ABSTRACT
Polarized epithelia play dual 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 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 anastomosing 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-Sarraillh 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 (Ts2, 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
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) 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.

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 (brv) (hereafter referred to as Mcrbrv) (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 (domain 1726 and 1745, according to TMHMM) (Krogh et al., 2001) (Fig. 1A). The Mcrbrv allele results from a CG to TA transition at nucleotide 8,079,766 of GenBank sequence AE014134.5, generating a Ser282 to Leu substitution in the conserved a-2-macroglobulin N-terminal domain (Fig. 1A; supplementary material Table S1). Ubiquitous da-GAL4>UAS-Mcr-RNAi expression of Mcr results in a hemizygous Mcr[brv] embryo (asterisk in D) and the ectopic salivary gland deposition in mutant animals (arrows in C-E). (D-G) Confocal optical sections of stage 17 w1118 (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 Mcrbrv embryo (asterisk in D) and the highly ordered trachea (arrows) in the Mcrbrv 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 tracheae (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<sup>−/−</sup> and Mcr<sup>Pex3</sup> mutant animals that is substantially reduced in lysates derived from Mcr<sup>E(Y)7427</sup> and Df(2L)Exel7034 late embryos (supplementary material Fig. S1A). On tissues, the antiserum recognizes a protein strongly expressed in wild-type and Mcr<sup>−/−</sup> stage 16 embryos that is slightly reduced in Mcr<sup>Pex3</sup> mutant embryos, strongly reduced in Mcr<sup>E(Y)7427</sup> mutant embryos and nearly absent in stage 16 Df(2L)Exel7034 mutant embryos (supplementary material Fig. S1B-F). Mcr protein in Mcr<sup>−/−</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 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 germarium, 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.

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).
shown). Interestingly, Mcr is not only mislocalized along the basolateral domain in T5f2 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 (Fig. 4F; 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 Mcr<sup>EY07421</sup> mutant animals. Similar to mutations in other genes encoding core SJ components (Oshima and Fehon, 2011), stage 15 Mcr<sup>EY07421</sup> embryos displayed rapid recovery of Nrx-IV-GFP after photobleaching in the epidermis (compare Fig. 4H with figure 7C in 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.

**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<sup>−</sup> 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 (Fig. 5E).

To examine imaginal disc SJs functionally we injected 10 kDa Rhodamine-dextran into the hemocoel of late third instar *w<sup>1118</sup>* 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>\textit{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 (Nrg\textsuperscript{17}). There is essentially no Mcr in any ectodermal epithelia cells of stage 16 Nrg\textsuperscript{17} 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 \textit{Mcr\textsuperscript{EY07421}} heterozygous animals (Fig. 6G), but is largely mislocalized to the apical surface in the salivary glands of stage 16 \textit{Mcr\textsuperscript{EY07421}} homozygous animals (Fig. 6H).

**DISCUSSION**

\textit{Mcr} in SJ organization

We have identified an essential developmental role for \textit{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. 3). Second, loss-of-function mutations in \textit{Mcr} (including RNAi) disrupt the organization of SJs, as demonstrated by the mislocalization of other core SJ proteins (Fig. 2). Third, \textit{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 \textit{Mcr} (Fig. 4). Fifth, FRAP analysis indicated that the mobility of the core SJ protein Nrx-IV in \textit{Mcr\textsuperscript{EY07421}} 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 \textit{Mcr} mutant cells (Fig. 5), indicating that Mcr functions cell-autonomously in SJ organization.

The results presented here also highlight a unique interdependence between \textit{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 \textit{Mcr} mutant animals, in which Nrg is predominantly at the apical domain at the expense of its SJ
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
t 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. Luschnig, 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 α-macroglobulins that function as broad-range protease inhibitors (Baumgartner et al., 1996), raising the possibility that 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. Luschnig, 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.

**MATERIALS AND METHODS**

**Drosophila strains**

Mcr is an EMS-induced mutation on the E(br)155 chromosome reported in Ward et al. (Ward et al., 2003). We recombined PBac{PB}/CG43320 to RNAi onto the Mcr chromosome in order to remove the linked uif allele (Zhang and Ward, 2009). P1iEPg2/T(3;1)83832 (hereafter Mcr) was obtained from the Bloomington Drosophila Stock Center (BDSC), Bloomington, IN, USA. Precise and imprecise excisions were obtained from Mcr chromosomes by crossing to Tm3; ey-2 Ser A2-3/Db [SBC 7; Df(2L)1] and carrying Chras-open reading frame (CRF) on the Bloomington stock background. FRAP analyses were performed as previously described (Wang and Ward, 2010). Mcr and Cor mutations were identified by convoluted trachea and mislocalization of Cor in hindguts. Mcr mutants were identified by absence of immunostaining with 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 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 PGE2ZTK 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 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, 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 CS56.9 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 3 (clone 7G10, DSHB) 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 1:300. 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 z-series 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). Using a 7% polyacrylamide gel for the separation. Immunoblots were detected with SuperSignal West Pico Chemiluminescent detection were performed as described (Zhang and Ward, 2003). The S. H., K. O., R. G. F. and R. E. W. designed the experiments. S. H., C. B., K. O., L. Z., S. H., T. M., B. L., R. G. F. and R. E. W. performed the experiments. S. H. and R. E. W. wrote the manuscript.

Funding

The project was supported by a Kansas IDeA Network of Biomedical Research Excellence (K-INBRE) Undergraduate Research Award (to B.L.); the Initiative for Maximizing Student Diversity (IMSD) and Post-Baccalaureate Research Experience (PREP) programs (to M.M.); and National Institutes of Health grants (P20 RR15563, P20GM103418, R01HD047570 to R.E.W.; and GM074063 to R.G.F.). Deposited in PMC for release after 12 months.

Supplementary material

Supplementary material available online at http://dev.biologists.org/lookup/suppl/doi:10.1242/dev.102152/-/DC1

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 Luschnig.

Competing interests

The authors declare no competing financial interests.

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Supplemental Information

Supplemental Figure Legends

Supplemental Fig. 1. Specificity of the anti-Mcr antibody. (A) Western blot of lysates from w^{1118} third instar wing imaginal discs, and w^{1118}, Mcr^{1}, Mcr^{EY07421}, Mcr^{Pex3} 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 w^{1118} (B), and Mcr^{1} (C), Mcr^{EY07421} (D), Mcr^{Pex3} (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 w^{1118} 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 Mcr^{1} mutant hindgut (C). Mcr levels are reduced and Cor is mislocalized in the hypomorphic allele Mcr^{Pex3} (E). Mcr protein is strongly reduced in Mcr^{EY07421} 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 w^{1118} 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 \textit{w}^{118} 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 \textit{w}^{118} 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 \textit{Mcr-RNAi} in the \textit{en-GAL4} and \textit{ap-GAL4} expression domains, and the functional disruption of the paracellular barrier in \textit{ap>Mcr-RNAi} discs. (A) Confocal optical section from the epidermis of a stage 16 \textit{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 \textit{UAS-Dcr/UAS-GFP;ap-GAL4/Mcr-RNAi} animal. Note that in both tissues, Mcr is substantially reduced specifically in the cells expressing \textit{Mcr-RNAi}. (C, D) Brightfield/fluorescence micrographs of late third instar \textit{w}^{118} (C) and \textit{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 \textit{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

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<tr>
<th>Genotype</th>
<th>% Embryonic lethality(^a) ((n))(^b)</th>
<th>% Faint denticle belts</th>
<th>% deposits in salivary glands</th>
<th>% Dorsal closure defect</th>
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<tr>
<td><em>Mcr</em>(^d)</td>
<td>100 (290)</td>
<td>99 ± 1</td>
<td>92 ± 3</td>
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<td><em>Mcr</em>(^{Ey07421})</td>
<td>100 ± 1 (304)</td>
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<td><em>Mcr</em>(^{Pex3})</td>
<td>90 ± 8 (285)</td>
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<td><em>Mcr</em>(^d)/Df(2L)Exel7034</td>
<td>99 ± 1 (361)</td>
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<td><em>Mcr</em>(^{Ey07421})/Df(2L)Exel7034</td>
<td>100 (385)</td>
<td>100</td>
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<td><em>Mcr</em>(^{Pex3})/Df(2L)Exel7034</td>
<td>100 (273)</td>
<td>100</td>
<td>79 ± 5</td>
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<tr>
<td><em>Mcr</em>(^d)/Mcr(^{Ey07421})</td>
<td>100 (288)</td>
<td>99 ± 1</td>
<td>61 ± 6</td>
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\(^a\) Mean ± s.e.m. from 3-5 independent experiments.
\(^b\) Total number of animals of indicated genotype that were scored.
Supplemental Fig. 1

A

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<th>w^{118} ID</th>
<th>w^{118} Emb</th>
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<th>Mcr^{270342}</th>
<th>Mcr^{pex3}</th>
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B

w^{118}

Mcr

C

Mcr^1

D

Mcr^{270342}

E

Mcr^{pex3}

F

Df(2L)Exel7034