Macroglobulin complement-related encodes a protein required for septate junction organization and paracellular barrier function in Drosophila

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ABSTRACT
Polarized epithelia play crucial roles as barriers to the outside environment and enable the formation of specialized compartments for organs to carry out essential functions. Barrier functions are mediated by cellular junctions that line the lateral plasma membrane between cells, principally tight junctions in vertebrates and septate junctions (SJs) in invertebrates. Over the last two decades, more than 20 genes have been identified that function in SJ biogenesis in Drosophila, including those that encode core structural components of the junction such as Neurexin IV, Coracle and several claudins, as well as proteins that facilitate the trafficking of SJ proteins during their assembly. Here we demonstrate that Macroglobulin complement-related (Mcr), a gene previously implicated in innate immunity, plays an essential role during embryonic development in SJ organization and function. We show that Mcr colocalizes with other SJ proteins in mature ectodermally derived epithelial cells, that it shows interdependence with other SJ proteins for SJ localization, and that Mcr mutant epithelia fail to form an effective paracellular barrier. Tissue-specific RNA interference further demonstrates that Mcr is required cell-autonomously for SJ organization. Finally, we show a unique interdependence between Mcr and Nrg for SJ localization that provides new insights into the organization of the SJ. Together, these studies demonstrate that Mcr is a core component of epithelial SJs and also highlight an interesting relationship between innate immunity and epithelial barrier functions.

KEY WORDS: Septate junction, Epithelia, Innate immunity

INTRODUCTION
Polarized epithelia play crucial roles as barriers to the outside world and in providing distinct compartments for organs to carry out essential metabolic functions in all metazoans. These functions require a physiologically tight epithelium to provide a barrier to the flow of small molecules between the apical and basal sides of the epithelium. This paracellular barrier is established and maintained by tight junctions (TJs) in the epithelium of vertebrate organisms, and by septate junctions (SJs) in invertebrate organisms. TJs localize along the lateral membrane in a region apical to the adherens junction and are characterized ultrastructurally as a series of anastamosing ribbons, where the plasma membranes of adjacent cells are in direct opposition (Farquhar and Palade, 1963). By contrast, the pleated SJs found in invertebrate epithelia localize basal to the adherens junction and are characterized by uniformly spaced rows of electron-dense septa between the plasma membranes of adjacent cells (Noirot-timothée et al., 1978). Despite these differences in subcellular localization and ultrastructure, the functional similarity and molecular conservation of key proteins, including members of the claudin and membrane-associated guanylate kinase (MAGUK) families, suggest that TJs and SJs are analogous structures. The localization of cell signaling and polarity proteins to TJs and SJs also suggests that these barrier junctions play crucial roles in organizing and orchestrating basic epithelial functions during development. Accordingly, disruption of SJs in embryonic epithelia and glia nearly always results in embryonic lethality, with characteristic defects in the epidermal cuticle (Lamb et al., 1998), tracheal size control (Behr et al., 2003; Paul et al., 2003), dorsal closure (Woods and Bryant, 1991; Fehon et al., 1994; Baumgartner et al., 1996), and embryonic paralysis due to a disrupted blood brain barrier (Baumgartner et al., 1996).

Genetic studies in Drosophila have identified more than 20 genes that function in the establishment or maintenance of SJs. The core constituents of the SJ include transmembrane proteins of the claudin family [e.g. Megatrachea (Pickel – FlyBase), Sinuous, Kune-Kune (Behr et al., 2003; Wu et al., 2004; Nelson et al., 2010)], cell adhesion molecules including Neurexin IV (Nrx-IV) (Baumgartner et al., 1996), Contactin (Cont) (Favre-Sarrailh et al., 2004), Lachesin (Llimargas et al., 2004), Neuroglian (Nrg) and Gliotactin, the α and β subunits of the Na⁺/K⁺ ATPase (Genova and Fehon, 2003), the GPI-linked protein Transferrin 2 (Tsf2, also known as Melanotransferrin) (Tiklova et al., 2010), and the cytoplasmic proteins Coracle (Cor; Cora – FlyBase) (Fehon et al., 1994) and Varicose (Wu et al., 2007). A second group of proteins is required for SJ assembly, but the proteins do not physically reside at the junction. This group includes several members of the Ly6 family of proteins (e.g. Boudin, Crooked and Coiled) (Hijazi et al., 2009; Nilton et al., 2010), as well as proteins that function in endocytosis and recycling such as Clathrin heavy chain, Dynamin (Shibire – FlyBase), Rab5 and Rab11 (Tiklova et al., 2010).

The biogenesis of SJs is a multistep process involving the synthesis and secretion of membrane-resident SJ proteins, followed by endocytosis and recycling of these proteins to the apical lateral plasma membrane during mid-embryogenesis (Tiklova et al., 2010). The final refinement of the SJ requires that each member of the core complex is present, suggesting that the SJ is a large, stable and
highly crosslinked protein complex. This was first appreciated when Ward et al. (Ward et al., 1998) showed that Nrx-IV and Cor physically interact and that their correct localization to the SJ was mutually dependent, and has been extended to other SJ proteins (e.g. Behr et al., 2003; Genova and Fehon, 2003; Paul et al., 2003; Faivre-Sarrailh et al., 2004; Tiklová et al., 2010). Fluorescence recovery after photobleaching (FRAP) experiments have revealed that SJ proteins are essentially fixed in the membrane by stage 14 of embryogenesis (Laval et al., 2008; Oshima and Fehon, 2011). Mutations in any core SJ gene increases the mobility of other SJ proteins (e.g. Ward et al., 1998) showed that Nrx-IV and Cor physically interact and that their correct localization to the SJ was interdependent with other SJ proteins, suggesting that SJ proteins are essentially fixed in the membrane by stage 14 of embryogenesis.

Here we identify a role for Macroglobulin complement-related (Mcr) during SJ biogenesis. We show that Mcr mutant epithelial tissues have defective SJ organization and function. We further demonstrate that Mcr localizes to epithelial SJs and that its localization is interdependent with other SJ proteins, suggesting that Mcr is a core structural component of the junction. Mcr belongs to a family of thioester-containing proteins (TEPs) that primarily serve additional essential developmental role in insects and highlight a potentially intriguing connection between epithelial barrier function and the innate immune response.

RESULTS
Identification and characterization of Mcr mutations
We recovered an EMS-induced allele in Mcr from a genetic screen of mutations that dominantly enhanced the malformed leg phenotype associated with a hemizygous mutation in broad (br') (hereafter referred to as Mcr') (Ward et al., 2003). Mcr encodes a 1760 amino acid protein with α-2-macroglobulin and LDL receptor class A domains and a predicted C-terminal transmembrane domain (Krogh et al., 2001). Fluorescence recovery after photobleaching experiments demonstrate that a member of the TEP family plays an additional essential developmental role in insects and highlight a potentially intriguing connection between epithelial barrier function and the innate immune response. Additional essential developmental role in insects and highlight a potentially intriguing connection between epithelial barrier function and the innate immune response.

Fig. 1. Mutations in Mcr are embryonic lethal with phenotypes associated with SJ defects. (A) Schematic of the Mcr gene and Mcr protein. The insertion site of Mcr' and the amino acid substitution in Mcr are shown. The region of Mcr used for antibody generation is indicated by a bracket. s, signal sequence; MG1, alpha-2-macroglobulin MG1 domain; A2M_N, MG2 domain; A2M_N_2, alpha-2-macroglobulin family N-terminal region; LDLα, low-density lipoprotein receptor A domain; A2M, alpha-2-macroglobulin family; A2M_comp, alpha-2-macroglobulin complement component; A2M_rec, alpha-2-macroglobulin receptor; TM, predicted transmembrane domain. (B-E) Cuticle preparations of a w1118 (wild-type) late embryo (B) and Mcr' (C), Mcr' (D) and da-GAL4>UAS-Mcr-RNAi (E) mutant embryos. Anterior is left and dorsal is up or facing. Note the dorsal hole in the Mcr' embryo (asterisk in D) and the ectopic salivary gland deposition in mutant animals (arrows in C-E).

(F,G) Confocal optical sections of stage 17 w1118 (F) and Mcr' mutant (G) embryos stained with Rhodamine-labeled wheat germ agglutinin (WGA), showing a highly convoluted trachea (arrows) in the Mcr' animal. Scale bars: 100 μm.
tracheal-specific expression of Mcr-RNAi [using breathless (btl)-
GAL4] recapitulated the tracheal length defects (Fig. 1E; data not
shown). Taken together, these results indicate that loss of
Mcr is responsible for these phenotypes.

Mcr is required for SJ organization and function
As the suite of phenotypes observed in Mcr mutants is commonly
associated with mutations in genes that function in the SJ, we
examined the organization and function of SJs in the embryonic
epithelia of Mcr mutant animals. In the ectodermal epithelia of stage
16 wild-type embryos, the SJ is localized to the apical part of the
lateral membrane in a region basal to the adherens junction, and can
be visualized by the localization of core SJ proteins including Cor
(Fig. 2A). Consistent with a defect in SJ organization, Cor is
mislocalized in the salivary glands, hindguts and tracheae of Mcr
mutant stage 16 animals (Fig. 2D-O). Interestingly, the
mislocalization of Cor is more pronounced in the hindguts and
trachea than in the salivary glands, and Mcr1 shows a stronger
phenotype in the salivary glands than the other Mcr mutations. The
correct localization of marginal zone (Crumbs; Fig. 2D′,G′,J′,M′)
and adherens junction (Armadillo and α-Catenin; data not shown)
proteins in Mcr mutant embryos indicates that Mcr is not required
for overall apical/basal polarity.

To test the barrier function of the SJs, we injected a 10 kDa
Rhodamine-labeled dextran into the hemocoel of stage 17 wild-type
and Mcr mutant embryos (Lamb et al., 1998). In wild-type embryos,
the SJs are physiologically tight by this stage, which prevents the
infiltration of the labeled dextran into the lumen of the trachea
(Fig. 2P′). In stage 17 Mcr1 mutant embryos, the dextran freely
passes between tracheal cells, filling the lumen with dye (Fig. 2Q′).
Taken together, these results indicate that Mcr is required to
establish or maintain a physiologically tight SJ in Drosophila
embryonic epithelia.

Mcr is expressed in ectodermal epithelia and localizes to
SJs
Developmental northern blot analysis revealed Mcr transcripts in
0- to 2-hour embryo lysates, suggesting a maternal contribution of
Mcr. Subsequently, Mcr levels are reduced in 2- to 4-hour embryo
lysates, but rise and peak between 4 and 12 hours of

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**Fig. 2. Mcr is required for SJ structure and paracellular barrier function.** (A-O) Confocal optical sections of salivary glands (A,D,G,J,M) and Cor (green, and in A′,D′,G′,J′,M′) and hindguts (B,E,H,K,N) and trachea (C,F,I,L,O) from stage 16 w1118 (A-C), Df(2L) Exel7034 (D-F), Mcr1 (G-I), McrEY07421 (J-L) and McrPex3 (M-O) embryos. The salivary gland in G is from the same animal as the hindgut in H, as are the salivary gland and hindgut shown in J and K. Apical surfaces face the lumen of the gland. The wild-type salivary gland epithelium is outlined by a dashed line in A. Note that Cor is mislocalized along the lateral membrane in Mcr mutant salivary glands (arrows), whereas Crb is unaffected. (P-Q′) Differential interference contrast (P,Q) and fluorescence (P′,Q′) photomicrographs of the tracheal dorsal trunk in stage 17 w1118 (P) and Mcr1 (Q) embryos that had been injected with a 10 kDa Rhodamine-labeled dextran. The labeled dextran does not cross the tracheal epithelium and enter the lumen in the wild-type embryo, but does in the Mcr1 mutant embryo (arrows). Scale bars: 20 μm.
embryogenesis, after which transcript levels are strongly reduced (Fig. 3A). We generated a polyclonal antibody against a non-conserved region of Mcr (indicated in Fig. 1A). Consistent with the predicted molecular mass of 203 kDa, we observe an ~225 kDa band from embryonic and imaginal disc lysates from wild-type, Mcr
\(^{−}\) and Mcr\(^{−}\) mutant animals that is substantially reduced in lysates derived from Mcr\(^{EYW\732}\) and Df(2L)Exel7034 late embryos (supplementary material Fig. S1A). On tissues, the antiserum recognizes a protein strongly expressed in wild-type and Mcr\(^{−}\) stage 16 embryos that is slightly reduced in Mcr\(^{−}\) mutant embryos, strongly reduced in Mcr\(^{EYW\732}\) mutant embryos and nearly absent in stage 16 Df(2L)Exel7034 mutant embryos (supplementary material Fig. S1B-F). Mcr protein in Mcr\(^{−}\) stage 16 embryos localizes to the lateral membrane, but is not enriched in the region of the SJ (supplementary material Fig. S1C). Taken together, these results indicate that the antiserum is specific for Mcr.

In wild-type embryos, Mcr protein is expressed in ectodermally derived epithelia, including the epidermis, salivary glands, trachea and fore- and hindgut, where it is associated with the plasma membrane (Fig. 3B-E). We first detect membrane-associated Mcr in some stage 9 embryos (data not shown), with strong expression apparent by stage 11 (Fig. 3B). At stage 13, Mcr is broadly localized along the basolateral membrane (Fig. 3C), and by stage 14 is enriched at the apical lateral membrane of the disc proper cells, which can be seen in deeper sections (G) where lateral membranes lie adjacent to the folds in the epithelium (arrow), and by rendering an xz transverse section from a confocal z-series (H). A higher magnification view (outset in H) shows that Cor localization extends more basally than Mcr (arrows), and that Mcr is also expressed on the apical surface in a domain independent of Cor (arrowhead; note that staining in the peripodial epithelium can be seen above this line). (I) Confocal optical section of a He-GAL4, UAS-GFP hemocyte stained with antibodies against Mcr (red) and GFP (green) and with DAPI (blue). Mcr is expressed in larval hemocytes, but is largely found inside the cell. (J) In the ovary, Mcr is most strongly expressed in stage 1 of the germarium (arrow) and in polar follicle cells (arrowhead), where it colocalizes with Fas3. Mcr is also expressed at lower levels in the follicle cells and at the membrane in the germ cells. Scale bars: 20 μm.
In addition, when we incubate wing imaginal discs with antibodies against Mcr prior to fixation, we observe an apical localization of Mcr in peripodial epithelial and disc proper cells, indicating that this pool of Mcr is expressed on the cell surface and does not represent an endosomal compartment (supplementary material Fig. S2D,E). In larval hemocytes of healthy wild-type third instar larvae, Mcr is primarily found inside the cell, presumably poised to be delivered to the membrane (Fig. 3I). Mcr does not appear to be expressed in embryonic hemocytes (data not shown).

In the ovary, Mcr is expressed in the gerarium, the ovarian follicle cells and in germ cells. In the gerarium, Mcr shows the highest level of expression in region 1 (Fig. 3J). Mcr is also strongly expressed in the polar follicle cells, where it colocalizes with Fasciclin 3 (Fas3) (Fig. 3J). Mcr expression remains strong in the border cells as they migrate through the nurse cells (data not shown). Finally, Mcr is expressed at low levels in the germ cells.

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The SJ localization of Mcr depends on other core SJ proteins
Proteins involved in SJ biogenesis typically display an interdependence in which the loss of any SJ protein results in the mislocalization of other SJ proteins. We therefore examined the localization of Mcr in stage 16 embryonic hindguts and salivary glands from animals with mutations in several SJ genes and compared the localization to that of Cor as a readout of SJ organization (Fig. 4). Mcr is mislocalized in the hindguts of every mutation we examined (Fig. 4B-E). In general, the mislocalization of Mcr is coincident with a mislocalization of Cor, although when the proteins were not equally mislocalized then Mcr was more likely to be enriched at the SJ than Cor (e.g. Fig. 4B,D). We also noted a wider variation in the mislocalization of Mcr in Nrx-IV4304 mutant hindguts than in other SJ mutants, with some mutant animals showing nearly completely wild-type Mcr localization, whereas others showed completely mislocalized Mcr (Fig. 4D; data not shown).
Mcr is required cell-autonomously for SJ organization

Given the observation that Mcr is secreted from S2 cells (Stroschein-Stevenson et al., 2006), we sought to determine whether Mcr is required cell-autonomously for SJ organization. We expressed Mcr-RNAi in posterior cells in each segment of the embryonic epidermis using en-gal4 (en)-GAL4 and examined stage 16 embryos for Mcr, Cor and En expression (Fig. 5A,B; supplementary material Fig. S3A). Mcr protein was strongly reduced specifically in the En+ cells (supplementary material Fig. S3A). Mcr protein was strongly reduced in the posterior cells, although the majority of the remaining protein appears to be membrane associated (arrow in A′, compared with Cor localization in A″). Note that Cor is not as enriched in the apical regions of Mcr-RNAi cells, indicating a disorganization of the SJ junction (B). (C-E) Confocal optical sections and and rendering from a confocal z-series from a late third instar UAS-Dcr-2; ap-GAL4/UAS-Mcr-RNAi wing imaginal disc stained with antibodies against Mcr (red, and in C-E′) and Cor (green, and in C″-E″). Dorsal is to the top in C and D and to the left in E, and the dorsal-ventral boundary is indicated by arrows in C. D is a higher magnification of the boxed region in C. Mcr is strongly reduced in the dorsal compartment of Mcr-RNAi wing discs, but the residual Mcr appears to align with Cor at the boundary between adjacent cells (arrows in D) and is apically enriched in the lateral membrane (arrows in E). Wild-type cells at the dorsal-ventral boundary have reduced Mcr expression along the membrane in contact with Mcr-RNAi cells (red arrowheads in D″). Scale bars: 20 μm.

Fig. 5. Mcr is required cell-autonomously for SJ organization in embryonic epithelia and larval wing imaginal discs. (A,B) Confocal optical sections (A) and and rendering from a confocal z-series (B) of the epidermis of a stage 16 UAS-Dcr-2; en-gal4/UAS-Mcr-RNAi embryo stained with antibodies against Mcr (red, and in A,B) and Cor (green, and in A″,B″). The zx rendering is along the white line in A. Mcr expression is strongly reduced in the posterior cells, although the majority of the remaining protein appears to be membrane associated (arrow in A′, compared with Cor localization in A″). Note that Cor is not as enriched in the apical regions of Mcr-RNAi cells, indicating a disorganization of the SJ junction (B). (C-E) Confocal optical sections and and rendering from a confocal z-series from a late third instar UAS-Dcr-2; ap-GAL4/UAS-Mcr-RNAi wing imaginal disc stained with antibodies against Mcr (red, and in C-E′) and Cor (green, and in C″-E″). Dorsal is to the top in C and D and to the left in E, and the dorsal-ventral boundary is indicated by arrows in C. D is a higher magnification of the boxed region in C. Mcr is strongly reduced in the dorsal compartment of Mcr-RNAi wing discs, but the residual Mcr appears to align with Cor at the boundary between adjacent cells (arrows in D) and is apically enriched in the lateral membrane (arrows in E). Wild-type cells at the dorsal-ventral boundary have reduced Mcr expression along the membrane in contact with Mcr-RNAi cells (red arrowheads in D″). Scale bars: 20 μm.

Mcr and Nrg show a unique interdependence for correct SJ localization

While conducting experiments to examine Mcr and Cor localization in imaginal discs expressing SJ RNAi transgenes in the dorsal wing compartment, we noticed that UAS-Dcr-2; ap>Nrg-RNAi had a

shown). Interestingly, Mcr is not only mislocalized along the basolateral domain in Tsg2 mutant embryos, but is also strongly enriched on the apical membrane, whereas Cor is only mislocalized along the basolateral domain (Fig. 4E). We observed that Mcr and Cor are similarly mislocalized in the salivary glands of all these mutant animals; however, the degree of mislocalization is often less extreme than in hindguts (compare 4G with 4D).

We next performed FRAP analysis to examine the mobility of the core SJ protein Nrx-IV in the epidermis of stage 15 McrEY07421 mutant animals. Similar to mutations in other genes encoding core SJ components (Oshima and Fehon, 2011), stage 15 McrEY07421 embryos displayed rapid recovery of Nrx-IV-GFP after photobleaching in the epidermis [compare Fig. 4H with figure 7C in Oshima and Fehon (Oshima and Fehon, 2011)]. Given the strong localization of Mcr at SJs in wild-type tissues, the mislocalization of Mcr in animals with mutations in other SJ genes and the effect of Mcr loss on Nrx-IV mobility, we conclude that Mcr is a core component of epithelial SJs.
nearly identical phenotype to that observed in UAS-Dcr-2; ap>McR-RNAi (Fig. 6A-C, compare with Fig. 5C-E). Specifically, Mcr is strongly reduced and Cor loses its apical enrichment in dorsal cells expressing Nrg-RNAi. Interestingly, the loss of Mcr in Nrg-RNAi cells included both the SJ-associated Mcr as well as that expressed on the apical plasma membrane (Fig. 6C). The Mcr protein that is expressed in these cells aligned with membranes at the boundary of neighboring cells and was apically enriched, similar to that seen in McrEY07421 cells.

To extend these observations, we examined Mcr localization in embryos mutant for a strong loss-of-function allele of Nrg (Nrg17). There is essentially no Mcr in any ectodermal epithelia cells of stage 16 Nrg17 embryos (Fig. 6D). Examining earlier Nrg mutant embryos revealed that Mcr is expressed and appears to show some membrane localization at stage 11 (Fig. 6E), but by stage 15 has largely disappeared from the lateral membrane and is mainly enriched on the apical surface (Fig. 6F). Finally, we examined Nrg expression and localization in Mcr mutant embryos. Nrg colocalizes with Mcr at the SJ in the salivary glands of stage 16 McrE107421 heterozygous animals (Fig. 6G), but is largely mislocalized to the apical surface in the salivary glands of stage 16 McrE107421 homozygous animals (Fig. 6H).

**DISCUSSION**

**Mcr in SJ organization**

We have identified an essential developmental role for Mcr for the establishment and/or maintenance of epithelial SJs. Five pieces of evidence indicate that Mcr is a core component of SJs. First, Mcr localizes to the SJ in embryonic and imaginal epithelia, where its pattern of localization during embryonic development mirrors that of other core SJ proteins (Fig. 5). Second, loss-of-function mutations in Mcr (including RNAi) disrupt the organization of SJs, as demonstrated by the mislocalization of other core SJ proteins (Fig. 2). Third, Mcr mutant animals fail to establish an effective paracellular barrier in embryonic tracheae (Fig. 2). Fourth, loss-of-function mutations in other core SJ genes result in the mislocalization of Mcr (Fig. 4). Fifth, FRAP analysis indicated that the mobility of the core SJ protein Nrx-IV in McrE107421 mutant epidermal cells is similar to that observed in mutations in other core SJ genes (Fig. 4). Using a series of RNAi experiments in which we could experimentally create a sharp boundary of Mcr-expressing and non-expressing cells in embryonic and imaginal tissues, we observed that Cor was mislocalized along the lateral membrane by stage 15 (arrows), (G,H) Confocal optical sections of salivary glands from a stage 16 McrE107421 heterozygous (G) and homozygous (H) animal stained with antibodies against Mcr. A higher magnification view of the apical plasma membrane expression of Mcr is also eliminated in the Nrg-RNAi cells (red arrowheads). (D) Confocal optical section of the hindgut from a stage 16 Nrg17 embryo stained with antibodies against Mcr (red, and in D′) and Cor (green, and in D″). Whereas Cor is mislocalized along the lateral membrane in the hindgut of the Nrg17 embryo, Mcr is nearly absent from the lateral membrane. (E-F) Confocal optical sections of a stage 11 (E) and stage 15 (F) Nrg17 embryo stained with antibodies against Mcr. A higher magnification view of the apical plasma membrane staining of Mcr is shown in F′. Note that Mcr is expressed and shows some membrane localization in the stage 11 Nrg embryo (arrows in E), but is largely lost from the lateral membrane at the expense of the apical plasma membrane by stage 15 (arrows). (G-H) Confocal optical sections of salivary glands in stage 16 McrE107421 heterozygous (G) and homozygous (H) animal stained with antibodies against Mcr (red) and Nrg (green, and in H′). Mcr and Nrg colocalize at the SJ in the heterozygous animal (arrow in G), whereas Nrg predominantly localizes to the apical surface in the Mcr mutant animal (arrow in H′). Scale bars: 20 μm.
localization (Fig. 6). We also noted that Mcr was enriched apically in Tsf2 mutant embryos, whereas Cor was only mislocalized along the lateral domain (Fig. 4), raising the possibility that Mcr, Nrg and Tsf2 might all work together to ensure proper localization of Mcr and Nrg. Further experiments are necessary to understand how these genes interact for correct distribution of each protein to the SJ.

These studies indicate that the SJ might not be as interdependent as previously thought, with every protein completely dependent upon each other for proper localization. Rather, the SJ might be composed of subcomplexes that may show strong interdependence among their members but less dependence on other subcomplexes. In this regard, we noted situations in which Cor was more strongly mislocalized than Mcr (Fig. 4), indicating that they might be in distinct subcomplexes. Similar suggestions about the organization of SJ have been made in the past (Nelson et al., 2010).

Mature SJs are composed of more than a dozen membrane and cytoplasmic proteins that appear to form a highly stable and crosslinked structure in the plane of the membrane. FRAP analyses by Oshima and Fehon (Oshima and Fehon, 2011) suggested that stable SJs require interactions between cells and not only within the plane of the membrane. Consistent with this idea, Genova and Fehon (Genova and Fehon, 2003) reported that Nrx-IV is strongly reduced in wild-type cells at the membrane in contact with cor mutant cells in wing imaginal disc clones. Here, we observed that Mcr expression was substantially reduced in many wild-type cells just at the membrane in contact with Mcr-RNAi cells (Fig. 5), suggesting that the disrupted SJ in the Mcr-RNAi cells had a non-cell-autonomous effect on adjacent wild-type cells. Altogether, these results indicate that stable SJs require intercellular interactions between SJ components. We propose that Mcr may function to organize the extracellular components in the SJ. Four lines of evidence motivate this speculation. First, the plasma membrane in wild-type cells at the boundary between Mcr-expressing and non-expressing cells often has substantially reduced Mcr expression (Fig. 5). Second, Mcr protein persists in Mcr-RNAi cells just at certain boundaries between two cells even as Cor is dispersed, raising the possibility that some form of intact SJ complex or subcomplex has been retained at this cell-cell contact (Fig. 5). Third, electron-dense septae are completely missing in Mcr mutant epithelia (S. Luschning, personal communication). Finally, the serum-soluble form of α-2-macroglobulins in invertebrates is a homodimer (Quigley and Armstrong, 1994), raising the possibility of an interaction between Mcr monomers expressed on opposing cells.

**Mcr in innate immunity**

Protein sequence analysis places Mcr in the TEP family. In vertebrates, TEPs include complement proteins that serve innate immune functions (Medzhitov and Janeway, 2002) as well as vertebrates, TEPs include complement proteins that serve innate immune role for Mcr. Consistent with this idea, primary role in the surveillance of pathogens in the hemocoel, which circulate through the hemolymph, recognizing engulfing invading microorganisms, and secrete anti-microbial peptides to further protect the animal (reviewed by Williams, 2007). When they encounter a foreign invader that is too large to engulf, for example a parasitic wasp egg, they initiate an encapsulation program to protect the larva. Encapsulation involves the coordinated assembly of adherent hemocytes into a polarized epithelium around the invading organism, followed by degranolation of crystal cells into the encapsulated compartment (Russo et al., 1996). The encapsulation epithelium expresses SJ proteins including Nrg and Cor (Williams et al., 2005; Williams, 2009) and has ultrastructural characteristics of an intact SJ (Russo et al., 1996). Although it has not been demonstrated to be physiologically tight, the ultrastructure suggests that it provides a paracellular barrier to the encapsulating epithelium, presumably to protect the larval internal organs from collateral damage from destructive agents secreted onto the invading organism. Thus, larval hemocytes can be thought of as ‘pro-epithelial’ cells, capable of a mesenchymal-to-epithelial transition. Ultimately, this pro-epithelial function of hemocytes is similar to that of the ectodermally derived epithelia of the embryo in physiologically partitioning the animal from threats in its environment, highlighting an interesting evolutionary connection between epithelial biology and innate immunity.

**Materials and methods**

**Drosophila strains**

Mcr is an EMS-induced mutation on the E(br)153 chromosome reported in Ward et al. (Ward et al., 2003). We recombined PBac(PB)CG43320 into the Mcr′ chromosome in order to remove the linked uif allele (Zhang and Ward, 2009). P[epoxy2]T(3;3)Ets275 (hereafter McrT(3;3)Ets275) was obtained from the Bloomington Drosophila Stock Center (BDSC), Bloomington, IN, USA. Precise and imprecise excisions were obtained from Mcr′ T(3;3)Ets275 by crossing to TM3, sc Ser 2-3/Df(3R)C7, ry, Mmp20A (an excision allele that leaves 25 nucleotides of the 3′ end of the P-element and 26 nucleotides that are an incomplete duplication of the Mcr sequence. The entire coding sequence of Mcr is intact, as is the upstream gene basigin. UAS-Mcr-RNAi (v100197) and UAS-Nrg-RNAi (v107991) strains were obtained from the Vienna Drosophila RNAi Center (VDRC), Vienna, Austria (Dietz et al., 2007). The Nrg-GFP FlyTrap line G00305 was obtained from the FlyTrap consortium (Yale University School of Medicine, New Haven, CT, USA) (Morin et al., 2001). fkh-GAL4, UAS-GFP (Wang et al., 2008) was obtained from Arash Bashirullah (University of Wisconsin, Madison, WI, USA). Df(2L)Exel7034, breathless (btl)-Gal4, apertus (ap)-Gal4, daughterless (da)-Gal4, engrailed (en)-Gal4, He-Gal4, actin (act)-Gal4 and UAS-Dcr-2 (on the X chromosome) were obtained from the BDSC, as were the SJ mutations crokG0657 (Nilton et al., 2010), kune1309 (Nelson et al., 2010), Nrg7D (Paul et al., 2003), Nrx-J1759 (Baumgartner et al., 1996), nrv2c13cl1649 (Buszczak et al., 2007), pck13281 (Behr et al., 2003), sinuemo (Wu et al., 2004) and Tsf2 (Triková et al., 2010). ContG056 (Faivre-Sarrailh et al., 2004) was obtained from Manzor Bhat (University of North Carolina, Chapel Hill, NC, USA). Mcr′ , Mcr107527, Mcr201, nrv2c13cl1649, actin-Gal4, ap-Gal4, btl-Gal4, en-Gal4 and Df(2L)Exel7034 were balanced with CyO, P[w], Df(3R)SBEF, TM6B, P[w], Df(3L)EF to allow for unambiguous identification of embryos. (Le et al., 2006). Tsf2, cro, kune, pck and sinu mutant embryos were identified by convoluted tracheae and mislocalization of Cor in hindguts. Nrg7D was identified by absence of immunostaining by Nrg-specific antibodies. Lethal phase and phenotypic analysis of the resulting dead Mcr mutant embryos was performed as described (Wang and Ward, 2010).
Generation of anti-Mcr and anti-Uif antibodies

We amplified nucleotides 1101 to 2091 of LD23292 (amino acids 321-650 of Mcr) by PCR with primers containing 5′ NdeI and 3′ XhoI linkers, and cloned them into NdeI/XhoI-cut pDZ1 plasmid (Estrada et al., 2009). The plasmid was transfected into E. coli BL21 (DE3) cells, from which the protein was overexpressed. The His-tagged Mcr protein was solubilized in binding buffer (20 mM Tris-HCl pH 8.0, 500 mM NaCl, 5 mM imidazole) with 5 M urea, and purified through Ni²⁺ affinity chromatography. Purified protein was used for antibody generation in guinea pigs and rats at the Pocono Rabbit Farm and Laboratory (PRF&L, Canadensis, PA, USA). An Uniflatable cytoplasmic domain-GST fusion protein was generated by PCR amplification of a uif cDNA isolated from leg imaginal discs and cloned into a pGEX2TK vector (GE Healthcare Bio-Sciences). The uif fragment encodes amino acids 3442-3557 of Uif. The fusion protein was overexpressed in E. coli BL21 (DE3) cells, and purified on glutathione sepharose 4 flow fast (GE Healthcare) according to standard procedures (Rebay and Fehon, 2000). Purified protein was used for antibody generation in mice at PRF&L.

Immunostaining, immunoblotting, dye exclusion experiments, FRAP analysis and northern blot analysis

Embryos, hemoocytes and imaginal discs were fixed and processed for antibody staining as described (Fehon et al., 1991). Embryonic staging was determined by gut morphology. For the experiment presented in supplementary material Fig. S2D, we dissected wing imaginal discs in PBS, tore a hole in the peripodial epithelium with forceps and incubated them in PBS plus primary antibody for 20 minutes prior to fixation. The following primary antibodies were used at the given dilutions for immunostaining: guinea pig anti-Mcr (described above) 1:400, mouse anti-Cor (clones C556.9 and C615.16 from the Developmental Studies Hybridoma Bank (DSHB) at the University of Iowa, Iowa City, IA, USA) 1:50, guinea pig anti-Cor 1:2000, mouse anti-Crb (clone C4q concentrate, DSHB) 1:100, mouse anti-Fasciclin 3 (clone 7G10, DSHB) 1:300, rat anti-a-Catenin (clone Dc1t1, DSHB) 1:10, mouse anti-Nrg [clone 1B7 (Bieber et al., 1989), gift from Nipam Patel, University of California, Berkeley, Berkeley, CA, USA] 1:100, rabbit anti-GFP (Clontech, 632677) 1:1000, and mouse anti-Uif (described above) 1:400. Rhodamine-labeled wheat germ agglutinin ( Molecular Probes/Life Technologies) was used at 1:400. Secondary antibodies (Jackson ImmunolResearchLaboratories) were used at 1:300. Confocal images were acquired on an Olympus FV1000 confocal microscope equipped with Fluoview software or a Zeiss LSM510 Meta laser-scanning confocal microscope. Photomicrographs were cropped and rotated, and xz renderings of confocal z-series stacks were performed in ImageJ (Schneider et al., 2012). Figures were compiled in Adobe Illustrator (version CS6).

Polyacrylamide gel electrophoresis, immunoblotting and chemiluminescent detection were performed as described (Zhang and Ward, 2003). The lateral mobility of cell adhesion molecule L1.

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Competing interests

The authors declare no competing financial interests.

Author contributions


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Supplementary material

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References


