Lumen Morphogenesis in C. elegans Requires the Membrane-Cytoskeleton Linker erm-1

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Summary

Epithelial tubes are basic building blocks of complex organs, but their architectural requirements are not well understood. Here we show that erm-1 is a unique C. elegans ortholog of the ERM family of cytoskeleton-membrane linkers, with an essential role in lumen morphogenesis. ERM-1 localizes to the luminal membranes of those tubular organ epithelia which lack stabilization by cuticle. RNA interference (RNAi), a germline deletion, and overexpression of erm-1 cause cystic luminal phenotypes in these epithelia. Confocal and ultrastructural analyses indicate that erm-1 functions directly in apical membrane morphogenesis, rather than in epithelial polarity and junction assembly as has been previously proposed for ERM's. We also show that act-5/ cytoplasmic actin and sma-1/β-H-spectrin are required for lumen formation and functionally interact with erm-1. Our findings suggest that there are common structural constraints on the architecture of diverse organ lumina.

Introduction

Tubulogenesis requires a concerted program of upstream regulators and downstream effectors to mold a polarized, junction-sealed epithelium into a tube (Lubarsky and Krasnow, 2003). Tubes extend basolaterally along extracellular matrix (Hardin, 2000). It is not clear what kind of matrix, if any, is needed on the apical/luminal side. Human Ezrin, Radixin, and Moesin (ERM), paralogous membrane-cytoskeleton linkers, and the Neurofibromatosis 2 (NF2) tumor suppressor Merlin, constitute the (M)ERM family of proteins, whose archetype is Protein 4.1. ERM proteins attach to the cell membrane by an N-terminal FERM (Protein 4.1/Ezrin/Radixin/Moesin) domain and to actin by their C terminus. In vitro, they have been implicated in cell shape, migration, adhesion, adherens junction, and stress fiber formation (Bretscher et al., 2002). These structural functions have recently received less attention than their presumed participation in various signaling pathways. An in vivo role in epithelial integrity, polarity, and junction formation was suggested for the Drosophila ERM ortholog Dmoe2sin, which was proposed to act by antagonizing rho signaling rather than by providing a structural function (Polesello et al., 2002; Speck et al., 2003). A role of ERM proteins in tubulogenesis has not yet been identified in any species in vivo. C. elegans has only one ancestral ortholog to the ERM family, facilitating in vivo analysis. Here we describe a structural role for the C. elegans ERM ortholog in luminal membrane morphogenesis, apparently independent of polarity and junction formation.

Results and Discussion

erm-1 Is Required for Lumen Morphogenesis in Tubular Organ Epithelia

We cloned two C. elegans (M)ERM family members by degenerate PCR and low stringency hybridization using human (M)ERM sequences (see Supplemental Results at http://www.developmentalcell.com/cgi/content/full/6/6/865/DC1). One molecule is more closely related to ezrin, radixin, and moesin (designated erm-1), and the other to neurofibromatosis 2 (designated nfm-1; Supplemental Figures S1 and S2). erm-1 and nfm-1 are the only family members in C. elegans, although there are additional FERM domain-containing genes (designated fmr-1–10). ERM-1 is unusually highly conserved compared to its human orthologs, with approximately 80% similarity in the N-terminal FERM domain, less homology in the central coiled-coil domain, and near identity in the C-terminal actin binding site (cloning, structural analyses, and novel splice variant, see Supplemental Results; Supplemental Figures S1 and S2).

We generated several transgenic lines expressing ERM-1-GFP/LacZ fusion proteins, including a rescuing C-terminal GFP fusion (see Experimental Procedures). ERM-1-GFP is enriched along the luminal surfaces of epithelia, but not in the remaining organ lumina of the pharynx, rectum, and excretory pore cell, which all secrete cuticle (Figure 1; for detail, see below and Supplemental Results). Conversely, NFM-1-GFP/LacZ fusion proteins are enriched at the basolateral surfaces of tubular epithelia (Figures 1F and 2E; Supplemental Figures S3G, S3H, S3K, and S3L). Both proteins are expressed in embryos, larvae, and adults. In situ RNA hybridization is consistent with this organ-specific expression pattern, and additionally detects early embryonic erm-1 RNA (see http://nematode.lab.nig.ac.jp), in accord with a maternal requirement (see below). erm-1 is thus expressed in organ epithelia that lack luminal stabilization by cuticle.

Reduction of ERM-1 levels by RNAi causes a cystic luminal phenotype in all tubular epithelia where it is expressed. erm-1 RNAi induces ~90% early larval lethality (depending on strength of RNAi; Experimental Procedures), ~80% intestinal and ~10% excretory canal cysts (visible by dissecting microscope), and, when conditionally induced, gonad tubulogenesis defects.

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Figure 1. ERM-1 Is Enriched at Luminal Surfaces

Here and elsewhere, animals are viewed from the side: anterior left, posterior right, dorsal top, ventral bottom. Gray boxes in schematics (iA, iiA, and iiiA) indicate areas magnified in images beneath. (iC and iiB) Nomarski images; (iD and iF) confocal sections; (iE, iiC, iiiB, and iiiC) confocal projections.

i. Intestine. (A) ERM-1 localizes to intestinal lumen (green). (B) Wild-type intestine with 20 bilaterally symmetrical cells in 9 rings (4 cells for first ring; blue), intestinal lumen (green), AJ (red); tube is rotated along longitudinal axis. (C–F) Anterior intestinal tube; apical (small arrowheads) and basal (large arrowheads) sides of tube indicated. ERM-1-GFP (D and E) is at the lumen, apical to but distinct from /AJM-1 (yellow in [E] from integration of sections, not overlap). ERM-1-GFP also seen in excretory canal in (E) (arrow). NFM-1-lacZ (F) is localized basolaterally.

ii. Excretory canals. (A) ERM-1 localizes to excretory canals (red; canals shown separated for clarity). (B and C) Anterior excretory canals: ERM-1-GFP outlines canals and also the intestinal (arrow), but not pharyngeal (arrowhead) lumen.

iii. Gonad. (A) ERM-1 localizes to gonad lumina: spermathecae, uterus, vulva (blue). (B) Integration of sections through uterine-vulval connection (ventral view); vulva opens to right: ERM-1-GFP outlines eggs in uterus and vulval opening (arrowhead). (C) Vulva (large arrowhead) at higher magnification (ventrolateral view; vulva opens to right): ERM-1-GFP is separate from complex junctional /AJM-1 pattern (yellow from integration of sections, not overlap); intestinal lumen at left (small arrowhead).

(Figure 2). A 969 bp erm-1 deletion from exons 6 to 7 removes part of the coiled-coil domain and generates a premature stop (tm677; Experimental Procedures; Supplemental Figure S1A). Characterization of this allele (see Supplemental Results) confirmed the RNAi phenotype and revealed a maternal requirement for erm-1: homozygous progeny of heterozygous parents survive to adulthood, but die with the erm-1 gonad phenotype, while >90% of progeny of homozygous parents die as early larvae with the erm-1(RNAi) intestinal and excretory canal phenotypes. An integrated erm-1 transgene (vjls2) rescues the mutant, and the full-length C-terminal GFP fusion transgene partially rescues and restores viability (see Supplemental Results). Mosaic expression of the extrachromosomal erm-1-GFP transgene in erm-1(tm677) animals showed that rescue of the different luminal phenotypes is conditionally dependent on the presence of the protein (Figures 2iA and 2iB). erm-1(tm677) is probably a null allele, given the molecular nature of the lesion, its similarity to the RNAi phenotype, and lack of an essential enhancement when placed in trans to a deficiency (data not shown).

The erm-1(RNAi) intestinal epithelium has a meandering and twisted luminal course with a mildly distorted tube structure. The twisting is so severe that the lumen often appears occluded, an impression accentuated by interspersed luminal cysts (Figure 2i). Excretory canal cysts grow large, but are less frequent in erm-1(RNAi) animals (Figure 2iC). This likely reflects the fact that most canals are not extended, as demonstrated by analysis of a vha-1/4-GFP transgenic line, made to label the excretory system (data not shown). erm-1 transgenic overexpression (confirmed by Western analysis; data not shown) causes a different type of septated excretory
Lumen Morphogenesis in *C. elegans* Requires *erm-1*

### i. Intestine

(A and B) Wild-type/erm-1(RNAi) L1s; compare intestinal luminal widths (arrowheads in all i panels); black arrows indicate basal sides of intestinal tube. (C and D) Integration of sections through intestinal AJ in wild-type/erm-1(RNAi) embryos; inset in (D) highlights distorted AJ ladder pattern; pharynx length (P) indicated in (C). (E and F) Wild-type/erm-1(RNAi) embryos; note correct placement of apicobasal markers in (F), but twisted lumen with “pseudo-obstructions” (falsely suggesting luminal discontinuity; large arrows), distorted cell bodies (small arrows); pharynx cut off in (E). (G and H) Wild-type/erm-1(RNAi) L1s; luminal course meanders in (H) with dorsoventral and right-left deviations/twisting; cell bodies are stretched (left arrow), hyperrotated (middle arrow), compressed (right arrow). Actin is correctly placed at the lumen (yellow, overlap with ICB4). (I and J) *erm-1*(RNAi) L1s; apical membrane and submembraneous markers (PKC-3, 1F52-2) correctly placed, but follow distorted luminal course; note “pears-on-a-string” pattern (arrow). (K and L) wild-type/erm-1(RNAi) L1s fed with DsRed-bacteria: note patency of luminal pseudo-obstructions ([L]; arrow).

### ii. Excretory canals

Arrowheads indicate cysts. (A and B) *erm-1*tm677 adult partially rescued by ERM-1-GFP: intestinal lumen (ERM-1-GFP present) is wild-type, excretory canal lumen (ERM-1-GFP absent) cystic. (C) *erm-1*(RNAi) L1 excretory canal cyst displacing pharynx and intestine, encompassing half of animal’s length. (D) Septated excretory canal cyst in transgenic adult overexpressing ERM-1; arrow indicates pharynx.

### iii. Gonad

(A) Wild-type adult hermaphrodite: D, distal gonad/germ cells; P, proximal gonad/oocytes; S, spermatheca; U, uterus/eggs; I, intestine. (B) *erm-1*tm677 adult with cystic uterine phenotype (arrowheads); protruding vulva (arrow). (C and D) Conditional *erm-1*(RNAi) animals: (C) escape of embryos (arrows) from anterior gonad (posterior gonad is intact); one embryo developing in head next to pharynx (large arrow in inset), one within proximal gonad. (D) Twisted gonad forming three-stranded braid with intestine; gonad arm twists indicated by small arrows, mid-uterus twist by large arrow.

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Canal cyst (Figure 2iiD). As *erm-1*(RNAi) animals die shortly after hatching, the *erm-1* gonad phenotype was assessed (1) by conditional *erm-1* RNAi (induced during larval development), (2) by conditional (larval) heat-shock expression of *erm-1* sense and antisense cDNAs, and (3) in homozygous progeny of heterozygous *erm-1(tm677)* hermaphrodites. In these animals, the tubular epithelia of vulva, uterus, spermathecae, and gonad arms are variably cystic, truncated, and twisted around the intestine (Figures 2iiIA–2iiID; Supplemental Figures S5A and S5B). Embryos escape from the uterus and spermathecae into the body cavity, and also develop inside the gonad arms (Figure 2iiIC), suggesting that affected epithelia fail to accommodate passing oocytes and retain sperm (Supplemental Figure S5).

ERM-1 is thus essential for lumen morphogenesis in distinct epithelia of the digestive, reproductive, and excretory systems. The multiorgan nature of this cystic
luminal phenotype, and its manifestation in intestinal and gonadal epithelia in particular, have not been reported in previous screens, indicating specificity for tubulogenesis. The only other C. elegans mutants with defects in tube formation were isolated in a screen for excretory cell pathology (Buechner et al., 1999).

**erm-1 Tubulogenesis Defects Are Not Caused by the Loss of Apicobasal Polarity, Apical Junction Assembly, or Epithelial Integrity**

Since ERM proteins have been implicated in apicobasal polarity and junction generation, we examined ERM-1-GFP’s position relative to apical membranes, their submembraneous cytoskeleton, and their junctions. The C. elegans intestine is a bilaterally symmetrical tube of 20 cells, derived through intercalation (see below; Figure 1iA). The epithelium is sealed by an apical junctional zone (AJ), combining features of tight-, adherens-, and septate junctions (Figure 1iB; Michaux et al., 2001). ERM-1-GFP, from the approximate time of intercalation onward, lies apically adjacent to but distinct from the AJ (identified by the junctional integrity marker AJM-1; Koppen et al., 2001), and overlaps with components of the submembraneous cytoskeleton, such as intermediate filaments (detected by α-IFB-2; J. McGhee, personal communication) and actin (Figure 1i; Supplemental Figures S3A–S3F; data not shown). The excretory canals are derived from a single cell and have no junctions, except for one focal junction to the excretory duct. ERM-1-GFP localizes along the four canals as they extend into the embryo, and in adulthood (Figures 1iiA–1iiC). The U-shaped gonad tube consists of four more complex but also single-layered epithelia which contain specialized junctions arranged for expansion (http://www.wormatlas.org/handbook/reproductivesystem/reproductivesystem1.htm; Figure 1ii). In the twisted spermathecal tube, ERM-1-GFP is also localized to the luminal side. In this specific tissue, it may partially overlap the pleated septate junctions closest to the lumen, thought to reversibly unzip when an oocyte enters (Supplemental Figures S3I–S3J). ERM-1-GFP remains separate from the apical membranes, from two, three, or four cells. These cells are twisted at the time of intestinal intercalation (Figures 1iB–1iD; Supplemental Figures S3A–S3D; data not shown). This demonstrated that the AJ remains functional in generating a patent and intact lumen (also confirmed by serial section TEM in larvae as well as in embryos, see below). Finally, the erm-1(RNAi) intestine always retains its normal number of cells (20) which do not invade neighboring tissue (100% of more than 500 L1 examined; not shown). In contrast, in the absence of Dmoesin, imaginal disc cells were noted to lose epithelial integrity and infiltrate adjacent tissues (Speck et al., 2003).

Taken together, these data suggest that the erm-1 tubulogenesis defects are not caused by a loss of epithelial integrity, apicobasal polarity, or AJ assembly. Such basic defects in epithelial morphogenesis would also be expected to result in an earlier disruption of development, as is typical for mutants with generalized AJ defects (Costa et al., 1998; Koppen et al., 2001; Legouis et al., 2000).

**erm-1 Is Required for Apical Membrane Morphogenesis along a Cell-free Space**

Rather than generating junctions and polarity, erm-1 could have a direct role in apical membrane morphogenesis by affecting membrane movement (through actin filament growth regulation at the leading edge), generation (via vesicle dynamics), or stabilization (through linkage to the cytoskeleton) (Bretscher et al., 2002). To assess tubulogenesis relative to membrane and junction morphology at higher magnification, we undertook serial section transmission electron microscopy (TEM) of erm-1(RNAi) animals through development. Leading edge extensions of intestinal cells drive intercalation, where a lower tier of cells inserts into an upper tier (Leung et al., 1999). During this process, a cruciform lumen constructed by four trapezoidal cells is restructured into an oval lumen formed by two cuboidal cells. It is not known if the lumen is remade or if cells migrate around it. In erm-1(RNAi) embryos, abnormalities are first visible during intercalation, when the lumen becomes assembled from uneven amounts of apical membranes derived from two, three, or four cells. These cells are twisted at their apical poles, stretching apical extensions (“fingers”) around the lumen, while basolaterally the cell bodies have moved to approximate the mature bilateral tube configuration (Figures 3A–3D; Supplemental Movie
Figure 3. Lumen Assembly and Scaffold Integrity Require ERM-1

TEM micrographs of luminal (L) cross-sections ([A]–[F]; slightly oblique section in [A], oblique section in [B]) of wild-type (A and C) and erm-1(RNAi) animals (B, D–F); AJ in all panels is morphologically intact (white arrows, insets in [A]–[D]). (A and B) early post-intercalation embryonic intestines: oval lumen (A) formed by two cells; note lush microvilli. Distorted lumen (B) assembled from apical extensions of multiple cells (shown from oblique angle; separated by the AJ); thin lumen arm is not a side-branch, but continuous with meandering lumen; note sparse microvilli (black arrowhead). (C and D) L1 intestines: oval lumen (C) formed by two cuboidal cells (numbered) with regular basolateral sides (black arrows), normal nucleus (N); note dense microvilli and smooth electron dense submembraneous cytoskeleton (white arrowhead). Cystic lumen (D) formed by three distorted cells (numbered; apical “finger” of cell 2 outlined in red): glycocalyx/luminal scaffold material (gray matter) fills the lumen; microvilli lost, submembraneous cytoskeleton rippled (white arrowhead); condensed nuclear material (black arrows) packed into membranes, taken up by vacuole (V), indicative of autophagic cell death. (E and F) Excretory canals. (E) Cystic canals at pharynx (P) level; note reduction of electron dense cytoskeleton around right lumen; compare with normal excretory duct lumina (arrowheads); few canaliculi (arrows) retained. (F) Cystic canal at intestinal level (intestine [I] is displaced); luminal scaffold material floats in lumen (arrows), canaliculi, submembraneous cytoskeleton are lost (inset, white arrowhead).

S1). This configuration is characteristic of mid-stage intercalation (Leung et al., 1999), suggesting that intercalation is incomplete. The resulting contortion of the epithelium explains the misshapen lumen, its twisted course, the abnormal AJ pattern, and the stretched cell bodies in an essentially intact tube structure (Figures 2iC–2iL). The AJ remains morphologically intact throughout development, and no intraluminal adhesions were detected in serial TEM sections of embryos and larvae (Figures 3A–3D, insets; data not shown). These data provide evidence of a direct morphogenetic role for erm-1 at the apical membrane in tubulogenesis. They also suggest that cells remodel the lumen during intercalation, and that this process requires erm-1.

Further evidence for a direct, junction-independent erm-1 function in lumen membrane morphogenesis comes from its essential role in the formation of the excretory canals, which lack junctions. Canal lumina form intracellularly by pinocytotic vacuole extension (Berry et al., 2003), requiring net luminal membrane generation and modeling, but no junction assembly. TEM confirmed that erm-1(RNAi) canals are either partially or not extended, or cystic (Figures 3E and 3F; Supplementary Movie S1). As luminal membrane generation, extension, and modeling occur simultaneously, it is difficult to distinguish which process is primarily disrupted.

We therefore next examined whether erm-1 might affect membrane stabilization, by tracking the lumen scaffold morphology with TEM. The lumen scaffold stabilizes the membrane and its microdomains (intestinal microvilli and excretory canal canaliculi), and contains the submembraneous cytoskeletal lattice, apical junction belt (intestine only), and, on the luminal side, glycocalyx (intraluminal polysaccharide coating). Structural scaffold defects were found in both erm-1(RNAi) excretory canals and intestine, growing progressively more severe over time. In the embryonic intestine, microvilli, though present, are smaller and sparser than in wild-type (Figure 3B). Later, the electron-dense undercoat (submembraneous cytoskeleton) becomes rippled (Figure 3D) or lost (Figures 3E and 3F). Microvilli, canaliculi, and associated glycocalyx disintegrate, and debris (presumed...
ERM-1's role in apical membrane morphogenesis lies in stabilizing the membrane onto the lumen scaffold, although erm-1 could also directly contribute to luminal membrane movement or expansion. In tubulogenesis, however, the apical scaffold may not only have a static function in stabilizing the membrane, but also a dynamic function in providing the matrix onto which the membrane is fastened during its expansion in apposition to a cell-free space. In Drosophila, the secretion of Piopio into the lumen helps form an intraluminal matrix essential for small tracheal tube morphogenesis (Jazwinska et al., 2003). Similarly, apical membranes might require stabilization onto a submembraneous matrix for tubule formation, particularly if they lack other types of stabilization, such as cuticle.

erm-1, act-5/Cytoplasmic Actin, and sma-1/β-H-Spectrin Functionally Interact in Lumen Morphogenesis
ERM-1’s highly conserved C-terminal actin binding site (Figure 4A) and its actin-dependent function in other organisms (Bretscher et al., 2002) suggested that it may build lumina by localizing actin to the luminal membrane. By confocal analysis, however, actin was not displaced from the membrane in erm-1(RNAi) animals (Figures 2iH, 4D, and 4E; Supplemental Figures S4E and S4F). To examine actin’s localization at higher magnification, we performed actin-immunogold-TEM. Gold particles are present in the sparse intestinal microvilli of erm-1(RNAi) embryos, although at reduced levels (0.22 particles/microvillus/EM section in erm-1(RNAi) versus 0.85 in wild-type; Supplemental Figure S6), compatible with an actin-mediated function of erm-1 at the luminal membrane and its microdomains. These studies do not distinguish between potential roles of erm-1 in actin organization at the membrane. However, they do demonstrate that actin can reach membrane microdomains in erm-1(RNAi) animals. In contrast, in the absence of Dmosin, actin was grossly displaced from the cell cortex (Poessel et al., 2002; Speck et al., 2003).

If erm-1’s essential function in lumen morphogenesis is mediated by actin, an actin loss-of-function phenotype at the lumen should resemble the erm-1 luminal phenotype. C. elegans has five closely related actins (act-1–5; Hresko et al., 1994), whose individual expression and potential functional overlap have not been fully characterized. To avoid interference with other actins and any essential functions in early development, we targeted the specific 3’UTR of act-5, the C. elegans cytoplasmic actin ortholog, by RNAi. act-5(RNAi) animals do exhibit an erm-1-like phenotype with cystic defects of the intestinal lumen, albeit at low penetrance, and L1 lethality (Figure 4C; data not shown).

We generated transgenic lines expressing a full-length act-5 C-terminal GFP fusion protein (ACT-5-GFP) and a GFP fusion to the act-5 start methionine (ACT-5ATG-GFP). ACT-5-GFP localizes exclusively to the excretory canal and intestine, where it colocalizes with ERM-1 at the lumen. In addition, ACT-5-GFP, but not ACT-5ATG-GFP, phenocopies erm-1’s twisted and cystic intestinal phenotype (Figure 4F; compare to Figure 2iJ) and L1 lethality. We next used mild erm-1 RNAi (reducing lethality from 90% to 20%; Experimental Procedures) to examine genetic interactions between erm-1 and act-5.

act-5-GFP/erm-1(RNAi) animals, but not act-5ATG-GFP/erm-1(RNAi) animals, exhibit an L1 lethality which
is greater than their combined lethality (Supplemental Figure S7), consistent with an enhancement of act-5 by erm-1 RNAi. Finally, ACT-5-GFP is not displaced from the lumen by erm-1 RNAi (Figure 4G), supporting our previous results that ERM-1 is not required to localize actin from the cytoplasm to the apical membrane.

Taken together, these data suggest that erm-1 functionally interacts with actin in lumen morphogenesis. The above findings, together with an apparent lack of apicobasal polarity or junctional defects in the absence of ERM-1, suggest that this function is primarily structural and takes place at the membrane. By contrast, in Drosophila, loss of Dmoesin was proposed to cause actin mislocalization secondary to apicobasal polarity defects (Speck et al., 2003), or apicobasal polarity defects secondary to actin misplacement (Polesello et al., 2002).

To further investigate a structural role for erm-1 in luminal membrane morphogenesis, we next examined spectrin, another scaffold component. Vertebrate spectrins crosslink the longitudinal microvillar actin bundles with each other and to the submembraneous cytoskeleton (Thomas, 2001). Protein 4.1 binds to the spectrin/actin lattice through a spectrin–actin binding site (SAB; Supplemental Figure S2A). erm-1 has no SAB, but could indirectly bind spectrin through actin. Moreover, it has been proposed that Dmoesin—which also lacks a SAB—forms a complex with spectrin in vivo, thought to reinforce apical junctions (Medina et al., 2002). sma-1 (small)/β-H-spectrin is apically expressed in epithelia, and regulates C. elegans body size through circumferential actin constriction in the epidermis (McKeown et al., 1998). It is also expressed in tubular epithelia, and sma-1 mutants have small excretory canal cysts (Buechner et al., 1999). We found subtle posterior intestinal lumen defects in sma-1 animals, and a fraction of extremely small, embryo-sized animals (“supersma”; Figure 4H; data not shown). Mild erm-1 RNAi was used to examine genetic interactions between erm-1 and sma-1(e30) or sma-1(ru18), two strong loss-of-function alleles (McKeown et al., 1998). sma-1(erm-1[RNAi]) animals demonstrate a dramatic enhancement in the number and relative size of intestinal and excretory canal cysts, the number of supersma animals, and lethality (Figure 4H; Supplemental Figure S7). Thus, two luminal scaffold molecules, act-5/actin and sma-1/β-H-spectrin, cooperate with erm-1 in lumen formation of diverse tubular epithelia not otherwise stabilized.

We have described an in vivo requirement of ERM proteins for lumen morphogenesis of tubular organ epithelia. erm-1 appears to function as a structural molecule in apical membrane morphogenesis, rather than as a regulatory molecule in the establishment of basic epithelial characteristics. erm-1 functions in lumen formation of nonstabilized epithelia with and without junctions, is required for the integrity of the luminal scaffoldls, and acts in concert with the submembraneous cytoskeletal molecules act-5/actin and sma-1/β-H-spectrin. Our observations favor the hypothesis that erm-1 stabilizes the luminal membrane onto the actin-spectrin cytoskeleton, but do not exclude a role in other aspects of luminal membrane morphogenesis. For otherwise nonstabilized epithelia, a submembraneous luminal matrix may be required to permit membrane expansion and movement along a cell-free space, such as a lumen.

Most human tubular organ epithelia express one or more ERM proteins (see Supplemental Results). Our results suggest that loss of human ERMs should interfere with organ functions which rely on intact tubes. A role of ERMs in vertebrate organ lumen morphogenesis may not have been previously recognized due to redundancies among ERM family members.

Experimental Procedures

Cloning of erm-1

See Supplemental Results at http://www.developmentalcell.com/cgi/content/full/6/6/865/DC1.

Genetics

The erm-1(tm677) deletion allele was kindly provided by S. Mitalipov (National Bioresource Project for the Nematode, Japan). We determined the deletion endpoints to lie from positions 14069 to 15037 (numbering for cosmid C01G8), generating a premature stop. After eight backcrosses to wild-type, strains ZZ1004 (unc-63[cb4] dpy-5[eb61]) and BA717 (spa-1[hr180]) were used to balance the allele, generating strains VJ311 (erm-1(tm677)/unc-63[cb4] dpy-5[eb61] I and VJ317 (erm-1(tm677) I, sdp2[2];f), respectively. For rescue experiments, strains VJ196 (vja2[erm-1(+) rol-6[sa102]] hi5-m(5[eb190]) V and VJ112 (unc-119(ed33) II; vjEx10[erm-1-GFP unc-119 (+)]) were crossed into VJ311, creating strains VJ330 (erm-1(tm677) I; vja2[erm-1(+) rol-6[sa102]] hmp-1::GFP)]) and VJ331 (erm-1(tm677) I; vjEx10[erm-1-GFP unc-119 (+)]) I, respectively. Strains BC700 (sDp4[4(4)e3(4)]) dpy-14[hr180]) I, VJ358 (bDp4)[bDp4];H11001 dpy-5[eb61] unc-13(e450)] I; sdp2[2];f), and KR522 (bDp4[bDp4]) dpy-5[eb61] unc-13(e450)] I; sdp2[2];f), the latter two gifts of A. Roepst, were used for deficiency testing. Strains used for marker/interaction studies were: SUG9 (cI51[ajm-1::GFP] IV, J1136 (unc-119[ed2498] II; vjEx24[unc-119 (+)] hmp-1::GFP]), AZ30 (sma-1[ru18] V), and CB30 (sma-1[e30]) V. Unless otherwise specified, strains were generously provided by the Caenorhabditis Genetics Center (CGC), University of Minnesota.

Characterization and Rescue of erm-1(tm677)

See Supplemental Results.

GFP/LacZ Expression Analysis and Transgenic Lines

Most erm-1 expression data were derived from a rescuing C-terminal GFP fusion construct that contained ∼3 kb of 5’ sequence cloned into vector pDF59.79 (all pPD vectors, A. Fire, S. Xu, J. Ahn, G. Seydoux, personal communication). Other GFP and lacZ fusion constructs with different amounts of promoter-, coding-, and 3’UTR sequence were made by PCR, ligating GFP at the 5’ end and an N-terminal GFP fusion construct that contained ∼3 kb of 5’ sequence cloned into pDF59.57 and an N-terminal GFP construct with 2.6 kb of 5’ sequence, cloned into pDF59.79, yielded similar results. All plasmids were coinjected with the rol-6 marker plasmid pRF4 into N2, or the unc-119 rescuing plasmid pDP#MM016B into unc-119 hermaphrodites (the latter were gifts of M. Maduro and D. Pilgrim), as previously described (Mello and Fire, 1995). The full-length erm-1 gene with ∼3 and ∼2 kb of 5’ and 3’UTR sequence, respectively, was cloned into pTopoXL (Invitrogen, La Jolla, CA). The full-length erm-1 cDNA was separately cloned in sense and antisense orientations into the pPD49.78 and pPD49.83 vectors under the control of heatshock promoters expressing in nervous system/hypodermis and gut, respectively. Transgenes were integrated by γ irradiation (Mello and Fire, 1995). Full-length (act-5-GFP) and ATG (act-5ATG-GFP) fusion constructs with ∼2 kb of 5’ sequence were made by PCR, ligating GFP at the C terminus and start methionine, respectively, and the PCR products directly coinjected with marker plasmids, as described (Hobert, 2002). A vha-1/4-GFP transgenic line, marking the excretory cell, was generated with vha-1 I and vha-4-GFP plasmids (pCV01 and pCV01, gifts of T. Oka and M. Buechner). Analysis was performed on epifluorescence and laser scanning confocal microscopes (Leica TCS4D with Leitz DMBR/E microscope and TCS-NT software).
Antibodies and Immunostaining

Antibody staining was performed as described (Miller and Shakes, 1995). The following primary antibodies were used: MH27 (α-AJMY1; 1:300), MH33 (α-IFB-2; 1:100), MH46 (1:100; all gifts of M. Hresko and R. Waterston), α-PAR-3 (1:50; gift of K. Kemphues), α-PKC3 (1:100; gift of M. Land and C. Rubin), ICB4 (1:50; gift of M. de Bono), α-actin (1:100; Sigma; St. Louis, MO), α-lacZ (1:100; Promega, Madison, WI). Conjugated FITC and TRITC (Sigma) were used as secondary antibodies. Protein extraction and Western blotting was performed as described (see Supplemental Results).

RNAi

The full-length erm-1 cDNA was used with an Ambion Megascript kit (Ambion, Austin, TX) to generate RNA, which was used for micro-injection, as described (Fire et al., 1998). For bacterial RNAi, the erm-1 cDNA was cloned into plasmid pPD129.36, transformed into HT115 (DE3) bacteria, and used for RNAi induction by feeding (Kamath et al., 2001). For act-5 RNAi, the 3’UTR of the act-5 gene (T25C3.2) was PCR-amplified, cloned into plasmid pPD129.36, and used to induce RNAi as above. Feeding approaches used 1–2 mM IPTG or 0.2% α-lactose (Sigma L-3625; E. Lambie, personal communication) as inducers. A continuum in intensity of phenotypes could be produced by variations in the RNAi conditions. Conditional RNAi approaches included (1) growth of wild-type embryos or L1s on erm-1(RNAi) bacteria and (2) induction of heatshock promoter-driven simultaneous erm-1 sense and antisense cDNAs during early larval development. For each experiment, at least 100 animals/plate were scored, and experiments were repeated three to six times and included IPTG and lactose in parallel. Each experiment also included the pPD129.36 parent plasmid in HT115 bacteria induced under identical conditions, as a negative control. GFP expression in an ERM-1-GFP background was extinguished by erm-1 RNAi, and related RNAs (erm-1, frm), and RNAs of other genes in the erm-1 operon did not phenocopy, confirming the specificity and efficiency of erm-1 RNAi (not shown).

Phenotypic Analysis and Feeding Experiments

See Supplemental Results.

Electron Microscopy

Whole animals with early larval phenotypes generated by RNAi were fixed for TEM using a microwave protocol (adapted from Paupard et al., 2001). Arrested larvae in buffered 2.5% glutaraldehyde were irradiated for 10 min at 4°C 12 min ON, 2 min OFF, etc., at 50% power at hotspot, fixed an additional 60 min at 4°C, rinsed in buffer and retained in 1% OsO4, 0.5% KFeCN6, then rinsed and en bloc stained in UAc, prior to Epon embedding. Five larvae of increasing phenotypic severity were serially thin-sectioned. Embryos, also generated by RNAi, were prepared by high-pressure freezing, freeze substitution into 1% OsO4 in acetone, and embedded in Epon. Thin sections were post-stained in UAc and PbCit for microscopy. For immunolocalization, specimens were microwave-fixed, embedded in LR Gold, and labeled on nickel mesh grids, as described (Paupard et al., 2001).

Acknowledgments

We thank L. Jander and P. Winge for help in the initial stages of the project; G. and T. Stepnheny and C. Nguyen for help with TEM; S. Mitani, A. Fire, M. Maduro, D. Pilgrim, A. Rose, M. Buechner, T. Oka, and T. Sternagle for plasmids and strains; M. Hresko, R. Waterston, K. Kemphues, M. Land, C. Rubin, and M. de Bono for antibodies; and A. Coulson for DNA fingerprinting. We further thank F. Solomon, H. Weinstein and A. Ezekowitz for continued support. This work was funded by the Charles H. Hood Foundation, NIH CA 74243 and CA 68475 to V.G., and NIH RR 12596 to the Center for C. elegans Anatomy (D.H.H.).

References


