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Extracellular leucine-rich repeat proteins are required to organize the apical extracellular matrix and maintain epithelial junction integrity in *C. elegans*

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SUMMARY

Epithelial cells are linked by apicolateral junctions that are essential for tissue integrity. Epithelial cells also secrete a specialized apical extracellular matrix (ECM) that serves as a protective barrier. Some components of the apical ECM, such as mucins, can influence epithelial junction remodeling and disassembly during epithelial-to-mesenchymal transition (EMT). However, the molecular composition and biological roles of the apical ECM are not well understood. We identified a set of extracellular leucine-rich repeat only (eLRRon) proteins in *C. elegans* (LET-4 and EGG-6) that are expressed on the apical surfaces of epidermal cells and some tubular epithelia, including the excretory duct and pore. A previously characterized paralog, SYM-1, is also expressed in epidermal cells and secreted into the apical ECM. Related mammalian eLRRon proteins, such as decorin or LRRTM1-3, influence stromal ECM or synaptic junction organization, respectively. Mutants lacking one or more of the *C. elegans* epithelial eLRRon proteins show multiple defects in apical ECM organization, consistent with these proteins contributing to the embryonic sheath and cuticular ECM. Furthermore, epithelial junctions initially form in the correct locations, but then rupture at the time of cuticle secretion and remodeling of cell-matrix interactions. This work identifies epithelial eLRRon proteins as important components and organizers of the pre-cuticular and cuticular apical ECM, and adds to the small but growing body of evidence linking the apical ECM to epithelial junction stability. We propose that eLRRon-dependent apical ECM organization contributes to cell-cell adhesion and may modulate epithelial junction dynamics in both normal and disease situations.

KEY WORDS: Epithelia, Extracellular matrix, *C. elegans*

INTRODUCTION

Polarized epithelial cells organize together to form many of the surfaces in our bodies, including the outer epidermis and the lining of many internal tubular organs such as the kidney, lung and gastrointestinal tract. Consequently, defects in epithelial development or maintenance underlie a variety of human diseases (Chamcheu et al., 2011; Wilson, 2011; Marchiando et al., 2010). Loss of epithelial character during epithelial-to-mesenchymal transition (EMT) is a key feature of tumor metastasis, the major cause of cancer morbidity (Kalluri and Weinberg, 2009; Polyak and Weinberg, 2009). Thus, it is important to understand how epithelial structures are formed and maintained.

Epithelial cells are linked by specialized junctions that hold the tissue together, create a paracellular barrier, and separate the apical and basolateral surfaces of the cells (Giepmans and van Ijzendoorn, 2009; Shin et al., 2006). Many junction components are evolutionarily conserved, although junction organization differs somewhat among organisms. In mammals, cadherin-based adherens junctions, which mediate cell-cell adhesion, are located basally to claudin-based tight junctions, which form the paracellular barrier and demarcate the apical and basolateral membrane surfaces. In *Drosophila*, adherens junctions are located apically to claudin-based septate junctions. In *Caenorhabditis elegans*, a single electron-dense structure, termed the 'apical junction', contains adjacent adherens junction-like and septate junction-like domains (Lynch and Hardin, 2009). Initial junction assembly depends on conserved polarity regulators such as the PAR, Crumbs and Scribble complexes (Goldstein and Macara, 2007). Once assembled, epithelial junctions are dynamic structures that must be frequently disassembled or remodeled during morphogenesis and tissue turnover (Acloque et al., 2009; Baum and Georgiou, 2011; St Johnston and Sanson, 2011). The mechanisms that control junction stability and dynamics are still poorly understood, but include changes in mechanical force transduced through cell-cell and cell-matrix adhesions (Papusheva and Heisenberg, 2010).

The basal and apical surfaces of epithelia contain different types of proteins and lipids, and each surface secretes and interacts with different factors in the extracellular matrix (ECM). Basal surfaces face towards the basement membrane and neighboring tissues. In simple planar epithelia, apical surfaces face towards the outside of the body, and in tubular epithelia apical surfaces face towards the lumen. Basal domains typically contain integrins, which link the actin cytoskeleton to basement membrane components such as laminins and collagens (Hynes, 2009). Apical domains contain other types of transmembrane proteins, such as zona-pellucida (ZP)-domain proteins and mucins, that interact with or contribute to the apical ECM (Bafna et al., 2010; Plaza et al., 2010). It has long been appreciated that the basal ECM influences epithelial cell polarity, cell shape and cell motility (Berrier and Yamada, 2007). The apical ECM, in contrast, has generally been viewed as a more passive protective barrier against pathogens and other

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environmental toxins. However, there is increasing evidence that the apical ECM also helps to shape epithelial cell morphology and can influence junction dynamics. For example, in the *Drosophila* trachea, a temporary chitinous apical ECM controls tube length (Devine et al., 2005; Tonning et al., 2005) and the ZP-domain proteins Piopio and Dumpy influence junction remodeling (Jazwinska et al., 2003). In humans, overexpression of the mucin MUC1 is observed in >90% of metastatic pancreatic ductal adenocarcinoma, and MUC1 can influence EMT in mouse models (Kufe, 2009; Roy et al., 2011). However, we still have a limited understanding of how the apical ECM contributes to epithelial morphology and junction dynamics.

We use the *C. elegans* excretory (renal-like) system as a simple model for epithelial tube development. The excretory system consists of three unicellular tubes: the large canal cell, which extends the entire length of the body, and the smaller duct and pore tube cells, which connect the canal cell to the outside environment to allow for fluid waste excretion (Nelson et al., 1983; Nelson and Riddle, 1984) (Fig. 1A). Each unicellular tube has an intracellular apical or lumenal domain and an extracellular basal domain, and the three tubes are connected in tandem via apicolateral junctions. The pore cell also contains an autocellular junction (Fig. 1A,B). All three tubes develop embryonically from initially non-epithelial precursors. Later in larval development, the original pore cell (G1)

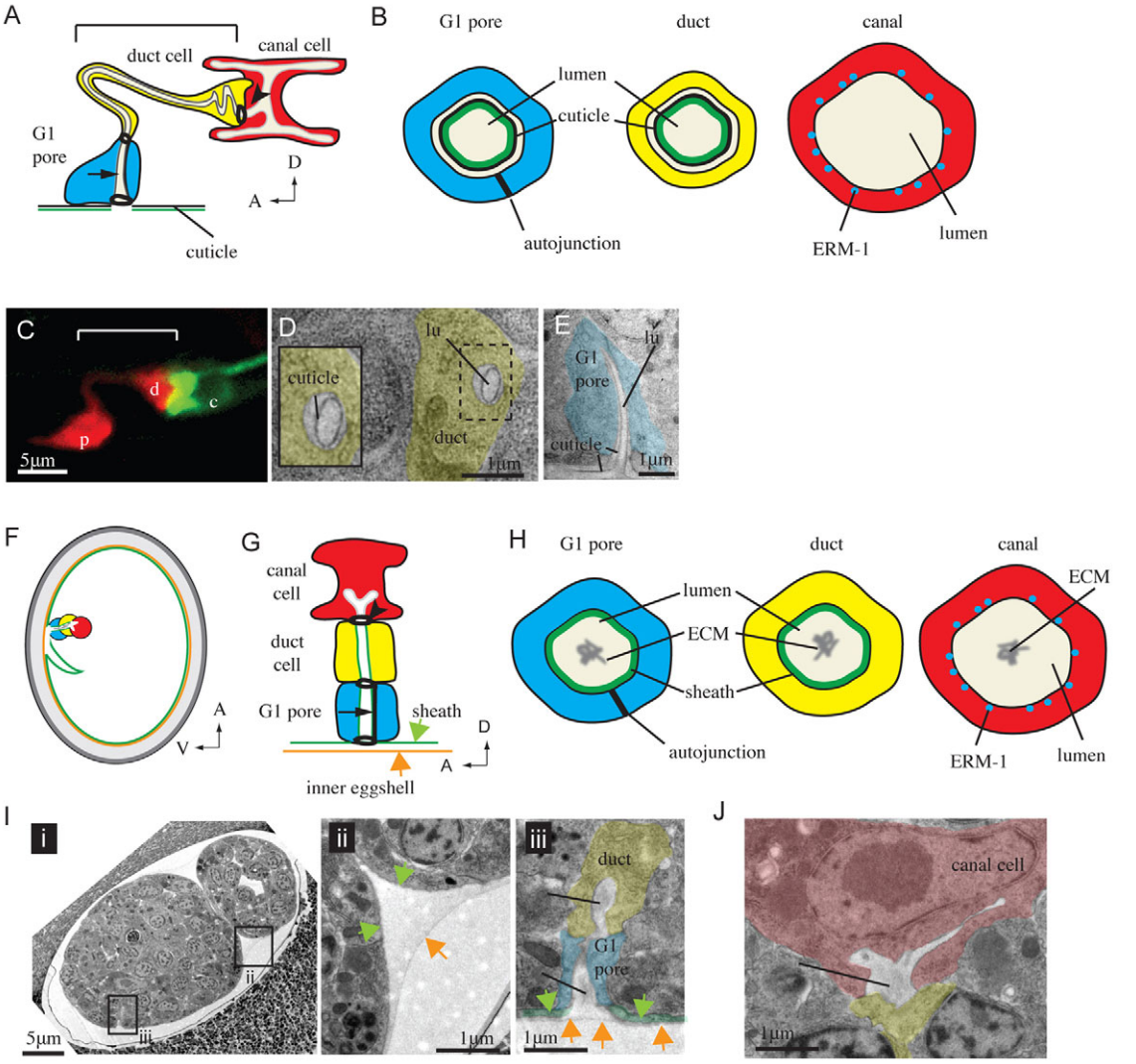

Fig. 1. The apical ECM differs between excretory tube types. (**A**,**B**) Schematics of the late threefold or early L1 excretory system. (A) Lateral view. (B) Cross sections. Cuticle lines the duct and pore lumen. In all schematics, canal cell is red, duct cell is yellow, and pore cell is blue. Green indicates the embryonic sheath, which at this stage makes up the outer layer of the cuticle. Dark lines and circles indicate apical junctions; arrowhead, duct-canal junction; bracket, duct cell body; arrow, pore autocellular junction. (**C**) Excretory system of early L1 larva. ERM-1::GFP lines the lumen of the canal cell (c), and is absent from the pore (p) and duct (d) cells (labeled with *dct-5p*::mCherry). Anterior is to the left and ventral down in all figures unless otherwise noted. (**D**,**E**) TEM of threefold embryo showing the duct cell lumen (lu) (D) and pore cell lumen (E). In (E) the cuticle reaches from the outside of the worm into the lumen of the pore cell. (**F-H**) Schematics of the developing excretory system at the 1.5-fold embryonic stage. (F) Whole embryo and eggshell layers. (G) Lateral view. (H) Cross sections. The inner eggshell layer (orange) surrounds the embryo. A thin sheath-like layer (green) lines the pore and duct lumen. The cells have recently undergone tubulogenesis and have not yet taken their mature shapes. Cuticle has not yet been secreted, and the lumen of all three tube cells contains a fibrous electron-dense material (ECM). (**I**,**J**) TEM of 1.75-fold embryo, showing the inner eggshell layer (orange arrows) and embryonic sheath layer (green arrows). Fibrous electron dense material (lines) is visible in the duct and pore cells (Iiii) as well as the canal cell (J). Embryo 'N2E6B' TEM (I-J) kindly provided by Shai Shaham (Rockefeller University).

withdraws from the organ to become a neuroblast, and is replaced by a second pore cell (G2) (Abdus-Saboor et al., 2011; Stone et al., 2009; Sulston et al., 1983). Thus, the excretory system is a simple model for studying lumen development and the dynamic control of epithelial junctions.

When searching for mutants that affect excretory duct and pore morphology, we identified two leucine-rich repeat transmembrane proteins, LET-4 and EGG-6, that localize to the apical domains of the duct, pore and epidermis. Here we show that LET-4, EGG-6 and a paralog SYM-1 are important both to organize the apical ECM and to maintain epithelial junction integrity.

MATERIALS AND METHODS

Strains and alleles

Strains were grown at 20°C and maintained under standard conditions (Brenner, 1974) unless otherwise noted. Bristol strain N2 was used as wild-type. Alleles used were: I: *mec-8*(*u218*) (Chalfie and Au, 1989). X: *let-4*(*mn105*) (Meneely and Herman, 1979), *unc-3*(*e151*) (Hodgkin, 1997), *sym-1*(*mn601*) (Davies et al., 1999). *egg-6*(*cs67*) was obtained after standard EMS mutagenesis of N2 (Brenner, 1974). Transgenes used were: *csEx146* (*lin-48p*:mCherry) (Abdus-Saboor et al., 2011), *fgEx11* (ERM-1::GFP) (Gobel et al., 2004), *jcIs1* (AJM-1::GFP) (Koppen et al., 2001), *mcIs46* (DLG-1::RFP) (Diogon et al., 2007), *qnEx59* (*dct-5p*::mCherry) (Abdus-Saboor et al., 2011), *saIs14* (*lin-48p*::GFP) (Johnson et al., 2001), *xnIs96* (HMR-1::GFP) (Achilleos et al., 2010), *qpIs11* (*vha-1p*::GFP) (Mattingly and Buechner, 2011).

Molecular analysis

let-4 was previously mapped to the right arm of the X chromosome (Meneely and Herman, 1979). *cs67* was mapped to chromosome I by linkage analysis and deficiency mapping (see Wormbase). Both genes were subsequently identified via transgenic rescue experiments. Gene structures were confirmed by sequencing C44H4.2/*let-4* cDNA clones yk8g5, yk134h6, yk1661a04, yk1708a10 and K07A12.2/*egg-6* cDNA clones yk117f12 and yk4a1.

let-4 and *egg-6* genomic rescue fragments were cloned from fosmids WRM0620cC02 and WRM0617dE11, respectively. GFP obtained from pPD103.87 (Addgene) was inserted into a native *Bam*HI site to generate LET-4::GFP (pVM4) or inserted into an engineered *Nhe*1 site to generate EGG-6::GFP (pMS204). Transgenic lines were generated by co-injecting each construct (2 ng/µl) with pRF4 (98 ng/µl).

For tissue-specific promoter constructs, *let-4* or *egg-6* cDNAs obtained from yk134h6 or yk117f12, respectively, were cloned into vectors pBG12 (*lpr-1p*), pKH11 (*dpy-7p*) or pHS4 (*lin-48p*), which are all derivatives of pPD49.26 (Addgene). For promoter information, see Gilleard et al. and Stone et al. (Gilleard et al., 1997; Stone et al., 2009). Transgenic lines were obtained by co-injecting each construct at 2-10 ng/µl with either pHS4 (*lin-48p*::mCherry) at 2.5 ng/µl or pIM175 (*unc-119*::GFP) at 90ng/µl. Additional details of plasmid construction are available upon request.

RNAi

let-4 double-stranded RNA (dsRNA) was synthesized using the Megascript RNAi Kit (Ambion), using as template a fragment of the *let-4* cDNA corresponding to exons 8-10 (Primers: oMS199 5′-GTAATACGACTCACTATAGGGCAGTCGTGAAGATGAGATTCGC-3′ and oMS200 5′-GTAATACGACTCACTATAGGGCGCAATAACTGGATCCAGGATTG-3′). dsRNA was injected into gravid hermaphrodites and embryos laid >16 hours later were scored. For *egg-6* RNAi, gravid hermaphrodites were fed HB101 bacteria containing clone JA:K07A12.2 (Kamath et al., 2003) and embryos laid >40 hours later were scored.

Immunostaining

L4 larvae were collected and fixed as previously described (Finney and Ruvkun, 1990). Primary antibody concentrations used were: MH4 (1:50) (Francis and Waterston, 1991); goat anti-GFP (1:50) (Rockland Immunochemicals); rabbit anti-DLG-1 (Segbert et al., 2004); P4A1 (mouse anti-PAR-3) (1:25) (Nance et al., 2003); rat anti-PKC-3 (1:150) (Tabuse et al., 1998). Secondary antibodies: Cy3 donkey anti-mouse (1:200); Cy3 donkey anti-rat (1:200); Cy3 donkey anti-rabbit (1:200); FITC donkey anti-goat (1:20) (Jackson ImmunoResearch).

Microscopy

Images were captured by differential interference contract (DIC) and epifluorescence using a Zeiss Axioskop (Jena, Germany) microscope with a Hamamatsu Chilled CCD camera (Hamamatsu City, Japan). Confocal microscopy was performed with a Leica TCS CP (Wetzlar, Germany). All confocal images were analyzed with Leica Confocal Software and ImageJ Software.

For transmission electron microscopy (TEM), 1.5-fold or late threefold embryos from wild-type or *let-4*(*mn105*) mothers were fixed by high pressure freezing followed by freeze substitution (Weimer, 2006), embedded in Eponate resin and cut into serial thin sections between 50 and 100 nm each. Sections were observed on a Philips CM10 transmission electron microscope (Amsterdam, The Netherlands) or a FEI-Tecnai T12. Serial section TEM images of a similarly processed 1.5-fold embryo, N2E6B, were provided to the Center of *C. elegans* Anatomy by Shai Shaham (Rockefeller University).

Hoechst permeability assay

L1 larvae of each genotype were collected in M9 and incubated in 2 µg/ml Hoescht dye 33258 (Sigma) for 15 minutes at room temperature, then washed twice with M9.

RESULTS

The apical ECM of the excretory duct and pore is contiguous with that of the epidermis

The apical ECM and cytoskeleton differ significantly between different tube types in the excretory system. The excretory canal cell resembles the *C. elegans* gut in that it is not lined by cuticle, but has a specialized apical cytoskeleton containing the FERM domain protein ERM-1 (Gobel et al., 2004; Van Furden et al., 2004) (Fig. 1B,C). It also requires a set of specialized '*exc*' gene products for its lumenal maintenance (Buechner et al., 1999; Buechner, 2002). By contrast, the duct and pore do not appear to express ERM-1 or most *exc* genes, but the mature duct and pore lumens are lined by a collagenous cuticle that is contiguous with that of the epidermis (Nelson and Riddle, 1984) (Fig. 1A-E). However, the bulk of cuticle secretion does not occur until late in embryogenesis (Costa et al., 1997; Johnstone and Barry, 1996), after the duct and pore have taken their mature shapes.

To examine the pre-cuticular duct and pore ECM, we analyzed existing transmission electron micrographs of 1.5-fold embryos (Fig. 1F-J). At this stage, the embryonic epidermis is lined by a thin apical ECM termed the 'embryonic sheath' (Priess and Hirsh, 1986), which later becomes an outer layer of the L1 cuticle (Costa et al., 1997). Outside the sheath, four additional ECM layers were visible that together constitute the eggshell and are secreted by the embryo soon after fertilization (Benenati et al., 2009; Rappleye et al., 1999). The innermost of these layers was a sac-like structure that encased the entire embryo; it was closely apposed to the sheath in most regions, but separated from the sheath at points where the embryo bends inward, including at the excretory pore opening (Fig. 1I). Within the nascent excretory pore and duct lumen, a very thin lining of gray material was visible that may correspond to a sheath-like ECM. The remainder of the duct and pore lumenal space, as well as the lumen of the canal cell, was filled with fibrous electron-dense material (Fig. 1I,J). This fibrous ECM material disappeared from the duct and pore by 6 hours later, at which time the cuticle lining of the duct and pore had been secreted (Fig. 1D,E). In summary, TEM analysis revealed the presence of two apical

ECM layers within the duct and pore before cuticle secretion; both of these layers are morphologically distinct from the innermost layers of the epidermal ECM with which they are in contact.

let-4 and *egg-6* encode transmembrane proteins with extracellular leucine-rich repeats

To identify genes important for excretory duct and pore development or maintenance, we searched for mutants with defects in these tubes. *let-4*(*mn105*) mutants previously were reported to have a rod-like lethal phenotype indicative of excretory system defects (Buechner et al., 1999; Meneely and Herman, 1979), making *let-4* a candidate of interest. We isolated *egg-6*(*cs67*) in an ethyl methane sulfonate (EMS) mutagenesis screen for rod-like lethal mutants (see Materials and methods); a second allele, *egg-6*(*ok1506*), was obtained from the *C. elegans* gene knockout consortium (Moerman and Barstead, 2008).

let-4(*mn105*) is a recessive, loss-of-function mutation and caused highly, but not completely, penetrant lethality (Fig. 2A). The majority of mutants died as early L1 larvae with excretory defects. A smaller percentage of mutants died as embryos. Approximately 2% of mutants were 'escapers' that survived to adulthood and were fertile, but exhibited defects in locomotion and egg-laying. The progeny of these escaper homozygotes had the same rate of lethality as progeny from heterozygous mothers, indicating that there was no maternal effect on lethality (Fig. 2A).

egg-6(*cs67*) and *egg-6*(*ok1506*) are also recessive loss-of-function mutations and both caused fully penetrant L1 lethality owing to excretory defects (Fig. 2A). Animals rescued for this zygotic lethality by an *egg-6*(+) transgene (see below) gave 100% dead embryos in the next generation, revealing a maternal *egg-6* requirement. Embryos lacking maternal *egg-6* arrested at the ~40 cell stage and had fragile eggshells (data not shown).

We positionally cloned *let-4* and found that it corresponded to the gene *sym-5*/C44H4.2, which encodes a predicted type I transmembrane protein with 14 extracellular leucine-rich repeat (LRR) domains and a short cytoplasmic tail (Fig. 2B,C). *let-4*(*mn105*) mutants had a C to T nucleotide change in the fourth exon of C44H4.2, introducing a stop codon into the 11th LRR. A 5.3 kb genomic fragment encompassing C44H4.2 and no other genes rescued *mn105* lethality. RNAi against C44H4.2 also recapitulated some aspects of the *let-4* phenotype (see below). Although C44H4.2 has been previously called *sym-5* (*synthetic lethal with mec-8*) based on genetic interactions with the *mec-8* splicing factor observed in RNAi experiments (Davies et al., 1999), the *let-4* gene name predates those studies. Therefore, we refer to C44H4.2 as LET-4.

We positionally cloned *cs67* and found that it corresponded to the gene *egg-6*/K07A12.2, which encodes an LRR transmembrane protein related to LET-4 (Fig. 2B,C). *egg-6* was independently identified and named based on its eggshell-defective RNAi phenotype (A. Singson, personal communication) (Carvalho et al., 2011; Sonnichsen et al., 2005). *cs67* mutants had a C to T

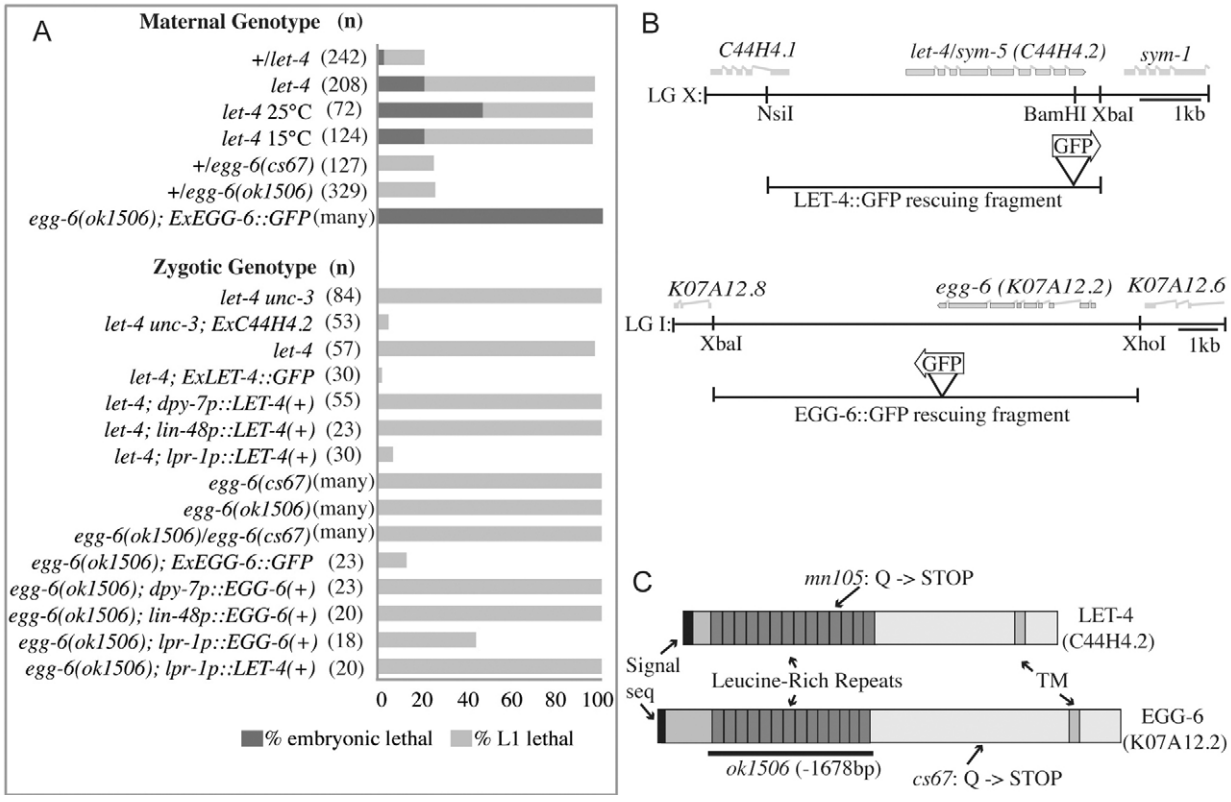

Fig. 2. *let-4* and *egg-6* encode related transmembrane proteins with extracellular leucine-rich repeats. (**A**) Genetic analyses and transgenic rescue experiments. For transgene rescue experiments, progeny were collected from *let-4*(*mn105*) homozygotes carrying *csEx173* (C44H4 cosmid +*sur-5p*::GFP) and a separately marked transgene of interest, or from *egg-6*(*ok1506*)/*hT2[qIs48]* heterozygotes carrying a marked transgene of interest. Rescue was determined by scoring each hatched worm for L1 lethality. For *let-4*; *lin-48p*::LET-4(+) rescue experiment, progeny were obtained from *let-4* heterozygous mothers carrying *lin-48p*::LET-4(+). (**B**) *let-4* and *egg-6* genomic regions and rescue fragments. Note that paralog *sym-1* is immediately adjacent to *let-4*. (**C**) Predicted protein structures and mutant lesions. The LET-4 and EGG-6 LRR domains share 49% similarity. GenBank accession numbers are JQ292907 (*let-4*) and JQ292908 (*egg-6*).

nucleotide change in the eighth exon of *egg-6*/K07A12.2, introducing a stop codon into the extracellular domain. *cs67* failed to complement *egg-6*(*ok1506*), which deletes 1678 bp of the coding region, completely eliminating the LRR domain. A 10.5 kb genomic fragment encompassing K07A12.2 and no other genes rescued *cs67* and *ok1506* zygotic lethality.

LET-4 and EGG-6 belong to the large family of extracellular LRR (eLRR) proteins, which includes many proteins involved in cell adhesion, ECM interactions and signaling. LET-4 and EGG-6 specifically belong to the 'eLRR only' or 'eLRRon' subgroup (Dolan et al., 2007), because they contain no other recognizable domains. Mice have 52 eLRRon proteins, including LRRTM1-3, which are involved in synaptic junction formation or stabilization (Brose, 2009; de Wit et al., 2009; Ko et al., 2009; Linhoff et al., 2009; Siddiqui et al., 2010) and the small leucine-rich proteoglycans (SLRPs), which modulate collagen matrix assembly (Kalamajski and Oldberg, 2010). In addition to LET-4 and EGG-6, *C. elegans* has 15 other members of the eLRRon family, including SYM-1 (Davies et al., 1999). The LRR domain of LET-4 is more similar to that of SYM-1 (53%) than to that of EGG-6 (49%), but all three proteins cluster together within the *C. elegans* eLRRon family (Dolan et al., 2007).

LET-4::GFP and EGG-6::GFP localize to the apical (luminal) side of the duct, pore and other external epithelia

To visualize the localization of LET-4 and EGG-6, we generated fusion proteins by inserting GFP at the LET-4 or EGG-6 C-terminus within our genomic rescue fragments. Both the LET-4::GFP and EGG-6::GFP fusion proteins rescued lethality of the corresponding mutants, indicating that all required regulatory elements were included in the transgenes and that the tagged proteins were functional (Fig. 2A,B).

LET-4::GFP and EGG-6::GFP were expressed in a subset of epithelial cells, including epidermal, vulval and rectal cells and the excretory duct and pore (Fig. 3; supplementary material Fig. S1). EGG-6::GFP was also observed in some neurons (supplementary material Fig. S1). Expression began around the ventral enclosure stage of embryogenesis and continued through larval development, but then decreased in adulthood. Expression was absent from internal epithelia such as the gut and pharyngeal tubes (Fig. 3C-F). LET-4::GFP was transiently expressed in the excretory canal cell at the 1.5-fold stage (Fig. 3C), but no longer visible in this cell by hatch. Notably, with the exception of the canal cell, the epithelia that expressed LET-4 and EGG-6 were those that would eventually become cuticle-lined.

In almost all epithelia where they were expressed, LET-4::GFP and EGG-6::GFP appeared strongly apically enriched (Fig. 3; supplementary material Fig. S1). In the excretory duct and pore, LET-4::GFP and EGG-6::GFP lined the luminal membrane (Fig. 3A,B; supplementary material Fig. S1). In the epidermis, both fusions were distributed across the apical surfaces of most dorsal and ventral epidermal cells but were observed more weakly or variably in the lateral (seam) epidermis (Fig. 3G-H). Neither fusion was strongly enriched at apical junctions based on co-visualization with DLG-1/Discs Large::mcherry (Fig. 3B,D,F-H). Both fusions partially overlapped with but did not strongly colocalize with transepidermal intermediate filaments at hemidesmosomes (supplementary material Fig. S1 and data not shown). Both fusions were present in many large puncta, potentially representing a vesicular compartment trafficking to or from the membrane. In summary, LET-4 and EGG-6 topology and apical localization

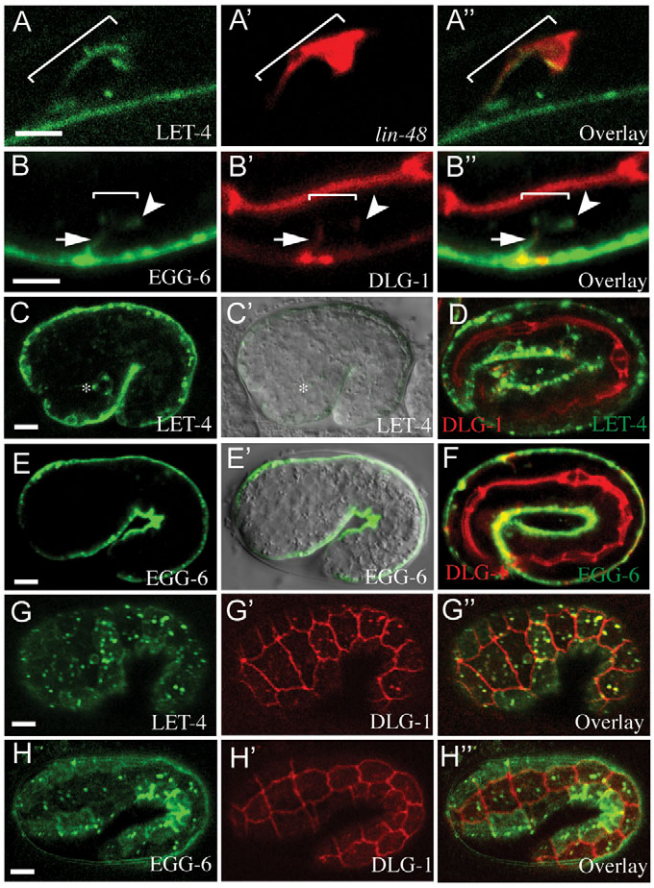

Fig. 3. LET-4::GFP and EGG-6::GFP localize to the apical domains of the excretory duct, pore and epidermal cells. (**A-B″**) In L1 larvae, LET-4::GFP and EGG-6::GFP localize apically within the excretory duct and pore. Symbols as in Fig. 1. (A) LET-4::GFP. (A′) *lin-48p*::mCherry labels the duct cell cytoplasm. (A″) Overlay. (B) EGG-6::GFP. (B′) DLG-1::mCherry marks apical junctions of the duct and pore cells. (B″) Overlay. (**C-H‴**) In 1.5-fold (C,E,G,H) and threefold (D,F) stage embryos, LET-4::GFP (C,D,G) and EGG-6::GFP (E,F,H) localize apically within the epidermis. Apical junctions are marked with DLG-1::mCherry. (C-F) Mid-plane. Note LET-4::GFP expression on the outer surface of the canal cell (asterisk in C). (G-H) Surface view. Scale bars: 5 μm.

suggest a configuration in which the LRR domains extend into the apical ECM, but localization is not limited to known sites of epidermal-apical ECM attachments.

Interestingly, the one exception to the apical localization of LET-4::GFP was the excretory canal cell. At the 1.5-fold stage, when LET-4::GFP was transiently expressed in the canal cell, LET-4::GFP localized uniformly around the plasma membrane and not to the developing internal lumen (Fig. 3C,C′). Although the significance of this expression is unclear, we speculate that the unique localization pattern reflects molecular differences in the apical domain of the canal cell versus the apical domains of other LET-4::GFP-expressing cells.

let-4 and *egg-6* are required to maintain junction and lumen integrity in the excretory duct and pore

The majority of *let-4*(*mn105*) mutants and all *egg-6*(*cs67*) or *egg-6*(*ok1506*) mutants arrested as L1 larvae with excretory defects (Fig. 2A). The overall morphology and junctional pattern of the

excretory system appeared initially normal in mutant threefold embryos, but became detectably abnormal shortly before hatching (Fig. 4; supplementary material Fig. S2). The first detectable abnormality was a swelling of the canal cell lumen in the region proximal to the canal-duct junction (Fig. 4C-E). Subsequently, the duct and pore cells separated from each other and the pore autocellular junction disappeared (Fig. 4F-H). Remnants of junction material sometimes remained at the separation points, suggesting junction breakage. The duct-canal junction and duct cell body remained intact, and the duct lumen often swelled considerably. Canal lumen swelling was a secondary consequence of defects in the duct and pore, as the excretory phenotype was rescued by *lpr-1p*-driven LET-4(+) or EGG-6(+) transgenes expressed specifically in the duct, pore and epidermal cells but not in the canal cell (Fig. 2A). Neither *let-4* nor *egg-6* was rescued by *dpy-7p*-driven transgenes expressed in the pore and epidermal cells or by *lin-48p*-driven transgenes expressed in the duct. Thus, *let-4* and *egg-6* are required in the excretory duct and pore, but not in the canal cell, and are required for both lumen and junction maintenance.

To confirm these interpretations for *let-4* mutants, and to visualize the narrow lumen of the canal cell, duct cell and pore cell directly, we performed TEM of serial thin sections. We analyzed three wild-type and nine *let-4* mutant embryos at the late threefold stage, surrounding the window when defects first become visible by light microscopy. In 5/9 *let-4* threefold embryos, all three tube cells were still connected and the lumen was continuous, with no apparent distortions. Intercellular apical junctions appeared normal (Fig. 4J), as did the cuticular lining of the duct and pore (Fig. 4K). Because 97% of *let-4* embryos eventually display excretory defects, we infer that these embryos would have displayed defects shortly thereafter, had they been allowed to mature. The absence of any detectable junction or luminal defect in these embryos indicates that initial steps of junction formation, lumen growth and cuticle secretion are fairly normal in *let-4* mutants.

In 4/9 *let-4* threefold embryos, the duct and pore appeared to have separated already, as we had also observed by confocal microscopy. In one of these embryos, the existing duct lumen and the canal lumen appeared normal. In another, the duct lumen appeared normal, but the canal lumen was greatly enlarged. In the remaining two embryos, the duct lumen diameter was enlarged proximal to the duct cell body, and the apical membrane in this region had separated from the cuticle lining (Fig. 4L). Because the cuticle ring had a normal diameter in these cases, we infer that lumen distortion occurred subsequent to cuticle secretion. In two embryos, we were able to trace the duct lumen to its premature termination within the duct process (Fig. 4M). We were unable to recognize the excretory pore cell in three of these embryos, suggesting that the pore lacked its characteristic autocellular junction and lumen. Our interpretation is that duct and pore separation leads to pore collapse and lumen retraction, and that duct and canal cell lumen swelling behind the break is a secondary

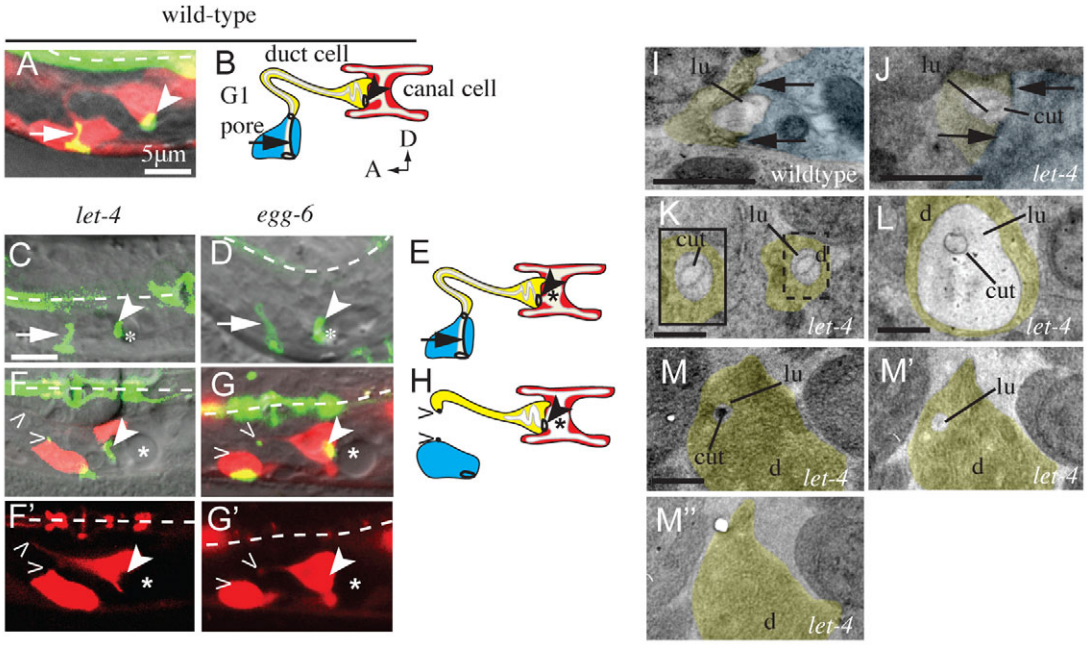

Fig. 4. *let-4* and *egg-6* are each required to maintain junction integrity between the excretory duct and pore. (**A-H**) Late threefold embryos (C,D) or early L1 larvae (A,F,G) expressing apical junction marker AJM-1::GFP. L1 larvae also express duct and pore cell marker *dct-5p*::mCherry. The arrowhead indicates the canal-duct junction; the arrow indicates the pore autojunction. The dotted line indicates the pharynx, which is also labeled by AJM-1::GFP. (A) Wild-type L1 larval excretory system. (B) Schematic interpretation of A. (C,D) Junction morphology initially appears normal in *let-4*(*mn105*) (C) and *egg-6*(*ok1506*) (D) mutants, but the canal cell lumen is beginning to swell (asterisk). (E) Schematic interpretation of C,D. (F,G) In older *let-4* (F) and *egg-6* (G) mutants, the duct and pore cells have separated (carets mark ends of cells) but contain junction remnants, and the pore autocellular junction has disappeared. Swelling of the canal cell lumen is more pronounced (asterisk). (F′,G′) Red channels from F and G, respectively. (H) Schematic interpretation of F,G. (**I-M″**) TEM images of the threefold excretory system. Duct cell is yellow; pore cell, blue; lu indicates lumen; cut, cuticle. (I,J) At threefold, 5/9 *let-4* embryos still appeared normal at the connection between the duct and the pore cell (compare wild-type (I) to *let-4* (J). The arrows indicate dark junctional material. (K) The duct cell lumen diameter also appeared normal (compare with wild-type, Fig. 1D). (L) In some *let-4* embryos that lacked duct-pore connectivity, the duct lumen behind the break was swollen and the apical membrane had separated from the cuticle lining. (M-M″) Adjacent serial sections documenting duct lumen termination. A bolus of cuticle-like material is present near the termination point (M). Scale bars: 5 µm in A-H; 1 µm in I-M.

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consequence of excretory fluid backup. Thus the primary defect in *let-4* mutants appears to be a failure to maintain the duct-pore intercellular junction.

Paralogs *let-4* and *sym-1* function redundantly to maintain epidermal junction integrity during embryonic elongation

A small proportion of *let-4* mutant or *let-4* RNAi embryos ruptured during elongation and failed to hatch (Fig. 2A, Fig. 5A). This phenotype reflected a semi-redundant role of *let-4* and its closest paralog, *sym-1*. Like LET-4, SYM-1 also is expressed in epidermal cells, but unlike LET-4, SYM-1 lacks a transmembrane domain and is secreted into the apical ECM (Davies et al., 1999). Whereas essentially all *sym-1* embryos developed normally, ~100% of *sym-1; let-4*(*RNAi*) embryos ruptured (Fig. 5A). The rare embryos that did not rupture swelled abnormally as they approached hatching, suggesting a defect in osmotic integrity (Fig. 5B). Similar osmotic defects were seen in *mec-8; let-4*(*RNAi*) embryos (Fig. 5A), the basis for the alternative *let-4* name '*sym-5*' (Davies et al., 1999).

egg-6 zygotic mutants did not rupture, *egg-6* RNAi did not show genetic interactions with *sym-1* (Fig. 5A), and *egg-6* apparently could not compensate for loss of *let-4* and *sym-1* despite being expressed in the epidermis (Fig. 3E,F,H). *lpr-1p*::LET-4(+) also failed to rescue *egg-6* excretory defects (Fig. 2A), indicating that

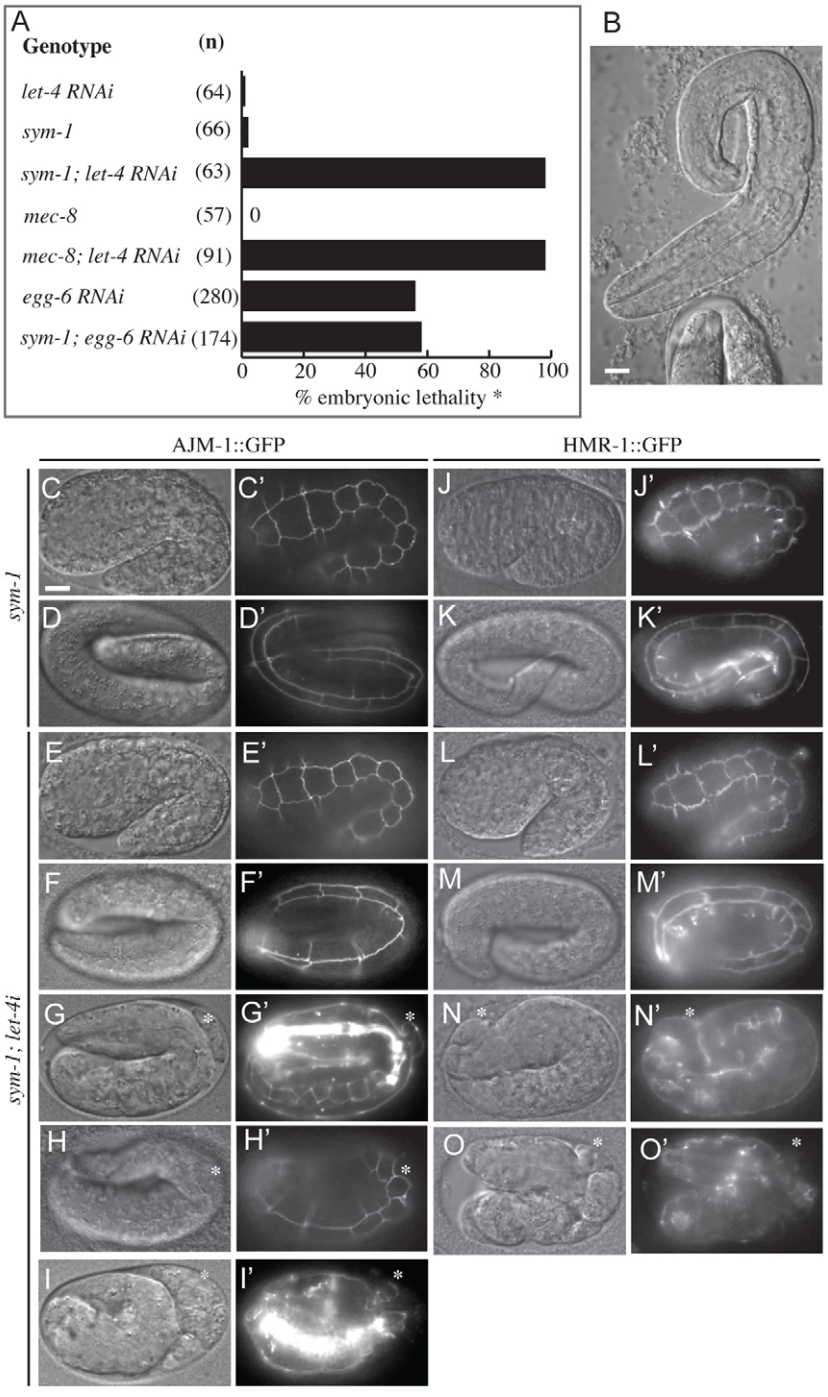

Fig. 5. *let-4* and *sym-1* are redundantly required to maintain junction integrity in the epidermis. (**A**) Quantification of embryonic lethality after *let-4* or *egg-6* RNAi. *Embryonic lethal phenotype varied. Most *mec-8; let-4*(*RNAi*) embryos swelled. *egg-6*(*RNAi*) embryos arrested at ~40 cell stage. (**B**) Rare *sym-1; let-4*(*RNAi*) embryos that did not rupture swelled as they approached hatching. (**C-O'**) AJM-1::GFP (C-I) and HMR-1::GFP (J-O) apical junction markers appeared normal in *sym-1* control embryos (C,D,J,K) and *sym-1; let-4*(*RNAi*) embryos (E,F,L,M) at the 1.5-fold stage (C,E,J,L) and at the threefold stage before rupture (D,F,K,M). (G,H,N) Most *sym-1; let-4*(*RNAi*) embryos began to extrude epidermal cells (asterisk) and rupture after elongating to the threefold stage. (I,O) Terminal arrest phenotype. Scale bars: 5 μm.

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let-4 cannot compensate for loss of *egg-6*. Thus, whereas LET-4 and SYM-1 have some redundant requirements, LET-4 and EGG-6 have unique requirements.

Epidermal rupture can be caused by excessive actin-myosin contractile activity during embryonic elongation (Wissmann et al., 1999; Diogon et al., 2007; Gally et al., 2009) or by defects in structural components of the epidermal junctions (Costa et al., 1998; Totong et al., 2007; Lockwood et al., 2008). In *sym-1; let-4*(*RNAi*) embryos, AJM-1::GFP and HMR-1/cadherin::GFP localized normally before rupture (Fig. 5E,F,L,M). Junctions did not appear distorted or break during the early steps of elongation as in known mutants with increased contractile activity (Diogon et al., 2007). Instead, in temporal analyses, most embryos elongated to the threefold stage before rupturing (Fig. 5F,M). Rupture occurred focally and was preceded by local junction distortions and small apical bulges as individual seam cells left the plane of the epithelium (Fig. 5G,H,N). After rupture, remaining non-ruptured regions of the epidermis still had normal junction morphology. Thus, as for the excretory system, we found no evidence for defects in junction establishment. Rather, LET-4 and SYM-1 are required to prevent epidermal junction breaks during the latter part of embryogenesis. Notably, this is the time frame when cuticle secretion begins and epidermal-ECM interactions must be remodeled (Costa et al., 1997).

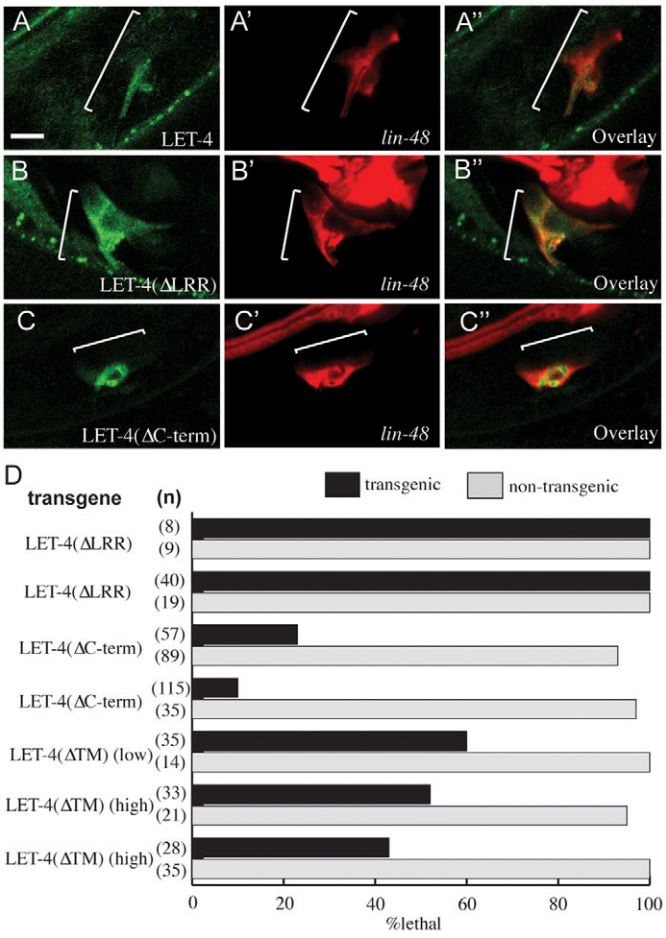

Fig. 6. The cytoplasmic and transmembrane domains of LET-4 are dispensable for function. (**A-C″**) L4 larvae expressing *lpr-1p*::LET-4 deletion constructs tagged with GFP. *lin-48p*::mCherry labels the entire cytoplasm of the duct cell. (A) LET-4::GFP localizes along the lumen of the duct cell. (B) signalseqGFP::LET-4(ΔLRR) is dispersed throughout the duct cell cytoplasm. (C) Similarly to A, LET-4(ΔC-term)::GFP localizes along the lumen of the duct cell. (**D**) *let-4*(*mn105*) rescue data. At least two independent transgenic lines were scored per construct, and non-transgenic siblings (gray) were scored as negative controls. low, injected at 2.5 ng/μl; high, injected at 5 ng/μl. LET-4(ΔLRR) contains LET-4 amino acids 398-773; LET-4(ΔC-term) 1-713; LET-4(ΔTM) 1-688. Scale bar: 5 μm.

The LET-4 transmembrane and cytoplasmic domains are dispensable for function

Some eLRRon proteins, including SYM-1, lack a transmembrane domain, and are secreted into the apical ECM (Davies et al., 1999). Furthermore, although EGG-6 has a predicted transmembrane domain, some proportion of EGG-6::GFP was still secreted, as it accumulated between the embryo and eggshell (supplementary material Fig. S1). To ask if LET-4 must be tethered to the membrane and to identify domains important for its function, we deleted the transmembrane (TM), cytoplasmic (Cterm) or extracellular LRR domains in the context of an *lpr-1p*::LET-4 transgene construct tagged with GFP to visualize localization within the excretory duct (Fig. 6). LET-4(ΔLRR) failed to rescue *let-4* lethality*;* furthermore, the fusion protein was not apically enriched (Fig. 6B,D). By contrast, LET-4(ΔCterm) efficiently rescued *let-4* lethality and appeared properly localized (Fig. 6C,D). LET-4(ΔTM) appeared toxic to embryos and we were able to obtain only a few transgenic lines with very low, undetectable levels of expression. LET-4(ΔTM) transgenes were apparently expressed, however, since they partially rescued *let-4* larval lethality (Fig. 6D). We conclude that the LRR domains are required for proper LET-4 function and localization, whereas the cytoplasmic domain is dispensable, and that tethering of LET-4 to the membrane is not absolutely required for function.

let-4, *egg-6* and *sym-1* are required for proper apical ECM organization

The above studies suggested that LET-4, EGG-6 and SYM-1 all might function extracellularly as part of the apical ECM. Although the excretory duct and pore lumen ECM appeared morphologically indistinguishable from wild type in *let-4* mutants (Fig. 4K, Fig. 7A), several abnormalities in the epidermal apical ECM were observed in eLRRon mutants. First, in 5/5 *let-4* embryos at the 1.5-fold stage, TEM analysis revealed a large gap between the inner eggshell layer and the epidermal embryonic sheath layer (Fig. 7A), suggesting disorganization of one or both of these layers. Second, in most *let-4* and *egg-6* mutant threefold embryos, many globular structures accumulated between the embryo and the eggshell (Fig. 7B-D). These globules contained cytoplasm, as they were marked by GFP in transgenic embryos expressing cytoplasmic GFP reporters (Fig. 7B). In *let-4* TEMs, these cytoplasts appeared membrane-bound and were positioned between the nascent cuticle and the inner eggshell layer (Fig. 7D), indicating that cell fragments had been shed before cuticle secretion; this is consistent with a defect in a protective function of the embryonic sheath. Third, most *let-4*, *egg-6* and *sym-1* mutant L1 larvae showed abnormal permeability to dye (Fig. 7E-J), indicating a defect in larval cuticle organization. A requirement for eLRRon proteins in apical ECM organization is further supported by the eggshell defects observed after depletion of maternal *egg-6* (Carvalho et al., 2011; Sonnichsen et al., 2005) (A. Singson, personal communication). In summary, eLRRon single mutants show penetrant and early, but relatively mild, defects in apical ECM

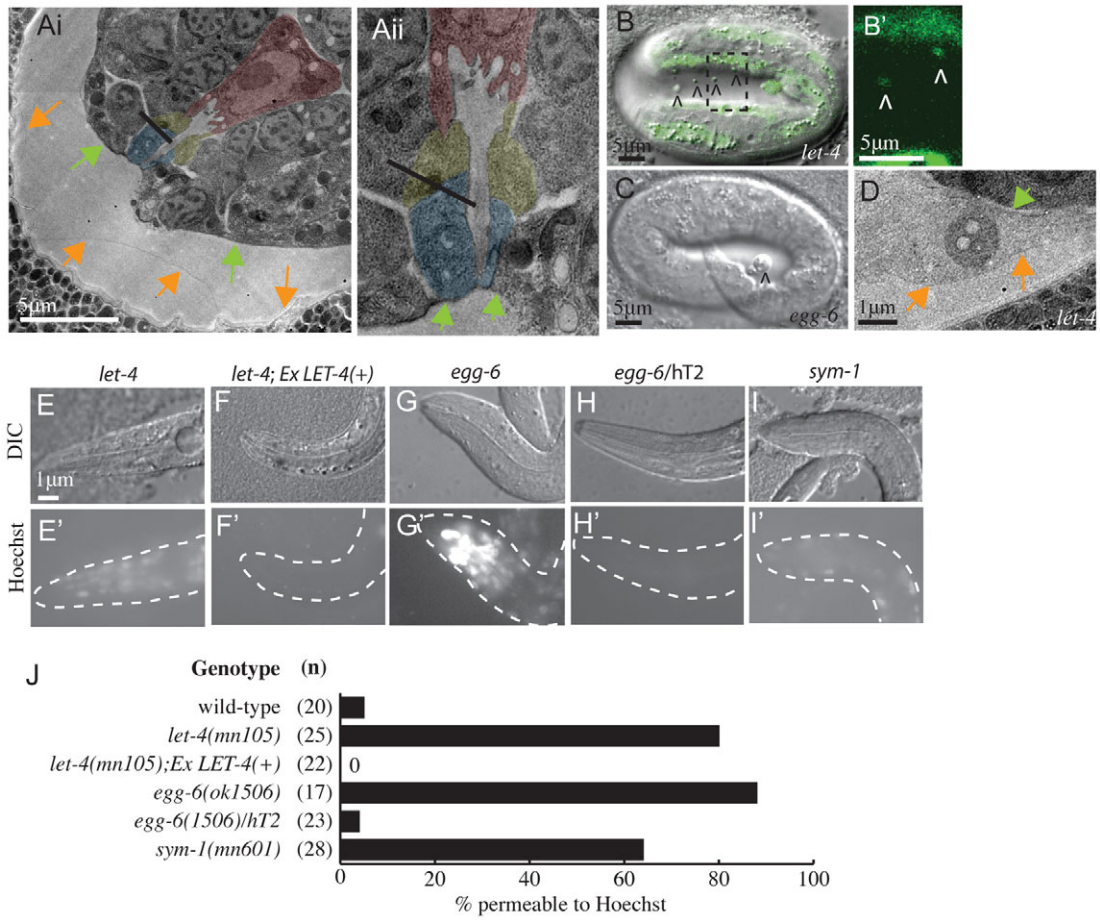

Fig. 7. *let-4*, *egg-6* and *sym-1* are required for proper apical ECM organization. (**A**) (i) TEM image of 1.5-fold *let-4* mutant, adjacent to the pore opening. Canal cell is colored red; duct cell, yellow; pore cell, blue. The distance between the inner eggshell layer (orange arrows) and the embryonic sheath layer (green arrows) was increased compared with wild type (Fig1I). (ii) At 1.5-fold, *let-4* lumen morphology and lumen ECM (line) were indistinguishable from wild type (compare with Fig. 1I,J). (**B-C**) DIC image of extra-embryonic cytoplasts in *let-4*(*mn105*) (B,B′) and *egg-6*(*ok1506*) threefold embryos (C). Cytoplasts are labeled with *vha-1p*::GFP in *let-4* (B′). (**D**) TEM image of extra-embryonic cytoplasts in a *let-4* threefold embryo. (**E-I′**) Hoescht dye permeability assay. DIC (E-I) and fluorescence (E′-I′) images of L1 larvae treated with Hoechst dye, 0.7 seconds exposure time. (**J**) Quantification of dye permeability. Scale bars: 5 μm.

organization. We propose that eLRRon proteins function primarily to organize the apical ECM, and that defects in apical ECM lead secondarily to defects in epithelial junction maintenance.

DISCUSSION

It has long been recognized that extracellular cues from contact with neighboring cells and the ECM influence epithelial polarity, cell shape and motility (Berrier and Yamada, 2007). Epithelial cells also secrete their own ECM factors. Whereas most studies have focused on the importance of basal ECM factors, the work presented here suggests a link between the apical ECM and maintenance of epithelial junction integrity in *C. elegans*. This work also identifies epithelial extracellular LRR only proteins as important components and organizers of the pre-cuticular and cuticular apical ECM.

Known roles for eLRRon proteins in matrix and junction organization

The eLRRon family of proteins includes 52 members in mice, 35 in *Drosophila* and 17 in *C. elegans* (Dolan et al., 2007), several of which are involved in ECM organization. In mammals, decorin and other secreted SLRPs bind directly to collagen and modulate collagen fibril assembly (Kalamajski and Oldberg, 2010). SLRP knockout mice have disorganized collagen fibrils and various tissue fragility phenotypes, and mutations in certain SLRPs are associated with similar syndromic conditions in humans (Ameye and Young, 2002; Schaefer and Iozzo, 2008). Although many SLRPs appear confined to stromal tissues, several are expressed in the kidney or other epithelia (Ross et al., 2003; Shimizu-Hirota et al., 2004). In *Drosophila*, the eLRRon protein convoluted is required in tracheal epithelial cells for proper apical ECM organization and tube length (Swanson et al., 2009).

Several transmembrane eLRRon proteins, including mammalian LRRTM1-3 and *Drosophila* capricious and tartan, promote synaptic junction formation or maintenance (Kurusu et al., 2008; Shinza-Kameda et al., 2006; Shishido et al., 1998; Taniguchi et al., 2000). Although synaptic junctions can be influenced by the ECM (Ackley et al., 2003; Fox et al., 2008), eLRRon junction phenotypes have not (thus far) been linked to any ECM defect. Instead, synaptic eLRRon proteins are believed to function as cell adhesion molecules (CAMs), in some cases by binding to other CAMs such as neurexins (de Wit et al., 2009; Ko et al., 2009; Siddiqui et al., 2010).

C. elegans eLRRon proteins reveal a link between apical ECM organization and epithelial junction maintenance

We showed here that the TM eLRRon proteins LET-4 and EGG-6 and their secreted paralog SYM-1 are required for both apical ECM organization and epithelial junction stability in the *C. elegans* epidermis and excretory duct and pore tubes. Potentially, these eLRRon proteins could play independent roles in both processes or could act in some common upstream process such as protein trafficking. However, as discussed below, several observations suggest that defects in apical ECM organization lead secondarily to defects in epithelial junction maintenance.

Like many other invertebrates, *C. elegans* has a tough outer exoskeleton or cuticle that lines the epidermis and other exposed epithelia, including the excretory duct and pore. The mature cuticle consists primarily of collagens and ZP-domain proteins termed cuticulins, and is coated by a lipid-rich epicuticle and a glycoprotein-rich surface coat (Page and Johnstone, 2007). The mature cuticle forms relatively late in embryogenesis; before that, the lipid- and glycoprotein-rich outer layers appear to comprise the early embryonic sheath ECM and are in direct contact with the epidermis at microfilament-based attachment sites (Costa et al., 1997; Priess and Hirsh, 1986). When the inner cuticle layers are secreted, the earlier sheath layers detach from the epidermis and are pushed outward, so the epidermis is subjected to a changing matrix environment. The cuticle is subsequently shed and re-synthesized at each larval molt, so matrix structure and membrane-matrix attachments must be constantly remodeled during development.

eLRRon proteins LET-4, EGG-6 and SYM-1 all localize to the apical domains of epithelia that are, or will eventually become, cuticle-lined, and they are important for the proper organization of both the pre-cuticular (embryonic sheath and eggshell) and cuticular apical ECM (supplementary material Table S1). Like the mammalian SLRPs, these eLRRon proteins may be structural components of the ECM that contribute to the strength and impermeability of the matrix by modulating associations among other matrix components such as collagens. Alternatively, or in addition, eLRRon proteins could affect protein-trafficking mechanisms that deliver other specific ECM components to the apical surface.

In addition to their roles in ECM organization, LET-4, EGG-6 and SYM-1 are required for apical junction maintenance (supplementary material Table S1). In mutants, junctions initially form in the correct locations and appear morphologically normal, but then rupture focally late in embryogenesis. As proposed for LRRTM1-3 or caps and tartan, the *C. elegans* eLRRon proteins may directly mediate cell-cell adhesion to stabilize junction integrity. However, such a model is difficult to reconcile with the broad apical localization patterns of the proteins and with the ability of LET-4 and SYM-1 to function in the absence of TM domains. We also note that internal epithelia with very different matrix composition do not express or require these eLRRon proteins. Furthermore, junction breaks in the epidermis (in *sym-1 let-4* RNAi mutants) and in the excretory system (in *let-4* or *egg-6* single mutants) occur after ECM defects are already apparent and soon after cuticle secretion. Therefore, we favor a direct role for eLRRon proteins in ECM organization, with secondary effects on junction integrity.

How might apical ECM organization affect epithelial junction stability?

Mutations in other *C. elegans* apical ECM components generally do not cause excretory or epidermal junction phenotypes such as those described here (Page and Johnstone, 2007), suggesting a relatively specific role for eLRRon proteins in junction integrity. Instead, mutations in individual cuticle collagen or cuticulin genes cause cuticle blistering or defects in body shape or cuticle patterning (Johnstone, 2000; Page and Johnstone, 2007). Mutations in glycosyltransferases that perturb the outer cuticle layer alter the susceptibility of larvae to bacterial infection and in some cases increase permeability (Partridge et al., 2008). Nevertheless, there have been prior indications that the apical ECM influences cell shape and connectivity. Embryos mutant for the cuticle collagen *sqt-3* elongate initially and then retract, revealing a requirement for cuticle to stabilize epidermal cell shape (Priess and Hirsh, 1986). Mutants for the tetraspanin *tsp-15*, which show various cuticle abnormalities, also show seam epidermal protrusions and junction connectivity defects (Moribe et al., 2004). Finally, *sec-23* mutations that impair cuticle secretion (and presumably secretion of eLRRon proteins as well) cause embryonic rupturing at the two- to threefold stage as described here for *sym-1; let-4*(*RNAi*) embryos (Roberts et al., 2003). It is possible that redundancy among the >170 cuticle collagens in *C. elegans* masks more widespread requirements for the cuticular ECM in maintaining junction integrity.

There are several mechanisms by which apical ECM organization might affect junction integrity. Our data are consistent with the idea that junction breakage in eLRRon mutants results from excessive forces placed on those junctions. The interconnected nature of the cuticular ECM may help bind together epithelial cells that share that ECM, evenly distributing force and reducing stress on individual junctions, much like the facings in sandwich structured composite construction (Allen, 1969). Alternatively, junction breakage could reflect inherent weaknesses in those junctions that were not detectable in our study. eLRRon proteins in the apical ECM are in a good position to interact with cadherins or with transmembrane apical polarity proteins such as Crumbs, and could potentially influence junction maintenance through such interactions. Finally, many ECM components, including SLRPs, affect signaling pathways that could alter gene expression and/or cytoskeletal organization to influence junction maintenance (Bulow and Hobert, 2006; Schaefer and Iozzo, 2008). Irrespective of specific mechanism, our findings suggest that changes in epithelial eLRRon expression or activity could contribute to junction remodeling during both normal and disease processes.

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Competing interests statement

The authors declare no competing financial interests.

Supplementary material

Supplementary material available online at
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