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Increased IP_3/Ca^{2+} signaling compensates depletion of LET-413/DLG-1 in *C. elegans* epithelial junction assembly

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ABSTRACT

The *let-413*/*scribble* and *dlg-1*/*discs large* genes are key regulators of epithelial cell polarity in *C. elegans* and other systems but the mechanism how they organize a circumferential junctional belt around the apex of epithelial cells is not well understood. We report here that IP_3/Ca^{2+} signaling is involved in the *let-413*/*dlg-1* pathway for the establishment of epithelial cell polarity during the development in *C. elegans*. Using RNAi to interfere with *let-413* and *dlg-1* gene functions during post-embryogenesis, we discovered a requirement for LET-413 and DLG-1 in the polarization of the spermathecal cells. The spermatheca forms an accordion-like organ through which eggs must enter to complete the ovulation process. LET-413- and DLG-1-depleted animals exhibit failure of ovulation. Consistent with this phenotype, the assembly of the apical junction into a continuous belt fails and the PAR-3 protein and microfilaments are no longer localized asymmetrically. All these defects can be suppressed by mutations in IPP-5, an inositol polyphosphate 5-phosphatase and in ITR-1, an inositol triphosphate receptor, which both are supposed to increase the intracellular Ca^{2+} level. Analysis of embryogenesis revealed that IP_3/Ca^{2+} signaling is also required during junction assembly in embryonic epithelia.

Introduction

Acquisition of cell polarity is essential to epithelial cells for carrying out their barrier/fence functions and for proper movements of epithelial sheets during morphogenesis (Knust and Bossinger, 2002; Nelson, 2003; Suzuki and Ohno, 2006). The formation of intercellular junctions is a hallmark of epithelial polarization. The zonula adherens is a belt-like junctional structure that encircles the apex of polarized epithelial cells and is called the apical junction in *C. elegans* (CeAJ) (McMahon et al., 2001). From genetic studies on *Drosophila* ectoderm, *C. elegans* hypodermis/intestine and mammalian cultured cells, it appears that three spatially restricted membrane associated protein-scaffolds are required for regulating the maturation of the zonula adherens in epithelial cells: the PAR-3–PAR-6–aPKC complex, the Scribble–Dlg–Lgl complex, and the Crumbs–Stardust–Patj complex (Legouis et al., 2000; Köppen et al., 2001; McMahon et al., 2001; Betschinger et al., 2003; Bilder et al., 2003; Plant et al., 2003; Tanentzapf and Tepass, 2003; Yamanaka et al., 2003; Aono et al., 2004; Bossinger et al., 2004; Harris and Peifer, 2005; Totong et al., 2007; Lockwood et al., 2008).

C. elegans LET-413, DLG-1 (the *Drosophila* Scribble and Discs large homologues, respectively) and AJM-1 play a fundamental role in establishing the CeAJ in the *C. elegans* embryo (Müller and Bossinger, 2003; Hardin and Lockwood, 2004; Labouesse, 2006), but to date the mechanisms how the LET-413 protein correctly localize the DLG-1–AJM-1 complex (DAC) and how the DAC then organizes a continuous junctional belt around the apex of epithelial cells are still elusive. During epithelial polarization in the embryo, patches of apical junction proteins become localized to the future apical cortex in a *let-413* and *par-6* dependent manner (McMahon et al., 2001; Totong et al., 2007). PAR-6 localizes apically in *C. elegans* epithelial cells and contains PB1, CRIB and PDZ domains that bind other polarity proteins such as the atypical protein kinase C (aPKC) PKC-3 (Hung and Kemphues, 1999; Joberty et al., 2000; Lin et al., 2000; Suzuki et al., 2001). The LET-413 protein is basolaterally expressed and contains one PDZ domain and 16 leucine-rich repeats with high homology to proteins known to interact with small GTPases (Legouis et al., 2000, 2003). The DLG-1 protein (a MAGUK) consists of three PDZ, one SH3,

Abbreviations: CeAJ, *C. elegans* apical junction; DAC, DLG-1–AJM-1 complex; dsRNA, double-stranded RNA; Emo, endomitotic oocyte; gof, gain-of-function; GUK, guanylate kinase; IP_3, inositol 1,4,5-triphosphate; lof, loss-of-function; MAGUK, membrane-associated guanylate kinase homolog; MTs, microtubules; NEBD, nuclear envelope breakdown; RNAi, RNA mediated interference.

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and one GUK domain, while the AJM-1 protein (*a*pical *j*unction *m*olecule) only displays a coiled-coil motif (Lockwood et al., 2008). After elimination of *let-413* function, the localization of the DAC is delayed, finally causing an embryonic lethal phenotype (Bossinger et al., 2001; Firestein and Rongo, 2001; Köppen et al., 2001; McMahon et al., 2001; Segbert et al., 2004). Removal of *let-413* function has been shown to allow lateral expansion of normally apical proteins (e.g. PAR-3 and PAR-6) and junctional proteins (Legouis et al., 2000; McMahon et al., 2001). Additionally, LET-413 is necessary for the maintenance of the entire terminal web or brush border assembly at the apical surface of the intestine, whereas removal of the DAC or components of the catenin–cadherin complex has little effect on the overall position or continuity of the terminal web (Bossinger et al., 2004). To explore a possible role for LET-413 and DLG-1 in development of the spermatheca, we depleted both proteins post-embryonically using RNAi.

We found that LET-413- and DLG-1-depleted worms most obviously exhibit defects in ovulation due to malfunction of the spermatheca. The spermatheca epithelium is a contractile accordion-like tube that contains sperm and is the site of oocyte fertilization. We discovered that in spermathecal cells LET-413 and DLG-1 are localized basolaterally and at the apical junction, respectively. We also found that the apical junction in the spermatheca is severely affected in LET-413 depleted worms due to mislocalization of DLG-1, the cell polarity protein PAR-3, and actin microfilaments. As a consequence, ovulation fails and results in sterility of worms. From these observations, we conclude that LET-413 and DLG-1 activities are necessary for the proper formation of the apical junction in the spermatheca and that defective ovulation results from failure of spermathecal cells to dilate. Finally, we showed that mutations, increasing IP_3/Ca^{2+} signaling in spermathecal cells can suppress the ovulation defects of *let-413(RNAi)* and *dlg-1(RNAi)* worms and rebuild the apical junctional pattern also. Remarkably, these mutations partially also rescue the embryonic epithelial phenotypes.

Material and methods

C. elegans strains, culture and GFP markers

Standard procedures for handling and maintaining *C. elegans* have been previously described (Brenner, 1974). Bristol strain N2 was used as wild type. Semi-synchronized L1s were obtained by treatment with alkaline hypochloride (2 vol 4 M NaOH:3 vol 13% NaOCl) (Wood, 1988). Strain SU93, AJM-1::GFP translational fusion (AJM-1 was formerly JAM-1), (Mohler et al., 1998); WM27 *(rde-1(ne219) V)*; NL2098 *(rrf-1(pk1417) I)*, (Sijen et al., 2001); and PS3653 *(ipp-5(sy605) X)*, (Bui and Sternberg, 2002); NL2099 *(rrf-3(pk1426) II)*, (Simmer et al., 2002); SU180 *(itr-1(jc5) jcIs1 IV)*, and PS2582 *(itr-1(sy290) unc-24 (e138) IV)* (Clandinin et al., 1998) were obtained from the *Caenorhabditis* Genetics Center stock collection (University of Minnesota, St. Paul, MN). The following markers were used: the pML801 plasmid, which is a functional *let-413::gfp* translational fusion; the pML808 plasmid, which is a functional *let-413::cfp* translational fusion; and the pML902 plasmid, which is a functional *dlg-1::gfp* translational fusion (Legouis et al., 2000; McMahon et al., 2001). *dpy-11(e224)let-413(s128)unc-42(e270)/rol-3(e754);ipp-5(sy605)* and *dpy-11(e224)let-413(s14531)/unc-42(e270)rol-3(e754);ipp-5(sy605)* and *dpy-11(e224) let-413(s1451)/unc-42(e270)rol-3(e754);ipp-5(sy605)* and *dpy-11 (e224)let-413(s1455)/unc-42(e270)rol-3(e754);ipp-5(sy605)* and *ipp-5 (sy605);Is[let-413::gfp;rol-6(su1006)]* strains were generated by classical genetic crossing.

RNA mediated interference (RNAi)

The following cDNA clones were obtained from Y. Kohara (Gene Network Lab, NIG, Mishima 411, Japan): for *Ce*MKLP1, see (Powers et al., 1998); for *crb-1*, yk74b1 (0.85 kB, XhoI fragment); for *dlg-1*, yk128b7 (1.5 kb, XhoI fragment); for *erm-1*, yk257f5 (1.6 kb, XhoI fragment); for *hmr-1*, yk611f6 (2.0 kb, XhoI–SacI fragment); for *let-413*, yk524b7 (2.5 kb, XbaI fragment); and for *par-3*, yk6c1 (1.0 kb, XhoI–HincII fragment). The RNAi clone for *itr-1* was obtained from the Ahringer RNAi library (Geneservice Limited). The 5′- and 3′-sequences are specific to the genes being tested, as determined by sequencing and BLAST searches (http://www.wormbase.org/). Each cDNA was inserted into the vector pPD129.36 for expression of dsRNA in *E. coli* strain HT115 (DE3). Induction and feeding were performed as previously described (Timmons and Fire, 1998; Timmons et al., 2001), except that 0.5 mM IPTG was used. HT115(DE3) bacteria harboring the "empty" KS+ based vector, L4440 (containing two T7 promoters flanking a polylinker), were used as a control for RNAi feeding experiments. To distinguish between essential functions during embryogenesis (F1 generation scored) and post-embryonic (P0 generation scored) development either young adults or L1 larvae were placed on RNAi feeding plates, which were replaced against fresh feeding plates after 1 and 3 days, respectively. To distinguish between essential functions in the intestine and elsewhere in the animal, we constructed a strain (OLB11) of worms in which RNAi functions only in the intestine (see Fig. S1).

Immunostaining, microscopy, and image processing

Larvae were transferred using a drawn-out pipette to a microscope slide coated with a thin layer of polylysine in a drop of distilled water. They were permeabilized by using the freeze–crack method (Strome and Wood, 1983) and fixed in 100% ice-cold methanol (10 min), ice-cold 100% acetone (20 min), ice-cold 90% ethanol (10 min), ice-cold 60% ethanol (10 min) and 30% ethanol (10 min at room temperature). Slides were washed twice for 10 min in TBT (20 mM Tris–HCl; 150 mM NaCl, pH8.0+0.1% Tween 20), incubated at 4 °C overnight with primary antibodies (see below) in blocking buffer (TBT plus 1% BSA and 1% nonfat dry milk powder), washed three times for 10 min each with TBT at room temperature, and finally incubated at room temperature for 1–3 h, with secondary antibodies (see below) in blocking buffer. Finally, slides were washed three times for 10 min each in TBT and mounted in Mowiol containing DABCO (1,4-diazabicyclo(2.2.2)octane; Sigma, Inc) as an antifade reagent.

Gonad dissecting was performed as described previously (Strome, 1986; McCarter et al., 1997). Gonads processed by freeze–cracking as described above for larvae.

The following primary and secondary antibodies were used at the dilutions (in blocking buffer) indicated: anti-DLG-1 (rabbit, 1:400), anti-ERM-1 (rabbit, 1:200), anti-PAR-3 (1:50), anti-GFP (rabbit or mouse, 1:100, Molecular Probes) and either Cy2-conjugated or Cy3-conjugated secondary antibodies (1:200, Jackson ImmunoResearch Laboratories).

Filamentous actin staining was performed according to Waterston et al. (1984). Briefly, worms were fixed in 2% formaldehyde in Na_2HPO_4 (0.1 M) buffer for 1 h 30 min then, permeabilized in chilled acetone for 2 min and incubated in PBS with Alexa 568-coupled-phalloidin (Molecular Probes) for 2 h. After washing once in PBS, worms were mounted in Mowiol containing DABCO (see above). Confocal images were analysed with the ImageJ software (http://rsb.info.nih.gov/ij/).

Immunofluorescence analysis of embryos was performed on a confocal laser scanning microscope (TCS-NT or TCSSP2, Leica) equipped with a 100×PL Fluotar oil immersion objective. Recorded images represent a Z-projection of optical sections taken 0.5 μm apart.

Differential interference contrast microscopy and digital image capture were performed using a Zeiss Axioplan 2 fitted with a Hamamatsu ORCA-ER camera.

Electron microscopy

Electron microscopy analysis of adults was performed as previously described (Legouis et al., 2000). Briefly, blocks of four to six animals