Our primary aim was better understanding of the regulation of neuronal cytoskeletal rearrangements during axonal growth by analyzing the role of the PCP navigation system in details, to shed light on the molecular mechanisms of this signaling pathway in the neuronal context during Drosophila mushroom body (MB) axonal pathfinding. In addition, we also wanted to investigate the redundancy between the formin type of actin assembly factors during development of the nervous system. During the course of these studies we made a number of unexpected observations that led us to two unplanned research directions as well. Notably, we found that two Diaphanous related formins, DAAM and FRL, play a redundant role during eye development, and we found that FRL plays an important role in formation of the nucleus positioning actin filaments during development of the egg chambers in the ovary.

Subsequently, I will summarize our results according to these tree major topics:

1. Investigation of PCP signaling during Drosophila mushroom body axonal pathfinding

2. Examination of DAAM and FRL redundancy in growth cones and during eye development

3. Investigation of the role of FRL during ovarium development

1. Investigation of PCP signaling during Drosophila mushroom body axonal pathfinding

Based on our preliminary studies, the Wnt/PCP signaling pathway plays a fundamental role during axon growth and pathfinding in the Drosophila MB neurons (and as shown by others, in numerous vertebrate contexts). Interestingly, we found that the two key PCP factors, Frizzled (Fz) and Strabismus (Stbm), regulate axonal growth in a lobe-specific manner, i.e. Fz directs axons into the dorsal direction, while Stbm promotes growth towards the medial direction (Fig.1.). We also found that the formin DAAM acts as the cytoskeletal effector of PCP signaling both in the dorsal and medial axons, although the mechanisms of the upstream processes remained unclear. Our plan was to investigate the regulation of PCP signaling through the Fz-Stbm and Fz-Wnt5 relationship. To facilitate these studies, during the first phase of my project we generated a set of transgenic lines carrying mutant PCP protein forms. These included a Fz mutant that lack most of the extracellular domain (FzΔCRD), two other Fz forms (Fz57 and Fz81) that are unable to bind Wnt5 due to point mutations, and a Stbm

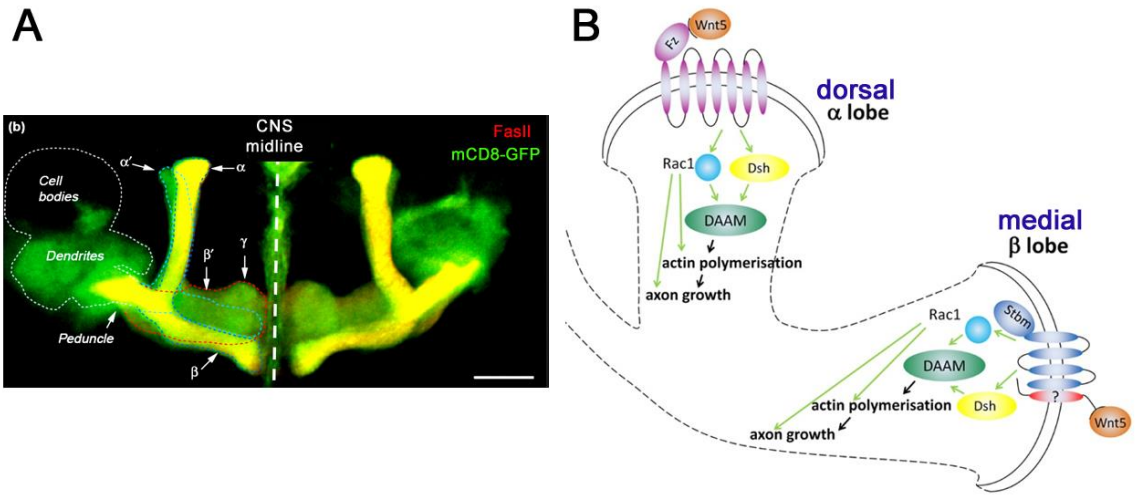

Figure 1. The structure of the mushroom body as illustrated by a composite confocal image (A) of adult MBs expressing mCD8-GFP (green) and stained with anti-Fas II (red), (adopted from Jefferis et al., 2002). (B) Hypothetic model for PCP signaling during axon growth in the MB (Gombos et al., 2015). Note that Fz is required for development of the dorsally running axon branches forming the α lobe, while Stbm is involved in development of the medially running axon branches, forming the β lobe.

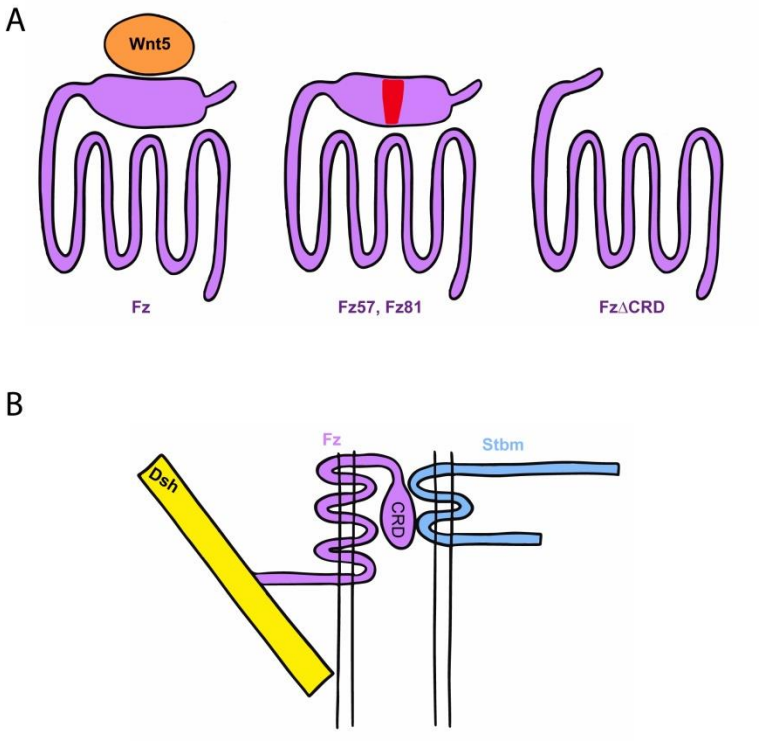

Figure 2. Schematic representation of the interaction between Wnt5 and Fz (A), and between Fz and Stbm (B), based on published data. In the case of the Fz mutants, the extra GSG amino acid insertion was placed after the 57th (Fz57) and 81st (Fz81) aa in the CRD domain. In the case of FzΔCRD the entire CRD domain was cut out (A).

mutant with impaired Fz binding (Stbm **F**ri**z**zled **b**inding **m**utant - Stbm^{Fzbm}) (Fig. 2.) to test them in various functional assays. During the second year we successfully employed the CRISPR/Cas9 system to create the two *fz* mutants mentioned above *in situ* by genome editing

of the *fz* locus. The transgenes were expressed in wild type as well as in the appropriate PCP mutant backgrounds, and the results collected allowed us to draw several important conclusions as to the functioning of the PCP system during MB axon guidance. Firstly, we established that Stbm and Wnt5 binding by Fz are both important for MB axon development, and in particular the interaction between Fz and Stbm is critical for medial lobe formation. We also revealed that absence of the Fz extracellular domain results in a strong LOF phenotype which is however weaker than the null phenotype. Thus, a truncated Fz receptor is partly functional with regards to axonal guidance in the MBs. Interestingly, we noticed a modest but significant difference between pan-neuronal and MB-specific rescue abilities of the mutant transgenes, suggesting that Fz acts non-cell autonomously and it is likely to be required in additional neurons than the ones of the MBs.

To get further insights, we also investigated the localization of the mutant and wild type proteins. In the case of the wild-type and fzΔCRD we found that these forms are mainly enriched in the axons. In contrast to this, Fz81 was not localized into the axons after the branching point, instead it was accumulated in the cell bodies, whereas Fz57 was observed in small amounts in the axons of young adults, but later the overexpressed protein was only found in the area of the cell bodies. We made a similar observation in the case of $Stbm^{Fzbm}$, i.e., unlike the wild type form, the Stbm mutant failed to accumulate in the axons and the signal was observed around the cell bodies. These observations were also supported by the experiments carried out in S2 cells. The Fz wild type form and the fzΔCRD localize into the lamellipodial and filopodial cellular protrusions, while the Fz81 and $Stbm^{Fzbm}$ mutant forms do not accumulate in these regions at all, and Fz57 exhibit a partial enrichment in these protrusions (Fig. 3.). Thus, we conclude that the protein-protein interactions between the PCP factors are crucial to establish stable signaling complexes in the MB axons, that will in turn dictate proper navigation of the axon branches.

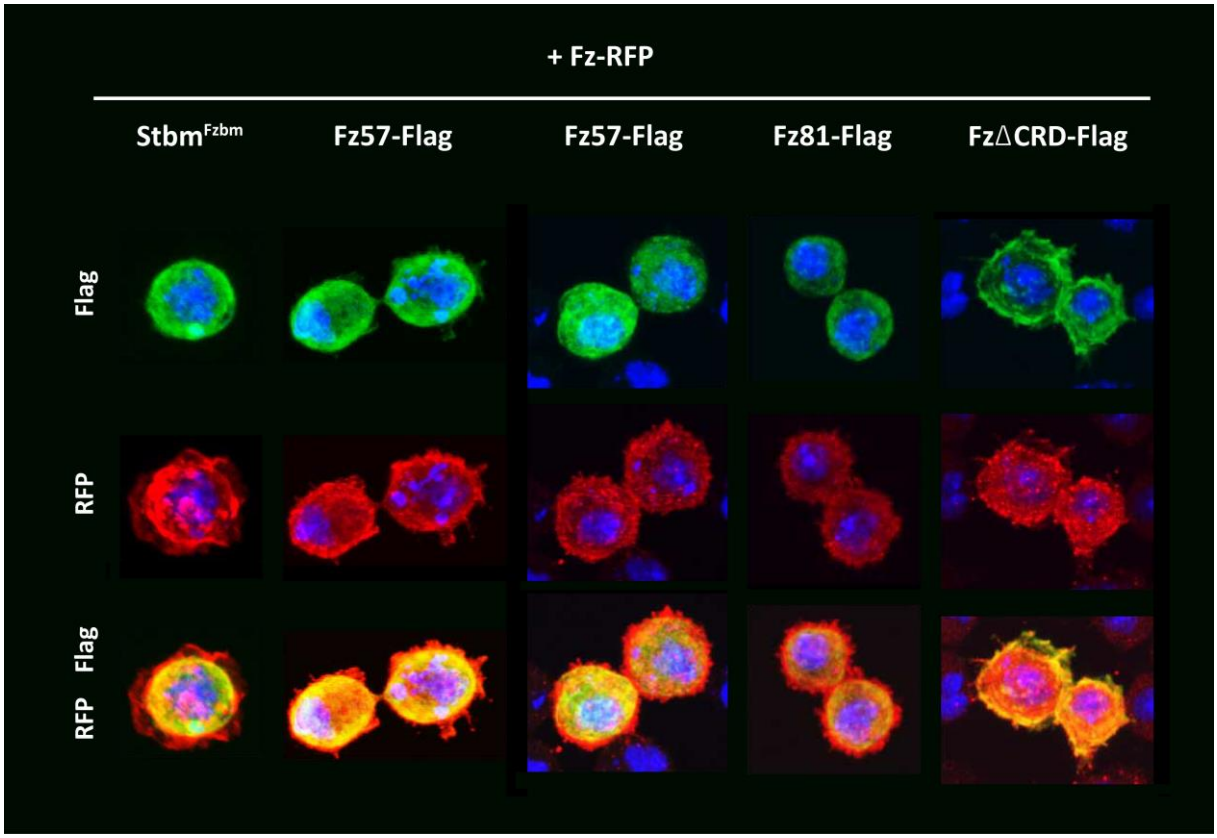

Figure 3. The localization of the mutant proteins in the S2 cells compared to the RFP tagged wild type Fz protein.

Besides understanding the Fz-Stbm interaction, we are interested to identify their potential co-receptors in the context of MB axonal development. We tested all the potential Wnt5-binding protein coding genes with genetic interaction analysis, and we found two promising candidates, Appl and Drl-2. We used the power of genetic epistasis analysis to determine the position of these candidates within the known PCP regulatory hierarchy. We found that Appl is likely to act downstream of Stbm during medial lobe specification. Consistent with this idea, the formin DAAM is epistatic to Appl, and we propose that Appl plays an important modulatory role in proper termination of the medial lobe axons at the CNS midline. In contrast to Appl, Drl-2 appears to play an early role in MB axon specification/growth as the lack of Drl-2 results in a premature termination of the neurites. Based on this, we can not exclude a later role as a Stbm/Fz co-receptor or signaling mediator, but further studies are required to clarify this issue. Overall, we already made significant progress in understanding PCP signaling specificity in development of the MB axons, yet a few new questions arose that need to be addressed before publication of these data.

2. Examination of DAAM and FRL redundancy in growth cones and during eye development

Besides focusing on the molecular mechanisms of the PCP navigation system, the second major aim of our study is analysis of the potential redundancy between members of the formin family of cytoskeleton regulatory proteins in axonal growth cones. To begin this project we collected genetic tools (mutants and RNAi lines) for all six *Drosophila* formin genes (*dia, DAAM, fhos, form3, capu* and *frl*). As controls, it was necessary to first test the LOF phenotypes in single mutants that was carried out both in the embryonic CNS and also in the MB neurons. These experiments revealed a role in axonal development for DAAM, Frl and Form3, while Dia is required for neuronal cell division. Subsequently, we started to analyze double mutant combinations to test for redundancy. Using these combinations we could further confirm our previous finding that DAAM and Frl are redundant in several neurons, and that Form3 is redundant with DAAM in the MB neurons. In line with our preliminary data, we collected further evidence suggesting that although DAAM and Frl are redundant, they are activated by different Rho GTPases, notably DAAM is mainly activated by Rac while Frl is activated by Cdc42 in neurons.

During the course of the above redundancy studies, we noticed that RNAi knockdown of FRL in a DAAM mutant background resulted in the formation of rough eyes (while the single mutants show wild type eyes). To further confirm this observation, we created an *frl* null mutant allele (designated as frl^{59}) by CRISPR/Cas9 mutagenesis, that subsequently allowed us to generate $DAAM^{Ex4}$; frl^{59} double mutants. Most of these animals exhibited pharate adult lethality, however, a few escapers hatched occasionally, all of which had rough eyes. Detailed examination of these double mutant eyes revealed an early defect in eye development, already evident at 18-20 hours after puparium formation (APF). When this phenotype was analyzed at the cellular level we found that the primary defect is a change in the shape and pattern of the interommatidial cells (IOCs), located in between the individual ommatidial cell clusters, leading to ommatidium fusions (Fig.4.). We revealed that the cell-cell connections in the lateral and basal regions of the IOCs are severely impaired. Consistent with this finding, we found that both proteins are present in the lateral and basal regions of the IOC membranes, with the addition that FRL is also found apically, while the DAAM staining is detectable in the membrane of the photoreceptor cells as well. We were able to rescue this

phenotype in the case of both formins by overexpressing the wild-type form of FRL or DAAM, either with *GMR-Gal4* (expressed in all eye cells) or with the IOC-specific *54C-Gal4* driver. No rescue was observed with the actin polymerization mutant form of the proteins (DAAM-I732A and FRL-I773A), which suggests that the actin nucleation and polymerization ability of both proteins is necessary for normal IOC development. In harmony with these results, actin intensity in the lateral membrane of the IOCs is significantly lower in the double mutants than in the case of wild type.

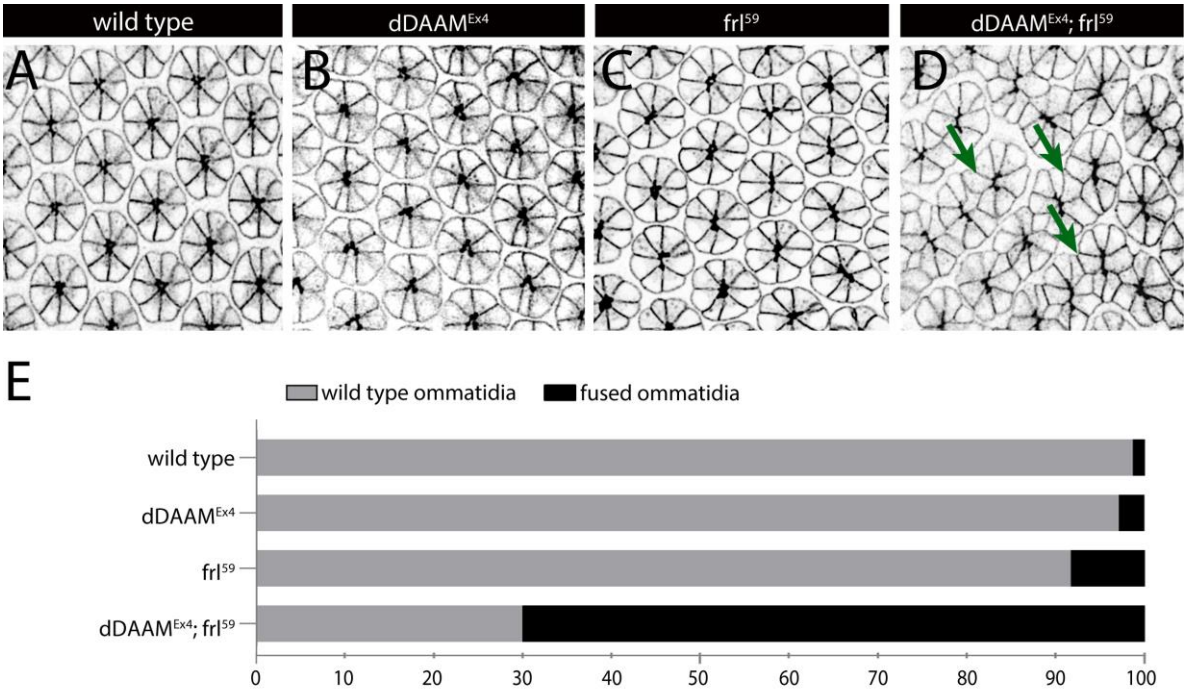

Figure 4. The combined absence of DAAM and FRL results in ommatidium fusions (green arrows).

To address whether the formin dependent actin filaments are involved in the formation of contractile acto-myosin cables, we tested the *zipper (zip)* gene encoding non-muscle myosin II. Curiously, the RNAi knock-down of *zip*, performed with an eye-specific driver, showed a similar phenotype as the $DAAM^{Ex4}$*;* frl^{59} double mutants. In addition, both the $DAAM^{Ex4}$ and frl^{59} single mutant alleles show a dominant genetic interaction with the amorphic zip^{2} allele. Therefore, these results support the involvement of FRL and DAAM in the formation of a basal and/or lateral acto-myosin system playing a crucial role in IOC cell shape determination and maintenance. Based on previous studies, members of the DRF formin family are activated by a Rho-type GTPase, thus next we investigated which GTPase is responsible for the activation of DAAM and FRL during eye development. Genetic analysis of the Drosophila Rho GTPases led to the conclusion that in this context both formins are activated by Cdc42

but not by Rac or RhoA. Collectively, these observations resulted in the discovery of a novel aspect of pupal eye development, which is formin dependent patterning of the basal and lateral regions of the retina. These results will be summarized in a manuscript that is nearly ready for submission.

3. Investigation of the role of FRL during ovarium development

Beyond the redundant effect on eye development, when we tested the new *frl* null mutant (frl^{59}) we found that the eggs of the homozygous animals were significantly smaller than the eggs of the wild type. Because the formin functions are poorly understood in the ovary, we decided to characterize this phenotype in more details. Each ovary is composed of several ovarioles that contain a chain of egg chambers representing the different stages of development. In the germarium (located at the anterior tip of the egg chambers), the germline stem cells divide asymmetrically to give rise to cystocytes, which undergo four cell divisions without completion of cytokinesis to form a cyst of 16 germ cells, connected by cytoplasmic bridges, called ring canals. One of these cells becomes the oocyte while 15 remaining cells become nurse cells. Each cyst is then enclosed by a polarized epithelium of somatic follicle cells. Nurse cells produce nutrients and macromolecules necessary for development of the oocytes. These materials are transferred into the oocyte in two stages: first by an early slow cytoplasmic transfer and later (after stage 10b) by a rapid dumping when the nurse cells transfer their entire cytoplasmic content into the oocyte, but the nuclei. This nurse cell dumping is driven by acto-myosin contraction, and it requires a dramatic remodeling of the actin cytoskeleton. A part of these cytoskeletal changes is the formation of the nucleus positioning actin filament cables running from the plasma membrane towards the nucleus. These filaments are initiated in filopodia/microvilli like cellular protrusions at the plasma membrane and growth until they reach the nuclear membrane. If these filaments are missing or damaged, dumping is reduced or stopped, mainly because the nuclei physically block the ring canals (places of the cytoplasmic transport). We found that in the absence of FRL the number of actin filaments starting from the membrane of the nurse cells decreased significantly in stage 10b egg chambers (Fig.5.). These filaments normally keep the nucleus in a stable, central position while the nurse cells contract and their cytoplasm is emptied into the oocyte. Based on antibody staining, the FRL protein is located at the (+) end of the actin filaments starting from the membrane, supporting the assumption that it is necessary for the

formation and growth of these filaments (Fig.6.). The observed phenotype can be rescued by expression of the full-length form of FRL. Former studies attributed a similar role to another cytoskeleton regulatory protein, Enabled (Ena). To understand the potential connection between Ena and DAAM, we analyzed the role of Ena as well. Based on these studies, we conclude that the primary role of Ena is to promote the formation of a specific nuclear positioning actin subpopulation initiated at the ring canals, while FRL is required for the cortical membrane initiated actin cable subpopulation (without affecting the ring canal attached cables). In addition, we found that in the absence of FRL much less Ena is localized in the membrane. Together, our observations suggest that the Frl and Ena proteins cooperate to build up a unique, nuclear positioning actin cable network that is key to prevent blocking of the ring canals, and henceforth, for an efficient transfer of the nurse cell materials into the oocytes. Based on our studies, FRL is under Cdc42 regulation in this tissue as well. The major conclusions of this work will be published in a manuscript that is also under preparation.

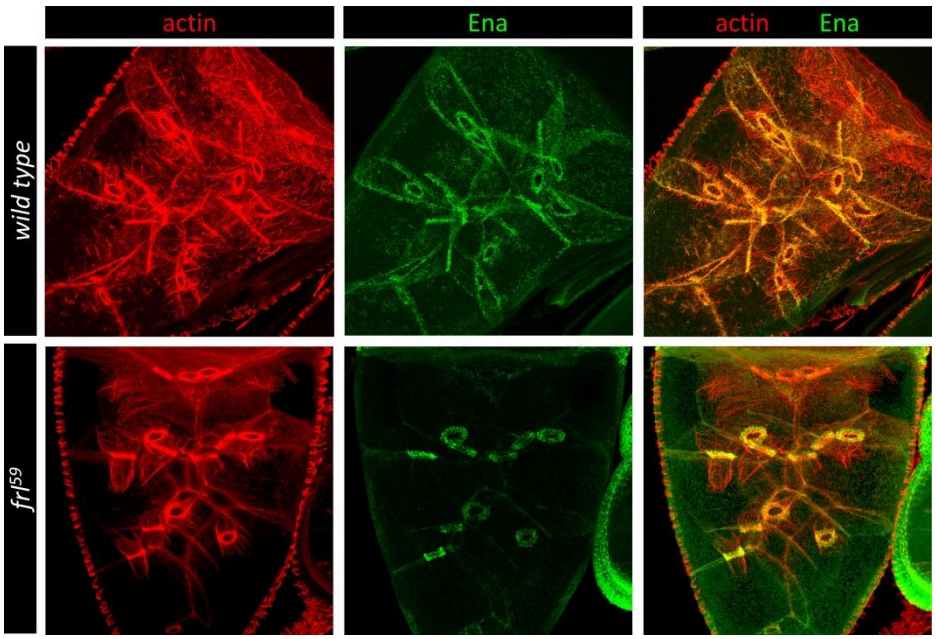

Figure 5. In the absence of the FRL protein (*frl*^{59}), the number of actin filaments running from the membrane is significantly reduced compared to the wild type

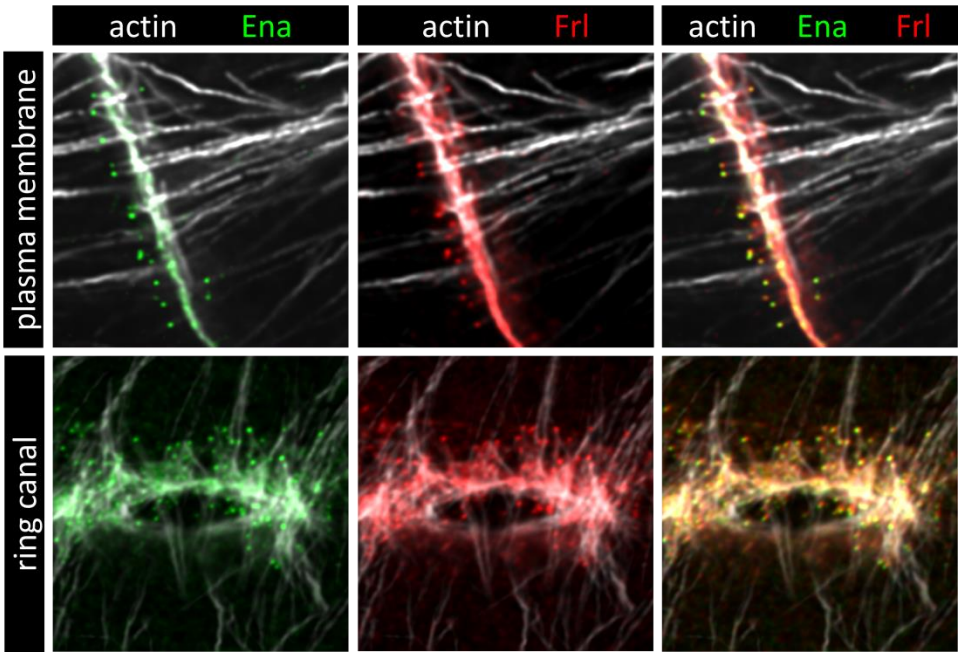

Figure 6. The DAAM and FRL proteins colocalize at the end of the actin filaments running from the plasma membrane or the ring canals.

In summary, I made significant advance as to the major goals of my application. I investigated the mechanisms of the Wnt/PCP signaling pathway during formation of the mushroom body, an important memory and learning center in the fly brain. In addition, I studied the neural role of formins, the cytoskeletal effector proteins of the PCP signaling cascade. These studies also led me to unexpected novel discoveries, and I advanced with two of these new lines resulting in exciting new findings, in particular with regard to neuro-epithelial development in the eye. My work contributed to the publication of 3 papers in respected international journals (Cell Reports, Development and Cells), and two additional manuscripts of mine are under preparation.