Eukaryotic cells possess a sophisticated membrane system to facilitate diverse functions. Whereas much is known about the nature of membrane systems in interphase, the organization and function of the mitotic membrane system are less well understood. In this study, we show that epsin, an endocytic adapter protein, regulates mitotic membrane morphology and spindle integrity in HeLa cells. Using epsin that harbors point mutations in the epsin NH2-terminal homology domain and spindle assembly assays in *Xenopus laevis* egg extracts, we show that epsin-induced membrane curvature is required for proper spindle morphogenesis, independent of its function in endocytosis during interphase. Although several other membrane-interacting proteins, including clathrin, AP2, autosomal recessive hypercholesterolemia, and GRASP65, are implicated in the regulation of mitosis, whether they participate through regulation of membrane organization is unclear. Our study of epsin provides evidence that mitotic membrane organization influences spindle integrity.

**Introduction**

Much progress has been made in understanding how the microtubule (MT)-based mitotic spindle captures and segregates chromosomes into daughter cells. However, it is not known whether membrane systems play a role in facilitating spindle organization in mitosis. In interphase, membrane organization and trafficking are regulated not only by membrane deformation, fusion, fission, and tethering systems but also by the MT cytoskeleton (McMahon and Gallop, 2005; Itoh and De Camilli, 2006). Interphase membranes also regulate MT organization. For example, the interphase trans-Golgi network functions as an MT-organizing center essential for both cell polarity and migration (Chabin-Brion et al., 2001; Efimov et al., 2007; Liu et al., 2007). Such an intimate relationship between MTs and membranes in interphase suggests that the two structures could regulate each other in mitosis to facilitate proper cell division.

Consistent with this idea, the ER and Golgi undergo dramatic reorganization during mitosis, with membrane tubules and vesicles being observed both within and surrounding mitotic spindles, suggesting a function for the spindle in the partitioning of mitotic membranes (Waterman-Storer et al., 1993; Axelsson and Warren, 2004; Altan-Bonnet et al., 2006; Lowe and Barr, 2007; Wei and Seemann, 2009). An increasing number of membrane-associated proteins have been shown to regulate different aspects of mitosis (Cao et al., 2003; Cao and Zheng, 2004; Royle et al., 2005; Sütterlin et al., 2005; Vong et al., 2005; Boucrot and Kirchhausen, 2007; Lehtonen et al., 2008). Moreover, a membranous mitotic spindle matrix has been isolated from *Xenopus laevis* egg extracts, which can stimulate MT assembly from pure tubulin in vitro (Tsai et al., 2006; Zheng and Tsai, 2006). These data suggest that proper organization of mitotic membranes could influence spindle assembly.

Endocytic proteins such as clathrin, AP2, and autosomal recessive hypercholesterolemia (ARH) induce membrane curvature either directly or indirectly by recruiting other proteins to drive endocytic vesicle formation. During mitosis, they localize to different places, with clathrin concentrating on the spindle, ARH localizing to centrosomes, and AP2 distributing throughout the cell (Royle et al., 2005; Boucrot and Kirchhausen, 2007; Lehtonen et al., 2008). Whereas clathrin appears to regulate kinetochoore MT bundling independent of its role in membrane deformation and endocytosis, how AP2 and ARH regulate mitosis remains unclear.

In this study, we focus on epsin, an endocytic adapter protein which directly binds and deforms membranes through its epsin NH2-terminal homology (ENTH) domain (Ford et al., 2002).
Another human cell line, MCF-10A, codepletion of epsin1 and -2 resulted in a similar level of mitotic defects to those observed in HeLa cells (Fig. S2).

Live imaging of HeLa cells expressing histone H2B–GFP showed that the time cells spent in prometaphase and metaphase, which was determined from the first sign of nuclear envelope breakdown to the first sign of anaphase chromosome separation (Fig. S1 C), was significantly increased in epsin1 RNAi–treated cells (Fig. 1 D). There was no obvious defect in chromosome segregation, as a majority of the cells proceeded through mitosis after a delay. Interestingly, a recent study also showed that deletion of NuMA (nuclear/mitotic apparatus protein) in mouse embryonic fibroblasts prevented spindle pole focusing without rendering obvious chromosome segregation defects (Silk et al., 2009).

Reduction of epsin alters membrane organization in mitosis

We provide evidence that the membrane deformation function of epsin affects spindle organization during mitosis.

Results and discussion

Reduction of epsin in human cells causes defects in spindle morphology

We used RNAi to determine whether endocytic adapter proteins might regulate mitosis in HeLa cells and found that epsin1 depletion caused a strong increase in mitotic cells with defective spindles (Fig. 1 C and Fig. S1, A and B). This was confirmed by two different siRNA oligonucleotides targeting different regions of epsin1, both of which reliably reduced epsin1 to 20–30% of the control levels in HeLa cells 48 h after siRNA transfection (Fig. 1 A). In contrast to control cells in which pericentrin staining was intense and focused at the spindle poles, epsin1 RNAi–treated cells showed a significant increase in splayed spindle poles and multipolar spindles (Fig. 1, B and C). There was no increase in the number of centrosomes in interphase cells (unpublished data). Although RNAi of epsin1 alone gave only a mild defect in spindle morphology in another human cell line, MCF-10A, codepletion of epsin1 and -2 resulted in a similar level of mitotic defects to those observed in HeLa cells (Fig. S2).

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Reduction of epsin alters membrane organization in mitosis

Next, we performed immunofluorescence staining for epsin1. Although clathrin was enriched on mitotic spindles relative to cytoplasmic staining, as previously reported (Royle et al., 2005),
we found that epsin1 was diffusely distributed in the mitotic cytoplasm, including the spindle region, with no obvious localization to centrosomes or spindle poles (Fig. S1 D). In addition, although epsin1 has been shown previously to interact with tubulin and MTs in rat brain lysate (Hussain et al., 2003), we could not detect any interaction between epsin1 and MTs or tubulin in mitotic Xenopus egg extracts (Fig. S3). Therefore, it is unlikely that epsin1 contributes toward spindle organization by directly regulating MT assembly dynamics in mitosis.

Epsin1 is known to induce membrane curvature through its ENTH domain in vitro (Ford et al., 2002). Thus, epsin1 may organize mitotic membranes, which in turn facilitates spindle morphogenesis. We used a membrane-permeable fluorescent dye, DiOC₆(3), to visualize mitotic membranes in live cells by confocal microscopy (Waterman-Storer et al., 1993). To identify mitotic cells, chromosomes were labeled with Hoechst 33258. In control RNAi–treated cells, the membrane network uniformly surrounded mitotic chromosomes, whereas epsin1 RNAi–treated cells often showed distorted and uneven membrane distribution (Fig. 2 A and Videos 1–4). The percentage of cells with abnormal membrane morphology was significantly higher with epsin1 siRNA treatment than with control siRNA treatment (control, 9.2 ± 3.8%; epsin1 siRNA, 45.8 ± 8.9%; Student’s two-tailed t test, P = 0.0016; n > 100 in each condition).

Next, we performed EM on control or epsin1 RNAi–treated cells (Fig. 2 B). Abundant membrane tubules and fragments were observed in control metaphase cells. These membrane structures usually surrounded condensed chromosomes in an even distribution (Fig. 2 B). However, in epsin1-depleted cells, uniform short membrane tubules were replaced by abnormally long membrane...
Cells with uneven KDEL signal were as follows: control, 15.7 ± 8% (n = 56); epsin1 siRNA2, 52.5 ± 2.3% (n = 64); Student’s two-tailed t test, P = 0.011. This is reminiscent of the membrane morphology defects observed in live cells by membrane dye labeling (Fig. 2 A). Therefore, epsin regulates the organization of the mitotic membrane network that contains ER membranes, which may in turn influence spindle integrity.

The membrane deformation function of epsin is required for proper mitotic spindle morphology

To further elucidate whether the membrane-bending activity of epsin1 is required for proper spindle morphology, we designed various epsin1 expression constructs for siRNA rescue. By introducing point mutations into the wobble codons of siRNA-targeting sequences, we generated RNAi-insensitive versions of epsin1 expression constructs that have the wild-type amino acid sequence. Because epsin1 is known to be phosphorylated by Cdc2 kinase on residue Ser357 during mitosis (Chen et al., 1999; Kariya et al., 2000; Rossé et al., 2003), we further introduced a single mutation at Ser357 to produce the phosphomimic and non-phosphorylatable forms Ser357D and Ser357A, respectively.

All three constructs were able to rescue the defects caused by epsin1 depletion when cotransfected with the siRNA (Fig. 4 and Fig. S1, E and F), suggesting that the mitotic function of epsin1 reduction did not cause obvious changes in the distribution of markers for Golgi (β-COP), nuclear pores (nucleoporin; nuclear pore complex; detected by MAb414), or nuclear lamina (lamin B2) in mitosis (Fig. 3 A). However, the evenly distributed mitotic ER network around the spindle (Puhka et al., 2007), which is revealed by the KDEL antibody in control cells, became disorganized upon epsin1 knockdown by RNAi (Fig. 3, A and B). Cells with uneven KDEL signal were as follows: control, 15.7 ± 8% (n = 56); epsin1 siRNA2, 52.5 ± 2.3% (n = 64); Student’s two-tailed t test, P = 0.011. This is reminiscent of the membrane morphology defects observed in live cells by membrane dye labeling (Fig. 2 A). Therefore, epsin regulates the organization of the mitotic membrane network that contains ER membranes, which may in turn influence spindle integrity.

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Epsin1 facilitates spindle organization independent of its endocytic function

To formally establish that epsin1 has a mitosis-specific role in spindle organization independent of its endocytic function in interphase, we used the cell-free spindle assembly assay using Xenopus egg extracts (Murray, 1991). Because spindle assembly in egg extracts is not confined within a cell cortex, we may not observe the same types of spindle defects observed in whole cells. Nonetheless, if epsin regulates spindle assembly independently of its role in endocytosis, we should observe spindle defects when its activity is inhibited.

We generated a polyclonal antibody against the ENTH domain of Xenopus epsin1 (XEpsin1; also known as MP90), which recognized the purified bacterially expressed XEpsin1-6His and a single band in egg extracts (Fig. 5 A). The retarded migration of the endogenous XEpsin1 on SDS-PAGE is most likely caused by posttranslational modifications such as phosphorylation and ubiquitination (Oldham et al., 2002; Polo et al., 2002; Horvath et al., 2007). Consistent with this idea, when purified XEpsin1-6His was incubated with egg extracts depleted of endogenous XEpsin1 (Fig. 5, D and E), it migrated to the same position as endogenous XEpsin1 (Fig. 5 A).

We estimated the endogenous XEpsin1 concentration to be ~70 nM in egg extracts. Because the ENTH domain of XEpsin1 appeared to be important for spindle organization (Fig. 4), the ENTH domain antibody might inhibit assembly of spindles when added to the egg extracts. We first incubated either the ENTH domain antibody or rabbit IgG purified from nonimmune rabbits with the cycled egg extract at a final IgG concentration of 0.15 mg/ml and then initiated spindle assembly using Xenopus sperm. We observed a reduction of normal spindle assembly and a corresponding increase in sperm associating with MT structures at all (Fig. 5, B and C).

Next, we immunodepleted XEpsin1 from cytostatic factor (CSF)–arrested egg extracts. Approximately 90% of the endogenous XEpsin1 was depleted, as judged by Western blot analysis (Fig. 5, D and E). Similar to ENTH domain antibody addition, we found that depletion of XEpsin1 also resulted in a significant reduction of normal spindle assembly compared with mock-depleted egg extracts and a corresponding increase in sperm associated with either no spindles or with defective spindles (Fig. 5, B and C).

Figure 4. Epsin1 regulators spindle integrity through its membrane-bending ENTH domain. (A) Domain structure of epsin1 expression constructs with the indicated mutations. The ENTH domain interacts with membrane and has membrane-bending activity. The ubiquitin-interacting motif (UIM) binds to ubiquitin. DPW motifs interact with AP2. The NPF motif interacts with proteins such as Eps15. The mutant epsin1Ser357D (S357D) mimics the mitotic phosphorylated form. The RNAi-insensitive rescue construct epsin1Ser357D-ins (S357D-ins) contains mutations in wobble codons in the sequence corresponding to siRNA2, which do not affect protein sequences. Mutant epsins epsin1L6E/Ser357D-ins (L6E/S357D-ins) and epsin1R63L/Ser357D-ins (R63L/S357D-ins) are derived from epsin1Ser357D-ins but contain an additional point mutation in the ENTH domain that abolishes the membrane-bending activity of epsin1. (B) Western blot analysis of epsin1 expression in HeLa cells. Epsin1Ser357D expression was inhibited by siRNA2 but not by control siRNA, whereas expression of epsin1Ser357D-Dins, epsin1L6E/Ser357D-ins, and epsin1R63L/Ser357D-ins was not affected by treatment with siRNA2. (C) Rescue of spindle morphology defects in HeLa cells by epsin1Ser357D-ins but not by epsin1L6E/Ser357D-ins or epsin1R63L/Ser357D-ins. The percentage of defective spindles, as judged by α-tubulin and pericentrin staining, was quantified in HeLa cells cotransfected with the siRNA oligonucleotide and each expression construct. The percentages of cells with normal spindle morphology from at least three independent experiments are shown, with >100 prometaphase and metaphase phase cells counted in each condition per experiment. Error bars show standard deviation. *, P < 0.05; **, P < 0.01; ***, P < 0.001.
elastic module to provide support for MTs during spindle assembly. A disorganized membrane network such as those caused by epsin reduction would exhibit uneven elasticity and deformability when subjected to forces from MTs, which could affect spindle morphology. We suggest that the membrane elasticity could be provided in part by the intermediate filament protein lamin B (Tsai et al., 2006; Ma et al., 2009). Consistent with this, we have found that lamin B balances motor activities during spindle assembly in *Xenopus* egg extracts (unpublished data).

Besides epsin, other proteins may also contribute to the organization of various membrane compartments in the mitotic cytosol. This may explain the relatively mild spindle defects we observed in epsin reduction. Proteins such as AP2, ARH, and GRASP65 have functions in mitosis (Sütterlin et al., 2005; Boucrot and Kirchhausen, 2007; Lehtonen et al., 2008). It will be interesting to determine whether they have a role in mitotic membrane organization as well. Other proteins such as BAR (Bin, amphiphysin, and Rvs) or F-BAR (FCH-BAR)...

**Figure 5. Epsin1 affects spindle assembly in Xenopus egg extracts.** (A) Characterization of XEpsin1 antibody. Rabbit polyclonal antibodies against the ENTH domain of XEpsin1 recognized a single band in Xenopus egg extract (XEE) by Western blot analysis. 6His-tagged XEpsin1 purified from bacteria showed an apparent smaller molecular mass than the endogenous protein. After incubating with the Xenopus egg extract that was immunodepleted of endogenous XEpsin1, the recombinant XEpsin1 migrated as a similar size as the endogenous protein [XEpsin1-6His in XEE]. (B) XEpsin1 antibody inhibits spindle assembly in cycled Xenopus egg extracts. Quantifications of percentages of MT structures formed in cycled extract after the addition of control antibody (+control rabbit IgG) or XEpsin1 antibody (+anti-XEpsin1) were performed by analyzing structures associated with >300 sperm in each reaction. Results from four independent experiments were averaged. (C) Examples of Xenopus sperm associated with normal spindle, abnormal MT structures, or no MT structure. MT structures were visualized by rhodamine-tubulin, and chromosomes were stained with DAPI. (D) Western blot analysis of XEpsin1 in extracts after immunodepletion. Different amounts of untreated egg extracts were loaded as standards. Mock-depleted extract [-IgG] had a similar level of XEpsin1 as nondepleted extract, whereas ~90% of XEpsin1 was depleted by XEpsin1 antibody (--XEpsin1). (E) Western blot analysis of XEpsin1 after depletion (--XEpsin1) and add back of recombinant wild-type or mutant XEpsin1 (--XEpsin1 + WT or --XEpsin1 + L6E) compared with mock-depleted extract. (F) Coomassie blue staining of SDS-PAGE to show the quality of purified wild-type (WT) and mutant (L6E) XEpsin1 proteins. (G) Effects of depletion/add back of XEpsin1 on spindle assembly in CSF extracts. Mock-depleted extract, XEpsin1-depleted extract (--XEpsin1), or XEpsin1-depleted extract with the addition of wild type or the L6E mutant of the XEpsin1-6His protein was allowed to form spindles by incubation with sperm DNA. The number of normal spindles was quantified and normalized against mock-depleted extract. Results from three independent experiments, each containing structures associated with >300 sperms per reaction, were averaged. *, P < 0.05; **, P < 0.01. (B and G) Error bars show standard deviation. Bar, 10 µm.

and used the proteins to rescue the XEpsin1-depleted egg extracts (Fig. 5, E and F). The addition of wild-type XEpsin1 but not XEpsin1L6E-6His resulted in a significant rescue of normal spindle formation in XEpsin1-depleted egg extracts (Fig. 5 G). Similarly, XEpsin1R63L-6His, which is defective in lipid binding, also failed to rescue the spindle defects caused by XEpsin1 depletion (unpublished data). Therefore, the spindle organization function of XEpsin1 requires its membrane-bending activity but is independent of its role in endocytosis during interphase.

We show that epsin regulates spindle morphology indirectly through its ability to regulate mitotic membrane organization. Although most studies of spindle assembly have focused on structures and forces produced within the spindle MTs, both our study (Fig. 2 and Videos 1 and 3) and previous work (Waterman-Storer et al., 1993; Axelsson and Warren, 2004; Altan-Bonnet et al., 2006) have shown that the mitotic spindle is built inside a highly interconnected membrane network, which could form an
domain—containing proteins, coat proteins such as COPI and COPII, and enzymes involved in lipid metabolism may also be involved in regulating mitotic membrane organization (McMahon and Gallop, 2005; Itoh and De Camilli, 2006). Our study of epsin and mitotic membrane should stimulate further investigation into how structures outside the spindle proper could affect MT organization within the spindle.

**Materials and methods**

**siRNAs, plasmids, proteins, and antibodies**

siRNAs for human epsin 1 [sirNA1, 5'-GGAGAACGCGCCGAGATCAT-3'; sirNA2, 5'-GGACCTTGTCGACGGTTC-3'; sirNA3, 5'-GGACTGGCGT-CAGGTCTA-3'], clathrin heavychain (5'-GCAATGCGTCGTTTGAAGA-3'), Eps15 (EGF receptor pathway substrate 15; 5'-AAACCGAGCTACAGA-TTAT-3'), Eps15R (Eps15 related; 5'-GCACTTGGATGAGATGAG-3'); Huang et al., 2004), and control siRNA (5'-GCTAAGCGGAAATCTGCTGA-3') as well as SMARTpool siRNA for epsin2 and -3 were purchased from Thermo Fisher Scientific. Full-length cDNA clones encoding human epsin 1 (IMAGE clone 5244118) and XEpsin 1 (IMAGE clone 3405036) were obtained from Open Biosystems. Wild-type human epsin 1 was cloned into the pEFG6/V5-His vector (Invitrogen). The phosphomimetic and nonphosphorylatable forms of epsin 1, Ser357D and Ser357A, respectively, were generated against the C-terminal region of mouse lamin B2 (aa 383–589).

**Cell culture and transfection**

Cells grown on coverslips were fixed 48 h after transfection with 4% paraformaldehyde for 15 min and permeabilized in TBS containing 0.1% Triton X-100 for 15 min. After blocking with 3% BSA in TBS, coverslips were incubated with DM-1a and M8 diluted in the blocking solution, followed by Alexa Fluor 488- and Alexa Fluor 594-conjugated secondary antibodies (Invitrogen) together with DAPI, and mounted. Images were acquired by MetaMorph (MDS Analytical Technologies) through a charge-coupled device camera (CoolSNAP HQ: Photometrics) on an inverted microscope (Eclipse TE2000; Nikon) with a Plan-Apochromat 60× NA 1.4 oil lens (Nikon). Confocal images were obtained by a confocal laser-scanning microscope (TCS SP5 DM6000B; Leica) with an HCX Plan-Apochromat 63× NA 1.4 oil lens. For mitotic timing measurements, cells were maintained in a liveCell chamber (Pathology Devices) were imaged by iPLab (BD) through a CoolSNAP HQ lens conjugated device microscope on an inverted microscope (TE2000; Nikon) with a Hoffman modulation contrast EWL Plan-Fluar 10x NA 0.3 lens 32–48 h after siRNA transfection of HeLa cells stably expressing histone H2B–GFP. To visualize live mitotic membranes, cells grown on the coverslip were labeled with Hoechst 33258 and DIOC6(3) (Invitrogen) as previously described [Waterman-Storer et al., 1993] 2 d after siRNA transfection. Mitotic cells with condensed chromosomes were picked randomly and imaged live on a TCS SP5 DM6000B confocal laser-scanning microscope with an HCX Plan-Apochromat 63× NA 1.4 oil lens at 1-µm intervals (z section). Cells were scored based on membrane morphologies. For 3D reconstruction, images were obtained at 0.118-µm intervals and analyzed with Imaris imaging software (Bitplane).

**EM imaging**

HeLa cells in 35-mm dishes were treated with siRNA for 72 h, followed by fixation in 3% glutaraldehyde, 1% formaldehyde, and 2 mM CaCl2 in 0.1 M cacodylate, pH 7.4, for 1 h, and subjected to uranyl acetate staining. For mitotic arrest, cells were fed with fresh medium containing 100 ng/ml nocodazole 14 h before fixation. Mitotic cells with condensed chromosomes were observed on an electron microscope (Tecnai 12; Philips). Images of mitotic cells were taken from control and epsin1 RNAi–treated cells. To quantify the membrane defects, all images were scrambled together and then analyzed blindly based on membrane morphology. Two siRNA oligonucleotides for epsin1 (siRNA1 and -2) produced essentially the same phenotype; results from siRNA2-treated samples are shown.

**Xenopus egg extract manipulations**

CSF-arrested extracts were prepared from Xenopus eggs collected and dejellied (Murray, 1991). The egg extracts were made by a crushing spin in an ultracentrifuge (LS-50; Beckman Coulter). For antibody addition experiments, extracts were drawn into interphase by incubating with 0.4 mM Ca2+ and 300 demembranated sperm/ml, energy mix (3.8 mM creatine phosphate, 0.5 mM ATP, 0.5 mM MgCl2, and 0.05 mM EGTA, pH 7.7), and 20 µg/ml rodamine-tubulin at 20°C for 60–90 min and monitored by nucleation formation. An equal volume of fresh CSF-arrested extract was added to the interphase egg extract along with purified XEpsin 1 antibody or control (1:2,500 dilution) for 15 min to final concentration) to cycle the egg extract back into mitosis. The reactions were incubated at 20°C for another 50–70 min to allow spindle formation before they were diluted into BR80 (80 mM K-Pipes, pH 6.8, 1 mM MgCl2, and 1 mM EGTA) containing 30% glycerol and spun onto 12-mm round coverslips through BR80 containing 40% glycerol. After fixation by cold methanol, the coverslips were stained with DAPI and mounted. Alternatively, fresh CSF-arrested extract was incubated with Dynabeads protein A (Invitrogen) coated with either control antibody or XEpsin 1 antibody for 1 h at 4°C. After retrieval of the magnetic beads, mock- or XEpsin-1-depleted extract was used for spindle assembly with sperm nuclei, energy mix, and rhodamine-tubulin. For rescue experiments, purified wild-type XEpsin 1-6His, mutant XEpsin 16L-6His, or XEpsin 163L-6His protein was added to the extract after depleting the endogenous protein and incubated on ice for 20–30 min before starting the spindle assembly reaction.
Online supplemental material
Fig. S1 shows mitosis in normal or siRNA-transfected HeLa cells. Fig. S2 shows mitotic defects in MCF-10A cells depleted of epsin family members. Fig. S3 shows a lack of interaction between epsin 1 and epsin 1 and tubulin in mitotic Xenopus egg extract. Videos 1 and 2 show membrane organization during normal mitosis and during mitosis in epsin 1-depleted cells, respectively. Videos 3 and 4 show 3D reconstructions of membrane organization during normal mitosis in a HeLa cell and during mitosis in an epsin 1-depleted HeLa cell, respectively. Online supplemental material is available at http://www.jcb.org/cgi/content/full/jcb.200902071/DC1.

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