

Membrane trafficking in *Drosophila* wing and eye development

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It is clear that membrane transport is essential to the proper sorting and delivery of membrane bound receptors and ligands, and secreted signaling molecules. Molecular genetic studies in Drosophila are particularly well suited to studies of membrane transport in development. The conservation of cell signaling pathways and membrane transport molecules between Drosophila and other species makes the results obtained in these studies of general interest. In addition, the ability to generate gain- and loss-of-function genetic mutations of various strengths, and the ability to generate transgenic flies that direct protein expression to tissues during development are of particular advantage. Several recent papers suggest that interesting and novel roles for membrane transport processes will be uncovered by studying classically defined membrane transport proteins in developmental contexts. Together these studies suggest that regulation of membrane transport may represent an additional mechanism to regulate the strength of cell–cell signaling during development.

Key words: exocytosis / endocytosis / SNARE / NSF / p47 / Hrs / signaling

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Introduction

The adult *Drosophila* eye and wing arise from primordial tissues known as imaginal discs. In larval stages the cells of the discs proliferate, and are specified and patterned. Thus the final adult structure is determined earlier in the fly's life. The transformation of the undifferentiated epithelial imaginal disc to a com-

plex patterned tissue requires an enormous amount of intercellular signaling. Therefore the *Drosophila* eye and wing have long served as developmental models of compartmentalization, cell specification and patterning. Indeed, founding members of several important signaling pathways that are conserved across many species, such as Wingless, Notch, and Hedgehog, were first identified by studying genetic mutants in *Drosophila* that perturb the development of these tissues.

In developmental contexts inter-cellular signaling occurs primarily by the interaction of transmembrane ligands with transmembrane receptors or by secreted signaling molecules interacting with receptors on cells near to, or far from, the source of the molecule. Therefore proper intra-cellular trafficking of secreted molecules, ligands and receptors is essential for proper inter-cellular signaling and thus development of the tissue.

Recent studies directed at understanding the mechanisms by which intracellular trafficking can effect development of the *Drosophila* eye and wing have revealed that subtle perturbations of the trafficking machinery can produce profound impacts on development of the adult structures. Collectively these studies reinforce the idea that regulation of the trafficking pathway may represent an additional layer of regulation in intracellular signaling pathways. The focus of this paper will be to review these recent advances in the study of intracellular trafficking in developmental contexts.

Trans-endocytosis of Notch and Delta in *Drosophila* eye development

The early finding¹ that *Drosophila* temperature sensitive mutants of the endocytotic protein dynamin,^{2,3} encoded by the *shibire* locus, give rise to developmental defects was among the first indications of the importance of membrane trafficking to development. Early work in the developing *Drosophila* eye showed that

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1084-9521 / 02 / \$- see front matter

internalization of the sevenless tyrosine kinase receptor and its ligand, bride of sevenless, was an important step in the development of the R7 photoreceptor.⁴ The importance of endocytosis to the Notch pathway was demonstrated by Seugnet *et al.*⁵ who showed a genetic interaction between *shibire* and *Notch* mutant alleles in bristle development. Thus, there were several indicators of the importance of endocytosis to development in general, and to the Notch pathway in particular.

The Notch signaling pathway, first described in *Drosophila*, is a widely conserved signaling cascade used in cell fate specification.^{6,7} The Notch pathway acts primarily by inhibiting cells within a field from adopting a particular fate, a process called lateral inhibition. In the 'core' Notch pathway ligands, such as Delta and Serrate in *Drosophila*, interact with extracellular EGF motifs of the Notch receptor. Activation of Notch leads to proteolytic cleavage of full-length Notch and translocation of the Notch intracellular domain to the nucleus where it acts with Suppressor of Hairless to activate the transcription of downstream target genes, such as the Enhancer of Split complex.^{6,7}

Recent work from Muskavitch and co-workers suggest that an important part of activating the Notch signaling cascade is endocytosis of the Notch extracellular domain into the ligand expressing cell.⁸ This process is called *trans*-endocytosis. While the dynamics of Delta trafficking have been known for some time,⁹ and that improper trafficking of Delta leads to impairment of Notch signaling,⁹ the direct influence of endocytosis of Delta upon activation of Notch signaling was not known.

Parks *et al.*⁸ first show that in developing wild-type eyes that the Notch intracellular domain (Notch^{ICD}) and the Notch extracellular domain (Notch^{ECD}), though initially expressed in the same cell, are differentially localized during development, implying their differential trafficking. Indeed Notch^{ECD} becomes localized in cells not previously expressing it. Furthermore, the Notch^{ECD} co-localizes with Delta in endocytic vesicles and the incorporation of Notch^{ECD} fails when the temperature sensitive mutant of dynamin, *sh^{ts1}*, is held at restrictive temperature for short periods of time. Thus, Notch^{ECD} is *trans*-endocytosed into neighboring cells that express Delta.

Using a genetic allele of Delta, *Δ^{RF}*, that exhibits trafficking defects they also show that the normal translocation of Notch^{ECD} is disrupted. Finally, another allele, *Δ^{CE9}*, which is a point mutant in the third EGF repeat of Delta, fails to initiate *trans*-

endocytosis of Notch in cell culture assay, and blocks Notch signaling when expressed in the developing wing.

These intriguing data show that endocytosis of Notch^{ECD} into the ligand expressing cell is required for activation of the Notch pathway in the Notch expressing cell. Several models could account for this possibility, including: (1) once Notch is bound to Delta, endocytosis of Delta exerts mechanical forces on Notch necessary to expose the cleavage site(s) required to release Notch^{ICD}; or (2) separation of Notch^{ECD} from Notch^{ICD} is required to release Notch^{ICD}. Consistent with this model is the observation that secreted Delta, and Delta lacking its intracellular domain act as suppressors of Notch signaling.^{10,11} In contrast, in at least one instance, an extracellular fragment of Delta is reported to activate Notch.¹²

While it is surprising that endocytosis of part of the receptor into the ligand expressing cell is necessary for activation of the signaling pathway, the similarity between *trans*-endocytosis of Notch and Delta and the endocytosis of the Boss/Sevenless ligand receptor complex⁴ suggests that internalization of large protein complexes may be generally important to developmental cell signaling.

The role of SNARE proteins in signaling at the *Drosophila* wing margin

While endocytosis has been recognized in the literature as an important contributor to development, molecules involved in the exocytotic pathway have recently come to the fore in developmental contexts. The SNARE (soluble NSF attachment protein receptors) proteins are a group of three proteins that are thought to form the minimal machinery required for fusion of transport vesicles with target membranes.¹³ The three proteins are from the VAMP/Synaptobrevin, Syntaxin and SNAP-25 families. Together they can form a very stable complex that consists of four parallel alpha-helices.^{14,15} It is currently thought that VAMP on the vesicle interacts with Syntaxin and SNAP-25 on the membrane to form a *trans*-membrane complex and that formation of this 'SNARE complex' may provide sufficient energy to cause fusion of lipid bilayers. One indication of the specific role of SNAREs in a developmental process was the finding that severe reduction in Syntaxin expression leads to the failure of cellularization of the *Drosophila* embryo.¹⁶

It follows that after membrane fusion the SNARE complex resides in a single membrane and that the complex needs to be broken apart to allow the proteins to be used in further rounds of membrane fusion. *N*-ethylmaleimide sensitive fusion protein (NSF) is an ATPase that can bind the SNARE complex through an adaptor called α -SNAP. Upon hydrolysis of ATP by NSF the SNARE complex breaks apart.¹⁷

A dominant negative form of NSF can be engineered by mutating a single amino acid in the ATP binding region of the ATPase domain.¹⁸ To analyze the role of SNARE dependent membrane trafficking in *Drosophila* wing development, a dominant negative *Drosophila* NSF2 (dNSF2) isoform was constructed¹⁹ and its expression directed to the developing wing margin using the Gal4–UAS system.²⁰ This resulted in developmental defects in the wing such that the wings were notched (Figure 1). Importantly, the wing phenotype was also enhanced by single copy mutations of the other SNARE genes, *synaptobrevin* and *syntaxin* confirming that other mutations affecting membrane trafficking can enhance the phenotype. The loss of wing margin phenotype is reminiscent of phenotypes obtained with certain mutant alleles of the *Notch*, *Serrate*, and *wingless* genes. Indeed, there was genetic enhancement of the wing phenotype when the *dNSF2* mutant transgene was introduced into *Notch*, and *wingless* mutant backgrounds indicating the *dNSF2* mutant disrupted these signaling pathways.

To confirm this, immunocytochemistry was performed on third instar imaginal wing discs to examine Wingless and Notch, and some of the downstream targets of these signaling pathways. Interestingly all of these markers for wing development were abnormal indicating signaling at the top of the cascade was disrupted. Direct evidence that both pathways are affected comes from the observation that both Wingless and Notch proteins are mislocalized in the dNSF2 mutant wing discs and that a direct target of the Notch pathway, the Vestigial boundary enhancer, is downregulated.

Perhaps the most interesting result to come out of the study of dNSF2 mutant wing discs is the finding that they provide an exquisitely sensitive background that can be used in modifier screens. In this study, *big brain* and *porcupine*, genes previously known to be involved in Notch and Wingless signaling respectively, were shown for the first time to be involved in wing margin development. Thus the partial disruption of membrane trafficking yields a genetic background which can be used to screen for molecules involved in the development of that tissue (Figure 2).

The dNSF2 study focused on wing margin development and the role of Notch and wingless proteins in that process. An outstanding issue that remains to be resolved is determining to what degree the dNSF2 mutants effect Notch and Wingless specifically, or is the effect one common to other signaling molecules.

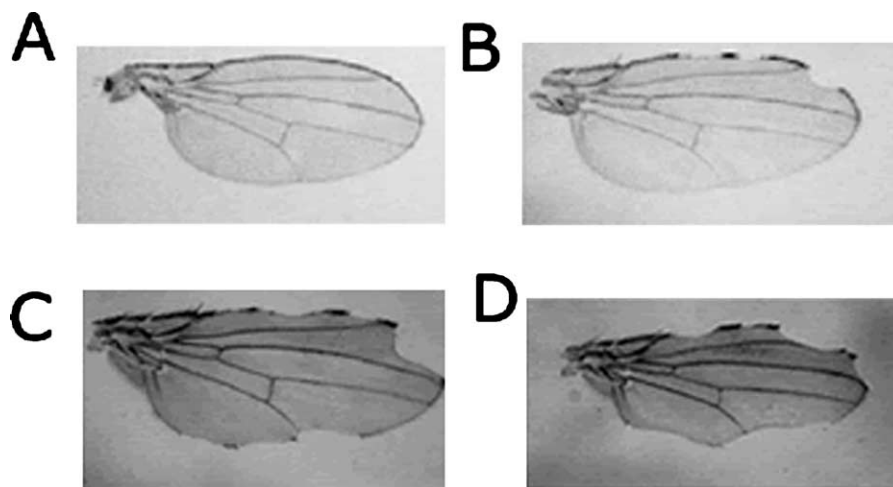


Figure 1. Dominant negative dNSF2 impairs wing margin development. (A) Wild-type *Drosophila* wing. (B) Wing from *Drosophila* expressing dominant negative dNSF2 along the wing margin. There are clear nicks in the wing. (C) The wing phenotype is enhanced when the dNSF2 transgene is placed in combination with a genetic mutant of wingless (*wg*¹⁻¹⁷) and with a mutant in (D) Syntaxin (*syx*^{L371}) showing genetic interaction with these two genes. These data were previously published.⁹

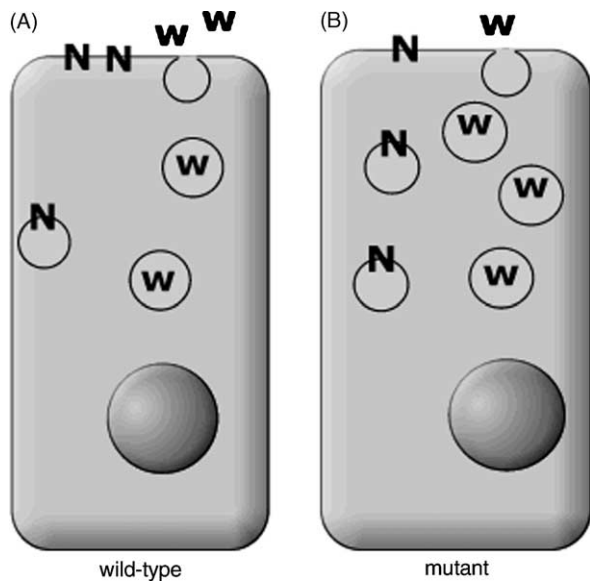


Figure 2. Model for the effects of dominant negative dNSF2 on wing margin development. (A) Notch (N) is normally delivered to the plasma membrane and wingless (w) is normally secreted from wing margin cells. (B) Sub-lethal disruption of membrane trafficking leads to reduced Notch and Wingless transport and the intracellular accumulation of the proteins. Further reductions in either the signaling pathway genes or membrane transport genes genetically enhances the phenotype.

For example, there are other secreted and transmembrane modulators of Notch signaling that could be involved in the phenotypes reported. These include the secreted proteins Fringe and Braniac, and the transmembrane modulator Big Brain. Whether these molecules are similarly affected remains unclear.

The generality of the effects on patterning may be tested by expressing the mutant dNSF2 in other domains. This study concentrated on the presumptive wing margin, which is also the dorsal-ventral boundary of the wing, but it could also be used to test anterior-posterior patterning. For example, the major A/P morphogen is the secreted protein Decapentaplegic²¹ (Dpp). It seems likely that disruption of Dpp secretion will have profound impacts on wing development just as disruption of the major D/V morphogen, Wingless, does. Lastly, the dominant negative dNSF2 transgene is likely to be a useful tool to study the importance of membrane trafficking in other developmental tissues. As one example, in *Drosophila* there is intercellular signaling between the germ line cells and supporting follicle cells important to oocyte development.²² Expression of the

mutant dNSF2 transgene in these cell types may reveal mechanisms of membrane transport important to development in that tissue.

Role of P47, an α -SNAP homologue, in *Drosophila* eye development

The adult *Drosophila* eye consists of an array of approximately 800 ommatidial units. Each ommatidium contains eight photoreceptor cells and four cone cells and a number of support cells. The photoreceptors are arranged in a trapezoidal array with their apical surfaces facing the interior of the structure. The rhabdomeres are apical surface membrane expansions of the photoreceptor cells containing the photosensitive molecule rhodopsin. Development of the rhabdomere requires a great amount of membrane trafficking to generate the expanse of membranous folds.

A very recent study has demonstrated the importance of another ATPase system involved in membrane fusion for development of the *Drosophila* eye.²³ The *eyes closed (eyc)* gene was isolated because of its effects on rhabdomere morphogenesis. The main phenotype of the *eyes closed* mutant is fragmented rhabdomeres with inappropriate adhesions joining adjacent rhabdomeres. Cloning of the *eyes closed* gene revealed that it encodes a homologue of p47, a co-factor that regulates the ATPase, p97.^{24,25}

Like α -SNAP and NSF, p47 and p97^{26,27} act on SNARE complexes to break apart unproductive *cis*-SNARE complexes allowing them to form functional *trans*-SNARE complexes. The main difference between the two ATPase molecules and their co-factors appears to be that α -SNAP and NSF act primarily on SNARE complexes resulting from heterotypic membrane fusion while p47 and p97 act on those complexes resulting from homotypic membrane fusion. Exceptions to this generality exist, and in fact, the NSF and p97 pathways may cooperate in the reassembly of mitotic Golgi fragments^{24,28} and both α -SNAP and p47 can bind to syntaxin-5 containing SNARE complexes.²⁹

Cellular analysis of photoreceptor development in *eyc* mutants revealed that the photoreceptors develop normally until about 55% of pupal development. At this time the photoreceptors normally release contacts between their apical surfaces whereas in the *eyc* mutants these contacts are abnormally maintained. This observation was confirmed by immunocytochemical analysis of adhesive proteins found at this junction, such as Armadillo and Crumbs, which showed that

these proteins persist at the junctions when they would normally be cleared.

Molecular analysis of the *eyc* ORF did not reveal any point mutations while analysis of the 3' end of the gene revealed two potential nucleotide substitutions. Similarly, a transposable P-element that is allelic to the original *eyc* mutation is inserted 3' to the ORF stop codon. Together these data suggest that the *eyc*¹ mutation is one that affects regulation of the gene. Interestingly, the phenotype of the *eyc* mutant was mimicked by misexpression of the wild-type protein under heat shock control. The severity of the heat shock induced phenotypes correlated with the number of heat shocks applied throughout development.

Since p97 has previously been implicated in trafficking of vesicles from the endoplasmic reticulum,^{26,30} the ER was examined in flies overexpressing *eyc*. An abundance of ER was found in those flies with a large increase in the number of ER stacks observed. This was further correlated with an increase in the amount of immature rhodopsin, assayed by Western blot. Together these results suggested a disruption in vesicle trafficking from the ER may lead to the *eyc*¹ phenotype.

Since *eyc* encodes a p47 homologue, which regulates the function the p97 ATPase, it seems likely that the *eyc*¹ phenotype may result from sequestering p97, preventing it from carrying out its normal functions. Previous *in vitro* studies on p97 function revealed the importance of a stoichiometric relationship between p47 and p97,²⁵ thus the overexpression of p47 will influence p97 function in the developing *Drosophila* eye. It also remains possible that the excess p47 stabilizes unproductive SNARE complexes as can occur when the yeast α -SNAP homologue, Sec17p is overexpressed.³¹

Like the study of dNSF2 dominant negative expression at the wing margin,⁹ the study of *eyc* revealed nuanced phenotypes that would not be predicted given the known function of the protein. Furthermore, it may be instructive to create transgenic lines with mutations in the p97 ATPase domain to dampen, but not eliminate, p97 activity.

While on the external surface the *eyc*¹ eyes appear normal, cellular analysis of the photoreceptors revealed alterations in membrane specialization and vesicular trafficking that could not have been observed in the complete loss-of-function mutants. It is presently not clear why overexpression of *eyc* leads to a failure of the photoreceptor cells to release their contacts at their apical surfaces. The failure of the cells to clear adhesive proteins likely implies the lack of the machinery normally used in this process. Fur-

ther analysis of the molecular mechanisms underlying this phenotype will yield interesting new functions for p47/p97 ATPase activity.

Hrs, a regulator of receptor tyrosine kinase trafficking

It is clear that manipulating the important components of exocytotic and endocytotic membrane trafficking pathways can have profound impacts upon development and this suggests that regulation of the transport pathway itself may be important for development. In support of this idea a recent study shows that hepatocyte growth factor-regulated tyrosine kinase substrate (Hrs), a regulatory protein that binds SNAP-25, one of the SNARE proteins, is an important regulator of tyrosine kinase receptor trafficking to the endosome.³²

Hrs was first identified as a protein that is phosphorylated in response to hepatocyte growth factor³³ and later identified as a SNAP-25 binding partner by yeast two-hybrid analysis.³⁴ Hrs has been shown to inhibit the formation of the SNARE complex *in vitro*³⁴ and expression of Hrs in PC12 cells inhibits Ca²⁺ dependent exocytosis³⁵ suggesting a role for this protein in exocytosis. However, Hrs is localized to endosomes, and can also interact with Eps15,³⁶ a protein required in receptor-mediated endocytosis, further suggesting that the protein may also be important for endocytosis.

Lloyd *et al.*³² investigate the function of Hrs by analyzing the *Drosophila* gene. While there was some localization of the protein to neuromuscular junctions in wild-type animals, there was no apparent functional phenotype at this synapse in mutants that live until pupae. Turning to the garland cells, a tissue with a high rate of membrane trafficking, they observed enlargement of endosomes by light microscopy and, by electron microscopy, failure of the endosomes to invaginate and form multivesicular bodies.

To examine the developmental consequence of Hrs loss, they next examined a fly strain in which the germline cells were homozygous mutant for the Hrs gene. Embryos lacking maternally derived Hrs are lethal. In such embryos the activity of two receptor tyrosine kinases, Torso and EGFR, was examined. Using a combination of immunocytochemistry and Western blot, it was apparent that there is misregulation of both receptors in Hrs mutants, with reduced degradation of Torso and EGFR leading to prolonged and spatially disrupted signaling from the two receptors.

These results suggest that, in *Drosophila*, Hrs has a major role in endosomal trafficking, and in development plays a role in attenuating signals from the tyrosine kinase receptors Torso and EGFR. Hrs has been proposed to have roles in exocytosis and endocytosis, as well as endosomal trafficking, and it therefore remains to be determined if, in other developmental contexts, Hrs may have other important modulatory roles.

Conclusion

The aim of this paper has been to advance the idea that membrane transport pathways may have a role in regulating the strength of intercellular signaling in developmental contexts. While proteins that modulate some of the signaling pathways directly are well known, for example Fringe, Numb and Big Brain are modulators of Notch signaling,³⁷ it is becoming evident that regulated changes in the efficiency of exocytosis or endocytosis may represent another layer of complexity in regulating the overall strength of cell–cell signaling.

Why might this type of regulation be important? One possibility is that if all receptors present on the cell surface are working at peak efficiency, one way to increase signaling strength is to add more receptors. This is analogous to the finding that rapid, NSF-dependent, trafficking of neuronal glutamate receptors is an important mechanism for controlling the strength of communication between neurons.³⁸ On the other hand, in the case of too much receptor activity, it may be beneficial to simply remove some of the cell surface receptors rather than trying to modulate the function of all of them. Thus regulated trafficking of receptors and ligands in development pathways will likely be important for the strength cell–cell signaling.

Another potentially important scenario for regulated trafficking is that receptors are critical to shaping the concentration gradients of secreted ligands (see Cadigan, this issue). It is therefore possible that regulated movement of these receptors may be a means of controlling the shape of morphogenic concentration gradients. Since molecules such as Wingless are known to be involved in regulatory feedback loops,³⁹ fine-tuning either secretion of the molecule or insertion of the membrane bound receptor may be an efficient way of maintaining signaling strength within a physiological range.

Future studies aimed at bringing together the roles of membrane transport pathways in specific develop-

mental contexts, and trying to understand the potential role of membrane transport regulation, will yield interesting new models on the importance of membrane trafficking to development.

Acknowledgements

I thank D. Ready, T. Lloyd and H. Bellen for providing preprints of their papers prior to publication and G. Boulianne and T. Lloyd for comments on the manuscript.

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