Macroglobulin complement-related, *coracle* and *Neuroglian* are required for *Drosophila* egg morphogenesis

By

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Abstract

Morphogenesis, along with cell growth and differentiation are crucial processes that define the body plan of an organism. Morphogenetic movements such as neural tube formation, gastrulation and organogenesis are accomplished by precisely timed and coordinated changes in cell shape and rearrangement. Throughout morphogenesis, epithelial cells maintain their physical connection with each other through intercellular junctions that reside along the lateral membrane of the adjoining cells.

Our lab previously demonstrated that core components of septate junctions (SJs), which are analogous to vertebrate tight junctions in that they provide an important occluding function to the epithelium, are essential for embryonic morphogenesis of *Drosophila melanogaster*. To extend these studies we are investigating the requirement of three core SJ genes *Macroglobulin complement-related (Mcr), coracle (cor)* and *Neuroglian (Nrg)* during morphogenetic events that occur in the *Drosophila* oogenesis. The *Drosophila* egg has been an excellent biological system to investigate aspects of cell and developmental biology because of its simple tissue structure and the number of morphogenetic changes occurring during oogenesis that are similar to those occurring during embryos development.

Based on the immunohistochemistry analysis, we find that *Mcr*, *cor* and *Nrg* are expressed throughout oogenesis. Mcr shows different expression pattern in the germarium region when compared to Cor and Nrg. High expression of Mcr is detected in the germline stem cells including germ line cells, whereas Cor and Nrg are expressed in the somatic follicle cells within the germarium. We then show that SJ proteins are essential for border cell migration as mutant

border cell clusters for *Mcr*, *cor* or *Nrg* display different border cell migration defects including migration delay and border cell cluster disassembly. These phenotypes demonstrate a role for SJ proteins in border cell cluster guidance and cohesion. Knocking down Mcr and Nrg in all the follicle cells early in oogenesis results in middle stages egg chambers that fail to pass mid-oogenesis checkpoint. Moreover, the later stages egg chambers mutant for Mcr, Cor or Nrg were significantly rounded when compared to the elongated wild type eggs. We also demonstrated that *Mcr* is not required for the correct localization of alpha-spectrin, aPKC, and Cor localization proteins in the *Drosophila* egg. Together, these observations indicate a role for SJ proteins in regulating morphogenesis that is independent of the occluding function.

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Chapter I: Introduction

Septate Junctions and their importance in morphogenesis

The generation of a fully sophisticated form of an organism from a single fertilized egg has been of great interest to developmental biologists. After fertilization, embryonic cells divide, differentiate and move in response to signaling cues as the embryo develops. These events are coupled with precisely timed and orchestrated morphogenetic events including changes in cell shape and rearrangements coincide with collective cell movement. Many morphogenetic processes are conserved in developmental events in multicellular organisms, and dysregulation of these processes can lead to developmental defects and embryonic lethality. In a genetic screen done by our lab aimed at identifying new mutations that dominantly enhanced a malformed leg phenotype in the fruit fly, *Drosophila melanogaster,* we identified a mutation in a transmembrane protein that was further characterized to be a core component of the septate junctions (SJs) (Fig. 1A-D) (Hall et al., 2014; Ward et al., 2003).

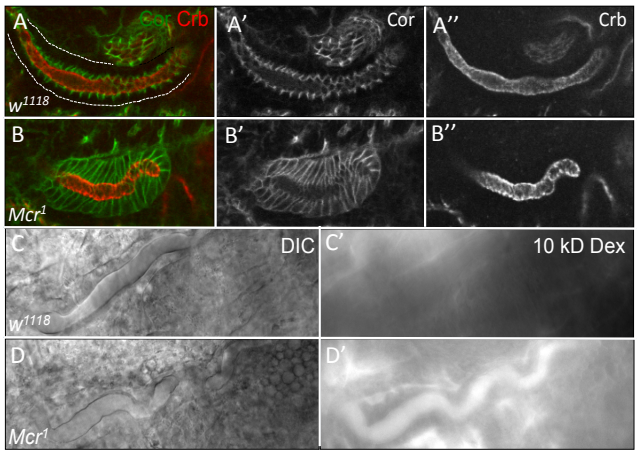

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Figure 1. ***Macroglobulin complement-related(Mcr)*** **is required for SJ biogenesis and formation.** Salivary glands from stage 16 wild type (A) and *Mcr*^{1} homozygous mutant embryo (B) showing that *Mcr* is required for the localization of the core SJ protein, Coracle, but not for apical-basal polarity. (A-B'') Compare Cor and Crumbs localization in wild type and *Mcr*^{1} mutant embryo. (C and D) Differential interference contrast and fluorescence (C' and D') photomicrographs of the trachea dorsal trunk in stage 17 of wild type (C and C') and *Mcr*^{1} homozygous embryos (D-D'). Note the diffusion of the 10-kDa rhodomaine-labeled dextran in the trachea of *Mcr*^{1} mutant embryo.

SJs are functionally homologous to vertebrate tight junctions (TJ) in providing an essential occluding function that seals epithelial cells together and prevent the diffusion of pathogens between adjacent cells. The SJ is found to reside just basal to the adherens junction and is distinguished by the ladder-like septa that span the lateral membrane between adjacent cells (Noirot-timothée et al., 1978). More than twenty proteins have been identified to form the SJ including transmembrane proteins of the Claudin family proteins Kune-Kune, Sinuous and

Megatrachea in flies, cytoplasmic proteins such as Coracle and Varicose, cell adhesion molecules including Neurexin IV, Contactin, Lachesin, Neuroglain, Glioactin and the ***α*** and ***β*** subunits of the Na+/K+ ATPase, and the GPI-linked protein Transferrin 2 (Behr et al., 2003;Nelson et al., 2010;Wu et al., 2004;Baumgartner et al., 1996;Faivre-Sarrilh et al., 2004;LIinagras et al., 2004;Genova and Fehon et al., 2003;Tiklová et al., 2010). A number of studies have shown that core SJ proteins are essential for embryonic survival and development. For example, embryos lacking Macroglobulin complement-related (Mcr) fail to survive embryogenesis with developmental defects including head involution (Hall et al., 2014) (Fig. 2). In addition, null *coracle* (*cor*) animals display dorsal closure defects (Fehon et al., 1994;Ward et al., 2001). Interestingly, dorsal closure and head involution processes occur prior to the formation of the occluding junction, suggesting a second function for these genes in controlling morphogenesis. Despite the extensive studies that have been conducted on SJ genes and their requirement in morphogenesis, the molecular mechanisms underlying the importance of these genes have not been investigated yet. Here, we are using the *Drosophila* ovary as a model system to uncover the role of these proteins in morphogenesis.

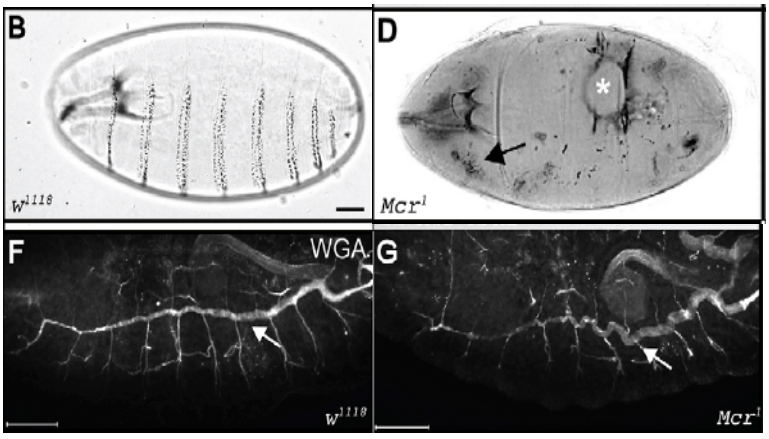

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Figure 2. *Mcr* is essential during embryogenesis. (B-D) cuticle preparations of wild type and of dead Mcr^1 homozygous embryos. Note the dorsal hole (asterisk) (D). (F-G) confocal optical images of wild type (F) and Mcr^1 mutant embryos stained with Wheat germ agglutinin (WGA) showing the long and highly convoluted trachea in *Mcr1* (G). Scale bar 100µm.

The *Drosophila* ovary as a model system for morphogenesis

The *Drosophila* ovary provides a genetically tractable system to study the molecular mechanisms underlying tissue and organ morphogenesis due to its simple structure and well-characterized morphogenetic processes (Horne-Badovinac and Bilder, 2005). The *Drosophila* ovary is comprised of only two type of cells; germ line cells and a surrounding layer of somatic cells, which are known as follicle cells. The follicle cells that encapsulate each egg chamber undergo a series of morphogenetic changes during egg development that promote egg chamber elongation, dorsal appendage and micropyle formation (Horne-Badovinac and Bilder, 2005;Haigo et al., 2011). The *Drosophila* ovary is also particularly amenable to various genetic tools such as the Gal4-UAS system that can be utilized to manipulate gene expression both temporally and spatially.

Each female *Drosophila* has two ovaries containing 16-22 bundles of ovarioles, which, in turn, consist of a series of progressively developing egg chambers that are connected to each other through a filament of cells called stalk cells. The eggs are assembled independently of each other in a well-organized and specialized structure at the anterior tip of the ovariole called the germarium, where the germline and somatic stem cells reside. The newly formed egg chamber is composed of 16 interconnected germline cells, 15 of which are trophic nurse cells while the other one forms the oocyte. Together this is referred to as a cyst. Each cyst is encapsulated by a monolayer of somatic follicle cells, which protects the germline cells from the environment, produces the yolk and secretes the chorion at the end of oogenesis (Mahowald et al, 1972;Horne-Badovinac and Bilder, 2005). Once a stage 1 egg chamber is pinched off from the germarium, it undergoes 14 morphological stages in order to give rise to the mature egg that will eventually be fertilized by a sperm. These stages are distinct from each other in terms of size, shape and many morphological features (King, 1970). Here, we are investigating the requirement of SJ proteins in two morphogenetic events; border cell migration and egg chamber elongation, which are two processes that are similar to those occur during embryo and larvae development.

Border cell migration

Border cell migration is a useful and genetically tractable system to study collective cell migration that has also become an excellent model system to study tumor metastasis and invasion (Montell, 2003). This process occurs during mid-oogenesis (stage8-10) in which a stationary pair of cells, polar cells (PCs), secrete *Unpaired*, a molecule that activates JAK-STAT signaling in the adjoining ~4-8 follicle cells. Upon the activation of this pathway, Slbo, the *Drosophila* homolog of C/EBP transcription factor, is expressed in the neighboring follicle cells and specifies them to become border cells (Montell et al., 1992). Then, border cells delaminate from the epithelium follicle layer and invade the neighboring nurse cells while carrying the polar cells in the center of the cluster. In order for the border cell cluster to move forward, one of the border cells becomes the leader and extends filapodia-like structure towards the oocyte. Border cells will continue to migrate through the nurse cells in response to chemical cues sent from the oocyte that guide the cluster to the posterior pole of the egg chamber (Fig. 3) (Cia et al., 2014;Montell et al., 2012). Mutations in genes that function in cell-cell adhesion, actin cytoskeleton, and extracellular signals that are crucial for the motility behavior of the border cells and lead to abnormal border cell migration (Horne-Badovinac and Bilder, 2005; Montell et al., 2003; Wang et al., 2006; Cia et al., 2014).

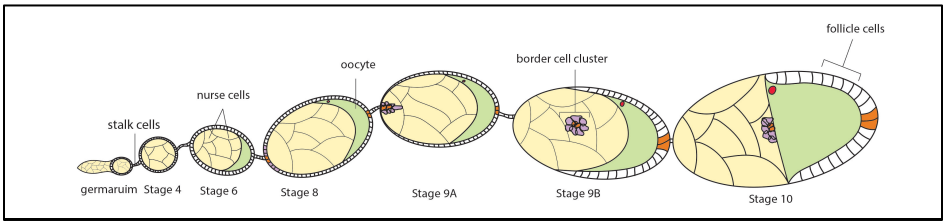

Figure 3. Schematic presentation modified from (Montell et al., 2003) of border cell migration process that occurs in mid-oogenesis of *Drosophila* ovary.

Egg chamber elongation

The *Drosophila* egg chamber undergoes another morphogenetic event during oogenesis that promotes the elongated shape of the egg. The egg chamber transforms its shape from spherical to ellipsoid upon the rotation of the follicular epithelium layer perpendicular to the anterior-posterior axis (Fig. 4). In order for an egg chamber to possess this elongated shape, it has to generate mechanical forces to establish the rotation process. This process occurs in three main steps as reviewed by (Cetera et al., 2015). Briefly, the basal actin bundles begin to align in the germarium as they orient parallel to each other within each cell. After the egg chamber is released from the germarium (Stage 1- 4), the follicle cells over the germline cyst start to rotate

perpendicular to the anterior-posterior axis. This rotation process was shown to be required for maintaining the actin bundles projection and arrangement. In the final step (Stage 5 - 10), secretion of the basement membrane protein Collagen IV polarizes its fibrils towards the leading edges of the follicle cells to where the rotation is projected, and this is important to maintain the same actin orientation as in the germarium. By stage 9, the rotation process ceases and the egg chamber starts to elongate upon oscillating contractions of actomyosin at the basal side of the follicle cells (He et al., 2010). It is worth mentioning that the rotation rate of the egg chamber is slow in between stage 1-5, but it significantly increases by stage 6-9 upon collagen IV secretion (Cetera et al., 2015).

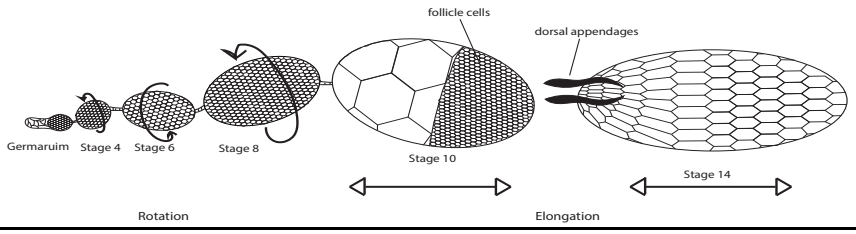

Figure 4. Schematic presentation of egg chamber elongation in the *Drosophila* ovary.

Septate Junction proteins in the *Drosophila* ovaries

SJs were shown to form in the *Drosophila immigrans* ovaries by stage 10B-14, which is during chorion formation, as they possessed the electron-dense continuous and discontinuous septa that span the plasma membrane between adjoining follicle cells (Mahowald et al., 1972). It was also shown by the transmission electron micrograph the presence of SJs in the developing epithelial of the *Drosophila* eggs (Muller, 2000). By the immunohistochemistry analysis, Cor was detected to be expressed in the follicle stem cells, cap cells and mature follicle cells by stage 8 (Bonfini et al., 2015;Gomez et al., 2012). Moreover, *Mcr* expression was observed in distinct regions in the *Drosophila* eggs including the germaruim, polar cells and border cells (Hall et al., 2014). Yet even though SJ proteins are known to be expressed in the *Drosophila* ovary, their role in egg morphogenesis has remained undiscovered. Our study provides an insight into the importance of SJ proteins and their highly correlation and involvement in morphogenesis.

Here we chose to study a select group of core SJ proteins in order to obtain a more thorough understanding of the role of SJ proteins in regulating morphogenesis during oogenesis. These proteins are the transmembrane proteins Mcr and cell adhesion molecule Neuroglian (Nrg) and the cytoplasmic protein Cor. We show that these SJ proteins are required for border cell migration and follicle cell rotation, two morphogenetic events that occur during *Drosophila* egg

development. Closer examination of SJ protein expression in the ovary indicates that Mcr, Cor and Nrg have similar expression profiles in the *Drosophila* egg, except in the germarium region. In addition, we found that knocking down SJ proteins only in the border cells by using RNA interference against Mcr, Cor or Nrg with Slbo-Gal4 driver affects border cell cluster cohesion and migratory direction. In addition, knocking down Mcr, Cor or Nrg in the follicle cells early in oogenesis results in rounded rather than elongated egg chamber. Mosaic analysis using an Mcr^{1} loss of function allele reveals that Mcr is not required for the correct localization of apico-basal polarity proteins alpha-spectrin, aPKC, and Cor. Together, these observations indicate a role of Mcr, Cor and Nrg in morphogenesis in the ovary that appears to be independent of their barrier function in this tissue. In addition, this function does not appear to act by regulating apical basal polarity.

Chapter II: Materials and methods

Drosophila strains

C204-Gal4 (3751), *C355-Gal4* (3750), *GR1-Gal4* (36287), *UAS-GFP* (1521), *UAS-Cor-RNAi* (28933), *UAS-Nrg-RNAi* (38215), *tub-Gal80* (7108) stocks were obtained from the Bloomington *Drosophila* Stock Center (BDSC). We mainly used the GAL4-UAS system to knockdown Mcr, Cor and Nrg, with the exception of FLP-FRT experiment. *hsFLP;FRT40A-UbiGFP* and *Mcr140AFRT/CyoYFP* (Ward et al., 2003). The *UAS-Mcr-RNAi* (v100197) strain was obtained from the Vienna *Drosophila* RNAi Center (VDRC).

Gal80 and mosaic clone experiments

Most of the experimental crosses were raised at 25^{o}C and shifted to 29^{o}C 2-3 days before ovaries were dissected, except for the Gal80 experiment in which *C204-Gal4* and *GR1-Gal4* lines were crossed to *Gal80ts* line and maintained at 25^{o}C. Flies with the appropriate genotype (*Gal80^{ts};C204-Gal4* or *GR1-Gal4*) were collected and crossed to the specific *UAS-SJ-RNAi* lines. The *C204-Gal4* line is expressed throughout *Drosophila* development, so we maintained the previous cross (*Gal80^{ts}*;*C204-Gal4* or *GR1-Gal4/UAS-SJ-RNAi*) at 18^{o}C to prevent Gal4 activity. To activate Gal4 and knockdown SJ proteins, virgin female flies with the appropriate genotype were collected along with males from the same genotype and maintained for 3 or 5 successive days at 29^{o}C. To generate mosaic clones of *Mcr1* homozygous mutant follicular cells, we used the FLP/FRT system. *hsFLP/w*;*FRT40A ubiGFP.nls* flies were crossed to *Mcr^{1}/CyoYFP*. Then, *hsFLP;FRT40,UbiGFP/Mcr^{1}* virgin flies were selected and heat shocked at 37^{o}C twice a day for 3 successive days. Ovaries were dissected after 24 hours of the last heat shock, fixed and stained as described below. For all the experimental crosses, vials were replaced each day throughout the heat shock process with fresh food and yeast to eliminate the lack of nutrient effect on egg production and morphogenesis.

Immunostaining

Ovaries from female flies were dissected in 1X PBS or S2 media at room temperature and fixed in 4% EM-graded paraformaldehyde (Polysciences) in 1X PBS for 20 minutes. Then, ovaries

were washed three times in 1X PBS for one minute each and permeabilized for 30 minutes in 1X PBS + 0.1% Triton + 1% Normal Donkey Serum (NDS). We incubated ovaries overnight at 4^{o}C with the following primary antibodies and concentrations. Guinea pig anti-Mcr was generated by our lab 1:400 (Hall, et al 2014), mouse anti-Coracle against the FERM domain (clone 566.9) and against C-terminal region (clone 615.16) 1:50 were obtained from Developmental Studies Hybridoma Bank (DSHB) at the University of Iowa as well as rat anti-Cadherin, DE- (clone DCAD2, DSHB) 1:30, mouse anti-Fasciclin III (clone 7G10, DSHB), mouse anti-spectrin (clone 3A9, DSHB) 1:10. Rabbit anti-aPKC (1:500, Santa Cruz Biotechnology). Mouse anti-Nrg (clone 1B7, Bieber et al., 1989) is a gift from Nipam Patel, University of California, Berkeley, Berkeley, CA, USA 1:100. Secondary antibodies were obtained from Jackson ImmunoReaseach Laboratories and were used at 1:800. Confocal images were obtained using TIRF spinning disc /Inverted confocal microscopy (Andor camera) and Epifluorescence microscopy in the Microscopy and Analytical imaging facility at the University of Kansas.

Statistical analysis

For the aspect ratio measurement, we measured the width (dorsal to ventral) and length (anterior to posterior tip) of stage 14 egg chambers. Then, the ratio of width:length was calculated for each genotype using Unpaired *t*-test. UAS-SJ-RNAi stage 14 egg chambers were compared to wild type egg chambers. For border cell migration experiment, we quantified control and SJ mutant stage 10 egg chambers from three independent experiments. Stage 10 egg chambers were identified by the intercalation of centripetal cells expressing GFP between the oocyte and the nurse cells and by the size of the oocyte as it occupies half of the egg chamber by stage 10. Then, undamaged stage 10 egg chambers were quantified. The four phenotypes were characterized as follow. Border cell cluster disassembly; one or more detached border cells between the nurse cells while the majority of the border cells reached the oocyte. Migration failure; border cell cluster that is already formed, but failed to migrate through the nurse cells and remained at the anterior tip of stage 10 egg chamber. Migration delay; observed border cell cluster that is in the middle of stage 10 egg chamber or in the first 25% of the anterior part of stage 10 egg. Border cell cluster that passed the oocyte was identified to be localized beyond the oocyte of stage 10 egg chambers. The mean of the percentage of each phenotypic group was then calculated along

with standard deviation and compared to the control (*Slbo,Gal4-UAS-GFP/+*). The statistical analysis was used is two-tailed Unpaired *t*-test for both @ 25^{o}C and @ 29^{o}C experiments.

Chapter III: Results

Examination of the localization of Macroglobulin complement-related, Coracle and Neuroglian proteins throughout oogenesis.

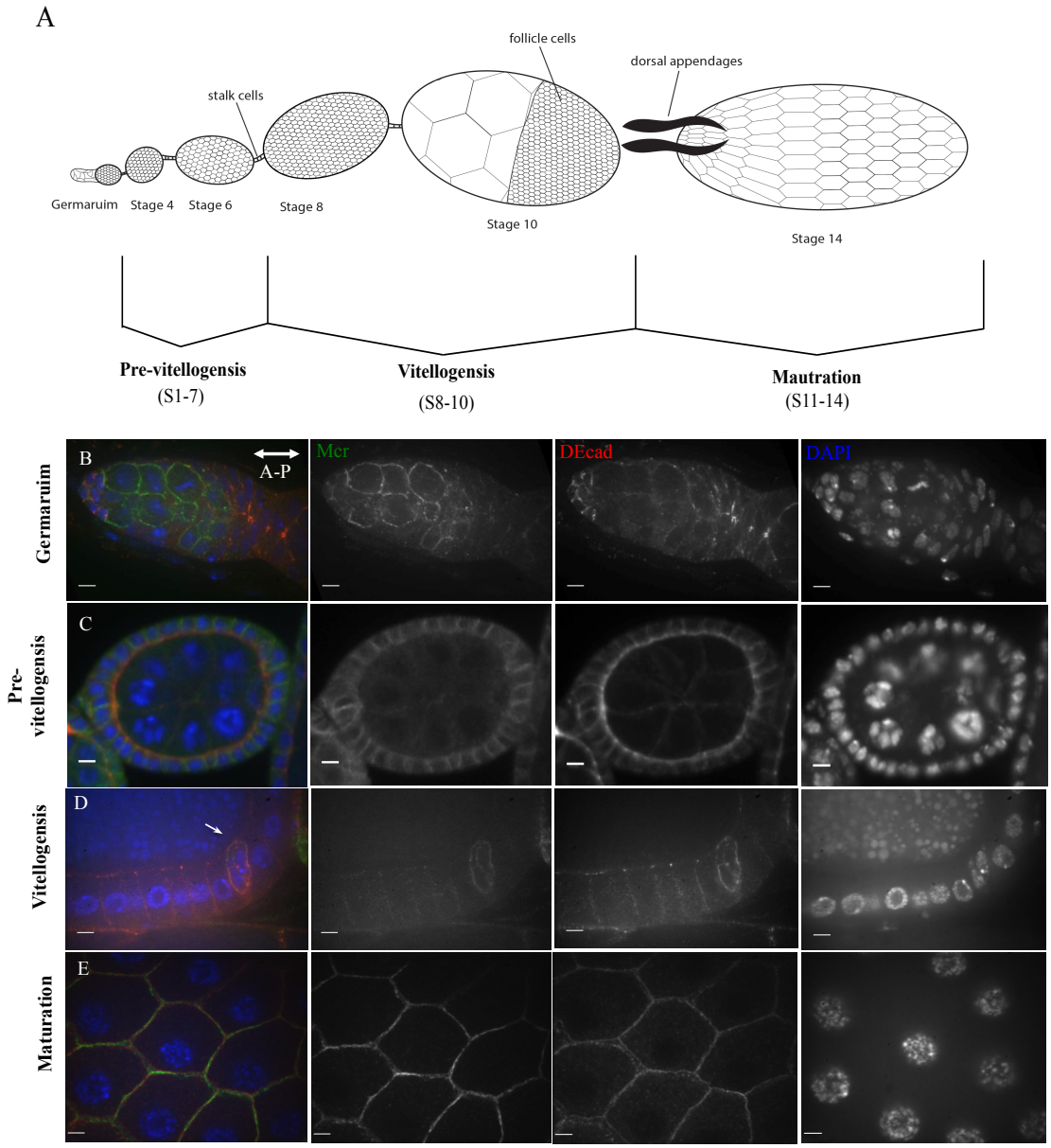

Figure 5. Macroglobulin complement-related expression pattern in the *Drosophila* ovary. (A) The drawing shows the main morphogenetic stages during oogenesis. (B) Mcr is expressed in the germline cells including germline stem cells. Note E-cadherin is slightly expressed in the germline cells. In the pre-vitellogensis stages, Mcr expression level is elevated along the lateral membrane of the follicle cells (C). (D) Mcr is detected in the polar cells (white arrow) and persists in the follicle cells until maturation stages (E). Note that anterior is to the left and posterior to the right. Scale bar 50µm.

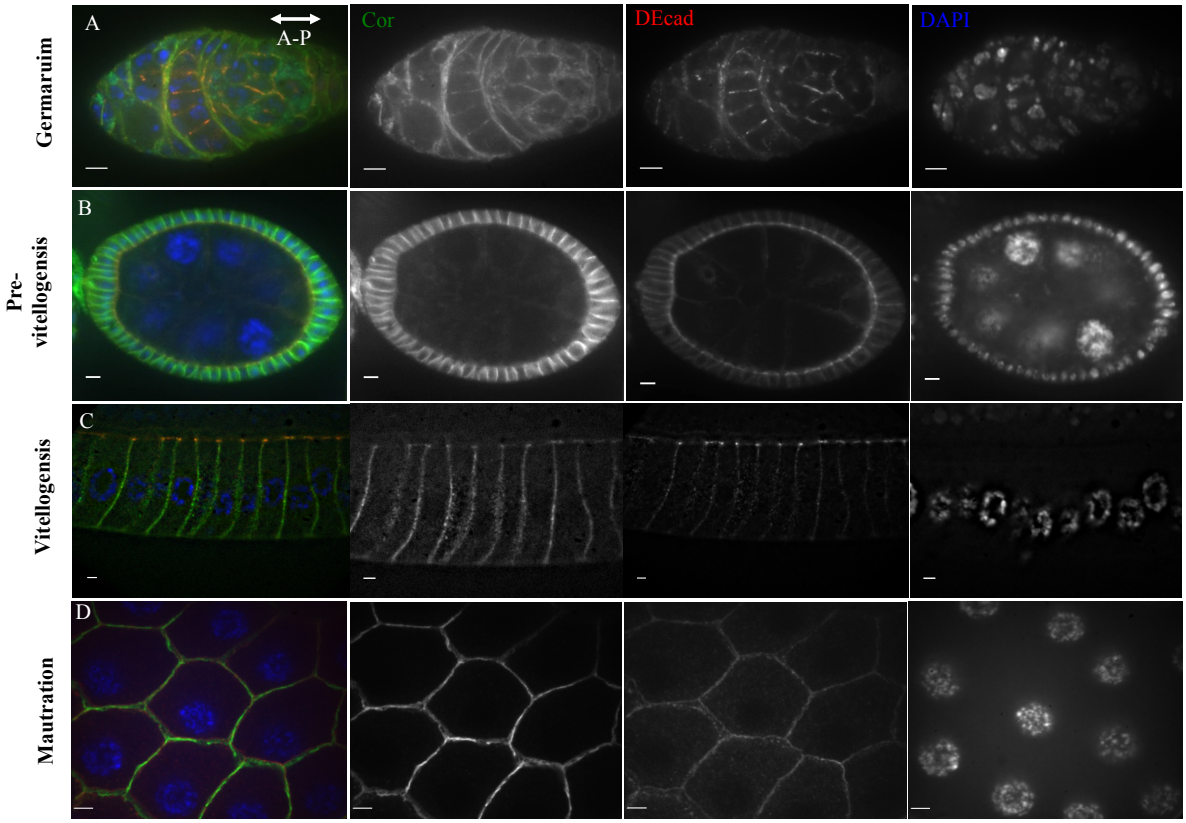

Figure 6. Coracle expression pattern in the *Drosophila* ovary. Cor is expressed in the germline stem cells and cap cells (cells in the most anterior tip of the germarium) with slight expression in the germline cells. In contrast to Mcr (Fig. 3B), Cor is expressed in the follicle cells within the germarium region (A). From stage 1-10, Cor is evenly expressed along the lateral membrane of the follicle cells (B-C). In the maturation stages, Cor expression was detected (D). Note that anterior is to the left and posterior to the right. Scale bar 5µm.

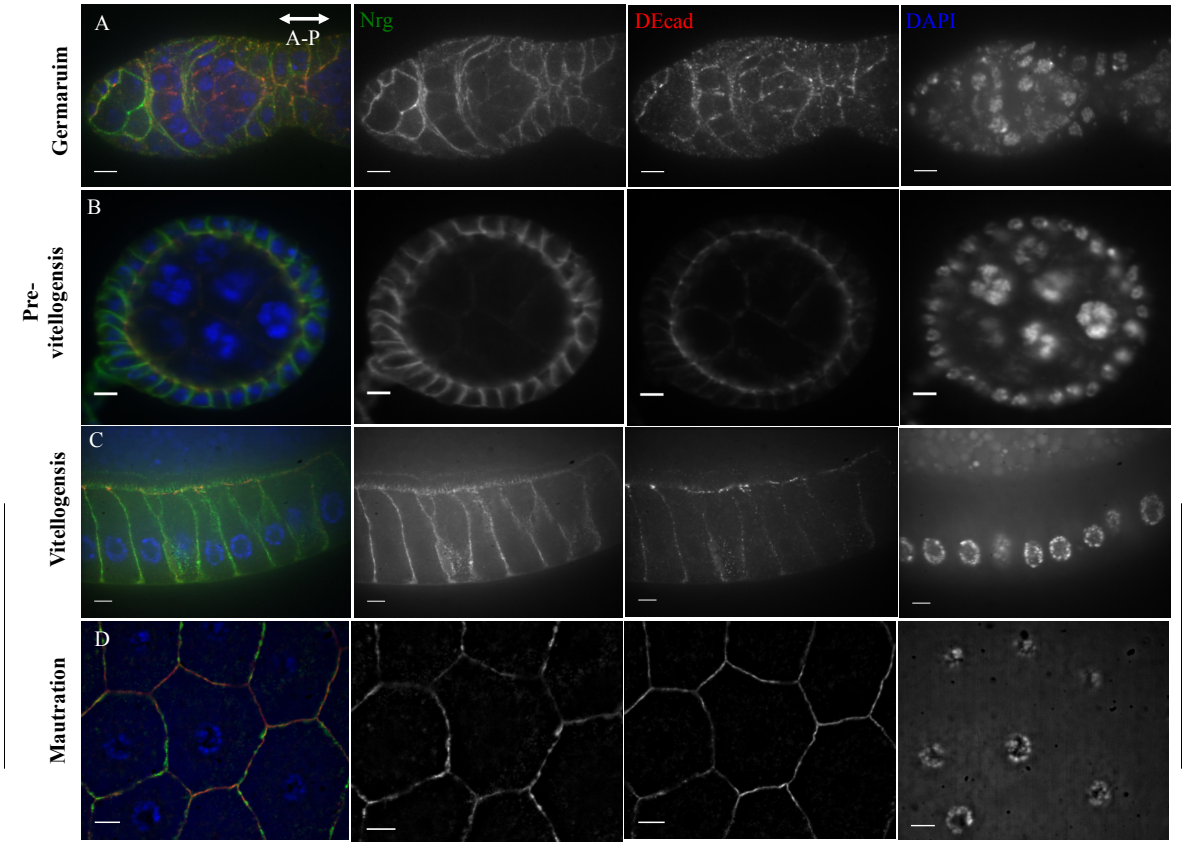

Figure 7. Neuroglian expression pattern in the *Drosophila* ovary. Nrg is expressed in the follicle cells within the germarium with slight expression in the germline cells (A). In the pre-vitellogensis and vitellogensis stages, Nrg is uniformly expressed along the lateral membrane of the follicle cells (B-C). From stage 11-14, Nrg is still expressed and localized at the membrane of the follicle cells. (D). Note that anterior is to the left and posterior to the right. Scale bar 5μm.

To elucidate potential functions of SJ proteins in the *Drosophila* ovary, we first wanted to examine the subcellular localization of Mcr, Cor and Nrg throughout *Drosophila* oogenesis. In order to facilitate the analysis of SJ proteins expression, we divided the developing egg chambers into four groups based on the morphogenetic events that occur during oogenesis. These groups consist of the germarium as well as pre-vitellogenesis (stages 1-7), vitellogenesis (stages 8-10) and maturation (stages 11-14) (King, 1970) (Fig. 5 A). w^{1118} wild-type *Drosophila* ovaries were separately stained with antibodies against Mcr, Cor or Nrg, and also stained with *D*E-cadherin to define the apical versus basal region along the lateral membrane of the follicle cells. Based on this immunohistochemistry analysis, we find that Mcr, Cor and Nrg show dynamic expression

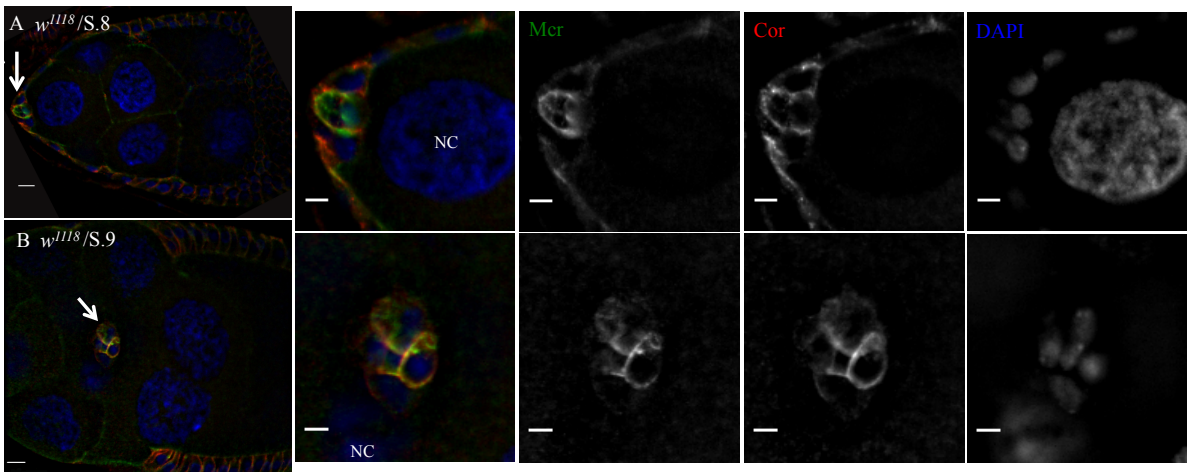

Figure 8. Mcr and Cor expression in the border cell cluster.
(A) Confocal images of the border cell cluster of stage 8 of oogenesis with high level of Mcr in the polar cells, whereas Cor is expressed in the border cells. After border cells cluster delaminates from the epithelium layer and migrate through the nurse cells (NC), both Mcr and Cor are evenly expressed in the border cells as shown in (B) of mid-stage9 egg chamber. Scale bar 10μm.

pattern throughout *Drosophila* oogenesis (Figures. 5-7). Specifically, high levels of Mcr are found in the germline cells including germline stem cells, but not in the follicle cells as in Cor and Nrg (Fig. 5 B) (Figures. 4-5 A). After the egg chamber is formed and pinched off from the germarium, levels of Mcr are reduced but remain in the germline cells (cells in the middle), whereas the levels are slightly up-regulated in the follicle cells (Fig. 5 C) (the cells surrounding the germline cells). From stage 8 to 10 of oogenesis, an increased level of Mcr was detected in a pair of differentiated follicle cells at the anterior and posterior poles of each egg chamber. These cells are called polar cells (white arrow in Fig.5 D). Cor and Nrg show similar patterns of expression to each other. Both are expressed in the germline stem cells with slight expression in the germline cells within the germarium. Note the expression of these proteins in the follicle cells within the germarium region and Cor being expressed in the cap cells (Figures. 6-7 A). In the pre- and vitellogenesis stages, Cor and Nrg are uniformly expressed along the lateral membrane of the follicle cells (Figures. 6-7 B-C). As the *Drosophila* egg matures, Mcr, Cor and Nrg continue to be expressed in the follicle cells (Fig. 6-7 D).

It was previously shown that Nrg is enriched in the border cells only in early stage 9 (Wang et al., 2006). Therefore, we wanted to see if Mcr and Cor are also expressed in the border cells. We found that Mcr is enriched in the polar cells in early stage 9, whereas Cor is evenly expressed in the border cells (Fig. 8 A). However, in the migratory phase (stage 9-10A), Mcr and Cor are both also expressed in the border cells (Fig. 8 B). Taken together, Mcr, Cor and Nrg are all expressed

throughout oogenesis, and show interesting expression in polar and border cells suggesting a potential role of these proteins in egg morphogenesis.

Mcr, Cor and Nrg are required for border cell migration.

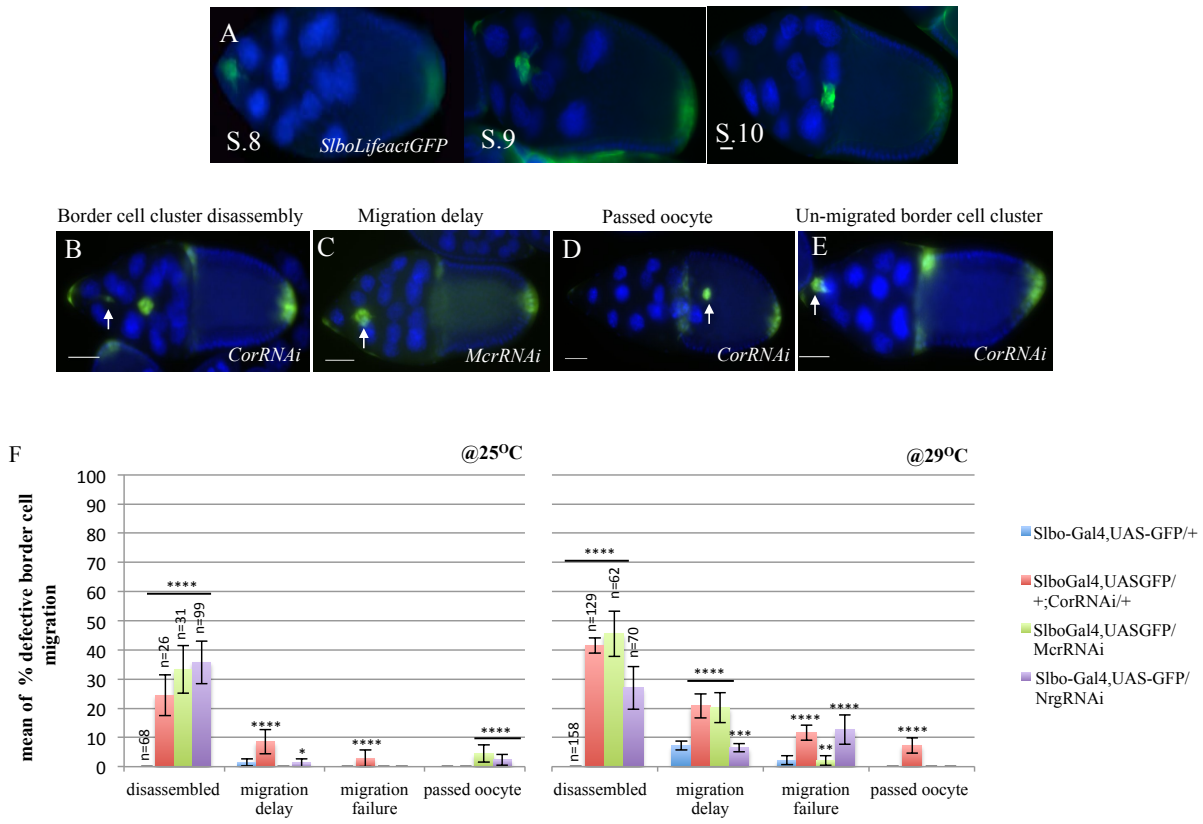

Figure 9. Mcr, Cor and Nrg are required for border cell migration. (A) wild type stage 8-10 eggs with *Slbolifeact-Gal4* driving the expression of GFP only in the migratory border cells. (B-E) Stage 10 of egg chambers showing defective border cell migration phenotypes in SJ mutant cluster (white arrows). (F) Quantitative analysis of border cell cluster at stage 10 egg chambers of both control (blue) and SJ mutant (red, green and purple with the indicated genotypes). Enhancing *RNAi* efficiency by raising the experimental crosses @29°C results in more penetrance phenotypes when compared to @25°C. Data are presented as mean ±SDM (F) Note that increasing the temperature enhanced the mutant phenotype, but also has an effect on wild type border cells (blue bars) (F). *UAS-SJ-RNAi* % was compared to control of the corresponding phenotypic group (**** =*P* value <0.0001, *** = *P* value =0.001, ** = *P* value =0.68, * = *P* value =0.03). Scale bar 100µm.

The classic cadherin member, *DE*-cadherin, was previously reported to be expressed in the polar cells and border cells throughout the process of border cell migration (Niewiadomska et al., 1999). Knocking down *DE*-cadherin in the border cells results in obstructing border cell migration without loss of the integrity of the border cell cluster, whereas reducing *D*E-cadherin specifically in the polar cells results in border cell cluster disassembly (Cia et al., 2014). These results raise the question of what holds border cells together in the absence of *D*E-cadherin. A number of SJ proteins are expressed in the migrating border cells such as Neurexin IV, Neuroglian, Contactin, and Gliotactin (Wang et al., 2006). We focused our functional analysis on Mcr, Cor and Nrg as we previously showed that they are expressed in the border cells and polar cells (Fig. 8) and (Wang et al., 2006). In order to test if SJ proteins are required for maintaining border cell cluster connection, we utilized GAL4-UAS system that allows for spatial and temporal knockdown. We used a cell-type-specific Gal4 driver (*Slbo-Gal4,UAS-GFP*) to reduce SJ proteins expression only in the border cells and visualize the cluster morphology and location in fixed stage 10 eggs by capturing the GFP signal (Fig. 9A). At this stage the border cell cluster in wild type egg chambers has reached the oocyte at the posterior end of the egg chamber. With adults expressing *SJ-RNAi* reared at 25^{o}C, we observed four phenotypes: border cell cluster disassembly, delayed border cell migration, failure of border cells to migrate and border cell cluster that passed the oocyte (Fig. 9B-E). All SJ-RNAi lines showed a higher penetrance of border cell cluster disassembly relative to the control (33.34%, 24.5%, 35.7% and 0%) respectively. Low percentage of migration delay was observed in *NrgRNAi* at 25^{o}C (1.3%), but it is not significantly different than the wild type mutant egg chambers (1.5%)(Fig. 9F). Only 2.9% of *corRNA*i mutant border cells failed to migrate towards the oocyte, whereas both *McrRNAi* (4.5%) and *NrgRNAi* (2.3%) border cells continue to migrate beyond the oocyte. There are a couple of possible reasons why the SJ-RNAi gave such a low percentage of defects in migrating border cells. First, SJ proteins that are made prior to the induction of *RNAi* may persist at the plasma membrane. Border cell migration takes ~6 hours to complete and this may simply not be enough time for protein turnover and thus *RNAi* may be insufficient to show a phenotype. Secondly, the *Slbo-Gal4* line we used may not be robust enough to produce a strong *RNAi* phenotype. Since *RNAi* often is more efficient at higher temperatures, we thought raising the experimental cross at 29^{o}C would enhance *RNAi* efficiency. Consistent with this expectation, we found that raising the temperature to 29^{o}C was sufficient to increase the penetrance of each

phenotype (Fig. 9F). In addition, we never observed control border cell clusters that passed beyond the oocyte or dissembled. It is important to note that the examined border cells are only stage 10 egg chambers, but we do observe the same phenotypes of border cell migration defects in later stages of oogenesis (Stage 11-13). Together, these observations suggest that SJ proteins are required for aspects of border cell migration, but further experiments are needed to verify these phenotypes statistically.

SJ proteins are required for egg chamber elongation and both Mcr and Nrg are essential to pass mid-oogenesis checkpoint.

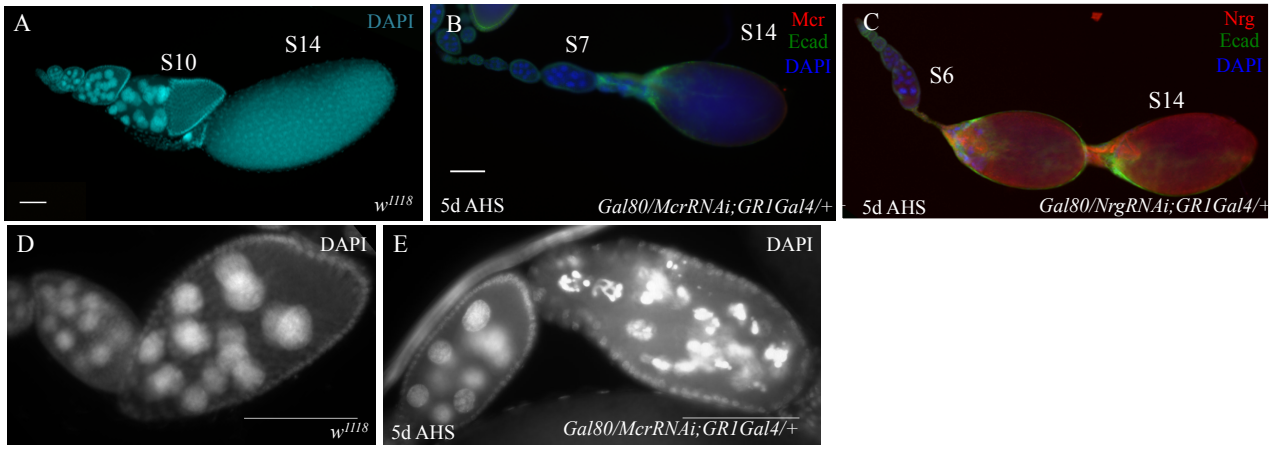

Figure 10. Mcr and Nrg are required to pass mid-oogenesis checkpoint. (A) Schematic representation of egg chamber elongation throughout oogenesis. Confocal images of wild type (B), Mcr (C) and Nrg (D) mutant ovarioles. (B) Wild type ovariole stained with DAPI to mark the nuclei. Mcr and Nrg mutant ovarioles stained with DAPI, Mcr or Nrg and D*E*-cadherin. Note the presence of middle stages of oogenesis in (B), but not in (C or D) (white asterisks). (E-F) Mutant stage 9 egg chambers that are missing in (*McrRNAi or NrgRNAi*;*GR1-Gal4*) ovarioles display DNA fragmentation upon Mcr or Nrg knock down. DAPI staining was used to mark the nuclei. Scale bar 100 µm.

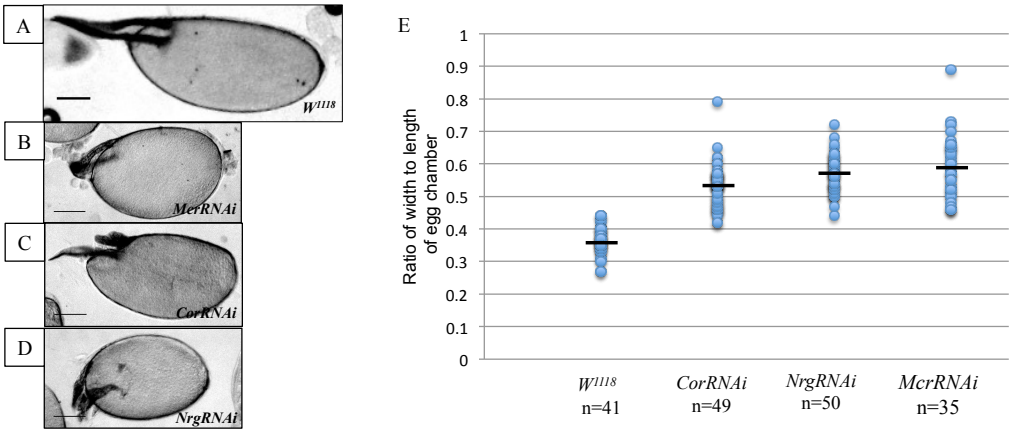

Figure 11. Mutant egg chambers for Mcr, Cor or Nrg failed to elongate. (A-D) Bright-field images of wild type (top) and SJ mutant (bottom) stage 14 egg chambers. Note the difference in the shape between wild type and SJ mutant egg chambers. (E) Quantitative analysis of the ratio of width to length of stage 14 wild type and SJ mutant egg chambers. The ratio of mutant eggs (*CorRNAi*, *NrgRNAi* and *McrRNAi*) is significantly different when compared to the control (W1118) with P value <0.0001.

The final elongated shape of the *Drosophila* egg requires a series of developmental events during oogenesis. One of these morphogenetic processes is follicle cell rotation in which the follicle cells rotate collectively with the aid of a molecular corset consisting of actin bundles and collagen IV fibrils that are polarized at the basal side of the follicle cells (Cetera et al., 2015). In order to investigate the role of SJ proteins in egg chamber elongation, we used a Gal4 driver (*GR1-Gal4*) that is expressed in all the follicle cells including follicle stem cells so that SJ proteins would be reduced early in egg development (Gupta and Schüpbach, 2003). *GR1-Gal4* driver is also expressed at earlier stages of development. Therefore, we used a temperature sensitive Gal80 line to suppress the Gal4 activity until adulthood. By shifting adults to 29^{o}C, the Gal80 is inactivated allowing for the *GR1-Gal4* driver to induce SJ-RNAi in the ovary. We found that Mcr and Nrg mutant egg chambers develop normally from stage 1-8. However, when the egg chambers reach stage 9 they undergo cell death resulting in missing middle stage egg chambers (Fig. 10B-E). Interestingly, we observed stage 14 egg chambers that are rounded rather than elongated. These rounded eggs must have come from egg chambers that were past the mid-oogenesis checkpoint when the *RNAi* was induced because they were able to continue their development normally until stage 14. To further examine this phenotype, we used the *c204-Gal4* driver to avoid mid-oogenesis checkpoint. This Gal4 driver is also expressed at earlier stages of development (Manseau et al., 1997) and so we also the used the Gal80 technique to suppress Gal4 activity until adulthood. *C204-Gal4* is expressed from stage 8-14 during which the follicle cell rotation ends and elongation of the egg chamber is dependent on actomyosin contraction at the basal side of the follicle cells as described by (He et al., 2010). Surprisingly, we found that reducing Mcr, Cor and Nrg from stage 8-14 results in rounded egg chambers (Fig. 11 A-D). We calculated the aspect ratio of width to length of the wild type and SJ mutant stage 14 eggs and found that the ratio of SJ mutant eggs is significantly different than the wild type eggs (*P* value <0.0001) (Fig. 11E). To confirm this phenotype, we used another Gal4 driver (*C355-Gal4*), which is expressed one stage earlier than the *C204-Gal4* line (Manseau et al., 1997), and we found that *C355-Gal4* gave similar results (data not shown). Taken together, these results indicate that SJ proteins are required for egg chambers to elongate properly.

Clonal analysis indicates that *Mcr* is dispensable for apico-basal polarity.

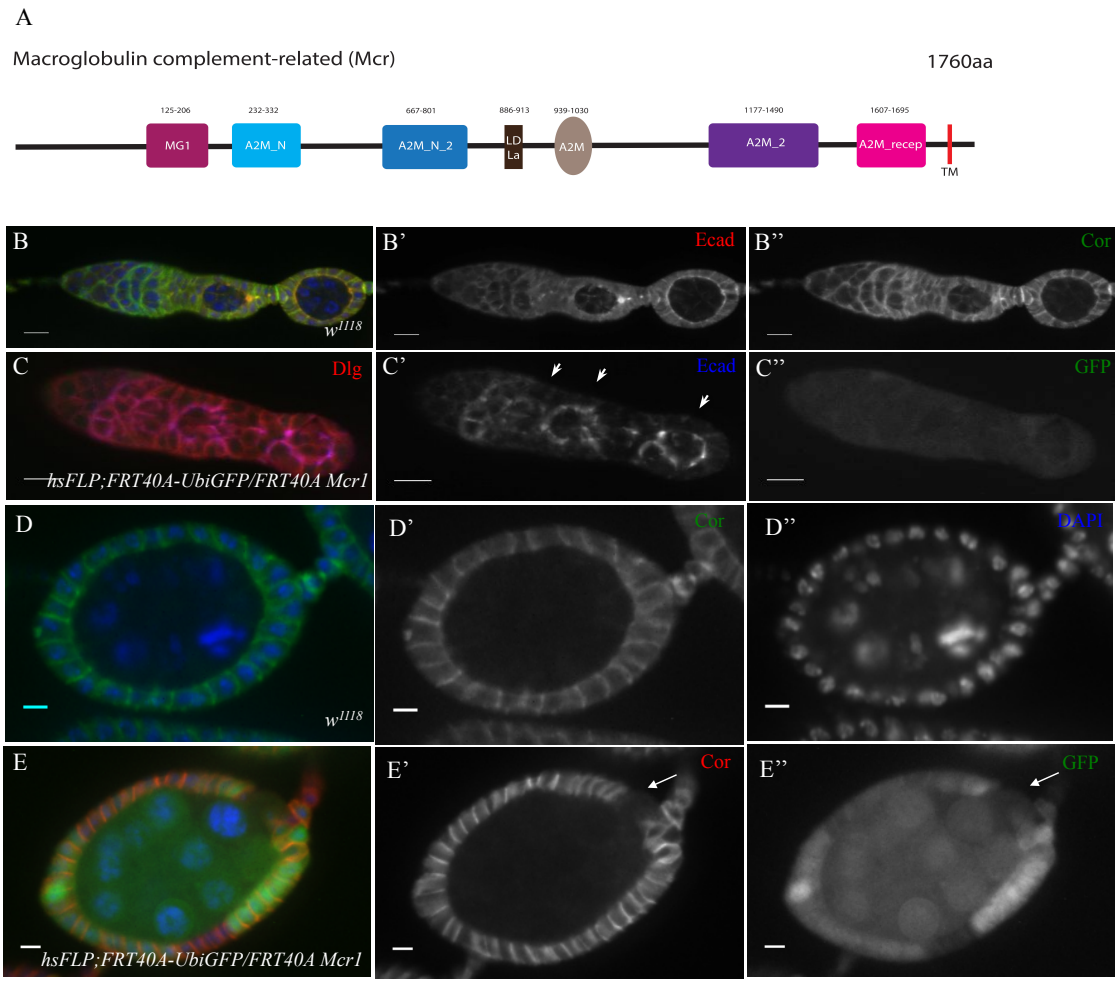

Figure 12. *Mcr* is required for egg development. (A) Schematic of Mcr proteins showing the different domains MG1 - alpha-2-macroglobulin MG1 domain; A2M_N, A2M_N_2, A2M_2 - alpha-2 - macroglobulin family N-terminal region; LDLa - low-density lipoprotein receptor A domain; A2M-alpha - 2-macroglobulin family; A2M_recep - alpha-2-macroglobulin receptor; TM - predicted transmmembrane domain (B-B'). Confocal images of wild type (B) and Mcr^1 mutant clones in germarium (C). Note the newly formed eggs failed to separate from the germarium in Mcr^1, which is the opposite to wild type egg chambers (arrows in C' with complete Mcr^1 clone in the germarium region). (D) Wild type egg chamber showing the connection between two egg chambers through the stalk cells and polar cells. (E) Mcr^1 clones only in the polar cells including stalk cells results in epithelium rupture or disassociation (white arrows), but not when Mcr^1 clone is induced in the main body of the egg. Scale bar 10µm.

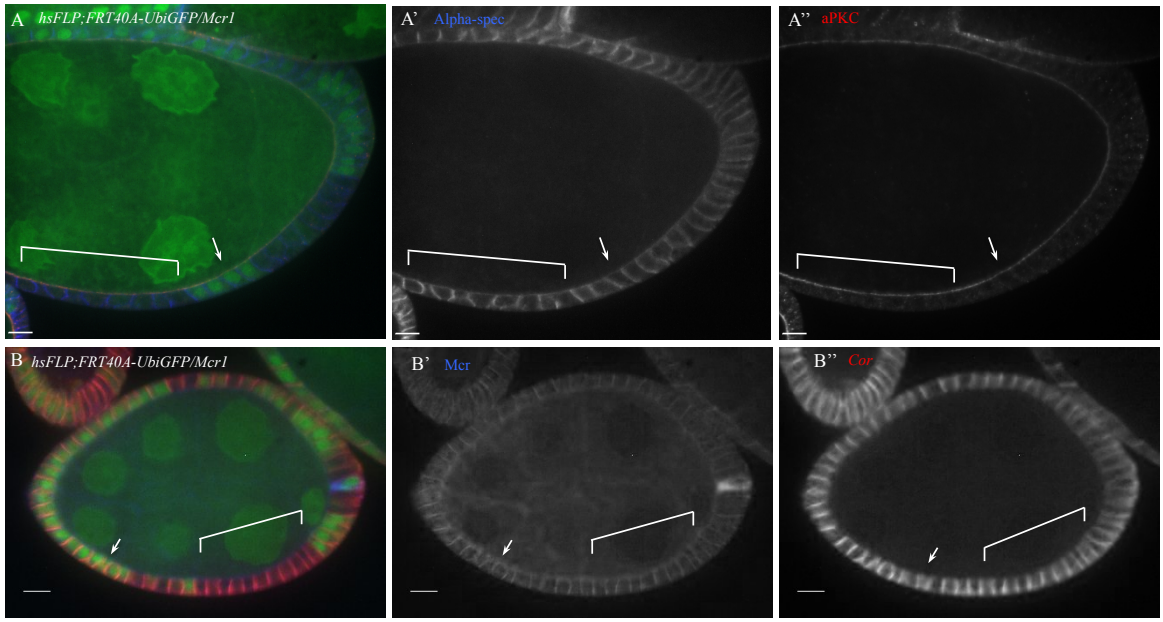

Figure 13. *Mcr* is not required for apical-basal polarity. (A) Egg chambers of *Mcr*^{1} mutant clones (GFP^{-}) in a wild type background (GFP^{+}). Note that aPKC and alpha-spectrin are not mis-localized in the mutant clones (bracket) when compared to wild type cells (arrow). (B) Cor is also unaffected in *Mcr* mutant clones. Scale bar 10μm.

Next, we examined the function of *Mcr* during oogenesis in greater detail using clonal analysis of a strong loss of function allele. *Mcr* encodes an ~200 kDa protein with α-2-Macroglobulin and LDL receptor class A domains (Fig. 12A) (Hall et al., 2014). We previously showed that *Mcr* encodes a core component of the SJ that is also required for essential developmental functions during embryogenesis (Hall et al., 2014). Mcr belongs to the thioester-containing protein (TEP) family, which is conserved among vertebrates and invertebrates, and thought to be involved in innate immunity (Mudiganti et al., 2010). To investigate the role of *Mcr* in egg chamber morphogenesis, we used the FLP-FRT system to generate *Mcr*^{1} clones in the follicle cells. *Mcr*^{1} is a strong loss of function allele. Several morphogenetic phenotypes were observed in *Mcr*^{1} clones depending upon where the clones were induced. *Mcr*^{1} clones in the germarium result in egg chambers that fail to separate properly from the germarium (Fig. 12 B-C). We stained ovaries with a D*E*-Cad antibody to outline the apical membrane of the follicle cells that is in contact with the germline cells (arrows in Fig. 12 C'). We also observed *Mcr*^{1} clones in the stalk cells including the polar cells that result in epithelium rupture (Fig. 12D-E). However, this

phenotype is not completely characterized. There is a potential that the cause of this phenotype is due to damage egg chambers during the dissection process that results in lose of GFP signal as explained by (Haack et al., 2013), but not due to eliminating Mcr. Further experiments will need to be done in order to confirm this phenotype. Since the hindgut, trachea and salivary glands of Mcr^{1} homozygous mutant embryos showed Cor mislocalization (Hall et al., 2014), we examined Cor localization in Mcr^{1} clones to determine if losing Mcr would also affect Cor localization in the follicular epithelium. We also examined a- serine/threonine kinase (aPKC) and alpha-spectrin proteins localization. Interestingly, we found no major difference in alpha-spectrin, Cor or aPKC localization between wild-type cells and Mcr^{1} clones (Fig. 13A-B''). Taken together, these observations indicate that *Mcr* is essential for egg chamber development, but dispensable for apical-basal polarity.

Chapter IV: Discussion and future directions

Here we show that core SJ proteins Mcr, Cor, and Nrg have essential morphogenetic role in the *Drosophila* ovary based on three major results. First, knocking down Mcr, Cor or Nrg in the migrating border cell cluster results in border cell migration defects. Second, Mcr and Nrg mutant eggs failed to pass mid-oogenesis checkpoint when knocked down early in oogenesis. In addition, reducing Mcr, Cor and Nrg in the follicle cells by stage 8 results in rounded rather than elongated egg chambers. Our work offers new insight into the important role SJ proteins has during *Drosophila* oogenesis.

SJ proteins are expressed in the *Drosophila* ovary

Although SJs were detected between adjoining follicle cells by electron microscopic studies, functional analyses of the SJs in the ovary have not been conducted yet (Mahowald, 1972;Muller, 2000). Here we show that three core components of the SJs are expressed in the *Drosophila* ovary. Closer examination of SJ proteins expression revealed unique expression patterns of Mcr, Cor and Nrg in the ovary as they have similar, but not identical expression patterns throughout oogenesis. In the ovary, Mcr, Cor and Nrg are localized along the lateral membrane of the follicle cells. Specifically, we found that Cor and Nrg have similar expression patterns. They are both expressed with similar level of expression in the germline cells within the germarium region, along the lateral membrane of the follicle cells (Stage 1-10), and the expression persists until the maturation stages (Stage 11-14) (Fig. 6-7 1-D). Cor was also detected to be expressed in the cap cells, which is consistent with a previous study (Fig. 6 A) (Bonfini et al., 2015). Mcr is strongly expressed in the germline cells including germline stem cells within the germarium region, but not in the follicle cells within the germarium (Fig. 5 B). In contrast to Cor and Nrg, Mcr is slightly expressed along the lateral membrane of the follicle cells with the strong expression in the polar cells (Fig. 5 C).

The examined SJ proteins are required for border cell migration

Our lab has recently demonstrated a crucial requirement of nine different SJ proteins in *Drosophila* embryos' development, which has led us to further examine the molecular mechanisms these proteins play during development (Hall et al., 2016). Following the expression pattern analysis of Mcr, Cor and Nrg in the ovary, we examined their requirement during *Drosophila* oogenesis. As mentioned earlier Mcr, Cor and Nrg are expressed in the border cells (Fig. 8A-B) and (Wang et al., 2006), which suggested a possibility of their requirement during border cell migration. Border cells are specialized follicle cells that contribute to the formation of the micropyle, a structure that facilitates sperm entry to the egg (Montell, 2003). Knocking down these proteins specifically in the border cells results in border cell cluster disassembly, migration delay and migration failure with percentage that is significantly different than the control (Fig. 9B-F). We do also see border cell cluster delay in the control, but the percentage (1.5% at 25^{o}C and 7.2% at 29^{o}C) is lower than *CorRNAi* (8.5% at 25^{o}C and 20.8% at 29^{o}C) and *McrRNAi* (0% at 25^{o}C and 20.22% at 29^{o}C) (Fig. 9 E). There are a number of proteins that are present in the border cell cluster not only to maintain the cluster cohesion, but also its direction. For example, mutation in DE-cadherin, an adhesion molecule expressed in the polar cells and border cells, results in migration failure or border cell cluster disassembly. Specifically, reducing DE-cadherin expression in the polar cells results in border cell cluster disassembly, whereas reducing the expression of DE-cadherin in the border cells affect their migration (Cia et al., 2014). These phenotypes are similar to what we observed in SJ mutant border cells. These results likely underrepresent the strength of the *RNAi* effect since the *RNAi* is induced right before border cells specification and migration (early stage 9). We will further confirm this result using clonal analysis or using the *C306-Gal4* line with GFP to mark the border cells throughout their migration. Since Mcr was shown to be expressed in the polar cells (Fig. 5C) and (Hall et al., 2014), we will examine the function of Mcr in the polar cells by using *Unpaired-Gal4* (*upd-Gal4*) that is only expressed in the polar cells (Bai and Montell, 2002).

Mcr, Cor and Nrg are essential for egg chamber elongation

We have demonstrated that Mcr, Cor and Nrg are required for egg chamber elongation, as reducing these proteins results in rounded rather than elongated egg chambers. Similar phenotypes were observed in several mutations that cause egg chambers elongation failure.

These mutations have been found to be in genes that regulate the interaction between the actin cytoskeleton and the extracellular matrix (ECM) proteins such as Dystroglycan, the receptor-like tyrosine phosphatase (Dlar), and beta-integrin subunit (Bateman et al., 2001;Frydman and Sprdling, 2001;Duffy et al., 1998;Cetera et al., 2015;Isebella and Horne-Badovinco, 2015). However, there is no evidence that SJ proteins have direct interaction with either the actin cytoskeleton or the ECM. Thus, we propose that SJ proteins function in intracellular communication within each follicle cells rather than direct interaction with actin or focal adhesion receptors.

Since reducing the expression of Nrg, Cor and Mcr by stage 8 of oogenesis results in round eggs, this has led us to hypothesize that the terminal phenotype we see is due to a disruption in actomyosin contraction at the basal side of the follicle cells. This notion is supported by two major results from our study. First, reducing Mcr and Nrg using (*GR1-Gal4*) results in a terminal phenotype of spherical eggs (Fig. 10 B-E) that are similar to what we observed in Mcr, Cor and Nrg mutant eggs with (*c204-Gal4*) (Fig. 11 A-E). Again, *c204-Gal4* line is expressed from stage 8-10 which coincides with basal myosin accumulation on a polarized actin network (Manseau et al., 1997;He et al., 2010). It was previously shown that inhibiting or enhancing actomyosin contraction, blocks or exaggerates egg chamber elongation, respectively (He et al., 2010). Moreover, SJ mutant embryos with dorsal closure defects show discontinues accumulation of myosin at the leading edge of the epidermal layers when compared to wild type embryos (Hall et al., unpublished). To better test this, we will use the strong loss of function allele Mcr^{1} to generate mosaic clones in the follicle cells and stain egg chambers with Spaghetti-squash antibody to mark the myosin regulatory light chain and phalloidin antibody to stain the basal actin filaments. We will also overexpress *Mcr* from stage 8-14 using *c204-Gal4* line to test if increasing *Mcr* would enhance egg chamber elongation.

Although SJ proteins are expressed throughout oogenesis, they do not appear to form the SJ until stage 10B as revealed by the ultrastructure analysis of the *Drosophila* ovary (Mahowald, 1972). Here, we show that Mcr, Cor and Nrg are expressed early in oogenesis (germarium) and have essential functions in border cell (Figs. 9) and in forming and shaping and the egg (Figs. 10-11). Taken together, these data suggest a unique requirement for SJ genes in morphogenetic events during oogenesis that is independent of their potential role in the occluding junction. This is

consistent with what we see in the embryo, as homozygous mutations in SJ genes lead to dorsal closure and head involution defects and these events also occur before the SJ is physiologically tight (Hall et al., 2014;Hall et al., 2016;Fehon et al., 1994).

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