In vivo analysis of exosome trafficking and function in *Drosophila melanogaster*

Senior thesis

Presented to

The Faculty of the School of Arts and Sciences
Brandeis University

Undergraduate Program in Biology
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In partial fulfillment of the requirements for the degree of Bachelor of Science

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May 11, 2016

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Table of Contents

1 Abstract 4

2 Introduction 5

2.1 Exosomes: function and therapeutic applications 6

2.2 Exosome cargo and targeting specificity 6

2.3 Exosomes and the endocytic process 7
  2.3.1 Rab proteins play a role in exosome trafficking in the donor cell 7
  2.3.2 SNARE machinery is necessary for exosomal trafficking from the donor cell 8
  2.3.3 Exosome uptake by the recipient cell 9

2.4 *Drosophila* as an *in vivo* model organism to study exosome function and trafficking 10
  2.4.1 Physiological function for neuronal exosomes in *Drosophila* 10
  2.4.2 Organismal level of exosome trafficking 11

2.5 Current methods and techniques in exosome research 12
  2.5.1 Biochemical approaches for exosome analysis 13
  2.5.2 Genetic and molecular biology approaches for *in vivo* exosome research 14

3 Methods 16

3.1 Molecular cloning 16
  3.1.1 Construction of pattB-13xLexAop2-Sun-5Myc-spGFP11 16
  3.1.2 Construction of pUAS-tattB-spGFP1-10-WPRE 16
  3.1.3 Construction of pUAS-tattB-prolact-3xHA-Lamp1TM-spGFP1-10 and pUAS-tattB-prolact-spGFP1-10-Lamp1TM-3xHA 16

3.2 Fly stocks 17
  3.2.1 Transgenic stocks 17

3.3 Antibodies 18

3.4 Immunohistochemistry and imaging 18

3.5 Transmission electron microscopy 18

3.6 S2 cell transfection, preparation and imaging 19
4 Results

4.1 Identification of a v-SNARE required for presynaptic exosome release at the larval NMJ 20
4.2 Identification of postsynaptic molecules involved in neuronal exosome uptake, trafficking and/or degradation 22
   4.2.1 Diversity of exosomal cargo trafficking revealed 26
4.3 Rationale for split-GFP reporter system to visualize exosomal trafficking in Drosophila 31
   4.3.1 Split-GFP reporter system in S2 cells 32
   4.3.2 Split-GFP reporter system at the larval NMJ 35

5 Discussion 42

5.1 Ykt6 is necessary for neuronal exosome trafficking 42
5.2 Post-synaptic Amphiphysin regulates exosome trafficking 43
5.3 Organismal exosome effects 45

6 References 47
1 Abstract

Exosomes are small secretory vesicles (50-150 nm in diameter) of endocytic origin that have been recently identified as a novel intercellular communication pathway. They carry select cargo that impact the recipient cell’s metabolism and signaling pathways. The tissue-specificity of targeting and intracellular trafficking after uptake to the target cell are not fully understood and likely involve novel molecular cross-talk. Given that cultured tissue cells are used for the majority of exosome research (out of necessity), the Drosophila melanogaster larval neuromuscular junction (NMJ) offers a truly unique in vivo experimental system to address the biology of exosomes including their formation, release, uptake and trafficking. Here, I identify the membrane remodeling protein Amphiphysin as required for exosome trafficking in the recipient muscle cell. Furthermore, I show that the v-SNARE Ykt6 plays a role in neuronal exosome release. Additionally, I provide evidence that suggests a cargo-dependent exosome trafficking mechanism in the recipient muscle cells. Finally, I also developed a split-GFP reporter system to visualize post-synaptic exosomal trafficking and describe its in vivo application.
2 Introduction

Exosomes have recently emerged as a novel and important component of intercellular signaling pathways. Exosomes are small secretory vesicles (50-150 nm in diameter) of endocytic origin, and carry select cargo that impact the recipient cell’s metabolism and signaling pathways. In the endocytic pathway, extracellular material is trafficked into early endosomes and packaged into multi-vesicular bodies (MVBs) that then can fuse with either lysosomes (resulting in degradation of the cargo), or with the plasma membrane (resulting in the release of intraluminal vesicles (ILVs) from the cell as small vesicles, including exosomes) (Vlassov et al., 2012).

Figure 1 Biogenesis and secretion of exosomes in the cell (Robbins and Morelli, 2014)
2.1 Exosomes: function and therapeutic applications

Exosomes represent a fundamentally novel signaling and communication pathway: they have been shown to take part in many physiological processes including immune responses, tumor progression, and neurodegenerative disorders (Zhang et al., 2015) by regulating key signaling pathways involved in apoptosis, metastasis, and angiogenesis. Given their endogenous origin and therefore “high biosafety profile” (El Andaloussi et al., 2013), the biomedical fields are exploring their tremendous potential as an alternative to viral drug delivery. However the mechanisms that drive exosome formation, cargo sorting, release, uptake and trafficking after internalization are still not well understood. Thus, elucidating these pathways would be key to our understanding their biological function and to develop the exosomal system for successful targeted therapies in many human diseases.

2.2 Exosome cargo and targeting specificity

The existence of different sets of exosomal surface/adhesion molecules and cargo suggests that exosomal content and surface molecules play a role in directing sorting and trafficking (Villarroya-Beltri et al., 2014). Proteomic profiling of exosomal proteins has provided evidence for the presence of both general and cell type-specific proteins in exosomes. All exosomes originate from the endocytic pathway, so they share proteins involved in MVB biogenesis such as membrane transport and fusion proteins like integrins or tetraspanins (e.g. CD63, CD9, CD82) (Braicu et al., 2015; Vlassov et al., 2012). These transmembrane proteins that have been reproducibly identified in exosomes derived from multiple cell lines are often used as exosomal markers. On the other hand, certain exosomes are enriched in specific proteins, lipids and RNAs that are absent in other exosomes derived from either the same or different cell lines (Smith et al., 2015; Villarroya-Beltri et al., 2014).

One interesting hypothesis is that exosomes function to deliver molecules that perform specific functions that are not normally inherent to the recipient cell. A proteomic analysis
detected that hepatic specific reporter ASGR was enriched in exosomes derived from non-tumorous hepatocytes (Conde-Vancells et al., 2008). These hepatocyte exosomes also contained proteins that play an important role in cellular detoxification such as cytochromes P450 and glutathione S-transferases. Many of the same detoxification enzymes were found in tissues from different parts of the body such as the lung stomach, small intestine and colon. This led the authors hypothesize that the exosomes function to facilitate intercellular protein exchange to confer these enzymatic activities to non-hepatocyte cells (Conde-Vancells et al., 2008). Overall, these studies support the existence of specialized mechanisms that control the sorting of molecules into exosomes and subsequent trafficking that is partially dependent upon receptor complexes on both the exosome and recipient cell.

2.3 Exosomes and the endocytic process

2.3.1 Rab proteins play a role in exosome trafficking in the donor cell

Endocytosis-related protein families such as Rab proteins, SNARES, and Sec1/Munc-18 related proteins (SM-proteins) are essential in facilitating vesicle fusion intracellularly (Mulcahy et al., 2014). Rab proteins are small lipid modified GTPases belonging to the family of Ras monomeric G proteins (Zerial and McBride, 2001) that are essential during the regulation of membrane trafficking, vesicle formation, transport, targeting and fusion within the endocytic system. Rab proteins are part of a complex that mediate the intracellular trafficking of vesicles and are essential in the trafficking prior to exosome release in the donor cell. Studies have shown that several Rab proteins are necessary in the exosome pathway - the first of which, Rab11 identified in K562 cell line, decorates the limiting membrane of MVBs (Savina et al., 2002). At the Drosophila larval neuromuscular junction (NMJ) the release of Evenness Interrupted (Evi), a transmembrane protein trafficked in exosomes, from the donor cell is dependent on Rab11 (Koles and Budnik, 2012). Additional Rab proteins are implicated in exosome trafficking, though not each protein that is essential in one cell line is essential in
another, including Rab27 (Ostrowski et al., 2010) and Rab35 (C et al., 2010). Rab27 and Rab35 knockdowns did not affect exosome release in S2 cells (Koles et al., 2012) though Rab27 was implicated to do so in HeLa cells (Ostrowski et al., 2010) and Rab35 in oligodendroglial cells (C et al., 2010). These observations hint at a certain specificity in this system that depends on exosome and cell surface/transmembrane proteins, content, and cell line origin to dictate exosomal trafficking and uptake activity.

Additionally, Nervous Wreck (Nwk), a membrane remodeling F-BAR/SH3 domain protein that plays a role in the endocytic process (Rodal et al., 2011), co-localizes with Rab11 (Rodal et al., 2008) and the mutant form of either protein leads to the lack of exosome release from the bouton at the *Drosophila* NMJ (K.K, A.Y., A.R., Unpublished data). This observation indicates that a complex endocytic sorting machinery may function in exosome trafficking in the neuron.

### 2.3.2 SNARE machinery is necessary for exosomal trafficking from the donor cell

*Figure 2.* Docking and fusion of a vesicle through a SNARE-mediated mechanism (Bryant et al., 2002)

Protein trafficking within the endosomal pathway requires the sorting, packaging and budding of vesicles followed by their transport, targeting, docking and fusion with the correct target membrane. SNAREs (Soluble NSF Attachment Protein Receptor) are required for these
intracellular fusion events, as they mediate plasma membrane fusion between the vesicle (containing vesicular v-SNARE) and target membranes (containing t-SNARE) (Littleton, 2000). The release of exosomes from the donor cell after MVBs fuse with the plasma membrane also depends on such v- and t-SNARE protein interactions, as VAMP7 (a v-SNARE) has been identified in K562 cells to be involved in this fusion process (Fader et al., 2009b). In Drosophila larval wing disc and in human cultured cells, the v-SNARE Ykt6 (of the Longin type), has been shown to be required for the exosomal release of Wg from the donor cell (Gross et al., 2012). At the larval NMJ, the t-SNARE Syntaxin1A (Syx1A) plays a role in the release of Evi containing exosomes from the donor neuronal cell (Koles et al., 2012). Highlighting the need for further research in this area, the cognate t- or v-SNARES of the listed proteins have not been identified yet, though the idea that Ykt6 might interact with Syx1A during neuronal exosome release is an intriguing possibility.

2.3.3 Exosome uptake by the recipient cell

Multiple mechanisms participate in exosome uptake by the recipient cell, including clathrin and other protein-dependent endocytosis, phagocytosis, micropinocytosis, and lipid raft-mediated uptake (Mulcahy et al., 2014). Membrane trafficking is heavily involved in exosome uptake and is integral to exosome activity and release. Endocytosis in particular plays an essential role in exosome uptake and intracellular trafficking post-uptake in the recipient cell. There is an expansive list of proteins that have been characterized to facilitate exosome-recipient cell interactions and subsequent endocytosis including clathrin, adhesion molecules (tetraspanins and integrins), and fibronecin (Mulcahy et al., 2014). Depolymerization of the actin filament network using cytochalasin D reduced exosome uptake, which supports endocytosis-dependent internalization, as it relies on a functioning cytoskeleton (Johnstone et al., 1987; Mulcahy et al., 2014; Svensson et al., 2013). After uptake, exosomes are engulfed
and either merge with endosomes that become lysosomes for subsequent degradation, or undergo transcytosis which involves moving exosomes to neighboring cells (Zhang et al., 2015).

2.4 *Drosophila* as an in vivo model organism to study exosome function and trafficking

![Diagram](image)

**Figure 3.** Pre-synaptic and post-synaptic exosome trafficking (Koles and Budnik, 2012)

2.4.1 Physiological function for neuronal exosomes in *Drosophila*

The *Drosophila* neuromuscular junction (NMJ) provides an exceptional model to visualize trafficking and the organism is easily genetically manipulated. Exosomal trafficking in the nervous system is an important area of study, as disruptions in intercellular communication
are implicated in many developmental and neuronal diseases (Zhang et al., 2015). The secretion and uptake of neuronal exosomes in vivo was first characterized in Drosophila at the larval NMJ (Koles et al., 2012; Korkut et al., 2009). An important player in this system is Evi, observed to be released at the synapse in vesicles that were characterized as exosomes (Koles et al., 2012). Evi is found in MVB’s at the pre-synapse and MVBs move to the presynaptic plasma membrane. Exosomes are released to the extracellular space after reaching the postsynaptic junction (Koles and Budnik, 2012). Wnt1 or Wingless (Wg) is a hydrophobic signaling molecule whose role has been implicated in neuronal development and in many diseases. It is found in the hydrophilic extracellular space in the larval nervous system, likely due to its packaging in exosomes. In the Wg trafficking model, Evi binds Wg and this allows for its transport via exosomes (Korkut et al., 2009). It is hypothesized that the secreted neuronal exosomes are taken up by muscle cells.

2.4.2 Organismal level of exosome trafficking

Moreover, exosomes have the ability of long distance trafficking throughout an organism. In a recent study, authors observed that increasing expression of molecular chaperones such as heat shock proteins Hsp40 and Hsp70 led to more efficient protein homeostasis in cultured cells and in Drosophila (Takeuchi et al., 2015). These molecular chaperones typically detect unnatural forms of the proteins and assist with their proper folding to prevent aggregation. They first established that Hsp40 is secreted through exosomes and then used muscle and fat body drivers to express these heat shock proteins in Drosophila in a mutant huntingtin (htt) background. The mutant htt protein causes photoreceptor degeneration in the eyes, which was used as an indicator for proteotoxicity. In these mutant htt flies, they saw suppression of photoreceptor degeneration, indicating that muscle or fat body exosomes were trafficked to the eye where the Hsps promoted proper folding of the mutant huntingtin proteins. Thus, they demonstrate that trafficking of molecular chaperones occurs through an exosomal pathway and
promotes “non–cell-autonomous” maintenance of protein homeostasis in an organism. This data provides evidence that exosome trafficking and delivery occurs not only across long distances, but also between different cell types to regulate protein homeostasis. With further in vivo studies, these results have tremendous implications for the use of exosomes in therapeutics as a targeted drug delivery molecule.

2.5 Current methods and techniques in exosome research

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<tr>
<td>Purification</td>
<td>Ultracentrifugation</td>
<td>(Momen-Heravi et al., 2013)</td>
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<td>Commercial precipitation reagent</td>
<td>(Van Deun et al., 2014)</td>
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<td>Sucrose gradient centrifugation</td>
<td>(Conde-Vancells et al., 2008)</td>
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<td>Microfiltration</td>
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<td>Antibody-coated magnetic beads</td>
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<td>Microfluidic devices</td>
<td>(Momen-Heravi et al., 2013)</td>
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<td>Exosome composition profiling</td>
<td>Raman spectroscopy (single exosome)</td>
<td>(Smith et al., 2015)</td>
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<td>Mass spectrometry</td>
<td>(Conde-Vancells et al., 2008)</td>
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<td>Microarray assessments</td>
<td>(Valadi et al., 2007)</td>
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<td>Proteome profilers (antibody arrays)</td>
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<td>(Mehdiani et al., 2015)</td>
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<td>Transmission electron microscopy (TEM)</td>
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<td>Enzyme linked immune-sorbent assays (ELISA)</td>
<td>(Franquesa et al., 2014; Wang et al., 2015a)</td>
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<td>Dynamic Light Scattering (DLS)</td>
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<td>Western blot (WB)</td>
<td>(Lässer et al., 2012)</td>
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<td>Delivery</td>
<td>Luciferin/Luciferase assay</td>
<td>(Soares et al., 2015)</td>
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<td>Visualization of exosome fate</td>
<td>Fluorescence microscopy</td>
<td>(Tian et al., 2010)</td>
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<td>TIRF microscopy</td>
<td>(Svensson et al., 2013)</td>
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Table 1. Current methods used to study exosome biogenesis, targeting, uptake, and post-uptake trafficking.
2.5.1 Biochemical approaches for exosome analysis

Visualizing exosomes using fluorescence microscopy in fixed and live imaging has been used in almost all studies on exosomes (Mulcahy et al., 2014). One method to follow exosome trafficking is through fluorescent lipid membrane dyes to stain membranes (Mulcahy et al., 2014). Subsequent entry of exosomes into recipient cells can be measured using methods such as flow cytometry, western blotting and confocal microscopy to measure fluorophore intensity (Mulcahy et al., 2014). However, it is possible that the fluorescent molecules affect normal exosomal uptake activity. Also there is a limited resolution with our current technology and single or groups of exosomes cannot be completely resolved if they are less than 200nm in diameter (Mulcahy et al., 2014). Using fluorescently tagged exosomal proteins as markers is a more specific way to visualize exosomes but again, there is a worry that these tags may affect normal exosome function and trafficking.

In light of these considerations, a challenge the field faces is to define novel and improved methods of exosome microscopy, profiling and visualization tools. Furthermore, it would be good to have more \textit{in vivo} studies of exosomal targeting. To use exosomes as a drug delivery vehicle, we should elucidate what directs exosomes to a target, especially to determine if injected exosomes could traffic to undesired destinations and if they could cause potential off-target effects. Also, it’s important to delve further into the function of specific proteins in exosome uptake and target specificity, such as fibronectin and tetraspanins. We should also keep an open mind to the possibility that there are other mechanisms that we have not yet characterized involved in the exosomal system. Thus, exosomes have tremendous potential in therapeutics, but it’s clear that exosome activity and trafficking is complicated, as exosome formation, sorting, secretion, uptake, and fate after internalization are still not well understood in all cases. Developing new tools to study exosome trafficking will be critical to elucidating the exosomal system.
2.5.2 Genetic and molecular biology approaches for in vivo exosome research

Novel systems to study exosome trafficking in vivo have been developed and provide a good foundation to elucidate these pathways. One of this includes using the Cre-loxP method to qualitatively identify cells that have taken up exosomes (Zomer et al., 2016). This system uses the exosomal delivery of Cre recombinase from Cre-expressing cells to switch DsRed in the recipient cell to enhanced GFP, differentially marking the reporter cells that have taken up the Cre-containing exosomes and the cells that did not take up exosomes. This is an improvement upon current methods to visualize exosome trafficking with pre-labelled exosomes with fluorescent tags, as fluorescent molecules may affect normal exosome function and trafficking.

Another powerful system that was first developed in C. elegans is the GRASP system, which consists of GFP split into two fragments: splitGFP1-10 and splitGFP11 (Feinberg et al., 2008). These fragments are non-fluorescent alone and reconstitute GFP fluorescence when they interact. To study the connection between motor neurons and primary sensory neurons in the Drosophila taste circuit, the authors adapted the GRASP system to be used in Drosophila (Gordon and Scott, 2009). They expressed the system in the olfactory system with spGFP1-10 in olfactory receptor neurons and with spGFP11 in second-order projection neurons. They imaged Drosophila brains and showed that GFP reconstitution can be detected using and without using immunofluorescence. In Drosophila, this efficacy of this spGFP reporter system has many important implications. By tagging an exosomal protein in donor cells and an endosomal reporter protein in recipient cells with either half of the construct, we can visualize exosome trafficking in the fly. To increase the sensitivity of this system, which may be a concern, multiple spGFP11 molecules can be tagged onto the protein of interest (Kamiyama et al., 2016). With more spGFP11 tags, the reconstitution event is expected to be significantly brighter.
Though studies have begun to make headway into dissecting these complicated pathways, there are still many unanswered questions about the exosomal system- what is driving exosomal specificity and targeting, especially across long distances? What players are required and conserved through the cell types for exosome formation, uptake, and intracellular trafficking? Optimizing our current technology used to study exosome sorting, uptake and trafficking would benefit these endeavors and to develop therapies. These novel genetic and molecular biology approaches provide a powerful tool to study exosome trafficking and the field is now poised to elucidate exosomes’ role in intercellular communication.
3 Methods

3.1 Molecular cloning

3.1.1 Construction of pattB-13xLexAop2-Sun-5Myc-spGFP11

To generate the pJFRC19 promoter driven Sunglasses-5Myc-spGFP11 stock, pJFRC1-13XLexAop2-IVS-myr::GFP (Addgene plasmid #26224, from Gerald Rubin, Janelia Farms, Ashburn, VA, USA) was digested using XhoI and XbaI. Gibson cloning was used to insert Sun-5Myc-spGFP11, amplified from pattB-nSyb-5Myc-spGFP11 (Kate Koles, Brandeis University, MA, USA, into this backbone. The result, pattB-13x-LexAop2-Sun-5Myc-spGFP11, was injected into fly embryos at the attP2 docking site. This construct is successfully expressed in S2 cells and in Drosophila.

3.1.2 Construction of pUASt-attB-spGFP1-10-WPRE

JFRC14 was cut with Kpn1 and EcoRI to excise GFP-WPRE, yielding the JFRC14 backbone (Addgene plasmid #26223, from Gerald Rubin, Janelia Farms, Ashburn, VA, USA). The WPRE woodchuck virus was reported to increase the expression level and stability of proteins from viral vectors (Pfeiffer et al., 2010). The WPRE sequence was PCR amplified along with 5’KpnI-3’EcoRI sites from pJFRC14 and cloned to the JFRC14 backbone. This was inserted into the pUASTattB vector (Bischof et al., 2007) to yield a 10xUAST-attB-WPRE-SV40 backbone. This was cut with Xhol-KpnI and a PCR amplified spGFP1-10 (Gordon and Scott, 2009) with 5’Xhol-3’KpnI sites was ligated into the 10xUAST-attB-WPRE-SV40 backbone. This yielded pUASt-attB-spGFP1-10-WPRE.

3.1.3 Construction of pUASt-attB-prolact-3xHA-Lamp1TM-spGFP1-10 and pUASt-attB-prolact-spGFP1-10-Lamp1TM-3xHA

To generate the pUAST promoter driven spGFP1-10-Lamp1TM-3xHA construct, synthetic gene blocks containing an N-terminal preprolactin signal peptide
(“MDSKGSSQKGSRLLLLVLVSNLLLCQGVVSTPVCP”) followed by split-GFP1-10 sequence followed by human Lamp1 protein transmembrane domain ("LIPIAVGGALGLVIAYLVRKRSHAGYQTI") and 3xHA-stop ("APYPYDVPDYASGYPYDVPDYAGSYPYDVPDYAS") tags were ordered from IDT DNA (Coralville, Iowa, USA). Similarly, the C-terminally tagged spGFP1-10 tagged Lamp1 synthetic gene fragment (Prolactin-3xHA-spGFP1-10) was ordered from IDT DNA. The C and N-terminally tagged spGFP1-10-Lamp1TM-3xHA fragments were Gibson cloned into KpnI and NotI digested backbone of pUASt-attB-spGFP1-10-WPRE.

3.2 Fly stocks

*Drosophila melanogaster* stocks were crossed and maintained at 25°C. The following lines were used: w¹¹⁸, double balancer lines sp/CyO;Dr/Tm6TbHu, sp/CyOGFP;Dr/Tm6SbTbHu Amphiphysin RNAi line (BL-39015), Df(2R)Exel7121 (BL-7869) and GMR57C10-nSyb-LexA/CyO (BL-52817) from Bloomington (Bloomington Drosophila Stock Center at Indiana University, Bloomington, IN, USA), *amph*²⁶ (Razzaq et al., 2001; Zelhof et al., 2001), pUASt-Evi-HA (Koles et al., 2012), UAS-TNT-Lc (Mathew et al., 2003), UAS-Ykt6-RNAi from Vienna Drosophila RNAi Center (Vienna, Austria), Amph-RNAi-8604 National Institute of Genetics Fly Collection, Japan), C380-Gal4 and C57-Gal4 from Vivian Budnik (UMASS Medical School, Worcester, MA, USA). Syt4-TSTEP knockin flies were generously shared by Dr. Kate Koles (unpublished reagent).

3.2.1 Transgenic stocks

pattB-13xLexAop2-Sun-5Myc-spGFP11 was injected at the attP2 docking site (Genetivision, Houston, TX, USA). pUASt-attB-prolact-3xHA-Lamp1TM-spGFP1-10 and pUASt-attB-prolact-spGFP1-10-Lamp1TM-3xHA were injected at the attP40 docking site (Genetivision, Houston, TX, USA).
3.3 Antibodies

α-GFP 3E6 (1:300) mouse monoclonal antibody was from Invitrogen (Carlsbad, California, USA). α-GFP MBL 598 (1:500) rabbit polyclonal antibody was from Medical & Biological Laboratories CO., LTD. (Naka-ku Nagoya, Japan). α-HA 3F10 (1:600) rat monoclonal antibody was from Roche (Indianapolis, IN, USA). α-myc 9E10 mouse monoclonal antibody (1:50) and α-Dlg 4F3 (1:500) mouse monoclonal antibody were from DSHB (Iowa City, Iowa, USA). α-Amph rabbit polyclonal antibody (1:1000) described in (Zelhof et al., 2001). α-HRP antibodies and secondary antibodies for imaging were conjugated to DyLight405, Alexa Fluor 488, Rhodamine Red X, or Alexa Fluor 647 (Jackson Immunoresearch, West Grove, PA, USA).

3.4 Immunohistochemistry and imaging

Wandering third instar larvae were dissected in HL3.1 (Feng et al., 2004) and fixed in a 4% paraformaldehyde in HL3.1 solution for 10 minutes. Larvae were washed three times for ten minutes and stained with primary antibodies in a PBT (PBS + 0.2% Triton X-100) solution overnight. After subsequent washes, larvae were incubated in secondary antibodies for one hour and washed. Larvae were mounted in Vectashield (Vector Labs, Burlingame, CA, USA). 0.3µm or 0.1µm Z-stacks were imaged using an Andor spinning-disk confocal microscope consisting of a Nikon Ni-E upright microscope equipped with 60× [numerical aperture (NA 1.4)] and 100× (NA 1.45) oil immersion objectives, Yokogawa CSU-W1 spinning-disk head and an Andor iXon 897U electron-multiplying charge-coupled device camera (Andor, Belfast, Northern Ireland). Images were analyzed and data analysis was conducted using Volocity (Improvision, PerkinElmer, Waltham, MA, USA). Statistical significance was calculated in Prism (GraphPad, La Jolla, CA) with an unpaired, two-tailed t-test.

3.5 Transmission electron microscopy

For TEM, samples were prepared and imaged as previously described (Karnovsky, 1965; Koles et al., 2015a).
3.6 S2 cell transfection, preparation and imaging

Drosophila Schneider 2 cells (S2) cultured in Schneider’s media supplemented with 10% fetal bovine serum were transfected using the standard Effectene® Transfection from Qiagen (Valencia, CA, USA). UAS constructs were co-transfected with actin-Gal4 and LexAop constructs were co-transfected with pMet-puro-nls-LexA-p65. 0.5-1.0µL of each construct was used in transfections. After induction with CuSO₄ for 18-24 hours, cells were incubated at 25°C for two days and harvested. Cells were fixed on a coverslip in a 4% paraformaldehyde in phosphate buffered saline (PBS) solution for 10 minutes. The fixed coverslip was washed and stained in primary and secondary antibodies in PBS. Samples were mounted in Vectashield (Vector Labs, Burlingame, CA, USA) and 0.3µm thick Z-stacks were imaged using a confocal microscope consisting of a Nikon Eclipse Ti microscope equipped with a 60x (NA 1.4) oil immersion objective and a Nikon C2 plus camera. Images were analyzed using Volocity (Improvision, PerkinElmer, Waltham, MA, USA).
4 Results

The Drosophila larval NMJ presents a powerful model system for understanding how membrane trafficking pathways contribute to exosome release from the donor cell (the neuron) and to exosome uptake in the recipient cell (the body wall muscle). Here I will present my discovery of several new components of the exosome traffic machinery, including a presynaptic v-SNARE, a postsynaptic membrane remodeling protein, and a role for postsynaptic t-SNAREs. Finally, I will describe a new assay I developed to track the fate of exosomes in the recipient muscle cell.

4.1 Identification of a v-SNARE required for presynaptic exosome release at the larval NMJ

Several reports indicated a role for the Longin-type v-SNARE protein Ykt6 in Drosophila exosome release in wing disc epithelial cells, human cell culture (Gross et al., 2012) and Drosophila fat bodies (Takeuchi et al., 2015). However, whether the same v-SNARE might also operate at the larval NMJs has remained an open question. α-HRP antibodies recognize a presynaptic neuronal membrane protein (Snow et al., 1987) that is released by neurons as postsynaptic α-HRP-debris (Fuentes-Medel et al., 2009). This debris co-localizes with known exosome markers such as Evi and Sunglasses (data not shown) so we used it as an exosome marker in our experiments. Here we examined whether HRP debris is affected by the RNAi-mediated knock-down of Ykt6 at the NMJ. We found a statistically significant decrease in HRP debris in flies expressing Ykt6-RNAi in the neuron (Figure 4). These data indicate that presynaptic Ykt6 plays a role in exosome trafficking. The remaining exosomes still present at the NMJ in the knockdown animals could be due to either the partial knockdown achieved by the RNAi reagent or to regulatory role of Ykt6 in the exosome release process. These possibilities will be addressed in future experiments.
Figure 4. RNAi-knockdown of Ykt6 at the pre-synapse decreases debris phenotype. Quantification of HRP debris intensity over bouton volume in the indicated genetic backgrounds. Images were taken at muscle 6/7 NMJ from segment A3. 7 larvae of each genotype were dissected and imaged. Scale bars are 8µm. There was a statistically significant difference in HRP debris between wild type flies and flies expressing Ykt-RNAi presynaptically with p=0.0005. Quantification was conducted using an unpaired, two-tailed t-test.
4.2 Identification of postsynaptic molecules involved in neuronal exosome uptake, trafficking and/or degradation

In the context of another project relating to the membrane deforming protein Past1 in the larval muscle (Koles et al. 2015a), we noticed that flies with a mutated form of the membrane-remodeling BAR domain protein Amphiphysin (amph$^{26}$) do not appear to have exosomes in their muscles, gauged by the absence of HRP positive debris. (Figure 5A). This was a striking observation as Amphiphysin is a postsynaptic molecule and so far no other molecules have been implicated in the postsynaptic uptake, trafficking or degradation of neuronally derived exosomes at the larval NMJ. All known molecules discussed so far function in the nervous system, i.e. the cell type from which exosomes originate. In order to confirm that this mutant phenotype was indeed due to loss of Amphiphysin and not to a second-site mutation, we analyzed HRP debris in the amph$^{26}$ mutant over a deficiency line that deletes the entire amph gene (Df(2R)Exel7121, BL#7869). We observed the same phenotype and quantifications confirm the decrease of post-synaptic HRP-debris in amph$^{26}$/Def(2R)Exel7121 flies (Figure 5A, B).
**Figure 5. amph<sup>26</sup> mutants show a reduced HRP debris phenotype in the post-synapse** (A) Projection of spinning-disk confocal images of larval NMJ in flies with the indicated genetic background with α-HRP staining. Muscle 4 NMJ is shown from segment A3. (B) Quantification of post-synaptic HRP debris intensity over bouton volume. Eight larvae were dissected for each genotype. Statistical significance of p<0.0001 when comparing wt to amph<sup>26</sup> mutants and wt to amph<sup>26</sup> deficiency flies. (C) Expression of tetanus toxin-light chain (TNT-Lc) in the muscle decreases post-synaptic debris phenotype. Quantification of HRP debris intensity over bouton volume in the indicated genetic backgrounds. Images for quantification were taken at muscle 4 NMJ from segment A3. There was a significant decrease in post-synaptic HRP debris in flies expressing TNT-Lc (p = 0.0007). Quantification was conducted using an unpaired, two-tailed t-test.
Amphiphysin antibody staining reveals strong post-synaptic localization of the protein in the subsynaptic reticulum area (Figure 7) as well as in the muscle T-bars and the muscle cortex, as previously described (Razzaq et al., 2001). This is in contrast to the mammalian homologue which is concentrated in the pre-synapse and is proposed to aid in synaptic vesicle endocytosis (David et al., 1996; Mathew et al., 2003). To rule out the possibility that the level of Amphiphysin expressed was below a detectable threshold and this was causing the lack of HRP debris, we turned to tissue-specific knockdown of Amphiphysin using two independent RNAi lines RNAi-8064 or RNAi-Trip39015 (Figure 6). When we expressed these lines only in the muscle with the strong muscle driver C57-Gal4, we observed the same depletion of HRP debris (Figure 6 A, Ci, Cii) (reproduced in several independent experiments by both RNAi lines, data not shown). To check that the UAS-RNAi lines were not leaking in a tissue-independent manner, we carried out the same experiments in the nervous system as well using the C380-Gal4 neuronal driver, but we did not see a change in either Amphiphysin staining levels or HRP debris level (Figure 6B, Ciii). Thus, the observed phenotype is due to the lack of Amphiphysin levels in the muscle.
Figure 6. Amph-RNAi knockdowns in the muscle and neuron. (A, B) Projection of confocal images of larval NMJ in flies of the indicated genetic background. (A) Post-synaptic expression of Amph-RNAi-8604 decreases post-synaptic HRP debris while (B) pre-synaptic expression of the same RNAi does not. Larvae were maintained at 29°C. Scale bars are 10 µm. Muscle 6/7 NMJ (A) and muscle 4 NMJ (B) are shown from segment A3. (C) Quantification of postsynaptic HRP debris in flies expressing indicated RNAi either pre- or post-synaptically. (Ci) Muscle 6/7 NMJ from segment A3 were imaged and quantified. (Cii) n= 7 for each genotype. Muscle 4 NMJ from segment A3 were imaged and quantified. (Ciii) n= 8 and n= 7 for the control and experimental genotypes, respectively. Muscle 4 NMJ from segment A3 were imaged and quantified. (D) Projection of confocal images of larval NMJ in flies of the indicated genetic background. α-Amph staining shows that Amph is not present in the post-synapse when Amph-RNAi8604 and RNAi-Trip39015 are expressed in the muscle. Quantification was conducted using an unpaired, two-tailed t-test.
While the above results confirm a role for Amphiphysin in larval muscle, it remains unclear whether the readout used for exosome trafficking and spreading in the muscle (i.e. HRP debris) is a broad universal marker of all exosomes, or if only a select subset of exosomal cargo happens to carry the HRP epitope and is present on some neuronal transmembrane proteins (Paschinger et al., 2008). To address these questions, we are currently in the process of analyzing different known exosomal cargo proteins to examine their localization in the amph^26 mutant background. Some of these proteins include Evi, Sunglasses and Syt4, which have

**Figure 7. Amphiphysin is localized around the bouton in the SSR area and in the muscle.** (A) Localization of Amphiphysin in wild-type flies. Scale bar is 10µm (B) Localization of Dlg and Amphiphysin in the larval muscle cortex.

### 4.2.1 Diversity of exosomal cargo trafficking revealed

While the above results confirm a role for Amphiphysin in larval muscle, it remains unclear whether the readout used for exosome trafficking and spreading in the muscle (i.e. HRP debris) is a broad universal marker of all exosomes, or if only a select subset of exosomal cargo happens to carry the HRP epitope and is present on some neuronal transmembrane proteins (Paschinger et al., 2008). To address these questions, we are currently in the process of analyzing different known exosomal cargo proteins to examine their localization in the amph^26 mutant background. Some of these proteins include Evi, Sunglasses and Syt4, which have
previously been reported to be released from larval neuronal terminals in exosomes (Koles et al., 2012; Korkut et al., 2013).

When we examined Evi in flies of the amph<sup>26</sup> mutant background, using the strong neuronal promoter C380-Gal4 to drive UAS-Evi-HA transgenic fly line, we noticed that Evi containing exosomes were in fact present and are released but did not spread away from the neuronal terminal after release (Figure 8A). This would suggest that Amphiphysin function is required for proper exosome trafficking in the muscle cells or alternatively, that the transferred exosomes are more rapidly degraded upon release from the presynaptic bouton. Our quantification of total Evi-HA levels in the two genotypes did not reveal a significant change in total Evi levels (Figure 8B) suggesting that the postsynaptic trafficking is likely affected rather than postsynaptic degradation. Also, these observations are congruent with the HRP debris data in amph<sup>26</sup> mutants, where we would not be able to differentiate between the pre- versus post-synaptic debris due to the fact that HRP is also used as the bouton marker. We will confirm this hypothesis using a myristoylated membrane marker in addition to the HRP label.
Figure 8. Evi containing exosomes are released but do not spread from the bouton in amph^{26} mutants (A) Projection of spinning-disc confocal images of larval NMJ in flies of the indicated genetic background. Muscle 6/7 NMJ are shown from segment A3. α-HA staining is much closer to the bouton in amph^{26} mutant flies than in wild type flies. (B) Quantification of Evi-HA intensity over bouton volume. 5 and 6 larvae were dissected for WT and amph^{26} genetic backgrounds, respectively. Scale bars are 3µm. There was no statistically significant difference between total Evi-HA amounts in the two genotypes. Quantification was conducted using an unpaired, two-tailed t-test.

Next we turned to analyzing the distribution of Syt4 in amph^{26} mutants using a novel unpublished reagent, Syt4-TagRFPT knock-in flies, which avoids the issues of overexpression artifacts through tagging at the endogenous loci (Koles et al., 2015b). Flies expressing Syt4-TagRFPT had Syt4 distribution around the bouton indistinguishable from endogenous Syt4, which is reported to be released from the neuron to the muscle. However, when we looked at Syt4 distribution in the amph^{26} mutant background, we did not observe the decrease of postsynaptic exosomes that we saw in the case of HRP debris or Evi-HA (Figure 9). Although
further experiments and larger number of NMJs are still need to be analyzed for conclusive results, these observations nevertheless hint at the possibility that different cargo might be trafficked and/or degraded in a cargo specific manner at the postsynaptic muscle.

Figure 9. Syt4 containing exosomes are not depleted in the amph\textsuperscript{26} background (A) Projection of spinning-disc confocal images of larval NMJ in flies of the indicated genetic background. The exosomal cargo protein Syt4 (Synaptogamin4) was endogenously tagged with TagRFPT and is expressed in the neuron. Muscle 6/7 NMJ are shown from segment A3. TagRFPT signals does not appear different in wild type and amph\textsuperscript{26} mutant flies. Scale bars are 6µm.

The larval neuromuscular junction is surrounded by several hundred nanometer wide membrane reticulum called SSR (subsynaptic reticulum), which is made up of membrane folds and invaginations of the muscle membrane opposing the synaptic bouton. We do not yet know whether exosomal cargo gets internalized to muscle cells or if they end up in the labyrinth of the SSR crevices without actually entering the muscle cell. Also, the SSR of the Amphiphysin mutants (Amphiphysin being a membrane deforming molecule) might be altered or completely missing the SSR folds, so the lack of exosome spreading could be explained by a this structural barrier. To address these considerations we examined the ultrastructure of the amph\textsuperscript{26} mutants by electron microscopy. Surprisingly, the structure of the SSR membrane in amph\textsuperscript{26} mutants was present and visually indistinguishable from that of the wild type (Figure 10). Therefore it is more likely that the uptake and/or trafficking and/or degradation of exosomal cargo is affected in these mutants as opposed to their ability to diffuse into the crevices of the SSR.
Finally, we tested whether a broad manipulation of membrane fusion in the postsynaptic muscle cell could phenocopy the Amph mutant. We tested HRP debris levels in flies expressing tetanus-toxin-light chain (TNT-Lc, which cleaves v-SNARES) in the muscle (Figure 5C), and found a decrease similar to that observed in amph\textsuperscript{26} mutants. Thus, v-SNAREs are required HRP debris/exosome trafficking and spreading at the NMJ, further defining important steps of postsynaptic traffic of exosomes.

\begin{figure}
\centering
\includegraphics[width=\textwidth]{figure10.png}
\caption{SSR membrane structure of wildtype and amph\textsuperscript{26} mutants are largely undistinguishable. TEM of NMJs from wild-type and amph\textsuperscript{26} larvae. Yellow arrowheads are pointing to the bouton. Black arrowheads are pointing to synaptic vesicles. Black arrowheads are pointing to synaptic vesicles. The surrounding membrane is the subsynaptic reticulum (SSR). Boxes indicate section that is magnified on the right of the respective image. Scale bars are 500µm.}
\end{figure}
4.3 Rationale for split-GFP reporter system to visualize exosomal trafficking in *Drosophila*

Developing a tool to investigate exosome trafficking pathways would aid in elucidating the exosomal system for targeted therapies in many human diseases. In *Drosophila* at the larval neuromuscular junction (NMJ), we know that exosomes are released at the synapse but we do not know their trafficking route or fate in the recipient cells. In order to visualize and study exosome trafficking, we generated a split-GFP reporter system to track the destination of exosomes. In this system, GFP (green fluorescence protein) is split into two parts: spGFP1-10 and spGFP-11, which are each non-fluorescent alone. Fluorescence is reconstituted when spGFP1-10 and spGFP-11 interact. An exosomal cargo protein, Sunglasses (Sun), was tagged with spGFP-11 and five Myc tags. The transmembrane domain of the lysosomal reporter (Lamp1) was tagged with spGFP1-10 and three HA tags. Sun was expressed specifically in neurons and Lamp1 in the muscle. Because we could not predict whether GFP reconstitution might occur in the lumen of lysosomes or on the cytosolic side of the lysosomal membrane, we generated two spGFP1-10-Lamp1 constructs that were tagged with spGFP1-10 at the C or N terminus. Lamp1 is a transmembrane protein and the C terminus is always cytoplasmic while the N terminus is always lumenal; thus the C and N-terminally tagged Lamp1 fusion proteins will be referred to as Lamp1-C and Lamp1-L, respectively. We hypothesize that neuronal exosomes would be sent to the muscle for intercellular communication and expected to observe GFP reconstitution in the muscle if exosomes are trafficked to muscle lysosomes.
Figure 11. Split-GFP reporter construct to study neuronal exosome uptake in muscle cells. Figure depicts the predicted membrane orientation of the reporters on the cell membrane as well as the intracellular organelle membranes.

4.3.1 Split-GFP reporter system in S2 cells

To test the efficacy of the splitGFP reporter system, we first expressed the system in Schneider 2 (S2) cells. These S2 cells were imaged using confocal microscopy. We first transfected the Sun-spGFP11 construct alone into S2 cells to observe its localization, which appeared to be distributed in compartments throughout the cytoplasm (Figure 12A). In S2 cells expressing both Sun-spGFP11 and cytoplasmic spGFP1-10, we observed an endogenous signal from the 488 channel, indicating a reconstitution event. Where Sun, tagged with Myc, co-localized with cytoplasmic spGFP1-10 recognized by GFP antibody, we also saw the endogenous reconstituted GFP signal (Figure 12B).
Figure 12. Reconstitution of GFP observed in cells co-expressing cytoplasmic spGFP1-10 and Sun-spGFP11. (A) Single confocal slices with representative localization of Sunglasses-Myc-spGFP11 in transfected S2 cells. (B) S2 cells co-transfected with Sun-spGFP11 and cytoplasmic spGFP1-10. Reconstituted GFP is observed where the two fusion proteins co-localize.

We also visualized Lamp1-spGFP1-10 in S2 cells, localized in what appears to be endocytic compartments, as expected (Figure 13). The absence of HA signal with the presence of spGFP1-10 signal near the nucleus in cells expressing the Lamp1-L construct suggests a potential cleavage event occurring in the cell (Figure 13). This pattern was not apparent in cells expressing the Lamp1-C construct, so the cleavage may be related to the orientation of the fusion protein. Then, we co-transfected S2 cells with the Sun and Lamp1-C or -L fusion protein constructs along with appropriate drivers. As we saw in S2 cells co-transfected with Sun-spGFP11 and cytoplasmic spGFP1-10, reconstitution was observed where the Lamp1-spGFP1-10 and Sun-spGFP11 fusion proteins co-localize (Figure 13). The intensity of reconstituted GFP appears weaker in cells expressing the Lamp1-L construct as expected based on their opposing transmembrane orientations (Figure 13B). We observe robust reconstitution of GFP where Lamp1, the lysosomal marker protein, and Sun, the exosomal cargo, co-localize in S2 cells. Thus, we establish that the split-GFP system is a viable tool for studying exosome trafficking.
Figure 13. Reconstitution of GFP observed in cells co-expressing Lamp1-C, -L and Sun-spGFP11.
Single confocal slices with representative localization of the Lamp1-C and -L fusion protein in transfected S2 cells. The absence of HA signal with the presence of GFP signal near the nucleus in cells expressing the Lamp1-L construct suggests a potential cleavage event (indicated by yellow arrowhead). (B) Reconstituted GFP indicates endogenous signal from this channel.
4.3.2 Split-GFP reporter system at the larval NMJ

We next expressed this split-GFP reporter system in *Drosophila* to visualize exosome trafficking at the larval NMJ. We observed localization of Lamp1 around the synaptic boutons on the postsynaptic side in larvae expressing Lamp1-C, but not in larvae expressing Lamp1-L (Figure 14A). Similar to the observations in S2 cells, the HA tag appears to be cleaved from the N-terminally tagged Lamp1 protein as spGFP1-10 is present near the nucleus while HA is not. We confirmed that Sun-spGFP11 expression using the neuronal driver nSybLexA (Figure 14B). α-Myc signals and α-HRP signals do not completely co-localize, which may indicate the existence of various exosome types that carry different cargo.
Figure 14. Localization of Lamp1-C and –L tagged with HA and spGFP1-10 and of Sunglasses tagged with Myc and spGFP11 at the larval NMJ. (A) Projection of confocal slices at the larval NMJ with Lamp1-C, –L overexpressed with the muscle driver C57-Gal4. Lamp1 localizes around the synaptic boutons on the postsynaptic side in larvae expressing Lamp1-C, but not in larvae expressing Lamp1-L. The HA tag appears to be cleaved from the N-terminally tagged Lamp1 protein as spGFP1-10 is present near the nucleus while HA is not. Scale bars are 10µm. White arrowhead indicates α-GFP signal without α-HA signal. (B) Sun-5Myc-spGFP11 overexpressed with the neuron driver C380-Gal4. Yellow arrowhead indicates co-localization of α-HRP and α-Myc signals. Scale bar is 5µm.
We expressed both halves of the split-GFP reporter construct in the transgenic flies and imaged them at the NMJ with spinning-disc confocal microscopy. We observed a strong co-localization of α-GFP and α-HA signals in the Lamp1-C constructs for both the experimental and control (Figure 15A). There was not as strong co-localization in the Lamp1-L constructs (Figure 15B), which may be explained by cleavage event of the fusion molecule similar to what we observed in S2 cells. We expected the α-GFP antibody to specifically recognize the full GFP molecule, but our data indicates that this antibody recognizes the spGFP1-10 molecule. We will look into using different antibodies to distinguish between the full and split GFP molecules.
Figure 15. α-GFP antibody 3E6 recognizes spGFP1-10. Single confocal slices with representative localization of Lamp1-C and –L at the NMJ in flies co-expressing the Lamp1-spGFP1-10 constructs with the Sun-spGFP11 construct. (A) Larvae co-expressing both halves of the spGFP construct: Lamp1-C in the muscle and Sun-Myc-spGFP11 in the neuron. (B) Larvae co-expressing both halves of the spGFP construct: Lamp1-L in the muscle and Sun-myc-spGFP11 in the neuron. The monoclonal α-GFP antibody 3E6 was used to detect the full GFP molecule, though it recognizes split-GFP1-10, as it is present in the controls. Yellow arrowheads indicate examples of co-localization of α-GFP and α-HA. Scale bars are 5µm. Exposure time in 500ms in the 488 channel with 30% laser intensity, 400ms in the 560 channel with 25% laser intensity, and 900ms in the 647 channel with 91% laser intensity for Lamp1-C constructs and 700ms with 21% laser intensity for Lamp1-L constructs.
Next, we left the green 488 channel unstained to observe any GFP reconstitution events from the interaction of the two split-GFP constructs. Again, we observed co-localization of endogenous GFP and α-HA in the Lamp1-C constructs not just in the experimental, but also the control crosses (Figure 16A). Interestingly, this co-localization does not appear in flies expressing either the control or experimental Lamp1-L constructs (Figure 16B). This observation indicates that the Lamp1-C construct exhibits a certain degree of auto-fluorescence that we are currently unable to differentiate from a reconstituted GFP signal. The lack of detectable auto-fluorescence signal from the Lamp1-L constructs indicates that the lower pH in the lumenal environment compared to the cytoplasm may be de-activating spGFP1-10-L auto-fluorescence. We are currently building a Sun construct that contains multiple spGFP11 molecules, which we hypothesize will lead to a brighter reconstituted GFP signal, to differentiate between spGFP1-10 auto-fluorescence and GFP reconstitution. Finally, we also stained with α-myc antibodies to confirm Sun-spGFP11 expression in the neuron and to observe its localization around the bouton (Figure 17).
Figure 16. spGFP1-10 tagged on the C-terminus (cytoplasmic side) of Lamp1 unexpectedly exhibits significant auto-fluorescence. Single confocal slices with representative localization of Lamp1-C and -L at the NMJ in flies co-expressing the Lamp1-spGFP1-10 constructs with the Sun-spGFP11 construct. (A) Larvae co-expressing both halves of the spGFP construct: Lamp1-C in the muscle and Sun-myc-spGFP11 in the neuron. (B) Larvae co-expressing both halves of the spGFP construct: Lamp1-L in the muscle and Sun-Myc-spGFP11 in the neuron. The 488 channel was left open to detect reconstitution of GFP fluorescence. Yellow arrowheads indicate examples of co-localization of endogenous GFP and α-HA. Scale bars are 5µm. Exposure time in 1s in the 488 channel with 92% laser intensity, 500ms in the 560 channel with 16% laser intensity, and 500ms in the 647 channel with 44% laser intensity.
Figure 17. Localization of Lamp1-C, -L and Sun spGFP constructs at the NMJ. Single confocal slices with representative localization of both halves of the split GFP reporter construct. (A) Larvae co-expressing both halves of the spGFP construct- Lamp1-C in the muscle and Sun-myc-spGFP11 in the neuron. (B) Larvae co-expressing both halves of the spGFP construct- Lamp1-L in the muscle and Sun-Myc-spGFP11 in the neuron. The 488 channel was left open to detect reconstitution of GFP fluorescence. Scale bars are 5µm. Exposure time in 2s in the 488 channel with 95% laser intensity, 800ms in the 560 channel with 33% laser intensity, and 700ms in the 640 channel with 45% laser intensity.
5 Discussion

In this work I have extended the *Drosophila* larval neuromuscular junction system to study the endocytic trafficking mechanism of exosomes, an emerging novel class of intercellular signaling organelles of endosomal origin.

5.1 Ykt6 is necessary for neuronal exosome trafficking

Given that SNARE mediated vesicle fusion requires the presence of cognate SNARE proteins on the vesicle and the target membrane, the observation that the t-SNARE Syx1A plays a role in exosome trafficking (Koles et al., 2012) suggested a requirement for a potential cognate SNARE on the multivesicular bodies that are likely to fuse with the neuronal membrane during exosome release. The v-SNARE Ykt6 which has been shown to regulate exosome trafficking in cultured S2 cells and in *Drosophila* fat body, and indeed when I tested Ykt6 knockdown in motor neurons I observed a significant decrease in the amount of α-HRP stained exosome in the muscle (Figure 4).

While Syntaxin1A and Ykt6 may indeed function as a cognate SNARE pair, further experiments are required to test this possibility. In non-neuronal cells, Ykt6 is largely localized in the cytoplasm and Golgi of cultured mammalian cells (Hasegawa et al., 2003; Zhang and Hong, 2001) where it is believed that Ykt6 aids in ER-Golgi transport by aiding in the docking and fusion of vesicles (Zhang and Hong, 2001). Ykt6 has not been characterized in *Drosophila* and the only known reagents are the RNAi lines. Determining the localization and expression pattern/level of Ykt6 in the nervous system and other tissue types would require either antibody generation or transgenic fly development. In addition, we have yet to determine the effect of Ykt6 knockdown on select well-defined exosomal cargo release, such as Evi, Sunglasses and Syt4. Furthermore, the v-SNARE VAMP7 has been implicated in MVB fusion and exosome
release in mammalian cells (Fader et al., 2009a) and is potentially a binding partner of Ykt6 (Flybase). In future experiments we will examine its contribution to exosome release as well.

In the future, it will be very interesting to examine the role of neuronal activity in the release of exosomes from presynaptic sites. Increased neuronal activity (via optogenetic stimulation) has been reported to increased neuronal debris shedding (Fuentes-Medel et al., 2009). Future experiments will address these questions, using well characterized mutants of neuronal excitability such as para
\textsuperscript{ts1}, a sodium channel subunit (Budnik et al., 1990) and seizure, encoding a potassium channel. In addition, we will induce enhanced neuronal activity by expressing the TrpA1 channel (Hamada et al., 2008) at different temperatures for different durations. Furthermore we will also address the role of presynaptic calcium levels in potentially contributing to exosome release using the mutant alleles of cacophony, a subunit of the Ca\textsuperscript{2+} channel α1 subunit gene (Savina et al., 2003). We will also test whether our observations under these different conditions apply to HRP debris and all other known exosomal cargo proteins, or whether some of these pathways might be cargo selective as some of our preliminary data are implying (see also Amph section below).

5.2 Post-synaptic Amphiphysin regulates exosome trafficking

One of the key observations from our studies was the discovery that the muscle endosomal system plays a role in exosome trafficking/degradation/uptake. By identifying the postsynaptic molecule Amphiphysin, a BAR domain (Bin-Amphiphysin-Rys) containing membrane deforming protein, we are beginning to unravel the trafficking routes and selectivity of the exosomal uptake pathway. Unlike the vertebrate Amph1, which is concentrated at synaptic sites and is required for endocytosis, the \textit{Drosophila} homolog is not detectable in neurons, but is expressed in muscle where it is required for proper t-tubule organization (Razzaq et al., 2001) and for SSR organization in cooperation with other membrane remodeling proteins (Koles et al. 2015a). Our antibody staining reveals a striking tubular staining pattern in
the cortex area of the muscle cells (Figure 7) as well as an enrichment in the SSR area of the NMJ (Figure 7). Furthermore, Amph does not seem to function in endocytosis at the postsynaptic membrane (Mathew et al., 2003). Instead, it’s been proposed to be necessary for cell adhesion molecule Fasciclin II (FasII) exocytosis from the muscle. Even though the total amount of FasII did not change in \textit{amph}\textsuperscript{26} mutant flies compared to wild type, less FasII was integrated into the plasma membrane. While endocytosis rates of FasII containing vesicles remained unchanged in these \textit{amph} mutants, it seems that the lack of amphiphysin significantly reduced the rates of FasII exocytosis into the plasma membrane (Mathew et al., 2003). Furthermore, this exocytosis step requires the function of unknown SNARE proteins, since expression of the tetanus toxin light chain in the muscle phenocopies the \textit{amph}\textsuperscript{26} mutant phenotype. Likewise, our results with the TNT-Lc muscle expression phenocopies the reduced HRP debris phenotype (Figure 5). We still cannot rule out that the reduced HRP debris we observe is due to enhanced degradation of the exosomal cargo(es) or to altered trafficking within the SSR area of the muscle. Ultrastructural analysis rules out the absence of SSR folds in \textit{amph}\textsuperscript{26} mutants, into which exosomes could diffuse, as the cause of this phenotype. Therefore the two most likely scenarios are the lack of endocytic trafficking in the muscle upon neuronal release or the enhanced degradation upon entry to the muscle.

Another intriguing observation from our \textit{amph}\textsuperscript{26} data is the potential cargo-specificity of exosomal trafficking. Evi-HA and HRP debris accumulate (or are rapidly degraded) without spreading to the muscle while endogenous level Syt4-TagRFP\textsubscript{t} in \textit{amph}\textsuperscript{26} mutants appears to spread almost like that in wild type flies (Figure 8, Figure 9). However these initial observations need to be corroborated by characterizing the trafficking of other exosomal cargo protein, such as Sunglasses, and controlling for the effect of protein overexpression in their exosomal cargo sorting (such as UAS-Gal4 driven Syt4 versus endogenous Syt4-TagRFP\textsubscript{t}). Furthermore, I will label the neuronal membrane with a myristoylated membrane marker to discriminate between
membrane bound and shed HRP debris in \textit{amph}^{26} mutants. I will also perform rescue experiments to confirm that Amphipysin is responsible for the observed exosomal debris phenotype with the caveat that UAS-Amph expression levels are significantly higher than endogenous Amph levels and may have negative effect on rescue (Mathew et al., 2003).

5.3 Organismal exosome effects

In light of recent studies, the exosomal system involves complex cargo and/or vesicle/target membrane proteins that dictate targeting and specificity. As previously mentioned, exosomes have the ability of long distance trafficking throughout an organism. Molecular chaperones expressed in fat body and muscle cells in \textit{Drosophila} were trafficked to the eye where they maintained proteostasis by regulating misfolded htt protein (Takeuchi et al., 2015). In a similar avenue, we designed our split-GFP reporter tool to visualize the delivery of neuronal exosomes to muscle lysosomes to elucidate the post-synaptic trafficking. In future experiments, we can adapt our system to observe the trafficking of exosomes derived from other cells, or the potential trafficking of neuronal exosomes to further locations in the larvae, such as hemocytes. We will also use different reporter proteins (early and late endosome) expressed in different genetic backgrounds (i.e. \textit{amph}^{26}) to study the exosomal trafficking pathway in more detail.

In our study, we establish that the split-GFP reporter system to study the trafficking of neuronally derived exosomes is viable at the larval NMJ, but it is currently not adequate to answer our biological questions. Using the neuronal driver nSybLexA, we expressed the Sun-5myc-spGFP11 construct specific to the neuron. We also see expression of both lysosomal reporter constructs, Lamp1-C and Lamp1-L specific to the muscle with our C57-Gal4 muscle driver. Therefore, both halves of the construct are expressed in the transgenic fly lines. However, we observe auto-fluorescence from the spGFP1-10 fragment that has not been previously described in the literature. In addition, the 3E6 \(\alpha\)-GFP antibody recognizes the
spGFP1-10 fragment as well as the full GFP molecule. These technical limitations will need to be overcome to use this system as we intend.

Thus, in our next round of experiments, we aim to differentiate between the auto-fluorescence we observe from spGFP1-10 and the reconstituted, endogenous GFP signal (Figure 16). We are currently building a Sun-spGFP11 construct that contains multiple spGFP11 molecules, which we hypothesize will lead to a brighter reconstituted GFP signal, to differentiate between spGFP1-10 auto-fluorescence and GFP reconstitution (Kamiyama et al., 2016). We will also use a GFP antibody that selectively recognizes the full GFP molecule, not the spGFP1-10 fragment.

In summary, we have shown that the Ykt6 v-SNARE is involved in neuronal exosome release and identified Amphiphysin as one of the first post-synaptic molecules that plays a role in the trafficking of neuronally derived exosomes. Our observations also support the possibility that exosomal trafficking involves a cargo-specific mechanism. In order to study post-synaptic exosomal trafficking, we turned to developing the split-GFP reporter tool to visualize these pathways. We observe GFP reconstitution of our reporter and exosomal cargo when co-expressed in S2 cells but have not achieved sufficient expression level/sensitivity at the *Drosophila* larval NMJ. Therefore we will further optimize this tool to track the endocytic trafficking of exosomal cargo in recipient cells. Data from these experiments will help elucidate these trafficking pathways, which will greatly aid in developing a model of the exosomal system.
6 References


