RESEARCH ARTICLE

The transmembrane protein Macroglobulin complement-related is essential for septate junction formation and epithelial barrier function in Drosophila

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ABSTRACT

Occluding cell-cell junctions in epithelia form physical barriers that separate different membrane domains, restrict paracellular diffusion and prevent pathogens from spreading across tissues. In invertebrates, these functions are provided by septate junctions (SJs), the functional equivalent of vertebrate tight junctions. How the diverse functions of SJs are integrated and modulated in a multiprotein complex is not clear, and many SJ components are still unknown. Here we report the identification of Macroglobulin complement-related (Mcr), a member of the conserved α-2-macroglobulin (α2M) complement protein family, as a novel SJ-associated protein in Drosophila. Whereas α2M complement proteins are generally known as secreted factors that bind to surfaces of pathogens and target them for phagocytic uptake, Mcr represents an unusual α2M protein with a predicted transmembrane domain. We show that Mcr protein localizes to lateral membranes of epithelial cells, where its distribution overlaps with SJs. Several SJ components are required for the correct localization of Mcr. Conversely, Mcr is required in a cell-autonomous fashion for the correct membrane localization of SJ components, indicating that membrane-bound rather than secreted Mcr isoforms are involved in SJ formation. Finally, we show that loss of Mcr function leads to morphological, ultrastructural and epithelial barrier defects resembling mutants lacking SJ components. Our results, along with previous findings on the role of Mcr in phagocytosis, suggest that Mcr plays dual roles in epithelial barrier formation and innate immunity. Thus, Mcr represents a novel paradigm for investigating functional links between occluding junction formation and pathogen defense mechanisms.

KEY WORDS: Epithelial barrier, Septate junction, Innate immunity, Thioester proteins, Alpha-2-macroglobulin, Complement, CD109, Drosophila melanogaster

INTRODUCTION

Epithelia form tissue barriers that separate functional compartments in organs. Occluding cell-cell junctions, which constrain the flow of solutes across the paracellular space, provide the epithelial diffusion barrier function. In vertebrates, tight junctions (TJs) form a seal between adjacent cells, thus providing a physical barrier that separates different membrane domains, restricts paracellular flux and prevents pathogens from crossing epithelia (reviewed by Shin et al., 2006). In invertebrates, septate junctions (SJs) represent the functional equivalent of vertebrate TJs. SJs and TJs differ in their ultrastructure, subcellular localization and molecular composition. However, both types of occluding junctions contain four-transmembrane-domain proteins of the Claudin family at the core of the barrier-forming complex (Behr et al., 2003; Hemphälä et al., 2003; Paul et al., 2003). In vertebrates, the paranodal junctions between axons and glia cells (axo-glial septate junctions, or AGSJs) resemble the ultrastructure and molecular composition of invertebrate SJs, suggesting that SJs originated early during metazoan evolution and have been functionally conserved (Hortsch and Margolis, 2003; Banerjee et al., 2006). In invertebrates, SJs between glial cells provide the functional equivalent of the vertebrate blood-brain barrier (Carlson et al., 2000). Furthermore, SJs were shown to contribute to the immune barrier in the gut (Bonnay et al., 2013).

Although SJs and AGSJs have been intensely studied, many questions about their molecular components and their mechanism of assembly remain open. SJs are large multiprotein complexes containing cytoplasmic and integral membrane proteins, several of which were identified through genetic screens in Drosophila (reviewed by Wu and Beitel, 2004; Furuse and Tsukita, 2006; Banerjee et al., 2008). Two distinct subtypes of SJs are known: pleated SJs (pSJs) are found in ectodermal organs (epidermis, salivary glands, foregut, hindgut, tracheal system), whereas the ultrastructurally distinct smooth SJs (sSJs) form epithelial barriers in endodermal organs, such as the midgut (Tepass and Hartenstein, 1994). SJs are also essential for limiting the expansion of tracheal tubes during embryogenesis (Behr et al., 2003; Hemphälä et al., 2003; Paul et al., 2003), thus providing a phenotypic readout that enabled the identification of additional SJ components (Beitel and Krasnow, 2000). Interestingly, the barrier-forming and tube size-limiting functions of SJs are genetically separable, suggesting that SJs play multiple roles in epithelial morphogenesis and barrier formation (Paul et al., 2007). SJs are required for the accumulation of the secreted chitin deacetylases Serpentine (Serp) and Vermiform (Verm) in the tracheal lumen, where these proteins are required for limiting tube expansion, presumably through modifying a chitin matrix (Luschnig et al., 2006; Wang et al., 2006). In addition, SJ-associated basolateral polarity proteins of the Scribble (Scrib)/Discs large 1 (Dlg)/Lethal (2) giant larvae (Lgl) complex limit tracheal tube elongation by restricting Crumbs (Crb)-dependent apical membrane expansion (Laprise et al., 2010). Despite these advances, the precise role of SJs in tracheal tube size control is not clear yet. Furthermore, it is not known how the diverse functions of SJs are integrated and modulated in different tissues and under different physiological conditions. Recent genetic and proteomic studies suggest that many SJ-associated proteins remain to be identified.
We identified Macroglobulin complement-related (Mcr) as a new SJ-associated protein. Mcr is a member of the widely conserved thioester protein (TEP) family, which includes α-2-macroglobulin (α2M) and complement proteins as key factors in innate immunity (Nonaka and Yoshizaki, 2004). These proteins contain a highly reactive intrachain thioester bond that mediates covalent binding to pathogen surfaces or to proteases. α2M, the most abundant serum protein in human blood, acts as an antiprotease that inactivates a broad spectrum of proteases. Upon proteolytic cleavage of a ‘bait’ region, α2M undergoes a conformational change and covalently binds the attacking protease to the exposed thioester (Borth, 1992). The α2M-protease complex is subsequently cleared by receptor-mediated endocytosis. Complement factors participate in opsonization of pathogens, targeting them for phagocytosis or lysis. Insect TEPs were first studied in Drosophila melanogaster and Anopheles gambiae and are involved in the response to pathogens and parasites, most notably Plasmodium (Lagacheu et al., 2000; Levashina et al., 2001). Drosophila contains six TEP family members, four of which (Tep1-4) are predicted secreted proteins expressed in hemocytes and thought to play roles in innate immunity (Lagacheu et al., 2000; Bou Aoun et al., 2011). Tep5 appears to be a pseudogene in Drosophila melanogaster (Lagacheu et al., 2001). Mcr (Tep6) is a diverged TEP family member with a mutated thioester motif and a predicted transmembrane domain not found in other TEPs, suggesting that the functions of Mcr are distinct from those of other TEPs. Mcr binds to the surface of Candida albicans cells and targets them for phagocytic uptake by cultured Drosophila S2 cells, thus resembling the opsonizing effect of α2M in vertebrates (Stroschein-Stevenson et al., 2006). However, it is not yet clear whether Mcr plays a similar role in pathogen clearance in vivo.

We report here a new and surprising role of Mcr in SJ-dependent epithelial barrier formation. We show that Mcr protein localizes to the lateral membranes of epithelial cells, where its distribution overlaps with known SJ components. Mcr is required in a cell-autonomous fashion for the correct localization of SJ components, indicating that membrane-bound rather than secreted Mcr isoforms are involved in SJ formation. Finally, Mcr is essential for SJ-dependent tracheal tube size control and epithelial barrier function. Our results, along with previous work (Stroschein-Stevenson et al., 2006), suggest that Mcr plays dual roles in epithelial barrier formation and innate immunity. These findings call attention to a potential link between pathogen defense mechanisms and the structure of occluding cell-cell junctions in epithelia.

RESULTS

A new gene required for tracheal tube length control

In a mutagenesis screen for genes involved in tracheal tube size control we isolated a complementation group with five lethal alleles showing overelongated tortuous tracheae (Fig. 1A,B; supplementary material Fig. S1). We named the locus luftschlange (luf). Four luf alleles (lufD13, lufD26, lufK101, lufK103) were embryonic lethal, whereas one allele (lufH23) showed weaker tracheal phenotypes and was larval lethal. The amorphic allele lufK103 was used for most analyses. The tracheal dorsal trunk (DT) in stage 16 lufK103 embryos was 20% longer than that of controls (DT metameres 6-8; Fig. 1G). This effect was not due to an increase in tracheal cell number, which was unchanged in luf embryos (data not shown). Instead, the apical surfaces of DT cells in luf embryos were axially longer compared with controls, suggesting that abnormal cell shapes caused the overelongated tracheal phenotype in luf embryos (Fig. 1H,I). In addition, luf mutants showed markedly reduced levels of Verm-RFP protein in the tracheal lumen (Fig. 1B; supplementary material Fig. S1), although the total amount of Verm-RFP detectable in embryonic extracts by immunoblot was unchanged (supplementary material Fig. S2). Furthermore, luminal chitin fibrils, which run parallel to the tube axis in the wild type, appeared disordered in luf mutants (Fig. 1E,F). Altogether, the morphological defects in luf embryos were reminiscent of mutants lacking SJ components (Behr et al., 2003; Wu and Beitel, 2004; Wu et al., 2004; Wang et al., 2006).

luf is allelic to the Mcr locus

We mapped the luf locus to the cytological interval 28E1-5E5. Within this interval, a lethal P-element insertion (McrEY07421; Fig. 2A) (Bellen et al., 2004) in the 5′UTR of the Mcr gene failed to complement all five luf alleles. Embryos carrying McrEY07421 in trans
to the luf alleles showed the luf tracheal phenotype (data not shown), indicating that luf is allelic to Mcr. Furthermore, tracheal-specific expression of Mcr dsRNA resulted in overelongated tubes resembling those associated with luf alleles (Fig. 1C,D). Together, these findings indicate that Mcr function is required in tracheal cells for limiting tube length and for luminal accumulation of Verm-RFP.

Mcr encodes a protein of 1760 amino acids with a predicted molecular weight of 203 kDa. It contains an N-terminal signal peptide sequence, a large extracellular part, a transmembrane domain, and a short cytoplasmic domain of 15 amino acids (Fig. 2B). The sequence contains an N-terminal signal peptide, a large extracellular part, a transmembrane domain, and a short cytoplasmic domain of 15 amino acids (Fig. 2B). The extracellular part resembles TEP/α2M family proteins with N- and C-terminal α-macroglobulin domains and a low-density lipoprotein receptor class A (LDLa) motif. The canonical thioester motif is mutated in Mcr, suggesting that Mcr does not function as a protease inhibitor (Stroschein-Stevenson et al., 2006; Bou Aoun et al., 2011). In addition, Mcr contains a predicted C-terminal transmembrane domain not found in other TEPs. The modified thioester motif and the transmembrane domain are conserved in insect Mcr/Tep6 homologs (Blandin and Levashina, 2004; Bou Aoun et al., 2011). Thus, Mcr represents a diverged member of the TEP family.

**EMS-induced Mcr alleles carry point mutations affecting Mcr protein**

McrKO103 and McrK10173 are amorphic alleles (data not shown) and contain premature stop codons predicted to result in truncated proteins of 329 and 1006 amino acids, respectively (Fig. 2A,B). Bands of the expected sizes (35 kDa and 130 kDa) were detectable in extracts from McrKO103 and McrK10173 embryos, respectively (supplementary material Fig S2). The remaining three alleles (McrD13, McrK101 and McrG249) carry missense mutations at residues that are conserved among insect Mcr/Tep6 homologs (Fig. 2B). The hypomorphic allele McrD13 contains a glutamate-to-lysine exchange at position 1274 inside the α-macroglobulin complement component domain. McrK101 contains a cysteine-to-tyrosine exchange at position 1717 N-terminal to the transmembrane domain. McrG249 contains an alanine-to-threonine exchange at position 1674 inside the α-macroglobulin receptor-binding domain. The amounts and electrophoretic mobility of Mcr proteins were unchanged in McrKO103, McrK10173 and McrG249 embryos (supplementary material Fig. S2), suggesting that the missense mutations in these alleles affect Mcr protein function or localization.

**Mcr is expressed in ectodermal epithelial tissues**

We analyzed the expression pattern of Mcr mRNA during embryogenesis by in situ hybridization. Mcr mRNA was expressed in epithelial tissues from embryonic stage 10 [5 hours after egg lay (AEL)] onwards (Fig. 2C-E). Mcr transcripts were first detectable in the hindgut and epidermis and subsequently in the tracheal pits at stage 11 (9 hours AEL). During later embryonic stages, Mcr mRNA was found in the epidermis, tracheal system, foregut, hindgut and salivary glands (Fig. 2F-J), but not in the endodermal midgut. Thus, Mcr is selectively expressed in ectodermal epithelial tissues.

To study the distribution of Mcr protein we used an antiserum raised against a peptide located near the N-terminus after the signal sequence (Stroschein-Stevenson et al., 2006). On immunoblots, a band of ~210 kDa corresponding to full-length Mcr protein was detected throughout embryogenesis (supplementary material Fig. S3). This band was present in syncytial blastoderm embryos (0-1 hour AEL), indicating the presence of maternal Mcr gene products in the egg. Mcr protein levels increased during germ band extension (6 hours AEL) and continued to rise until the end of embryogenesis. As the levels of full-length Mcr protein increased, several bands of lower molecular weight appeared, which might represent degradation products or processed forms of Mcr protein (supplementary material Fig. S3).

**Mcr protein localizes at the lateral plasma membrane of epithelial cells**

Mcr protein was detectable in embryonic epithelia from germ band extension onwards (supplementary material Fig. S3). Mcr protein...
was enriched on lateral cell membranes of salivary gland, epidermis, tracheal and hindgut epithelial cells (Fig. 3). Double labeling with alpha-Spectrin to outline lateral cell boundaries indicated that Mcr levels are highest in the apical third of the lateral membrane (Fig. 3A). As this distribution was reminiscent of SJ components, we analyzed the localization of Mcr relative to SJ proteins. Mcr signals overlapped with the SJ-associated cytoplasmic ERM (Ezrin, Radixin and Moesin) protein Coracle (Cora) (Fehon et al., 1994) in salivary gland, epidermal and tracheal cells (Fig. 3B-D). However, in all cell types Mcr signals extended slightly further apically than Cora signals. In salivary glands, Mcr lined the apical cell surfaces facing the lumen, in addition to its distribution on lateral membranes (Fig. 3B). Mcr protein was also present on lateral cell membranes in larval imaginal discs, as well as in larval and adult trachea, foregut and hindgut, but not in the midgut and Malpighian tubules (data not shown). Interestingly, besides epithelial cells, we found Mcr protein also at the plasma membrane of embryonic germ cells and adult spermatocytes, which do not exhibit epithelial features such as SJs (Fig. 3E; data not shown). Consistent with previous work (Stroschein-Stevenson et al., 2006), we did not detect Mcr protein in cultured S2 cells. However, upon transient transfection of an Mcr cDNA, Mcr protein was detectable at the plasma membrane, as well as in intracellular puncta in S2 cells (Fig. 3F). Surprisingly, although Mcr is expressed in larval hemocytes (Bou Aoun et al., 2011), we were unable to detect Mcr transcripts and Mcr protein in embryonic hemocytes (data not shown). Taken together, Mcr is an unusual TEP that localizes to lateral membranes of ectodermal epithelial cells and to the plasma membrane of germ cells.

We analyzed the subcellular distribution of Mcr protein in the five mutant alleles in epidermis, salivary gland, hindgut, tracheal and germ cells (supplementary material Fig. S4). Mcr signals were abolished in McrK103 embryos and only residual signal was detectable in McrD13 embryos. By contrast, Mcr levels in the hypomorphic allele McrH25 and in the strong allele McrK101 were comparable to those of wild-type embryos. However, in both of these mutants Mcr protein was mislocalized basolaterally in salivary gland and hindgut cells. In McrG249 embryos, membrane localization of Mcr was lost and the protein instead accumulated intracellularly (supplementary material Fig. S4D). These findings suggest that correct membrane localization of Mcr protein, presumably mediated by the predicted transmembrane domain, is essential for Mcr function.

Localization of SJ components depends on Mcr

The Mcr mutant phenotype and the localization of Mcr protein suggested that Mcr is involved in the assembly or maintenance of SJs. Mutations in SJ components lead to mislocalization of other SJ proteins and to breakdown of the entire complex (Ward et al., 1998; Genova and Fehon, 2003). We therefore examined the localization of the cytoplasmic SJ components Cora, Dlg (Woods and Bryant, 1991) and Scrib (Bilder and Perrimon, 2000) and of the membrane proteins Fasciclin III (FasIII) (Snow et al., 1989), Neurexin IV (Nrx-IV) (Baumgartner et al., 1996) and Neuroglian (Nrg) (Genova and Fehon, 2003) in salivary gland, tracheal, epidermal and hindgut cells of McrK103 embryos (Fig. 4; data not shown). The distribution of all SJ components tested was affected in Mcr embryos. The apicolateral accumulation of Cora and FasIII...
was lost and the distribution of these proteins extended along the entire lateral membrane (Fig. 4B,D). Similarly, Dlg, Scrib-GFP and Nrx-IV-GFP were mislocalized basolaterally in Mcr\textsuperscript{K103} embryos, although these proteins were still partially enriched in the apicolateral zone (Fig. 4C,E,F). By contrast, Nrg-YFP was completely lost from lateral membranes of all epithelia in Mcr\textsuperscript{K103} embryos (Fig. 4G). Interestingly, a fraction of Nrg-YFP localizes at the apical membrane in wild-type salivary glands (Fig. 4G’). In Mcr mutants, this apical pool of Nrg-YFP appeared increased at the expense of basolateral Nrg-YFP, suggesting that Nrg-YFP increases in the apically localized pool (arrowheads) of Nrg-YFP increases in Mcr\textsuperscript{K103} mutants. (G’) Low-magnification projection of the salivary gland in G. Scale bars: 5 μm in A-G; 10 μm in G’.

Fig. 4. Mcr is essential for the correct localization of SJ components. (A-G) Confocal sections of salivary glands in fixed (A-D) and living (E-G) stage 15 embryos. In Mcr\textsuperscript{K103} heterozygous control embryos (upper rows), Cora (B), Dlg (C), FasIII (D), Scrib-GFP (E), Nrx-IV-GFP (F) and Nrg-YFP (G) are localized at the level of SJs in the apicolateral membrane. In Mcr\textsuperscript{K103} mutants (lower rows), in which Mcr protein is not detectable (A), the localization of Cora, Dlg, FasIII, Scrib-GFP and Nrx-IV-GFP extends basally and enrichment of these proteins at SJs is reduced or lost. Nrg-YFP is completely absent from lateral membranes in Mcr\textsuperscript{K103} embryos, whereas the apically localized pool (arrowheads) of Nrg-YFP increases in Mcr\textsuperscript{K103} mutants. (G’) Low-magnification projection of the salivary gland in G. Scale bars: 5 μm in A-G; 10 μm in G’.

SJ components are required for the correct localization of Mcr
We examined whether the localization of Mcr protein was also dependent on the presence of SJ components. We analyzed mutations in megatrachea (mega; pickel – FlyBase) (Behr et al., 2003), sinuous (simu) (Wu et al., 2004), Nrx-IV and Nrg (Fig. 6; data not shown). Mutations in these core SJ components cause SJ protein mislocalization, loss of SJ structure and loss of barrier function. Cora was partially mislocalized basolaterally in sinu\textsuperscript{06524} mutant salivary glands and hindguts (Fig. 6B,F’) and uniformly mislocalized along lateral membranes in Nrx-IV\textsuperscript{4304} and Nrg\textsuperscript{903} mutant salivary glands and hindguts (Fig. 6C,G,H). Similarly, the distribution of Mcr extended basolaterally in sinu\textsuperscript{06524} and Nrx-IV\textsuperscript{4304} mutant epithelia. However, Mcr protein remained partially enriched apicolaterally even in cells in which Cora was uniformly mislocalized (Fig. 6F,G), suggesting that Mcr might localize partially independently of Nrx-IV and Sinu. Strikingly, Mcr protein was strongly reduced in all epithelia in Nrg\textsuperscript{903} mutant embryos, and residual Mcr protein localized to intracellular puncta (Fig. 6D,H; data not shown). Similarly, depletion of Nrg-YFP using anti-GFP nanobodies (Cauaius et al., 2011) in Nrg-YFP embryos caused loss of Mcr membrane staining in the nanobody-expressing cells (supplementary material Fig. S5). Thus, Mcr and Nrg mutually depend on each other for recruitment to, or maintenance at, the plasma membrane. Taken together, Mcr is required for the correct localization of SJ components and vice versa.

Mcr is required during SJ assembly
To test whether Mcr mutations affect the assembly or maintenance of SJs, we monitored the distribution of the cytoplasmic SJ protein.
Cora and the integral membrane protein FasIII throughout SJ maturation in wild-type and in McrK103 embryos (Fig. 7). In wild-type hindgut epithelia, Cora starts to localize at apicolateral membranes at stage 14 and subsequently extends basally during SJ maturation (Fig. 7A). By contrast, McrK103 mutants show mislocalized Cora along the entire basolateral membrane from stage 14 (Fig. 7B). Similarly, FasIII was mislocalized from stage 14 in McrK103 embryos (Fig. 7C,D). Consistent with these findings, Mcr remains partially enriched apically even in cells in which Cora is severely mislocalized (note the green apical Mcr signals in F″, G″). In Nrg17 mutants, Mcr signals are dramatically reduced. Residual Mcr is detectable in intracellular puncta. Apical is up in E-H. Scale bars: 10 μm.

Fig. 7. Mcr is required during SJ assembly. (A-D) Confocal sections of embryonic hindguts stained for Cora (A,B) and FasIII (C,D). Apical is up in all panels. Embryonic stage is indicated. In McrK103/+ heterozygous control embryos, Cora (A) and FasIII (C) localize at apicolateral membranes during stage 14 and continue to accumulate apicolaterally during subsequent stages (the basolateral front of accumulation is marked by arrowheads). In McrK103 mutants, Cora (B) and FasIII (D) are not restricted to the apicolateral prospective SJ region at stage 14 but are instead distributed throughout the lateral plasma membrane. This distribution persists during subsequent stages. (E,F) Transmission electron microscopy sections of apicolateral membranes in hindgut cells of stage 17 embryos. Note that the ladder-like septa corresponding to pSJs in the wild type (E) are absent in McrK103 mutants (F). Scale bars: 5 μm in A-D; 200 nm in E,F.

**DISCUSSION**

We describe a new and unexpected function of the complement-related protein Mcr in epithelial barrier formation. Insect TEPs are thought to function as secreted factors in innate immunity, although the functions of individual TEPs are not well understood (Blandin...
and Levashina, 2004; Bou Aoun et al., 2011). We found that Mcr, a member of the diverged Tep6 subfamily, is a membrane-localized protein required for the assembly of SJs. This conclusion is based on three lines of evidence. First, we show that Mcr protein localizes at the lateral membrane of epithelial cells, where its distribution overlaps with that of known SJ components. Second, loss of Mcr function leads to morphological, ultrastructural and epithelial barrier defects resembling those of mutants lacking SJ components. This finding explains why mutations in Mcr, unlike other TEP genes, cause embryonic lethality (Bou Aoun et al., 2011). Third, Mcr is required in a cell-autonomous fashion for the correct assembly of SJ proteins at apicolateral membranes, and, conversely, SJ components are required for the correct membrane localization of Mcr.

Our finding that Mcr protein localizes at the plasma membrane and does not spread away from the producing cells suggests that full-length Mcr is an integral membrane protein, presumably anchored in the membrane by its predicted transmembrane domain. This notion is further substantiated by the recent mass spectrometry-based identification of Mcr along with several membrane-associated SJ proteins as components of the Claudin complex (Jaspers et al., 2012). However, earlier work had shown that Mcr is secreted by S2 cells into the culture medium (Stroschein-Stevenson et al., 2006). We were not able to detect secreted Mcr protein in the embryonic hemocoel or in tubular organ lumina, suggesting that the majority of Mcr protein is stably associated with the plasma membrane. However, secreted Mcr protein species might be present at low concentrations and may be poorly immobilized by chemical fixation, whereas the membrane-bound locally concentrated pool of the protein is readily detected by immunofluorescence. Consistent with this view, localized Mcr signals become visible only after the onset of zygotic Mcr expression, although Mcr protein of presumably maternal origin is detectable by immunoblot throughout embryogenesis. These results and the presence of multiple Mcr-specific bands on immunoblots suggest that Mcr protein is processed into different cleavage products. Proteolytic cleavage may yield Mcr isoforms lacking the C-terminal transmembrane domain. Such Mcr cleavage products may be released from the plasma membrane through proteolytic shedding. Indeed, αM proteins are proteolytically processed by attacking proteases, which are subsequently inhibited through covalent thioester bond formation with the αM protein (Borth, 1992). The protease bait region is expected to render Mcr sensitive to protease cleavage, although attacking proteases are presumably not inactivated due to the mutated thioester motif. Thus, proteolytic processing of Mcr may result in multiple secreted and membrane-bound protein isoforms, which could carry out distinct functions. Secreted Mcr protein was shown to mediate opsonization of C. albicans cells and to promote their uptake by S2 cells in tissue culture (Stroschein-Stevenson et al., 2006). Secreted Mcr isoforms might play a similar role in pathogen clearance by hemocytes in vivo. Conversely, we show that membrane-localized Mcr is involved in SJ assembly and epithelial barrier function. Our finding that Mcr is required autonomously in tracheal cells suggests that integral membrane isoforms, rather than secreted Mcr protein, mediate tracheal tube size control and epithelial barrier function. However, these results do not formally exclude an autocrine requirement of secreted Mcr protein in tracheal cells.

The presence of Mcr in embryonic germ cells prompted us to test whether Mcr plays a role in gonad development. Although we did not detect defects in embryonic germ cell migration and gonad coalescence in zygotic Mcr mutants (data not shown), potential functions of Mcr in these processes might be masked by maternal Mcr gene products in the embryo. However, we found Mcr to be essential in the female germline for oogenesis, thus precluding the analysis of Mcr mutant germline clones.

The dynamics of SJ formation and the order of assembly of SJ components are not well understood. Our results suggest that Mcr plays an early role during SJ assembly at the lateral plasma membrane. Such a role is consistent with our finding that Mcr membrane accumulation precedes the accumulation of Corta at epidermal cell membranes. Mutations in all known SJ components cause mislocalization of all other components of the complex, indicating a high degree of mutual interdependence between SJ proteins (Ward et al., 1998; Oshima and Fehon, 2011). Similarly, we showed that Mcr is required for the correct localization of SJ components, and that Mcr localization depends on core SJ components. Furthermore, we found the immunoglobulin and fibronectin domain-containing transmembrane protein Nrg to be essential for maintaining membrane localization or stability of Mcr protein. Nrg might bind to the extracellular part of Mcr and thereby protect it from proteolytic cleavage. Alternatively, Nrg could be required at the transcriptional or translational level for Mcr expression. However, the abnormal punctate distribution of residual Mcr protein in Nrg mutants suggests that Nrg is required post-translationally, rather than for Mcr mRNA or protein synthesis. Although it is not yet clear whether Mcr and Nrg interact directly, the strict mutual dependency between the two proteins suggests that they act together to mediate an essential early step in SJ assembly. We speculate that Mcr might interact with other SJ-associated proteins, including Nrg, through its extracellular domain, because the small (15 amino acid) cytoplasmic domain of Mcr is not conserved among insects and lacks identifiable protein-binding motifs, such as the PDZ consensus binding sites that are found in several SJ components, including Gliotactin, Nrg, Nrx-
IV and the Claudins. The identification of binding partners of Mcr will be instrumental for understanding the molecular hierarchy of the SJ assembly pathway.

The discovery of a Macroglobulin complement-related protein as a component of SJs was surprising, since α2M proteins have generally been thought of as secreted factors involved in innate immunity, and functions of α2M as epithelial cell surface proteins have to our knowledge not been described thus far. *Anopheles* Tep1, which is the best-studied insect TEP, binds to bacteria and *Plasmodium* cells and targets them for phagocytosis (Levashina et al., 2001; Blandin and Levashina, 2004; Blandin et al., 2008). *Drosophila* Tep2 and Tep3 are required for phagocytosis by S2 cells of *E. coli* and *Staphylococcus aureus*, respectively, and Mcr is specifically required for phagocytosis of *C. albicans* (Stroschein-Stevenson et al., 2006). Our finding that Mcr is a cell surface protein required for epithelial barrier formation has potential implications for the evolution and function of occluding cell-cell junctions. SJs not only serve as a paracellular diffusion barrier, but they also prevent pathogens from crossing epithelial barriers and invading the organism. Mcr as a complement-like protein may therefore be strategically well placed on epithelial cell surfaces to provide a first line of defense against invading pathogens. This might be particularly relevant also for the gonads, where Mcr protein localizes on germ cell membranes. Interestingly, the process of pathogen encapsulation by hemocytes in insects involves the formation of SJ-like structures (Gupta and Han, 1988; Russo et al., 1996). Larger pathogens, such as parasitoid wasp eggs, are recognized by plasmacytomas and lamellocytes, which attach to and spread around the wasp egg, thus separating the pathogen from the hemocoel and enabling melanization of the capsule. This process involves the formation of an epithelial-like layer of multiple plasmacytocytes surrounding the capsule (Russo et al., 1996). Interestingly, the encapsulating plasmacytocytes are connected by SJs, which contain Cora and Nrg (Russo et al., 1996; Williams, 2009). Although this process has not been characterized in detail, it is likely that additional SJ components, possibly including Mcr, are involved in the formation of cell-cell junctions between immune cells. SJ-like structures were also observed between mammalian lymphocytes and macrophages upon encapsulation of pathogens (McIntyre et al., 1976; Siebert, 1979). These findings suggest an intriguing link between innate immunity and the formation of occluding cell-cell junctions. It is tempting to speculate whether mammalian CD109, a GPI-linked cell surface glycoprotein of the α2M family found on a subset of hematopoietic and myoepithelial cells (Hasegawa et al., 2007), might play similar roles to Mcr in SJ-like structures were also observed between mammalian lymphocytes and macrophages upon encapsulation of pathogens (McIntyre et al., 1976; Siebert, 1979). These findings suggest an intriguing link between innate immunity and the formation of occluding cell-cell junctions. It is tempting to speculate whether mammalian CD109, a GPI-linked cell surface glycoprotein of the α2M family found on a subset of hematopoietic and myoepithelial cells (Hasegawa et al., 2007), might play similar roles to Mcr in SJ. In *Drosophila*. Although the function of CD109 is largely unknown, it will be interesting to explore whether membrane-bound α2M proteins mediate functions in epithelial barrier formation and innate immunity in vertebrates and invertebrates.

**MATERIALS AND METHODS**

*Drosophila* strains and genetics

Fly stocks are described in FlyBase unless mentioned otherwise: *w*, *Abd-B-Gal4* 199 (de Navas et al., 2006), *UAS-Gal4* (Shiga et al., 1996), *hh-Gal4*, *UAS-lacZ* (gift from Peter Gallant, University of Würzburg, Germany), *UAS-dicer2* (VDRC 60014), *UAS-Mcr* (VDRC 100197), *UAS-a-Cat-GFP* (Oda and Tsukita, 1999), *UAS-mCherry-NLS* (Caussinus et al., 2008), *UAS-Serp-CBD-GFP* (Luschling et al., 2006), *UAS-smyr-tTomato* (Bloomington 32223), *UAS-NSlmb-hh-GFP* (Caussinus et al., 2011), *Df(2L)Exel7034* (Bloomington 7807), *Mcr* (Bellen et al., 2004), *mega0012* (Bloomington 11471), *Nyc* (Bloomington 5595), *Nyc-II* (Bloomington 4380), *sinu0624* (Bloomington 11690), * Scrib-GFP*, *Nyc-I-V-GFP* (Buzsacz et al., 2007) and *Nyc-YFP* (DGRC 115188). Mcr mutations were induced by ethylmethanesulfonate (EMS) mutagenesis of a *btl-Gal4 UAS-GFP UAS-Verm-RFP* chromosome (Förster et al., 2010) and recombined onto FRT40A chromosomes. The *Jf/Mcr* locus was mapped to 28E1-E5 by non-complementation of *Df(2L)Trf-C6R31* and *Df(2L)Exel7034* and by complementation of *Df(2L)TE29Aa-11*. For tracheal-specific RNAi, UAS-RNAi transgenes and UAS-dicer2 were co-expressed using *btl-Gal4*.

**Molecular biology**

Genomic DNA was extracted from 20 homoygous *Mcr* embryos (identified by the absence of *CyO Dfd-YFP* (Le et al., 2006)) for each *Mcr* allele. The *Mcr* coding sequence, including exon-intron boundaries, was amplified from *Mcr* mutants and from the parental strain. PCR products were sequenced on both strands. UAS-Mcr was generated by amplifying the *Mcr* coding sequence from the *Mcr* full-length cDNA Ld2392 (BDGP) using oligonucleotides (5′-3′, restriction sites are underlined): EcoRI-Mcr-F, ATATGAATTCCGACAAATTAGTGGCAGCCTTG; and NotI-Mcr-R, TTATCGCGCGCCCTGTTGACAGTTGCATCATG. The fragment was inserted between the EcoRI and NotI sites of pUAS-TattB (Bischoff et al., 2007). The construct was integrated into the attP2 site at 68A4 using *φC31* integrase (Bischoff et al., 2007).

**In situ hybridization**

A 937 bp fragment containing the *Mcr* 3′ UTR was amplified from genomic DNA using oligonucleotides containing the T7 RNA polymerase promoter either on the forward or reverse primer. PCR products were used as templates for *in vitro* transcription. Antisense and sense RNA probes were generated using digoxigenin-labeled UTP (Roche) and were detected using alkaline phosphatase-conjugated anti-digoxigenin Fab fragments (1:2000; Roche).

**Immunofluorescence**

Embryos were fixed in 4% formaldehyde in PBS/heptane for 20 minutes and deivellinized by shaking in methanol/heptane or ethanol/heptane (for anti-E-Cad immunostainings). The following antibodies were used: chicken anti-GFP (1:500; Abcam, ab13970), mouse anti-GFP (1:300; Clontech, JLB), rabbit anti-Mcr [1:1000; gift from A. Johnson, UCSF (Stroschein-Stevenson et al., 2006)], guinea pig anti-Cora [1:200 (Lamb et al., 1998)], mouse anti-Dig (1:500; 4F3, DSHB), mouse anti-Fasll1 (1:50; 7G10, DSHB), mouse anti-alpha-Spec (1:50; 3A9, DSHB), guinea pig anti-Uif [1:1000 (Zhang and Ward, 2009)] and rat anti-DE-Cad (1:100; DCAD2, DSHB). Goat secondary antibodies were conjugated with Alexa 405, Alexa 488, Alexa 568 (Molecular Probes) or Cy5 (Jackson Immunoresearch). Chitin was detected using Alexa Fluor-SNAP-tagged chitin-binding domain from *Bacillus circulans* chitinase A1, which was prepared as previously described (Caviglia and Luschnig, 2013).

**Tissue culture**

S2R+ cells were cultured on glass coverslips in Schneider’s medium at 25°C. Cells were transfected with pUAS-TattB-Mcr using Fugene HD reagent (Promega). Forty-eight hours after transfection, cells were fixed with 4% formaldehyde/PBS and stained with anti-Mcr (Stroschein-Stevenson et al., 2006) and Hoechst 33342.

**Light microscopy and image analysis**

Imaging was performed using an Olympus FV1000 confocal microscope with 40×/1.3 NA and 60×/1.35 NA objectives. Images were processed using Huygens Deconvolution (SV1), ImageJ (v1.42), Imapix (v7.3.0; Bitplane) and Adobe Photoshop. For live imaging, embryos were dechorionatated, mounted on glue-coated coverslips and covered with Voltalef 105 oil. The three-dimensional path of the DT lumen and apical cell outlines were traced using Imapix. Lumenal length was measured in living embryos between fusion joints of DT metameres Tr5/6 and Tr8/9. P-values were calculated using Student’s two-tailed, unpaired t-test.

**Electron microscopy**

Dechorionatated stage 17 embryos were high-pressure frozen (Leica EM HPM100) and freeze-substituted (Leica EM AF52) in 1% OsO4 in water-free acetone. Subsequently, embryos were block-stained in 1% uranyl
aceta in water-free acetone for 1 hour at 4°C and embedded in Epon/Araldite (Sigma). Ultra-thin (50-70 nm) transverse sections of posterior abdominal segments were post-stained with lead citrate and imaged in an FEI Tecnai G2 Spirit transmission electron microscope.

**Immunoblotting**

Embryos were homogenized in Laemmli buffer. Equivalents of ten embryos per lane were loaded on 7.5% polyacrylamide SDS-PAGE gels. Proteins were transferred to nitrocellulose membranes by electroblotting. Membranes were probed with rabbit anti-Mcr [1:1000 (Stroschein-Stevenson et al., 2006)], mouse anti-α-Tubulin [1:50,000, Sigma, DM11A] and guinea pig anti-Verm [1:2500 (Wang et al., 2006)].

**Dextran injections**

Rhodamine-labeled 10 kDa dextran (Molecular Probes) was injected as described (Lamb et al., 1998) into wild-type and Mcr-10/10 embryos carrying btl-Gal4 UAS-GFP. Embryos were imaged 20 minutes after injection.

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**Competing interests**

The authors declare no competing financial interests.

**Author contributions**

T.B., D.F. and S.L. designed and performed the experiments, analyzed and interpreted the data, and wrote the paper.

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