to colocalize with regions of the cells showing the densest tubulin staining (Fig. 7). Mitotic cells in multicellular embryos exhibited centrosome, kinetochore and spindle staining through at least the 28-cell stage (n = 23, older embryos are not retained in these samples). The latter observation suggests that CLS-2 continues to play a role in mitosis throughout early embryogenesis.

**CLS-2 has HCP independent roles in the one-cell embryo**

HCP-1 and HCP-2 are CENP-F related proteins in *C. elegans* that play a critical role in both mitosis and meiosis. HCP-1 and HCP-2 are redundant, but embryos depleted simultaneously for HCP-1 and HCP-2 (hereafter referred to as HCP-1/2) show the same chromosome misorientation and weak spindle phenotypes during the first division that were observed after CLS-2 depletion. CLS-2 associates with HCP-1/2 in embryonic extracts, and analyses of multiple kinetochore proteins indicated that HCP-1/2 recruits CLS-2 to kinetochores (Cheeseman et al., 2005; Dumont et al., 2010). To determine if HCP-1/2 also play a role with CLASPs in spindle positioning, we examined embryos after depletion of HCP-1/2 by RNAi. DIC imaging of the embryos during the first division showed that the spindle poles separated abruptly after NEB and spindle poles oscillated independently, as previously reported (Table 2). The spindles in these embryos did not collapse or exhibit position-defects, even after extended RNAi treatment (Table 2). Similarly, other kinetochore mutants show a phenotype of abrupt separation of spindle poles at metaphase without spindle pole collapse (Cheeseman et al., 2004; Grill et al., 2001; Oegema et al., 2001), while we observe spindle pole collapse in 25–75% of *cls-2* single RNAi embryos (Table 2). These results suggest that CLASPs have HCP-1 and HCP-2 independent functions in the one-cell embryo.

**Discussion**

Our work demonstrates that CLS-1 and CLS-3 are partially redundant with CLS-2 during nuclear rotation, spindle length...
maintenance and spindle displacement in *C. elegans* embryos. This redundancy explains why a role for CLS-1 and CLS-3 in early embryos was not uncovered in earlier studies that targeted these genes singly with RNAi. Depletion of CLS-1 and CLS-3 simultaneously did not produce spindle positioning defects, nor did we observe any clear difference in the phenotypes produced by cls-2; cls-1 RNAi versus cls-2; cls-3 RNAi. The simplest explanation for these results is that all three CLASPs function interchangeably in regulating astral microtubules at this stage, but that there are higher levels of CLS-2 than of CLS-1 and CLS-3 in the embryo. Alternatively, the CLS-2 protein could possess functional differences that cause it to have a more dominant affect on microtubule dynamics in the one-cell embryo. Future analyses will be needed to resolve this issue.

In *C. elegans*, other kinetochore mutants show the weak spindle phenotype with premature spindle pole separation and absence of central spindle microtubules during anaphase, similar to what was reported for cls-2 in this and previous studies. However, those kinetochore mutants do not appear to exhibit the spindle collapse that we document here (Cheeseman et al., 2004, 2005; Grill et al., 2001; Oegema et al., 2001; Krueger and Rose, unpublished). Further, we showed that hcp-1; hcp-2(RNAi) embryos do not exhibit spindle pole collapse even after extended RNAi treatments. One explanation for the spindle collapse phenotype is that CLASPs play an additional role in microtubule bundling in the central spindle, as described for *S. pombe* and *Drosophila* CLASPs in mitosis, and *C. elegans* and Xenopus CLASPs in meiosis (Bratman and Chang, 2007; Dumont et al., 2010; Hannak and Heald, 2006; Maiato et al., 2002). Consistent with this view, we found that CLS-2 localizes to the central spindle during anaphase after the chromosomes have segregated. Another explanation for the spindle collapse phenotype is that it results from the loss of microtubule contacts with the cortex which reduces pulling forces on the spindle poles. The absence of spindle collapse and other spindle positioning phenotypes in hcp-1; hcp-2 embryos is also consistent with this model and indeed the two possibilities are not mutually exclusive.

Cortical pulling forces on astral microtubules are regulated by polarity cues throughout the cell cycle in the one-cell embryo, and such pulling forces are necessary for rotation, spindle displacement and anaphase spindle pole separation (Galli and Van Den Heuvel, 2008; Conczy, 2008). We found that PAR-6 localization is normal in the majority of CLASP RNAi embryos, and that the spindle moves towards the posterior as in wild type, albeit with more variable timing. In contrast, there was a more severe change in microtubule-cortex contacts in the CLASP double RNAi treatments compared to cls-2 single RNAi, which does correlate with the stronger spindle positioning defects. These results strongly suggest that the nuclear rotation and excessive spindle displacement phenotypes are caused by altered microtubule-cortex contacts.

To characterize the effects of CLASP depletion in more detail, we examined microtubule plus-end growth using an EBP-2::GFP reporter. The cls-2 and cls-2; cls-1(RNAi) embryos exhibited slightly increased polymerization rates on average. A similar effect was observed after depletion of mammalian CLASPs and was proposed to result from an increase in the soluble tubulin pool (due to the loss of long microtubules), which could enhance microtubule polymerization rate (Mimori-Kiyosue et al., 2005). We found no change in the number of EBP-2::GFP comets at a distance of 10 μm from the centrosome in cls-2(RNAi) embryos compared to wild type, as previously reported (Srayko et al., 2005). Nonetheless, there were fewer EBP-2::GFP comets growing within 2 μm of the cortex in cls-2(RNAi) embryos and double CLASP depleted backgrounds, similar to what we observed for GFP::tubulin marked microtubules. Although we cannot directly measure the rate of microtubule catastrophe and rescue in our system, this decrease in the number of growing microtubules reaching the cortex is consistent with a role for *C. elegans* CLASPs in suppressing catastrophe and promoting microtubule rescue, as reported for CLASPs in other organisms (Al-Bassam et al., 2010; Slep, 2009). We therefore propose that microtubules grow towards the cortex in CLASP depleted embryos, but that fewer microtubules maintain this growth and reach the cortex.
to interact with asymmetrically activated force generators. A stochastic loss of connections with different regions of the cortex would then lead to the variable affects on spindle positioning and spindle collapse that were observed. Alternatively the failure in rotation and the excessive posterior spindle displacement observed in CLASP depletion embryos could suggest that CLASPs specifically affect microtubules at the anterior cortex. It has been shown that posterior pulling is active starting at prometaphase, but the anterior spindle pole is tethered until late metaphase without active pulling (Labbe et al., 2004). However, it is unlikely that CLASPs function as part of this tether, because loss of the tether should result in early spindle movement, and CLASP depletion resulted in variable or late posterior displacement (Fig. 2). Rather, if the tether is intact but microtubule contacts are lost stochastically in clasp(RNAi) embryos, then sometimes spindles would move excessively towards the posterior in response to the active pulling forces, and the timing would be variable. Consistent with this view, microtubule-cortex contacts

---

**Fig. 7.** Localization of CLS-2 in the early embryo. (A) Western blot of embryo extracts probed with anti-CLS-2 and anti-tubulin antibodies. (B) Confocal images showing CLS-2 immunolocalization or merged CLS-2, α-tubulin and DAPI. The display levels for metaphase and multicellular control embryos shown are lower to allow visualization of kinetochore staining. Arrowheads point to centrosome staining, arrows to central spindle staining. Scale bar is 10 mm.
were reduced in all regions of clasp(RNAi) embryos, and we did not observe any posterior movement of the spindle in clasp(RNAi) embryos in which asymmetric cortical forces were attenuated by loss of lin-5 activity. Excessive posterior displacement movements have also been reported in zyg-8 mutants which have shorter microtubules beginning at metaphase, and in embryos treated with nocodazole at metaphase (Gonczy et al., 2001). Interestingly however, in those embryos the spindles stay in the posterior, whereas in clasp(RNAi) embryos, the anterior spindle pole moves back to a more normal position. Microtubules in zyg-8 and nocodazole treated embryos appear uniformly short. In contrast, some microtubules are long enough to contact the cortex in clasp(RNAi) embryos. Thus at anaphase, when centrosomes normally nucleate more microtubules and anterior cortical pulling becomes active again, interactions of the microtubules with the cortex in CLASP embryos may allow the anterior spindle pole to move anteriorly once again and the spindle poles to separate.

In summary, we have demonstrated a role for CLASPs in regulating astral microtubules during spindle positioning in mitosis. The three C. elegans CLASPs appear to function interchangeably to regulate microtubules in the one-cell embryo. This is similar to what has been seen in mammalian cells where CLASPs were shown to function redundantly to regulate microtubule stability (Maia et al., 2003; Mimori-Kiyosue et al., 2005; Pereira et al., 2006) during interphase and mitosis. However, the C. elegans CLASPs may have separable roles as well. The kinetochore and meiosis defects caused by cl-2 depletion appear fully penetrant (Cheeseman et al., 2005; Dumont et al., 2010), while the spindle positioning and spindle maintenance phenotypes we observed were more severe after double depletion. These data raise the possibility that CLS-2 has a unique function at the kinetochore compared to CLS-1 and CLS-3. Further, CLS-1 is observed were more severe after double depletion. These data raise the possibility that CLS-2 has a unique function at the kinetochore compared to CLS-1 and CLS-3. Further, CLS-1 is predicted to have two TOG-like domains, similar to mammalian CLASPs, whereas CLS-2 and CLS-3 are only predicted to have one. CLS-1 could therefore have unique functions. In addition, promoter fusions to CLS-1 and CLS-3 show differential expression in larvae and adults (Hunt-Newbury et al., 2007; Lynch et al., 1995). Future work comparing each of these proteins’ biochemical activities and cellular roles will yield important insights into the different functions of the CLASP family of proteins.

Acknowledgments

We thank Geraldine Seydoux for strains and Frank McNally for strains and the cl-2 cDNA. Other strains were provided by the Caenorhabditis Genetics Center (funded by the NIH National Center for Research Resources). Dae Hwi Park, Adam Hayashi and Hai Chi Pham provided valuable technical assistant. We thank Frank McNally and Jawdat Al-Bassam for comments on the manuscript, and members of the Rose and McNally labs for helpful discussions. This research was supported by NIH R01GM68744 (partially funded via the ARRA) to L.R., an NSF Predoctoral Fellowship to E.E. This research was supported by NIH R01GM68744 (partially funded via the ARRA) to L.R., an NSF Predoctoral Fellowship to E.E.

References


