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

Sonia Hall1, Courtney Bone1,⁎, Kenzi Oshima2,⁎, Liang Zhang1,⁎, Molly McGraw1, Bethany Lucas1,⁎, Richard G. Fehon2 and Robert E. Ward, IV1,⁎⁎

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 metazoa. 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 epithelia 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 (Tfs2, also known as Melanotransferrin) (Tiklová 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 (Tiklová 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 (Tiklová 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
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 (bv’r) (hereafter referred to as Mcr1) (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 (aminolic acids 1726 and 1745, according to TMHMM) (Krogh et al., 2001). Mutations in any core SJ gene increases the mobility of other SJ proteins at stage 14, further demonstrating the highly stable and interdependent nature of the SJ complex.

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 functions related to innate immunity (Medzhitov and Janeway, 2002). There are six TEPs encoded in the Drosophila genome, five of which, including Mcr, are expressed in larval hemocytes (Bou Aoun et al., 2011), suggesting a role in innate immunity. Our 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.

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 at stage 14, further demonstrating the highly stable and interdependent nature of the SJ complex.

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 (bvr) (hereafter referred to as Mcr1) (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 (amino acids 1726 and 1745, according to TMHMM) (Krogh et al., 2001). Mutations in any core SJ gene increases the mobility of other SJ proteins at stage 14, further demonstrating the highly stable and interdependent nature of the SJ complex.

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 functions related to innate immunity (Medzhitov and Janeway, 2002). There are six TEPs encoded in the Drosophila genome, five of which, including Mcr, are expressed in larval hemocytes (Bou Aoun et al., 2011), suggesting a role in innate immunity. Our 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.
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 development.
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<sup>1</sup> and Mcr<sup>Pex3</sup> mutant animals that is substantially reduced in lysates derived from Mcr<sup>EY07421</sup> and Df(2L)Exel7034 late embryos (supplementary material Fig. S1A). On tissues, the antisera recognizes a protein strongly expressed in wild-type and Mcr<sup>1</sup> stage 16 embryos that is slightly reduced in Mcr<sup>Pex3</sup> mutant embryos, strongly reduced in Mcr<sup>EY07421</sup> mutant embryos and nearly absent in stage 16 Df(2L)Exel7034 mutant embryos (supplementary material Fig. S1B-F). Mcr protein in Mcr<sup>1</sup> 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 antisera 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).

Mcr colocalizes with Cor in the apical region of the 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).

(Fig. 3F-I) consistent with the RNA expression patterns reported by Bou Aoun et al. (Bou Aoun et al., 2011). In imaginal discs, Mcr localizes along the apical lateral membrane in disc proper cells and in the peripodial epithelium (Fig. 3F-H). Although the majority of Mcr colocalizes with Cor in imaginal discs, we note that Cor extends further basally than Mcr in these cells and that Mcr is localized in an apical domain independently of Cor (outset in Fig. 3H). This apical Mcr domain is found in both imaginal discs and embryonic epithelia and appears to be on the apical surface since it does not colocalize with adherens junction proteins including α-Catenin and Armadillo, but does colocalize with the apical plasma membrane protein Uninflatable (Zhang and Ward, 2011)
(supplementary material Fig. S2A-C). 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 germarium, the ovarian follicle cells and in germ cells. In the germarium, 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.

**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-IV^G4304^ 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 engrailed (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. S3), whereas Cor was expressed at wild-type levels. Confocal z-sectioning revealed that Cor extended more basally along the lateral membranes in Mcr-RNAi cells and was less enriched apically (Fig. 5B), suggesting a disruption of SJ organization in these cells.

We next expressed Mcr-RNAi in the dorsal compartment of the wing imaginal disc using apterous (ap)-GAL4. Mcr protein is strongly reduced specifically in the cells expressing Mcr-RNAi (supplementary material Fig. S3B), whereas Cor is expressed at normal levels but is not enriched in the region of the SJ (Fig. 5E). The residual Mcr protein found in the Mcr-RNAi cells is not uniformly localized around the cell, but is instead enriched along the border between a cell and one or two of its neighbors (Fig. 5D). These persistent Mcr-containing clusters are also enriched apically in the cell, typically in a region that would correspond to the SJ.

To examine imaginal disc SJs functionally we injected 10 kDa Rhodamine-dextran into the hemocoel of late third instar w1118 and ap>Mcr-RNAi animals and examined wing imaginal discs for the presence of labeled dextran. The lumen between the peripodial epithelium and the disc proper rapidly filled with dye in the ap>Mcr-RNAi discs, indicating a functional disruption of the SJ in these cells, whereas little or no dye infiltrated the wild-type discs (supplementary material Fig. S3C,D). Taken together, these results indicate that Mcr is required cell-autonomously for SJ organization, and that Mcr is required for the SJ paracellular barrier in imaginal discs.

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
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 Mcr-RNAi 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).

The results presented here also highlight a unique interdependence between Mcr and Nrg for proper SJ localization. Mcr is localized both at the SJ and on the apical membrane (supplementary material Fig. S2), and mutations in Nrg alter the relative distribution of Mcr to these locations. Specifically, Mcr can be found at the lateral membrane in stage 11 Nrg mutant embryos, but is predominantly apically localized in the epithelia of animals through stage 15, after which it largely disappears (Fig. 6). We observed a similar alteration of Nrg distribution in Mcr mutant animals, in which Nrg is predominantly at the apical domain at the expense of its SJ 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 in all Mcr mutant cells (Fig. 5), indicating that Mcr functions cell-autonomously in SJ organization.

The results presented here also highlight a unique interdependence between Mcr and Nrg for proper SJ localization. Mcr is localized both at the SJ and on the apical membrane (supplementary material Fig. S2), and mutations in Nrg alter the relative distribution of Mcr to these locations. Specifically, Mcr can be found at the lateral membrane in stage 11 Nrg mutant embryos, but is predominantly apically localized in the epithelia of animals through stage 15, after which it largely disappears (Fig. 6). We observed a similar alteration of Nrg distribution in Mcr mutant animals, in which Nrg is predominantly at the apical domain at the expense of its SJ 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).
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 corΔ 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 SJ requires 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 embryos, whereas Mcr expressed on the apical plasma membrane of ectodermal epithelia, whereas Mcr expressed on the apical plasma membrane of ectodermal cells can provide a similar function in those compartments in direct contact with the outside world.

The encapsulation response of larval hemocytes suggests another connection between Mcr and innate immunity. Larval hemocytes 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 degranulation 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(bri)153 chromosome reported in Ward et al. (Ward et al., 2003). We recombined PBac(PB)CG4332260748 onto the Mcr chromosome in order to remove the linked uitΔ allele (Zhang and Ward, 2009). P[EpGal4]3917023 (hereafter Mcr3917023) was obtained from the Bloomington Drosophila Stock Center (BDSC), Bloomington, IN, USA. Precise and imprecise excisions were obtained from Mcr2 by crossing to TM3, ry; Ser2-3/3; Sb SCR7, ry. Mcr3917023 is 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 (Dietzl 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, en-grailed (en)-Gal4, He-Gal4, actin (act)-Gal4 and UAS-Drk-2 (on the X chromosome) were obtained from the BDSC, as were the SJ mutants crokG10557 (Nilton et al., 2010), kune399 (Nelson et al., 2010), Nrg10 (Paul et al., 2003), Nrx-IV4304 (Baumgartner et al., 1996) and balancer chromosomes reported in Williams et al., 2009), nrv2ZCL1649 (Buszczak et al., 2007), pck40081 (Behr et al., 2003), and sinu104 (Wu et al., 2004) and Tsf2 (Tiklová et al., 2010). Conts0654 (Faivre-Sarrailh et al., 2004) was obtained from Manzor Bhat (University of North Carolina, Chapel Hill, NC, USA). Mcr2, Mcr3917023, Mcr3917023, ap-Gal4, actin-Gal4, ap-Gal4, bal-Gal4, en-Gal4 and Df(2L)Exel7034 were balanced with CyO, P[w+]. Dfd-EYFP and Nrx-IV4304 was balanced with TM6B, P[w+]. Dfd-EYFP to allow for unambiguous identification of embryos (Le et al., 2006). Tsf2, crok, kune, pck and sinu mutant embryos were identified by convoluted tracheae and mislocalization of Cor in hindguts. Nrg10 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).

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 α-macroglobulins that function as broad-range protease inhibitors (Armstrong and Quigley, 1999). Interestingly, Mcr contains a serine in place of a cysteine in the conserved thioester motif suggesting a novel function for Mcr. Nevertheless, using an S2 cell assay with an S2 cell assay with GFP-labeled Candida albicans, Stroschein-Stevenson et al. (Stroschein-Stevenson et al., 2006) identified Mcr in an RNAi screen of genes required for the phagocytosis of C. albicans. Although these experiments need to be confirmed in vivo, they suggest an innate immune role for Mcr. Consistent with this idea, Mudiganti et al. (Mudiganti et al., 2010) found that both Tep2 and Mcr were significantly upregulated 5 days after infection with the alphasivirus Sindbis. Perhaps Mcr expressed in hemocytes plays a primary role in the surveillance of pathogens in the hemocoel, which is in contact with the basal surfaces of all ectodermal epithelia.
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 pEukXhol-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 6 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 Uninflatable 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 fast flow (GE Healthcare) according to standard procedures (Rebay and Feohon, 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, hemocytes 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 Cq4 concentrate, DSHB] 1:100, mouse anti-Fasciclin II 1:300, rat anti-a-Catenin (clone Dcat1, 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 ImmunoResearch Laboratories) were used at 1:800. 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 z-stack 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 claudin-like megatrachea is essential for antibody generation in guinea pigs and rats at the Pocono Rabbit Farm and Laboratory (PRF&L), using a 7% polyacrylamide gel for the separation. Immunoblots were washed and chemiluminescent detection were performed as described (Zhang and Ward, 2003).

Acknowledgements

We thank Greg Beitel, Arash Bashirullah, Manzoor Bhat, the Bloomington Drosophila Stock Center and the Vienna Drosophila RNAi Center for fly stocks; Nipam Patel and the Developmental Studies Hybridoma Bank for antibodies; and Brian Ackley for the use of his Olympus FV1000 confocal microscope. We appreciate helpful comments and the sharing of unpublished results from Stefan Luschning.


Supplemental Information

Supplemental Figure Legends

**Supplemental Fig. 1. Specificity of the anti-Mcr antibody.** (A) Western blot of lysates from w¹¹¹⁸ third instar wing imaginal discs, and w¹¹¹⁸, Mcr¹, Mcr⁰, Mcr⁰ and Df(2L)Exel7034 late embryos (17-22 hours after egg laying) probed with antibodies against Mcr and α-Catenin. (B–F) Confocal optical sections showing the hindguts of stage 16 w¹¹¹⁸ (B), and Mcr¹ (C), Mcr⁰ (D), Mcr⁰ (E), and Df(2L)Exel7034 (F) mutant embryos stained with antibodies against Mcr (red and in panels B’–F’) and Cor (green, and in panels B”–F”). In the w¹¹¹⁸ hindgut, Mcr co-localizes with Cor in the region of the SJ (arrow), whereas Mcr is strongly expressed but mislocalized coincidently with Cor in the Mcr¹ mutant hindgut (C). Mcr levels are reduced and Cor is mislocalized in the hypomorphic allele Mcr⁰ (E). Mcr protein is strongly reduced in Mcr⁰ mutant hindguts (D), and is nearly absent in Df(2L)Exel7034 mutant hindguts (F), whereas Cor is mislocalized in both of these embryos. Preimmune serum fails to recognize the ~225 kDa protein on westerns of wild type lysates, and shows no immunoreactivity on wild type embryos or imaginal discs (data not shown). Scale bars = 20μm.

**Supplemental Fig. 2. Mcr’s localization is distinct from the adherens junction, but does include the apical plasma membrane.** (A, B) Confocal optical sections from a stage 16 hindgut (A) and an epithelial fold in a w¹¹¹⁸ third instar wing imaginal disc (B) stained with antibodies against Mcr (red, and in panels A’ and B’) and α-Catenin (green, and in panels A” and B”). In the fold of the wing disc the apical surfaces face each other and the lateral membranes can be readily observed. Note that Mcr and α-Catenin do not overlap in the embryonic hindgut or imaginal disc.
The apical plasma membrane expression of Mcr can be observed in both tissues (arrows). (C) Confocal optical section from an epithelial fold in a w1118 third instar wing imaginal disc stained with antibodies against Mcr (red, and in panel C’) and Uninflatable (green, and in panel C”). Note that Mcr and Uif puncta are intermingled in the deepest part of the fold (representing the apical surface; arrow). (D, E) Confocal optical section and xz rendering from a w1118 third instar wing imaginal disc that has been incubated with antibodies against Mcr (red, and in panel D’) and Cor (green, and in panel D”) prior to fixation and further processing so that only surface exposed epitopes can be stained. In this section parts of both the peripodial epithelium (PE) and the disc proper (DP) can be observed. Note that Mcr is exposed on the apical surface of both the PE and the DP and that the Mcr antibody was able to reach the lateral membrane in the PE during the 20-minute incubation, whereas the cytoplasmic protein Cor was not accessible. Scale bars = 20µm.

**Supplemental Fig. 3.** Control experiments showing the specificity of Mcr-RNAi in the en-GAL4 and ap-GAL4 expression domains, and the functional disruption of the paracellular barrier in ap>Mcr-RNAi discs. (A) Confocal optical section from the epidermis of a stage 16 UAS-Dcr;en-GAL4/Mcr-RNAi embryo stained with antibodies against Mcr (red, and in panel A’) and En (green, and in panel A”). (B) Confocal optical section of the wing imaginal disc from a UAS-Dcr/UAS-GFP;ap-GAL4/Mcr-RNAi animal. Note that in both tissues, Mcr is substantially reduced specifically in the cells expressing Mcr-RNAi. (C, D) Brightfield/fluorescence micrographs of late third instar w1118 (C) and ap>McrRNAi (D) wing imaginal discs from animals that had been injected with 10 kDa rhodamine-labeled dextran prior to dissection. Note that the labeled dextran fills the space between the disc proper and peripodial epithelium and can be seen pooling in the folds of the ap>McrRNAi disc, whereas very little dextran has infiltrated the wild type disc. Scale bars = 20µm.
**Supplemental Table 1**  
**Lethal phase and terminal phenotypic analysis of Mcr mutations**

<table>
<thead>
<tr>
<th>Genotype</th>
<th>% Embryonic</th>
<th>% Faint denticle</th>
<th>% deposits in</th>
<th>% Dorsal closure</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>lethality(a (n)^b)</td>
<td>belts</td>
<td>salivary glands</td>
<td>defect</td>
</tr>
<tr>
<td>(Mcr^d)</td>
<td>100 (290)</td>
<td>99 ± 1</td>
<td>92 ± 3</td>
<td>20 ± 1</td>
</tr>
<tr>
<td>(Mcr^\text{EY07421})</td>
<td>100 ± 1 (304)</td>
<td>100</td>
<td>76 ± 7</td>
<td>0</td>
</tr>
<tr>
<td>(Mcr^\text{Pex3})</td>
<td>90 ± 8 (285)</td>
<td>100</td>
<td>53 ± 11</td>
<td>11 ± 4</td>
</tr>
<tr>
<td>(Mcr^d/Df(2L)\text{Exel7034})</td>
<td>99 ± 1 (361)</td>
<td>97 ± 3</td>
<td>51 ± 10</td>
<td>7 ± 1</td>
</tr>
<tr>
<td>(Mcr^\text{EY07421})</td>
<td>100 (385)</td>
<td>100</td>
<td>96 ± 1</td>
<td>1 ± 1</td>
</tr>
<tr>
<td>(Df(2L)\text{Exel7034})</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(Mcr^\text{Pex3})</td>
<td>100 (273)</td>
<td>100</td>
<td>79 ± 5</td>
<td>4 ± 1</td>
</tr>
<tr>
<td>(Df(2L)\text{Exel7034})</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(Mcr^d/Mcr^\text{EY07421})</td>
<td>100 (288)</td>
<td>99 ± 1</td>
<td>61 ± 6</td>
<td>3 ± 1</td>
</tr>
</tbody>
</table>

\(^a\) Mean ± s.e.m. from 3-5 independent experiments.  
\(^b\) Total number of animals of indicated genotype that were scored.
Supplemental Fig. 2
Supplemental Fig. 3