Mechanisms of Synaptic Development and Premature Aging in Drosophila: A Dissertation

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MECHANISMS OF SYNAPTIC DEVELOPMENT AND PREMATURE AGING IN DROSOPHILA

A Dissertation Presented

By

YIHANG LI

Submitted to the Faculty of
University of Massachusetts Graduate School of Biomedical Sciences, Worcester
in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

September, 20th, 2016

NEUROSCIENCE
MECHANISMS OF SYNAPTIC DEVELOPMENT AND PREMATURE AGING
IN DROSOPHILA

A Dissertation Presented
by

YIHANG LI

This work was undertaken in the Graduate School of Biomedical Sciences
Program in Neuroscience

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that the student has met all graduation requirements of the School

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Dean of the Graduate School of Biomedical Sciences

09/20/2016
“There are no facts, only interpretations.”

Friedrich Nietzsche
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ABSTRACT

Development and aging, two fundamental aspects of life, remain key biological processes that researchers try to understand. *Drosophila melanogaster*, thanks to its various merits, serves as an excellent model to study both of these processes. This thesis includes two parts. In the first part, I discuss our finding that the presynaptic neuron controls a retrograde signaling pathway by releasing essential components via exosomes. During synaptic development, postsynaptic cells send retrograde signals to adjust the activity and growth of presynaptic cells. It remains unclear what the mechanism is which triggers the release of retrograde signals; and how presynaptic cells are involved in this signaling event. The first part of this thesis demonstrates that a retrograde signal mediated by Synaptotagmin4 (Syt4) depends on the anterograde delivery of Syt4 protein from the presynaptic neuron to the muscle compartment likely through exosomes. This trans-synaptic transfer of Syt4 is required for the retrograde control of activity-dependent synaptic growth at the *Drosophila* larval neuromuscular junction.

In the second part of this thesis, I talk about our discovery that the disruption of nuclear envelope (NE) budding, a novel RNA export pathway, is linked to the loss of mitochondrial integrity and premature aging in *Drosophila*. We demonstrate that several transcripts, which are essential for mitochondrial integrity and function, use NE-budding for nuclear export. Transgenic *Drosophila* expressing a LamC mutation modeling progeroid syndrome (PS), a premature aging disorder in humans, displays accelerated aging-related phenotypes.
including progressive mitochondrial degeneration as well as decreased levels of a specific mitochondrial transcript which is normally enriched at NE-budding site. The PS-modeled LamC mutants exhibit abnormal lamina organization that likely disrupts the egress of these RNAs via NE-budding. These results connect defective RNA export through NE-budding to progressive loss of mitochondrial integrity and premature aging in *Drosophila*. 
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<tbody>
<tr>
<td>ACh</td>
<td>Acetylcholine</td>
</tr>
<tr>
<td>ALS</td>
<td>Amyotrophic Lateral Sclerosis</td>
</tr>
<tr>
<td>AMPA</td>
<td>α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid</td>
</tr>
<tr>
<td>aPKC</td>
<td>atypical Protein Kinase C</td>
</tr>
<tr>
<td>Blw</td>
<td>Bellwether</td>
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<tr>
<td>ChR2</td>
<td>Channelrhodopsin2</td>
</tr>
<tr>
<td>CMT2A</td>
<td>Charcot-Marie Tooth Type 2A</td>
</tr>
<tr>
<td>DCM</td>
<td>Dilated cardiomyopathy</td>
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<tr>
<td>DFz2</td>
<td>Drosophila Frizzled-2</td>
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<tr>
<td>DFz2C</td>
<td>C terminal fragment of DFz2</td>
</tr>
<tr>
<td>DLG</td>
<td>Discs-Large</td>
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<td>DLM</td>
<td>Dorsal longitudinal muscles</td>
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<tr>
<td>Drp1</td>
<td>Dynamin related protein 1</td>
</tr>
<tr>
<td>EDMD</td>
<td>Emery-Dreifuss muscular dystrophy</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>EJP</td>
<td>Excitatory Junctional Potential</td>
</tr>
<tr>
<td>EM</td>
<td>Electron microscopy</td>
</tr>
<tr>
<td>ER</td>
<td>Endoplasmic reticulum</td>
</tr>
<tr>
<td>ETC</td>
<td>Electron Transport Chain</td>
</tr>
<tr>
<td>Evi</td>
<td>Evenness Interrupted, aka Wntless (Wls)</td>
</tr>
<tr>
<td>EV</td>
<td>Extracellular vesicle</td>
</tr>
<tr>
<td>Fis1</td>
<td>Fission protein 1</td>
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<tr>
<td>FNI</td>
<td>Frizzled Nuclear Import</td>
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<tr>
<td>FPLD</td>
<td>Familial partial lipodystrophies</td>
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<tr>
<td>GFP</td>
<td>Green Fluorescent Protein</td>
</tr>
<tr>
<td>HGPS</td>
<td>Hutchinson-Gilford progeria syndrome</td>
</tr>
<tr>
<td>HRP</td>
<td>Horseradish Peroxidase</td>
</tr>
<tr>
<td>HRS</td>
<td>Hepatocyte Growth Factor (HGF)1-regulated tyrosine kinase substrate</td>
</tr>
<tr>
<td>HV</td>
<td>Herpesvirus</td>
</tr>
<tr>
<td>IF</td>
<td>Intermediate filament</td>
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<tr>
<td>IFM</td>
<td>Indirect flight muscle</td>
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<tr>
<td>Ig</td>
<td>Immunoglobulin</td>
</tr>
<tr>
<td>ILV</td>
<td>Intraluminal vesicle</td>
</tr>
<tr>
<td>INM</td>
<td>Inner nuclear membrane</td>
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<tr>
<td>LEM</td>
<td>Lap2/Emerin/MAN</td>
</tr>
<tr>
<td>LGMD</td>
<td>Limb girdle muscular dystrophy</td>
</tr>
<tr>
<td>LINC</td>
<td>Linker of nucleoskeleton and cytoskeleton</td>
</tr>
<tr>
<td>LTD</td>
<td>Long Term Depression</td>
</tr>
<tr>
<td>LTP</td>
<td>Long Term Potentiation</td>
</tr>
<tr>
<td>Marf</td>
<td>Mitochondrial assembly regulatory factor</td>
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<tr>
<td>Mfn</td>
<td>Mitofusin</td>
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<tr>
<td>MHC</td>
<td>Myosin Heavy Chain</td>
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MiD49/51 mitochondrial dynamics protein of 49kDa and 51kDa
Miro Mitochondrial Rho
mtDNA mitochondrial DNA
MuSK muscle, skeletal receptor tyrosine kinase
MVB Multivesicular Body
NE Nuclear Envelope
NMJ Neuromuscular Junction
NPC Nuclear Pore Complex
NpHR Halorhodopsin from N. pharaonic
ONM Outer nuclear membrane
OPA1 Optic atrophy 1
PABP2 Poly(A) binding protein 2
PD Parkinson's disease
PDZ Postsynaptic Density-95/Discs-Large/Zona Ocludens
PINK1 PTEN-induced putative kinase 1
PS Progeroid syndrome
RNAi RNA interference
RNP Ribonucleoprotein
ROS Reactive Oxygen Species
S2 Drosophila Schneider-2
SNARE Soluble NSF attachment receptor
SOD Superoxide dismutase
SSR Subsynaptic reticulum
STD Short-term depression
STF Short-term facilitation
STP Short-term plasticity
Syt4 Synaptotagmin4
UAS Upstream activating sequence
Wg Wingless
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CHAPTER I

Introduction
Overview

Development and aging, two fundamental aspects of life, remain key biological processes that scientists try to understand. This thesis represents my novel contribution to our knowledge of both aspects by focusing on specific areas including synaptic growth, exosome-dependent intercellular communication, mitochondrial wellness and premature aging, using *Drosophila melanogaster* as a model system. In the first half of the introduction, I will talk about what we know about synaptic plasticity, the use of *Drosophila* larval neuromuscular junction (NMJ) to study synaptic development and plasticity, the function of Wnt signaling in NMJ development, and the involvement of exosomes in this process. In Chapter 2, I will describe the work we did to understand how the presynaptic neuron controls a retrograde signaling pathway by releasing essential components via exosomes, which is essential for the activity-induced synaptic growth. In the second half of the introduction, I will talk about the recent discovery of a novel RNA export pathway called nuclear envelope budding, and our current knowledge of mitochondria and premature aging. In Chapter 3, I will talk about our work which connects the defective RNA export through nuclear envelope budding to loss of mitochondrial integrity and premature aging in *Drosophila*. Finally, in Chapter 4, I will discuss the significance, implication and limitation of both studies.
Synaptic plasticity

In the human brain, thousands of neurons form a complicated network through connections called synapses. Neurons communicate with each other by releasing or receiving neurotransmitters at these connections. Synapses are highly dynamic structures, which can be strengthened or weakened. The ability of synapse to change efficacy, both functionally and structurally, is called synaptic plasticity. The existence of brain plasticity was first demonstrated by Ramon y Cajal in the late 19th century, when he discovered that axons of pyramidal neurons in the cat brain cortex were able to regenerate and form new circuits after injury (DeFelipe, 2006). The mechanism of synaptic plasticity was introduced by Donald Hebb in 1949. In his book “The organization of behavior”, he described plasticity as increased synaptic efficacy caused by repetitive stimulation of the postsynaptic cells by the presynaptic cells (Hebb, 1949).

Synapses are most plastic during developmental stages while the neural network is constantly shaped and reshaped. Synaptic modulation made at early developmental stages can lead to long-term neuronal and behavioral changes. One example is the experiment conducted by Hubel and Wiesel on the kitten visual system, in which they discovered that lack of visual input in the young animal can lead to developmental failure of the visual cortex and permanent blindness. Deprivation of visual input in older cats did not cause any long-term
defects, which raised the concept of “critical period” during developmental stages when the synapses are most elastic (Wiesel and Hubel, 1963a, b)

The other aspect illustrated by Wiesel and Hubel’s studies on kittens is that function of the nervous system can be influenced by activity. Activity-dependent plasticity at the synaptic level was further supported by the discovery of long-term potentiation (LTP) by Lomo and Bliss in 1970s (Bliss and Lomo, 1973). In their study, Lomo and Bliss reported that high-frequency stimulation of the prefrontal path induced long lasting increases in transmission efficiency at synapses on dentate granule cells. It was found that N-Methyl-D-Aspartate (NMDA) receptors, a type of glutamate receptor, were required for LTP induction (Collingridge et al., 1983). The NMDA receptor is not only glutamate-gated but also a Mg$^{2+}$-dependent voltage sensitive ion channel. At resting potential, Mg$^{2+}$ blocks the ion channel of NMDA receptors. The release of glutamate from the presynaptic neuron activates ionotropic α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptors, another glutamate receptor, triggering the depolarization of the postsynaptic membrane. The change in voltage potential in the postsynaptic membrane relieves the Mg$^{2+}$, together with the presence of synaptic glutamate, activates NMDA receptors and triggers the channel opening (Mayer et al., 1984; Nowak et al., 1984). The activated NMDA receptor allows Ca$^{2+}$ influx and the elevated cellular Ca$^{2+}$ level leads to CaMKII autophosphorylation and activation. Activated CaMKII enhances the conductance and density of
postsynaptic AMPA receptors through direct phosphorylation of the AMPA receptor subunits, modulating trafficking and anchoring of AMPA receptors at synapses and enhancing gene expression (Lisman et al., 2002; Lisman et al., 2012). LTP is believed to be the basis of learning and memory, although controversy remains. Direct supporting evidence includes: inhibitory avoidance training in rats induces LTP in a subset of hippocampal synapses mimicking the effects of high frequency stimulation (Whitlock et al., 2006), and disruption of LTP through inhibiting PKM, a downstream molecular player of LTP, leads to the loss of spatial memory in rats (Pastalkova et al., 2006). However, evidence pointing to the opposite direction was also found. Kikusui and colleagues reported that NMDA receptor antagonist blocks hippocampal CA1 LTP but does not affect spatial working memory in rats (Kikusui et al., 2000). These results suggest the relationship between memory and LTP is rather complicated and depends on the paradigm and the brain region involved.

Long-term depression (LTD), low-frequency stimulation that induced depression of synaptic efficacy, the opposite of LTP, was also discovered (Ito and Kano, 1982). LTD has been found in many regions of the brain with the ones in hippocampus and cerebellum best characterized (Massey and Bashir, 2007). Hippocampal LTD requires NMDA receptors and postsynaptic Ca\(^{2+}\), which further regulates open channel probability and internalization of postsynaptic AMPA receptors (Malenka and Bear, 2004).
Short-term plasticity (STP) including short-term facilitation (STF) and short-term depression (STD) is another form of synaptic plasticity besides LTP and LTD. Similar to LTP/LTD, STP modulates synaptic efficacy in an activity-dependent manner but in a much shorter time scale (milliseconds to minutes) and the synaptic efficacy alteration is temporary. Unlike LTP and LTD, which mainly involve postsynaptic modification, STP arises from increased or decreased release of neurotransmitters from the presynaptic terminal upon repetitive stimulation (Deng and Klyachko, 2011). It has been suggested that STP could be involved in working memory formation and decision making (Deng and Klyachko, 2011).

Activity-dependent synaptic efficacy alteration also involves structural changes of the synapse. For example, the size, density and morphology of dendritic spines, where most excitatory synapses occur in mammalian brain, can be altered by activity (Hering and Sheng, 2001). Induction of LTP in hippocampal CA1 leads to new spine formation (Engert and Bonhoeffer, 1999). Low-frequency stimulation induced LTD leads to dendritic spine retraction or shrinkage (Nagerl et al., 2004; Zhou et al., 2004), which can be reversed following high-frequency stimulation induced LTP (Zhou et al., 2004).
**Drosophila larval NMJ: a model system to study synaptic development and plasticity**

The *Drosophila* larval neuromuscular junction (NMJ) has been serving as an excellent model to study synaptic development and plasticity. This is an easily accessible model system with a simple stereotypical structure. Most of the larval body is composed of abdominal segments (A1-A8), which contain many of the same muscles in a segmentally repeated pattern. Within each segment, there are 30 muscles innervated by 32 motor neurons, with well characterized morphology. The muscles, motor neurons and the innervations are so stereotyped that the same NMJ can be identified and compared among different flies (Collins and DiAntonio, 2007). Each NMJ comprises branches of synaptic boutons formed at motor neuron terminals, which are surrounded by a complex infolded muscle membrane system called subsynaptic reticulum (SSR). The mature boutons contain active zones, which are the sites of neurotransmitter release.

The developing *Drosophila* larval NMJ is a highly plastic system. From first instar to late third instar, the muscle volume increases by more than 100-fold (Griffith and Budnik, 2006). In order to compensate for the muscle growth, the NMJ needs to expand by forming new branches and boutons at the nerve terminal, and construct postsynaptic apparatus at the muscle side. The proper coordination between the pre- and the post-synapse ensures the formation of new mature synapses with functional neurotransmission sites.
NMJ is mostly glutamatergic, sharing many components in common with the vertebrate central nervous system. Upon stimulation, motor neurons release glutamate, which activates glutamate receptors on the muscle surface, causing depolarization of the muscle membrane. This evoked response in muscle can be detected with electrophysiological recording, referred to as amplitude excitatory junctional potentials (EJPs). The development and plasticity of the larval NMJ is activity dependent. Hyperexcitable mutants including eag and Sh exhibit excessive motor neuronal terminal branching and bouton formation (Budnik et al., 1990). Both spaced K\(^+\)-depolarization and high-frequency nerve stimulation can lead to nascent bouton formation and increased spontaneous release frequency (Ataman et al., 2008). These features of the Drosophila larval NMJ make it a valuable system to study both short-term and long-term plasticity.

The Drosophila model has many advantages as a model system, including short life cycle, simpler genetics than vertebrate models and availability of research tools. The Drosophila life cycle takes approximately 10 days from egg laying to mature adult, about 1 day for eggs to hatch, 4 days of larval development, and another 4-5 days of metamorphosis before eclosion. The life span of the adult Drosophila is approximately 2-3 months in wild-type flies, under laboratory rearing conditions. One of the most commonly used tools in Drosophila is UAS/GAL4 system (Brand and Perrimon, 1993). GAL4 is a transcription activator discovered in yeast Saccharomyces cerevisiae. GAL4 regulates transcription by
binding to an Upstream Activating Sequence (UAS) element of the target gene (Duffy, 2002). The GAL4 sequence can be driven by different promoter sequences, leading to a large array of *Drosophila* stocks expressing GAL4 in tissue and temporally specific patterns. The UAS element can be fused with different coding sequences, such that when combined in the same fly, tissue specific expression of a target sequence is achieved. Thousands of *Drosophila* GAL4-driver lines and UAS lines have been reported and made publically available for research purposes. Over the years, two other binary expression systems have been developed, the Q-System (Potter et al., 2010) and the LexA/LexAop system (Lai and Lee, 2006), which both express a protein that drives expression when bound to its target. This allows for the expression of multiple transgenes in multiple different tissues, within the same organism.

**Wnt signaling at NMJ**

Wnt family members are a group of morphogens that are crucial for early development by controlling events including cell proliferation, cell fate determination, cell polarity and movement and programmed cell death in all animal embryos (Ciani and Salinas, 2005) (Bejsovec, 2013). 19 Wnt family members have been identified in vertebrates while 7 homologs are found in *Drosophila* (Swarup and Verheyen, 2012). The best characterized *Drosophila* Wnt is
Wingless (Wg), also known as *Drosophila* Wnt-1. Similar to vertebrate Wnts, Wg functions in various cellular events and regulates different developmental processes including body patterning, wing imaginal disc development, and larval neuromuscular junction development. The Wnt receptors include the typical Frizzled (Fz) receptors and the non-conventional receptors such as Derailed and ROR2 (receptor tyrosine kinase-like orphan receptor 2) (Korkut and Budnik, 2009). To achieve its multi-functional roles in a diversity of cellular process, Wnts have very complicated downstream signaling, including at least five major signaling pathways: 1 Canonical pathway; 2 Divergent canonical pathway; 3 Planar cell polarity pathway; 4 Calcium pathway and 5 Frizzled nuclear import (FNI) pathway (Korkut and Budnik, 2009; Speese and Budnik, 2007).

Besides body patterning and early embryogenesis, Wnts are also critical for neural development at several neural developmental stages including nervous system patterning, axon guidance, dendrite morphogenesis and synapse formation (Ciani and Salinas, 2005). The first *in vivo* evidence of vertebrate Wnts playing a role in synapse formation was reported by the Salinas group. Hall and colleagues discovered that Wnt7a, secreted by granule cells, controls mossy fiber axonal remolding and morphological maturation of glomerular rosettes in a retrograde manner (Hall et al., 2000). A similar function of Wnt3 as a retrograde signal was later demonstrated in the synapses formed between axons of dorsal root ganglia neurons and motor neurons (Krylova et al., 2002).
 NMJ formation is also modulated by Wnt signaling in both vertebrate and invertebrate model systems. Within the vertebrate NMJ, motor neuron innervates the muscle surface where the muscle membrane forms invaginations called post-junctional folds, also known as endplates. In these junctions, motor neurons release acetylcholine (ACh) as the excitatory neurotransmitter, which activates ACh receptors (AChRs), clustered at endplates, to control muscle contraction. AChR clusters form on the muscle surface even before motor neuron innervation, but in a less concentrated way (Lin et al., 2001; Yang et al., 2001). Upon motor neuron innervation, the preformed AChRs are recruited to the postsynaptic sites and stabilized to form mature AChR clusters (Flanagan-Steet et al., 2005; Lin et al., 2001). Both the preformed and mature AChR clustering require MuSK (muscle, skeletal receptor tyrosine kinase) signaling. Before innervation, MuSK prepatterns the muscle ensuring the local concentration of AChR clusters at prospective synaptic regions; while upon innervation, Agrin, a heparan-sulphate proteoglycan released by the motor neuron, stabilizes AChR clusters through stimulating MuSK (Burden et al., 2013). The first evidence suggesting Wnts are involved in vertebrate NMJ development came from the finding that Agrin induced AChR clustering requires Dishevelled1 (Dvl1), a scaffolding protein downstream of several Wnt signaling pathways, which was found to interact with MuSK (Luo et al., 2002). Another downstream player of canonical Wnt signaling that is required for Agrin-dependent AChR clustering is APC (adenomatous
polyposis coli) (Wang et al., 2003), which interacts with multiple targets including β-catenin (Neufeld et al., 2000; Rosin-Arbesfeld et al., 2003), microtubules, the microtubule plus-end binding protein EB1, actin filaments and PSD93 (postsynaptic density 93) (Rosenberg et al., 2008; Su et al., 1995; Temburni et al., 2004). Further evidence suggested a direct role for Wnt signaling, and not just convergent downstream molecules. Wnt3 enhances Agrin induced AChR clustering in chick wing NMJ and cultured myotubes (Henriquez et al., 2008). Wnt11r is critical for growth cone guidance and prepatternning of AChR clusters in zebrafish embryos (Jing et al., 2009). Both of the above studies showed that the Wnts influencing NMJ are acting through non-canonical signaling pathways (Henriquez et al., 2008; Jing et al., 2009). There is also evidence showing that Wnt ligands can play negative roles in NMJ formation in vertebrates. Wnt3a can suppress Agrin induced AChR clustering when applied to cultured myotubes. (Wang et al., 2008). Studies in C. elegans have also demonstrated both positive and negative roles of Wnt signaling molecules at the NMJ. Shen group reported that LIN-44/Wnt sequestered LIN-17/Frizzled receptors to the undifferentiated subdomains of motorneuron axon. Downregulation of Wnt signaling led to ectopic presynapstic assembly in these subdomains, indicating an antisynaptogenic role of Wnt signaling (Klassen and Shen, 2007). Maricq group, however, discovered that Wnt-signaling mutants exhibited abnormal accumulation of AChRs at subsynaptic region on the muscle side, which led to synaptic current reduction.
and behavioral defects, suggesting Wnt signaling positively regulates NMJ transmission though inducing translocation of AChRs to synaptic region (Jensen et al., 2012). These discoveries in vertebrates and worms suggest that the functions of Wnt family members in NMJ development are rather complicated, and involve several different signaling pathways.

The roles of Wnt in Drosophila NMJ development were first reported by Budnik lab as they discovered that Wg, the Drosophila Wnt1, is secreted by motor neurons at the larval NMJ (Packard et al., 2002). A temperature sensitive allele of wg (wg\textsuperscript{ts}), in which Wg is trapped in the endoplasmic reticulum (ER) at restrictive temperature, showed dramatically reduced NMJ expansion, abnormal boutons devoid of active zones and lacking postsynaptic SSR. These abnormal boutons, known as ghost boutons, were later characterized as immature or undifferentiated boutons, which are also observed in normal development, although at a very low frequency (Ataman et al., 2008). In the wg\textsuperscript{ts} mutant, the differentiation of nascent boutons into mature boutons is disrupted, which leads to reduced bouton number and accumulation of ghost boutons.

Drosophila Frizzled-2 (DFz2) presents both pre- and post-synaptically at the larval NMJ, which triggers two different signaling pathways upon Wg activation. In the motor neuron, Wg activates the divergent canonical pathway regulating the microtubule cytoskeleton remodeling through GSK3β (glycogen synthase kinase
3 beta) inhibition (Korkut and Budnik, 2009; Packard et al., 2002). On the other side, Wg binds to DFz2 receptor on the muscle surface and triggers the frizzled nuclear import (FNI) pathway, in which DFz2 is internalized. *Drosophila* GRIP (Glutamate receptor-interacting protein), a 7-PDZ protein binds to DFz2 and facilitates the transportation of DFz2 along microtubules to the perinuclear region (Ataman et al., 2006; Mathew et al., 2005). The C-terminal of DFz2 is then cleaved and imported into nucleus, where it regulates nuclear export of mega ribonucleoprotein particles (megaRNP) containing mRNA critical for postsynaptic development (Mathew et al., 2005; Speese et al., 2012). Disruption of the FNI pathway by interfering the trafficking of DFz2 results in impaired NMJ expansion and increased accumulation of ghost boutons. Thus, Wnt signaling controls NMJ development in both vertebrate and invertebrate model systems, though very different signaling pathways are involved.

**Retrograde signaling at Drosophila NMJ**

At the *Drosophila* NMJ, there is not only anterograde signaling such as Wg signaling, but also retrograde signaling sent by the muscle cells to control the presynaptic differentiation.
One of the best characterized retrograde pathways at the Drosophila NMJ is BMP (bone morphogenic protein) signaling. Gbb (glass bottom boat), the homolog of BMP in Drosophila, is secreted by the muscle and functions as a retrograde ligand on the presynaptic receptor (McCabe et al., 2003). Downregulation of Gbb leads to abnormally small NMJs (McCabe et al., 2003). Gbb binds to a tetrameric receptor complex composed of two Type-I receptors: Saxophone (Sax) and Thickveins (Tkv), and two Type-II BMP receptors: Wishful thinking (Wit) and Punt (Put), all are serine/threonine kinases. The activated receptors phosphorylate Mad (Mother against decapentaplegic), the R-Smad (receptor regulated Smad) in Drosophila, which further interacts with Medea, the non-phosphorylated Smad (co-Smad) in fly, to form a phosphor-Mad complex (Keshishian and Kim, 2004). This phosphor-Mad complex is then translocated to nucleus and regulates transcription of trio, a Rho-type guanidine exchange factor (GEF), which controls synaptic growth (Ball et al., 2010).

A later study from Littleton lab discovered that Synaptotagmin4 (Syt4) controls a retrograde signaling pathway at Drosophila embryonic NMJ (Yoshihara et al., 2005). As a member of the Synaptotagmin family, Syt4 is a transmembrane protein localized to postsynaptic vesicles. Upon Ca\textsuperscript{2+} influx, Syt4 functions as a Ca\textsuperscript{2+} sensor and triggers postsynaptic vesicle fusion, which induces a retrograde signal that enhances presynaptic function. It was also demonstrated that the Syt4-dependent retrograde signaling regulates presynaptic function through
presynaptic cAMP- PKA (dependent protein kinase A) pathway (Yoshihara et al., 2005). Later on, Barber and colleagues further demonstrated that Syt4-dependent retrograde signaling also plays a role in the development of the larval NMJ as well as central nervous system synapses (Barber et al., 2009).

Exosomes at the NMJ

Extracellular vesicle (EV) is a general term referring to all membrane-surrounded vesicles that are released by cells to the extracellular environment, including exosomes, ectosomes and/or microvesicles and apoptotic bodies. The presence of EVs was discovered around late 1960s to early 1980s (Anderson, 1969; Colombo et al., 2014; Dvorak et al., 1981; Trams et al., 1981), however, the nomenclatures of the subtypes of EVs are still under debate. One of the most common classifications of EV subtypes depends on the size of the vesicle. Exosomes typically fall into the range of 50-100nm diameter. Ectosomes are usually larger with diameters ranging from 100-350nm. Many use the term microvesicles, which includes ectosomes, referring to vesicles with a broader diameter range 100-1000nm, but exclude apoptotic bodies (1000-5000nm size range).

While ectosomes or microvesicles are shedding vesicles derived from the plasma membrane, exosomes are released intraluminal vesicles (ILVs) upon exocytosis
of multivesicular body (MVB). These ILVs initiate from inward budding of late endosomal membrane, while the late endosome turns into MVB (Cocucci and Meldolesi, 2015).

One leading challenge in the field of EVs is to specifically isolate exosomes vs other microvesicles from culture and/or body fluid. The traditional way to isolate exosomes is based on differential centrifugation. Successive centrifugations with increased speed allow for the elimination of larger vesicles and cell debris, while small vesicles like exosomes can be sedimented at 100,000g. However, this method faces the challenge that small protein aggregates, or other vesicles within a similar size range can be sedimented together with exosomes. An improved method is to couple differential centrifugation with sucrose gradient, which separates protein aggregates from lipid-containing vesicles based on the density difference. However, all of the current methods only allow for enrichment of exosomes but cannot guarantee purity. Many publications use the term exosomes to refer to the small EVs in their studies, which are actually a mixed population of EVs (Colombo et al., 2014). To be consistent with the literature, however, I will continue using the term “exosomes” in the following part of my thesis.

Exosomes were first described by (Trams et al., 1981) and (Harding et al., 1983), followed by an electron microscopy (EM) study describing these vesicles (Pan et
al., 1985). However, for more than a decade, exosomes were only considered as a way to discard unwanted components from cells. It was not until 1996 that the significance of exosomes in intercellular communication was first recognized. Raposo and colleagues reported that exosomes released by B lymphocytes contain major histocompatibility complex class II molecules, and are capable of stimulating class II restricted T cell clones in vitro (Raposo et al., 1996). Later, a similar observation was made in dendritic cells, which release antigen presenting exosomes and trigger specific T cell activation (Zitvogel et al., 1998). The idea of exosomes being an extension of antigen presenting immune cells became widely accepted. In 2001, Wolfers and colleagues showed that tumor cells can secrete exosomes containing tumor specific antigens and these exosomes, when taken up by dendritic cells, activates the dendritic cell induced T cell dependent antitumor effects (Wolfers et al., 2001).

Although immune and tumor cells are the major systems in which exosomes have been characterized, later studies demonstrated that neurons and glia can also secret exosomes (Faure et al., 2006; Fevrier et al., 2004; Fruhbeis et al., 2013). Faure and colleagues discovered the presence of exosomes in rat cortical neuron culture, and these exosomes contain neuronal specific markers suggesting they originate in neurons. The release of these exosomes from cortical neurons can be stimulated by depolarization using medium containing high K⁺ (Faure et al., 2006). Fruhbeis and colleagues later showed that
oligodendrocytes secrete exosomes in response to neuronal activity. These oligodendrocyte secreted exosomes are internalized by cultured cortical neurons through endocytosis, and the cargos delivered by exosomes are functionally retrieved by recipient neurons (Fruhbeis et al., 2013).

The idea of exosomes as a mediator for intercellular communication is also supported by the discovery that the Wg molecule is transported with exosomes at Drosophila larval NMJ (Korkut et al., 2009). Wnts, as secreted glycoprotein ligands, mediate both short-range and long-range signaling (Swarup and Verheyen, 2012). As mentioned earlier, Wg is secreted by motor neurons and functions by binding to DFz2 receptors on the muscle surface at the larval NMJ. How does Wg, a highly hydrophobic molecule with several pamitoyl modifications, get transported in the extracellular environment? In 2006, two groups independently reported that Evi/Wls (Evenness interrupted/Wntless), a multipass transmembrane protein, is required for Wg secretion in Drosophila S2 cells and wing discs (Banziger et al., 2006; Bartscherer et al., 2006). Later, the Budnik group showed that Evi is also required for Wg transfer at larval NMJ (Korkut et al., 2009). Evi is localized both pre- and postsynaptically at larval NMJ. Expression Evi-RNAi in the motoneurons not only reduces Evi levels presynaptically but also postsynaptically, suggesting Evi is transferred from the motoneurons to the muscle compartments. In evi mutant, Wg accumulates in motoneuron cell bodies and both pre- and postsynaptic Wg level is significantly decreased,
suggesting that Evi is required for Wg secretion from the motorneurons. In addition, evi mutant larvae showed decreased bouton number and accumulation of the immature “ghost” boutons at the NMJ, similar to mutants affecting Wg/FNI signaling. These results indicate Evi is required for Wg secretion and transfer at the larval NMJ (Korkut et al., 2009). Moreover, in the motorneurons, Evi is localized to the MVB where exosomes originate. (Korkut et al., 2009). When expressed in S2 cells, Evi is found present in S2 cell-secreted exosomes (Koles et al., 2012). These data suggest that the transfer of Wg from pre- to the post-synapse at the larval NMJ is mediated by Evi, likely through exosome.

The release of exosomes requires membrane fusion between the MVB and the plasma membrane. Up until now, the major machinery for exosome release identified includes Rab proteins, a small GTPase family controlling different steps in vesicle trafficking; and SNARE (Soluble NSF Attachment protein REceptror) proteins, which control vesicle fusion. Koles and colleagues conducted a dsRNA screen on S2 cells and discovered that Rab11, a small GTPase, and syntaxin1A, a SNARE protein, are required for Evi-containing exosomes release in S2 cell culture and at the NMJ (Koles et al., 2012). Other studies found that Rab27a, Rab27b and Rab35 control exosomes release in oligodendrocytes and HeLa cells (Hsu et al., 2010; Ostrowski et al., 2010). Interestingly, Rab27a, Rab27b or Rab35 does not affect Evi-containing exosome secretion from S2 cells (Koles et al., 2012), while Rab11 controls exosome release from human erythroleukemia
cells (Savina et al., 2002), but does not affect exosome release from HeLa cells (Ostrowski et al., 2010). Apart from syntaxin1A, another SNARE protein Ykt6 was identified from a separate screen done in Drosophila S2 cells (Gross et al., 2012). Knockdown of Ykt6 inhibits the release of Wnt-containing exosomes from S2 cells (Gross et al., 2012). However, neither Ykt6 nor syntaxin1A is required for exosome release in the HeLa cell model (Colombo et al., 2014). These inconsistencies indicate the heterogeneity of exosome release based on the tissue and cell types.

One interesting question about exosome is: what does it carry? Exosomes have been found to contain both protein and RNA. Numerous proteomic studies have been performed on exosomes purified from a variety of cell types. It is not surprising that depending on the cell types and tissue origin, exosomes have different protein compositions. However, while some proteins are ubiquitous in exosomes regardless of their origins, including heat shock proteins, 14-3-3, tetraspanins and translation machinery, others such as those enriched in nucleus, mitochondria or Golgi complex are not (Colombo et al., 2014; Koles and Budnik, 2012; Thery et al., 2002). The first evidence showing RNA present in exosomes was reported by (Valadi et al., 2007). In this study, Valadi and colleagues reported the presence of both mRNA and microRNA in exosomes secreted by mast cells using microarray techniques. They further demonstrated that mRNA in exosomes remains translationally active after being taken up by recipient cells.
Specific miRNAs transferred through exosomes were also reported later (Montecalvo et al., 2012; Pegtel et al., 2010). These studies also showed that the expression of reporter genes in the recipient cells is downregulated by the acquired miRNA from exosomes, suggesting the exosomal miRNAs are functionally active.

In order to deliver the content into the target cells, exosomes need to be taken up by the recipient cells. Most studies have shown that exosomes are taken up as intact vesicles, which can be through receptor dependent endocytosis (Fruhbeis et al., 2013; Tian et al., 2013), macropinocytosis (Fitzner et al., 2011) or phagocytosis (Feng et al., 2010), likely depending on the recipient cell type. Some studies, however, have proposed an opposing model in which exosomes fuse with the plasma membrane of recipient cells directly to release their content (Del Conde et al., 2005; Parolini et al., 2009).

In the first part of my thesis, I will demonstrate how exosome release from motorneurons controls a Syt4-dependent retrograde signaling during NMJ development.

Nuclear envelope budding pathway as a novel RNA export pathway
All eukaryotic cells have a nucleus, which is a double membrane surrounded structure containing genetic material. The double membrane, known as nuclear envelope (NE), separates the content of nucleus from the cytosol. Based on conventional understanding, the communication between cytosol and nucleus is through the nuclear pore complex (NPC). The NPC is a large protein complex composed of multiple copies of around 30 distinct proteins, collectively called nucleoporin (Nup), which adds up to around 500 polypeptides (Fradkin and Budnik, 2016). Proteomic studies and computational modeling have revealed the structure of NPC as a barrel with a central pore surrounded by layers of proteins forming an inner ring, an outer ring and a membrane ring, with the central pore serving as the connecting tunnel between cytosol and nucleoplasm (Hoelz et al., 2011). Both passive diffusion and facilitated transport are supported by the NPC. Ions, small molecules and proteins less than 40kD may pass the NPC more freely, while proteins and ribonucleoproteins beyond the size limit require specific transporters, or interaction with NPC associated elements (Wente and Rout, 2010).

The internal diameter of the NPC is around 39nm. However, some viral capsids and ribonucleoprotein (RNP) particles have diameters far beyond this limit with some more than 100nm (Wente and Rout, 2010). For RNP particles, it is possible that they are assembled in the cytosol from smaller particles that can fit through the NPC. One alternative mechanism proposed by Mehlin and colleagues is
based on the observation that the Balbiani ring RNP particle, which has a diameter of ~50nm, can reorganize and adopt an elongated conformation before entering the pore (Mehlin et al., 1992).

Herpes viruses (HVs), however, use a NPC-independent pathway to export their capsids from the nucleus. After infection and replication, newly synthesized and packed HV capsids need to leave the host cell nucleus. These HV capsids are on average ~125nm in diameter, which makes it difficult for them to squeeze through the NPC. Instead, HV capsids use an alternative pathway, in which they disrupt the lamina and get enveloped by the inner nuclear membrane (INM), allowing them to enter the perinuclear space in an INM coated form. These enveloped capsids are then de-enveloped as the INM coat fuses with the outer nuclear membrane, which allows the naked capsids to be released into the cytosol (Bigalke and Heldwein, 2015; Fradkin and Budnik, 2016; Johnson and Baines, 2011; Mettenleiter et al., 2013).

A recent study by Speese and colleagues discovered that this nuclear envelope (NE) budding is not a virus specific mechanism, but also an endogenous pathway used by mega ribonucleoprotein particles (megaRNPs) to exit the nucleus (Speese et al., 2012). This finding was initiated with the observation that the C terminus of DFz2 (DFz2C), the downstream component of the FNI pathway, when cleaved and imported into the muscle nucleus, associates with nuclear
lamina and forms a frame-like structure at the periphery of the nucleus (Figure 1.1A). The major component of larval muscle nuclear lamina is LamC, the Drosophila A-type Lamin. Both LamC and DFz2C are essential for the formation of these LamC/DFz2C foci, as lamC null or muscle knockdown of LamC showed largely decreased foci number and increased ghost bouton number, a phenotype consistent with disrupted FNI pathway. Further deconvolution analysis revealed that these foci contain individual DFz2C granules, typically forming clusters, associated with the nuclear envelope (Figure 1.1A). Ultrastructural study using electron microscopy (EM) confirmed that these DFz2C/LamC foci observed at light level are invaginations of the inner nuclear membrane with accumulation of electron dense granules present at the perinuclear space between the INM and ONM (Figure 1.1B). These electron dense granules are bounded by membrane, which correlates well with the observation that HV capsids are bounded by INM when localized within the perinuclear space. Unlike HV capsids, however, these granules do not contain DNA as no co-localization between DFz2C/LamC foci with DNA dye was observed. Instead, these foci show co-localization with PABP2 (polyadenylate-binding protein2), an mRNA binding protein, suggesting the presence of RNA in these foci. Further in situ hybridization study using an oligo-d(T) probe confirmed the presence of mRNA in the foci. Bernhard’s regressive EDTA (rEDTA) stain was performed to verify the presence of RNA in the electron dense granules at EM level. EDTA selectively binds electron dense uranyl
acetate from DNA but not from RNA, which leads to the elimination of electron density caused by DNA (Bernhard, 1969). The retention of electron dense staining in the large electron dense granule between INM and ONM evidence the presence of RNA in these granules. *In situ* hybridization based on a candidate approach for mRNAs known to function in postsynaptic development showed that specific transcripts including *par6* and *magi* were found present in the foci. *LamC* null or animals with *LamC-RNAi* expressed in muscle showed dramatically decreased postsynaptic level of *par6* and *magi* transcripts and protein, suggesting megaRNP granules leave the nucleus and presumably get transported to postsynaptic site for local translation. The release of granules was demonstrated using E36, a RNA specific dye, coupled with fluorescent live imaging. Samples were then fixed and labeled with *LamC* and DFz2C antibody. Images from fixed samples were then superimposed with live images. It was revealed that E36 labeled puncta emerge from LamC foci and are released into the cytosol (Speese et al., 2012).

These membrane bound electron dense granules highly resemble the enveloped HV capsids at the perinuclear space, raising the possibility that these granules exit the nucleus using a mechanism similar to NE-budding. As mentioned earlier, HV capsids need to dissociate the lamina before contacting the INM. It has been demonstrated that HVs use their own kinases or recruit cellular kinases, such as protein kinase C (PKC), to phosphorylate lamina protein (Marschall et al., 2011;
Muranyi et al., 2002). Interestingly, atypical PKC (aPKC), a nonconventional PKC in *Drosophila*, showed nuclear immunoreactivity. Downregulation of aPKC using RNAi largely reduced the LamC/DFz2C foci number, suggesting aPKC is required for foci formation. Expression of PKM, a constitutively active version of aPKC, increased the phosphor-LamC level, and increased the number and size of LamC/DFz2C foci, suggesting aPKC regulates phosphorylation of nuclear lamina. These data further support the idea that megaRNP granules in the *Drosophila* larval body wall muscle use a NE-budding pathway for nuclear exit akin to herpes virus (Speese et al., 2012). Another protein, TorsinA, has been found to be involved in the viral capsid egress (Maric et al., 2011). TorsinA belongs to AAA-ATPase superfamily, which is localized at both the perinuclear space and ER maintaining the nuclear envelope structure and ER function respectively (Gerace, 2004; Goodchild et al., 2005; Laudermilch and Schlieker, 2016). Mutation of TorsinA has been linked to DYT1 dystonia, a neurological movement disorder in human (Granata and Warner, 2010). In DYT1 dystonia patients, a 3-nucleotide deletion in DYT1 gene leads to the deletion of a glutamate residue in TorsinA protein (Tanabe et al., 2009). Knockin mice expressing dystonia-modeled Tor1A^{ágag/ágag} mutant displayed accumulation of vesicular structure at the nuclear envelope (Goodchild et al., 2005). A later study by Jokhi and colleagues discovered *Drosophila* Torsin, the only fly ortholog of TorsinA, controls NE-budding of megaRNP (Jokhi et al., 2013). Knockdown of
Torsin or expressing a mutant form of Torsin modeling dystonia patients leads to accumulation of RNP granules at perinuclear space similar to what has been observed in dystonia mouse models. These RNP granules are stuck at INM after envelopment as they are still tethered to the INM. Expressing a substrate trap Torsin E-Q mutant with a miniSOG tag, a small tag that allows visualization of the protein by TEM (Shu et al., 2011), showed enrichment of Torsin at the neck of stuck granules, indicating a possible scission role of Torsin. Consistent with the model, torsin mutant and knockdown showed reduced postsynaptic par6 and magi levels and increased ghost bouton formation (Jokhi et al., 2013).

These evidences support the presence of a novel RNA export mechanism separated from NPC, known as NE-budding pathway, which is important for large RNP exit from nucleus (See Figure 1.2 for the overview of the NE-budding pathway).

**Mitochondria and aging**

The link between mitochondria and aging has been speculated, studied and debated for decades. Numerous studies imply that mitochondrial function declines with aging. Such evidence includes: reduced ATP generation, accumulation of reactive oxygen species (ROS), loss of mitochondrial mass, increased mtDNA mutations, and altered mitochondrial morphology in aged
animals. The idea that mitochondrial dysfunction is associated with aging has been widely accepted, however, no causative relation has been concluded. One popular theory regarding mitochondria and aging is that due to the electron transport chain (ETC) reaction that involves oxidative reactions constantly, mitochondria are a major source of ROS, or free radicals. During aging, mitochondrial function declines, causing increased ROS production. The free radical theory of aging, first raised by Harman in 1956 (Harman, 1956), proposes that the accumulation of free radicals with age causes the damages to biomolecules and cellular components, which eventually leads to cell senescence and aging. The ETC consists of five protein complexes (Complex I-V) located on the inner membrane of mitochondria, which conduct a series of redox reactions to convert acetate and \( O_2 \) into \( H_2O \) and \( CO_2 \) while generating the energy to phosphorylate ADP into ATP. In the chain reactions of oxidative phosphorylation, superoxide is generated as a byproduct. Less harmful by itself though, superoxide can be converted to more toxic free radicals such as hydroxyl radical (\( OH^- \)) and peroxynitrite anion (ONOO-), especially when the antioxidative system is compromised. ROS can cause damage to a variety of cellular components including protein, lipids and DNA. For example, free radicals can oxidize guanidine, change its electron status and cause nucleotide mismatching during DNA replication, which eventually leads to mutations. ROS can also cause inter- and intra-strand DNA crosslink, or DNA-protein crosslink, which
leads to a diversity of cellular damages such as DNA break, inhibited transcription and replication, etc. (Cadet and Wagner, 2013; Jena, 2012). Moreover, ubiquitin proteasome disassembly can be induced by increased ROS level caused by mitochondrial dysfunction, which results in accumulation of proteasome substrates (Huang et al., 2013; Livnat-Levanon et al., 2014; Ross et al., 2015). The accumulation of oxidative stress, DNA damage and proteasome failure leads to the development of various age related disease including cancer, cardiovascular diseases, and neurodegeneration. Mice deficient in superoxide dismutase (SOD2), which serves as a primary defense against superoxide, exhibited severe myocardial injury, neurodegeneration and premature death (Lebovitz et al., 1996); while transgenic Drosophila with SOD overexpression showed increased life span (Sun et al., 2002). However, opposing evidences have also been reported showing actual benefits of ROS in cellular functions and organism longevity (Ristow and Schmeisser, 2011). Mild increase of ROS by inhibiting or downregulating respiratory chain components can extend life span in C. elegans (Lee et al., 2010; Sanz, 2016; Yang and Hekimi, 2010). Nevertheless, little evidence so far has shown that ROS can extend longevity in fruit flies or mammals (Sanz, 2016).

Mitochondria have their own genome known as mtDNA which includes 37 genes with 13 of them encoding proteins involved in oxidative phosphorylation and the rest encoding transfer RNA (tRNA) and ribosomal RNA (rRNA). Each cell has
numerous mitochondria, with each containing several copies of mtDNA. The mtDNA genes have a much higher mutation rate, possibly due to the higher replication frequency. This leads to a mixed mtDNA population within a single cell or even a single mitochondrion, which is called heteroplasmy (Wallace and Chalkia, 2013). The amount of mtDNA mutations increases with age. Increased deletions and point mutations of mtDNA have been observed in the human brain, liver and colonic crypt stem cells with advanced age (Bratic and Larsson, 2013). Direct evidence suggesting mtDNA mutation as a driving force of aging has been shown in mouse models. Transgenic mice with mutated subunit of mtDNA polymerase (PolgA\textsuperscript{mut}), which causes proof-reading deficiency, display a range of premature aging related phenotypes, including alopecia, kyphosis, osteoporosis, cardiomyopathy, reduced fertility and shortened life span (Trifunovic et al., 2004). The increased amount of mtDNA in these PolgA\textsuperscript{mut} mice were found not associated with elevated ROS markers or cell proliferation, but correlated with increased apoptotic markers (Kujoth et al., 2005).

The link between mitochondria and aging is further strengthened by the fact that mitochondrial dysfunction is highly associated with neurodegenerative disorders such as Amyotrophic Lateral Sclerosis (ALS) and Parkinson’s disease (PD). Mutation of SOD1 is responsible for a familial form of ALS, a neurodegenerative disease featured with loss of motor neurons in brainstem, cortex and spinal cord. EM analysis showed abnormal mitochondrial morphology in human postmortem
skeletal muscle, motor cortex and spinal cord (Tan et al., 2014). SOD1 mutant mice displayed fragmented mitochondrial network, impaired axonal transport of mitochondria, compromised electron transport chain activity and Ca$^{2+}$ buffering capacity preceding motor neuron degeneration and onset of disease related symptoms (Cozzolino et al., 2015; Tan et al., 2014). On the other side, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), an impurity of desmethylprodine production, induced rapid onset of Parkinsonism in a group of people who consumed a contaminated batch of opioid drug (Langston and Palfreman, 1995). The postmortem examination confirmed the loss of dopaminergic neurons in substantia nigra (Langston and Palfreman, 1995). Although non-toxic by itself, MPTP can be metabolized to MPP$^+$, a neurotoxin which inhibits complex I of the mitochondrial respiratory chain, by monoamine oxidase after entering glial cells (Tipton and Singer, 1993). Since then, MPP$^+$ injected mice have been used as Parkinson’s disease model and mitochondrial defects have been repeatedly observed in MPP$^+$ treated mouse brain. A similar chemical is rotenone, used as pesticide due to its inhibitory effect on mitochondrial ETC complexes. Rodents administrated with rotenone developed Parkinsonism-like symptoms: presence of Lewy-body like inclusion bodies together with selective neurodegeneration in nigrostriatal system (Betarbet et al., 2000; Cannon et al., 2009). Genetic studies of PD also reveal the strong correlation between mitochondrial dysfunction and onset of the disease. Mutations of PINK1 and parkin have been found in early-
onset familial PD patients. PINK1 is a kinase localized to the outer membrane of mitochondria with its kinase domain facing the cytoplasm (Zhou et al., 2008), while parkin, an ubiquitin E3 ligase, has been found associated with mitochondria (Li et al., 2011). *Drosophila pink1* or *parkin* null mutants displayed infertility, reduced life span, muscle and dopaminergic neuron degeneration and morphologically abnormal mitochondria, while parkin overexpression rescues phenotypes seen in *pink1* mutants suggesting a common signaling pathway (Clark et al., 2006; Greene et al., 2003; Park et al., 2006). PINK1 is stabilized at impaired mitochondria and parkin is selectively recruited to damaged mitochondria to promote mitophagy (Geisler et al., 2010; Narendra et al., 2008; Narendra et al., 2010). It was further demonstrated that PINK1 phosphorylates Miro, a component of motor/adaptor complex mediating mitochondria axonal transportation, and induces the Parkin-dependent degradation of Miro, which leads to arrested mitochondrial motility (Wang et al., 2011). The interaction between PINK1, Parkin with Miro is enhanced by dissipation of mitochondrial membrane potential (Wang et al., 2011). These results indicate that PINK1 and Parkin participate in the quality control of mitochondria by isolating and sequestrating damaged mitochondria presumably for mitophagy degradation.

This also raises another interesting feature of mitochondria, mitochondrial dynamics. There are two aspects of mitochondrial dynamics: fission/fusion and motility. Mitochondria constantly fuse and divide, which is an important process
for maintaining mitochondrial homeostasis. In mammalian systems, mitochondrial fusion is mediated by mitofusins (Mfn1 and Mfn2), which are GTPases associated with the mitochondrial outer membrane and control outer membrane fusion; OPA1 (Optic Atrophy 1), another GTPase controls inner membrane fusion. Mitochondrial fusion enables content exchange between mitochondria. As stated above, mitochondria exist in a heteroplasmic way, with some mitochondria having more mtDNA mutations than others. During cell division, these mitochondria can be separated into daughter cells unevenly leading to one daughter cell containing more mtDNA mutations. Fusion is theorized to play a protective role by allowing mitochondria with damaged or mutated mtDNA to dilute their damage by exchanging content with healthy mitochondria, which homogenize the heteroplasmic mitochondrial population and prevent genetic drift in daughter cells (Chan, 2006). This is believed to be critical for mitochondrial health and proper cellular function. Mice with a skeletal muscle specific knockout of Mfn1 and Mfn2 exhibit swollen mitochondria with collapsed cristae structure (Chen et al., 2010). These double knockout mice also displayed an increase of mtDNA point mutations and deletions compared to their littermate, suggesting a lack of mitochondrial content mixing is responsible for mtDNA instability (Chen et al., 2010). Mutations of Mfn2 cause Charcot-Marie Tooth Type 2A (CMT2A) in humans, which features axonal peripheral neuropathy, muscle atrophy and sensory loss at distal limbs (Chan, 2012). Fission, the opposite motion of fusion,
is mainly controlled by Drp1 (dynamin related protein 1), together with Fis1 (fission protein 1), MiD49 and MiD51 (mitochondrial dynamics protein of 49kDa and 51kDa) in mammalian cells. Drp1 is a dynamin-like GTPase with cytosolic localization but recruited to the mitochondrial outer membrane upon fission. Several fission factors located on the mitochondrial outer membrane including Fis1 and Mff (mitochondrial fission factor) have been found functioning as recruiters of Drp1 (Mozdy et al., 2000; Otera et al., 2010). Other pro-fission factors such as MiD49 and MiD51 have also been identified, as knockdown both of these two results in elongated mitochondria, while the underlying mechanism remain unclear (Chan, 2012; Palmer et al., 2011). Mitochondrial division is critical for separating mitochondria during mitosis. The function of fission, however, is not limited to the biogenesis of mitochondria. Excessive fission is found associated with enhanced apoptosis, caused by release of cytochrome c. Inhibiting Drp1 function prevents loss of mitochondrial membrane potential, reduces cytochrome c release from mitochondria and blocks apoptosis (Frank et al., 2001). Consistent with this, downregulation of Fis1 also inhibits apoptosis, while depletion of OPA1 sensitizes the cells to apoptosis induction (Lee et al., 2004). Mitochondrial fission is also linked to mitophagy. In Drp1 deficient mouse embryonic fibroblast, parkin-dependent mitophagy is inhibited (Tanaka et al., 2010). Moreover, Parkin/PINK1 mediates Mfn1 ubiquitylation and degradation upon induction of mitochondrial depolarization in neuroblastoma cell lines.
(Tanaka et al., 2010). These results suggest that fission is critical for degradation of damaged mitochondria through mitophagy likely through keeping mitochondria smaller and easier for mitophagosome engulfment (Chan, 2012).

The direct link between mitochondrial fission/fusion and aging is not clear. Evidences supporting either favored fission or fusion in aged organisms have been reported. Muscle cells derived from aged rats displayed smaller mitochondria and increased level of fission proteins (Iqbal et al., 2013). In *C. elegans* body wall muscle, mitochondria exhibit age-dependent fragmentation and volume loss (Regmi et al., 2014). Hepatic cells and skin fibroblasts from aged human show decreased mitochondrial size and number (Solmi et al., 1994; Tauchi and Sato, 1968). On the other side, giant or swollen mitochondria were observed in human skeletal muscle cells and lymphocytes (Beregi and Regius, 1987) as well as in *C. elegans* (Yasuda et al., 2006) at the ultrastructural level. Most of these studies, however, are based on the morphological analysis, which does not necessarily reflect fission/fusion dynamics.

Apart from constant fission and fusion, mitochondria also move around in the cell. Mitochondrial transportation is especially important for highly polarized cells such as neurons to ensure proper local mitochondria density. Mitochondria can be transferred along microtubules in either anterograde or retrograde manner in axons, while in dendritic spines, growth cones and synaptic boutons,
mitochondria can be transported along actin filaments (Lovas and Wang, 2013). Miro/Milton/Kinesin-1 complex is the most well characterized motor/adaptor complex mediating transportation of mitochondria from the soma to the axonal terminal. Miro is associated with mitochondria through its transmembrane domain, which is inserted in the mitochondrial outer membrane. Milton interacts with Miro directly and connects Milton to kinesin-1, which moves on microtubules towards the (+)-end. Misregulated mitochondrial motility has been implicated in several neurological diseases including Parkinson’s disease, Alzheimer’s disease, Schizophrenia, CMT-type2A, ALS and Huntington’s disease with detailed mechanisms unclear (Lovas and Wang, 2013). However, how mitochondrial motility is affected during normal aging and whether it contributes to aging remains obscure.

**Lamin, Laminopathies and premature aging**

As mentioned earlier, nuclei in eukaryotic cells are separated from the cytoplasm by the nuclear envelope, a double membrane structure. The outer nuclear membrane (ONM) is continuous with the endoplasmic reticulum (ER). Underneath the inner nuclear membrane, the nuclear lamina forms a meshwork like structure. The major components of nuclear lamina are lamin proteins. Lamins can be classified into two categories: A-type and B-type lamins. In human,
A-type lamins includes LaminA, LaminC and two other less ubiquitous variants LaminC2 and LaminAΔ10, which are encoded by a single gene, *LMNA*, through alternative splicing. B-type lamins are encoded by two genes *LMNB1* and *LMNB2*, which give three proteins: LaminB1 from *LMNB1* and LaminB2, B3 from *LMNB2* (Dechat et al., 2008; Schreiber and Kennedy, 2013). B-type lamins are expressed in all cell types and are crucial for early embryonic development, while A-type lamins are mainly expressed in differentiated somatic cells (Burke and Stewart, 2002).

Lamins belong to the type-V intermediate filament (IF) family of proteins, the only IF localized to the nucleus. All lamins have an N-terminal globular domain, a central α-helical rod domain followed by a C-terminal immunoglobulin-fold (Ig-fold) domain. LaminB1, LaminB2 and LaminA have a CAAX (C=Cysteine, A=Aliphatic, X=any) motif at the C-terminal end that requires posttranslational modifications to form the mature proteins. The CAAX motif is first farnesylated at the cysteine residue, followed by the cleavage and removal of AAX residues. The cysteine is further carboxylated in all the three lamins, while LaminA requires another cleavage by Zmpste24 (Zinc metalloprotease related to Ste24p) to remove 15 more amino acids in order to form mature LaminA (Dechat et al., 2008).

In order to form the nuclear lamina, a 20-50nm thick protein meshwork underneath INM, lamins need to assemble into higher-ordered structure. It is not
clear how lamins organize to form the meshwork-like structures in vivo due to the
difficulties of in situ imaging of the dense nuclear lamina at high resolution. In
vitro studies have demonstrated that lamins form homodimers through the coiled-
coil interaction between central rod domains. These dimers further associate with
each other in a head-to-tail manner to form polymers, and these polymers
associate laterally into beaded filaments or fibers and eventually into paracrystal
structures depending on the assembly conditions (Gieffers and Krohne, 1991;
Heitlinger et al., 1991; Stuurman et al., 1998).

Lamins, as major components of nuclear lamina underneath nuclear inner
membrane, provide critical mechanical support for the nucleus. Numerous
studies have reported nuclear deformity in lamin deficient cells or cells
expressing lamin mutations. LMNA−/− cells displayed misshapen nuclei, reduced
nuclear stiffness and increased cell fragility under mechanical strain (Broers et al.,
2004; Lammerding et al., 2006; Lammerding et al., 2004). Fibroblasts from
Lmnb1 deletion mutant mice exhibit striking nuclear dysmorphology and nuclear
envelope lobulation (Vergnes et al., 2004). Lamins, like other scaffolding proteins,
interact with many structural proteins including LEM (Lap2, emerin, MAN)-
domain proteins, Nup153, nuclear actin, and SUN-domain proteins. Loss of LEM-
domain protein such as emerin also leads to fragile nuclei in mouse skeletal
muscle (Ozawa et al., 2006) and abnormal nuclear shape in mouse embryonic
fibroblasts (Lammerding et al., 2005), suggesting lamins functions together with LEM-domain proteins to ensure structural support of the nucleus.

As nucleoskeletal components, LEM-domain proteins are also critical for the chromatin organization. Loss of function studies in *C. elegans* (Liu et al., 2003) and mammalian cells (Huber et al., 2009) demonstrated that LEM-domain proteins are essential for chromatin organization though in a redundant manner (Brachner and Foisner, 2011). Most LEM-domain proteins contain transmembrane domains inserted into INM. Their LEM domains tether chromatin to the nuclear periphery by binding to DNA or chromatin proteins directly, or through their interaction with BAF (Barrier to Autointegration Factor), a chromatin binding protein (Barton et al., 2015; Wilson and Foisner, 2010). Lamins by themselves have been found to interact with chromatin typically at regions with low gene expression (Guelen et al., 2008). Consistent with this, downregulating lamins or expressing lamin mutations also leads to chromatin organization defects (Dittmer and Misteli, 2011). *Lmna*−/− mouse embryonic fibroblasts exhibit loss of peripheral heterochromatin and abnormal condensation of chromosome territories (Galiova et al., 2008). Expressing a mutant LaminA with 50 amino acid deletion (Lamin∆50), which mimicks HGPS patient progerin, results in downregulated heterochromatin markers and altered histone methylation patterns (Shumaker et al., 2006).
Besides LEM-domain protein, lamins also interact with SUN-domain (Sad1 and UNC-84 homology domain) proteins, another group of integral membrane proteins localized at the inner nuclear membrane (INM). SUN-domain proteins further interact with KASH-domain (Klarsicht, ANC-1 and SYNE1 homology domain) proteins, another group of integral nuclear membrane proteins with cytoplasmic domains interacting with cytoskeleton proteins (Tzur et al., 2006). SUN-domain and KASH-domain proteins form the linker of nucleoskeleton and cytoskeleton (LINC) complex, providing intracellular force transmission (Crisp et al., 2006; Ho and Lammerding, 2012). The LINC complex, together with lamins, controls nuclear positioning, cell migration and polarity (Ho and Lammerding, 2012).

Apart from structural support, lamins are also involved in cellular signaling and gene expression. Lamins interact with transcription regulators such as pRb (retinoblastoma protein), c-Fos (FBJ osteosarcoma oncogene), SREBP1 (sterol regulatory element-binding protein 1), Oct-1 (POU class 2 homeobox 1), MOK2 (Zinc-finger protein 239) either transiently or stably (Wilson and Foisner, 2010). Interactions with either A- or B-type lamin sequester some of these transcription factors and prevent their activation (Dittmer and Misteli, 2011; Wilson and Foisner, 2010). For example, the transcription factor c-Fos is sequestered and inactivated by LaminA/C until the activated MAP kinase signaling molecule ERK1/2 binds to LaminA/C and phosphorylates c-Fos. Downregulation of
LaminA/C leads to constitutively active c-Fos dependent transcriptional activity (Gonzalez et al., 2008). Consistently, upregulated MAPK signaling was also observed in mice expressing LMNA loss-of-function mutation (Muchir et al., 2009). In addition, lamins likely participate in DNA replication through interaction with proliferating cell nuclear antigen (PCNA) at replication foci (Shumaker et al., 2008).

Lamin mutations in human are associated with a spectrum of diseases collectively called laminopathies. Most of the laminopathies are linked to mutations of LMNA. This is likely due to the fact that the proper function of B-type lamins are essential for early embryonic development and mutations of LMNB1 and LMNB2 result in early embryonic abolishment. One mysterious but also interesting fact is that mutations of LMNA, a single gene, lead to many different kinds of laminopathies affecting various tissues. LMNA mutations are found in patients with striated muscle diseases, lipodystrophy syndromes, accelerated aging disorders, peripheral nerve disorders and bone diseases (Schreiber and Kennedy, 2013). Some of the laminopathies are caused by mutations of lamin-associated proteins. For example, Emery-Dreifuss muscular dystrophy (EDMD), characterized by progressive muscle weakening and wasting beginning in childhood, was initially identified as X-linked muscular dystrophy caused by mutation of emerin, a LaminA/C binding protein (Bione et al., 1994). Several cases of autosomal forms of EDMD, which is clinically identical to the X-linked
form of disease, were later reported and genetically mapped to mutations in the *LMNA* gene (Bonne et al., 1999; Brown et al., 2001; Raffaele Di Barletta et al., 2000). Other myopathies linked to *LMNA* mutations include limb girdle muscular dystrophy (LGMD) and dilated cardiomyopathy (DCM) (Capell and Collins, 2006; Schreiber and Kennedy, 2013). These myopathies have been attributed to a variety of mutations located throughout the *LMNA* gene. Interestingly, most of the autosomal EDMD mutations are mapped to the central rod domain of LaminA/C, the domain responsible for dimerization, suggesting the assembly of LaminA/C protein may be affected in these patients (Capell and Collins, 2006). *LMNA* point mutations which affect fat storage and metabolism causing familial partial lipodystrophies (FPLD) fall mostly into two exons: 8 and 11, which encode the C-terminal region of LaminA/C protein, suggesting disruptions of the C-terminal Ig-fold might be responsible for lipodystrophy onset (Burke and Stewart, 2002).

Perhaps one of the most devastating laminopathies characterized so far is Hutchinson-Gilford progeria syndrome (HGPS), an extremely rare (1 in 8 million) premature aging disorder. HGPS was initially described by Jonathon Hutchinson in 1886 and by Hasting Gilford in 1897 independently. Patients are typically born normal but within one year start to develop aging-like phenotypes including hair loss, wrinkled skin, atherosclerosis, cardiovascular problems and musculoskeletal degeneration. Death usually occurs during early teen years. The genetic factors of HGPS were not identified until 2003. Two groups reported that
more than 90% of HGPS patients analyzed carried a de novo single nucleotide substitution (C1824T a.a.G608G) in the LMNA gene. Although the substitution from C to T does not affect the encoded glycine residue, this mutation creates an aberrant splicing donor site within exon11 (De Sandre-Giovannoli et al., 2003; Eriksson et al., 2003). As mentioned earlier, pre-laminA protein is farnesylated at the CAAX motif and the last 18 amino acids at C-terminal are removed in two steps by endopeptidases to form mature LaminA. The aberrant splicing removes the second cleavage site resulting in a Lamin mutant with retained farnesylation which cannot be processed to mature LaminA (known as progerin) (Eriksson et al., 2003). The farnesylated progerin targets the nuclear envelope causing abnormal nuclear shape, nuclear envelope blebbing, nuclear lamina thickening, loss of peripheral heterochromatin and nuclear pore clustering in a passage and dose dependent manner (Goldman et al., 2004; Yang et al., 2005). Zmpste24, a metalloproteinase, was discovered to be responsible for the second cleavage of farnesylated prelaminA (Bergo et al., 2002). Zmpste24-deficient mice exhibited premature aging related phenotypes including hair loss, premature death, muscle weakness, and osteogenesis resembling some of the clinical features of HGPS patients (Bergo et al., 2002; Pendas et al., 2002). One theory is that the toxicity of progerin is at least partially due to the permanent farnesylation. This theory has been supported by evidence provided by several independent groups including: 1. cellular defects such as misshapen nuclei and nuclear blebbing
caused by progerin can be rescued by farnesyltransferase inhibitors (Young et al., 2006); 2. HGPS mouse models showed enhanced survival and improved physiological conditions after administration of farnesyltransferase inhibitors (Young et al., 2006); 3. Knock in mice generating non-farnesylated progerin, in which the -CSIM sequence was modified to -CSM, displayed lower frequency of misshapen nuclei, normal body weight and survival without bone disease (Yang et al., 2011).

Besides nuclear deformation and abnormal nuclear envelope morphology and function, studies have demonstrated other altered cellular pathways in HGPS cells including decreased cell proliferation (Goldman et al., 2004), enhanced DNA damage/compromised DNA repair system (Liu et al., 2005), faster telomere shortening (Decker et al., 2009), increased ROS (Viteri et al., 2010) and abnormal mitochondrial morphology and function (Rivera-Torres et al., 2013; Xiong et al., 2016). These pathways may interplay with each other making the disease mechanism even more complicated.

In the initial discovery of LMNA mutation as the cause of HGPS, one HGPS patient carried a heterozygous missense mutation E145K instead of the most common G608G silent mutation. This raised the possibilities that other mutations of LMNA may cause progeria as well. There are so far at least 21 point mutations of LMNA (http://www.umd.be/LMNA/) other than G608G that have been
discovered causing progeria or progeroid (a more general term for premature aging disorders as the term “progeria” is frequently used to mean specifically HGPS). These progeroid causing LMNA mutations are found throughout the whole gene including those in the N-terminal globular domain P4R, T10I; those in the central rod domain such as S143F, E145K, E159K, E262K, D300N and those in the C-terminal Ig-fold domain like R471C, P485R, R527C. The progeria/progeroid cases caused by non-typical mutations other than G608G are referred to atypical progeroid/progeria syndrome (APS) or simply progeroid syndrome (PS). To be consistent, progeroid syndrome (PS) will be used in this thesis to refer to progeria/progeroid syndromes caused by LMNA point mutations other than G608G.

Fibroblasts from PS patients carrying LMNA point mutations did not show accumulation of progerin or attenuated cellular defects upon farnesyltransferase inhibitor treatment, suggesting other disease causing mechanisms exist (Garg et al., 2009). Fibroblast from patient carrying LaminA/C-E145K displayed lobulated nuclei and mislocalization of heterochromatin, centromere and telomere (Taimen et al., 2009). Different from progerin caused HGPS, misshapen nuclei cannot be rescued by treatment with farnesyltransferase inhibitor, suggesting a different pathogenic mechanism of E145K (Taimen et al., 2009). In vitro assembly study indicates altered higher order assembly of LaminA/C-E145K, consistent with the fact that the E145K mutation falls in the central rod domain (Taimen et al., 2009).
Dermal fibroblasts from PS patient carrying the S143F mutation exhibited numerous nuclear envelope blebs at both light and EM level. Several LEM-domain proteins, nesprin and RNA polymerase II were found mislocalized in S143F fibroblast while the NPC was not disturbed morphologically. Interestingly, these phenotypes are ameliorated in a subpopulation of the patient fibroblasts with nesprin-2 giant expression, suggesting the LINC complex is involved in the disease mechanism (Kandert et al., 2007). The variation of cellular phenotypes from PS patient carrying different LMNA mutations indicates the heterogeneity of disease causing mechanisms.

Several transgenic mouse lines have been generated to study laminopathies including knockout animals LMNA$^{-/-}$, Zmpste24$^{-/-}$ and animals with knock-in mutation such as LMNA$^{H222P/H222P}$ and LMNA$^{L530P/L530P}$. Invertebrate models of laminopathies, however, are very limited, possibly because most of the invertebrate model systems do not have differentiated A- and B-type lamins (Stuurman et al., 1998). Drosophila is the only invertebrate model known to have both A- and B-type lamins (Stuurman et al., 1998). The B-type lamin in Drosophila is LamDm0, which is ubiquitously expressed and contains a CAAX motif at the C-terminus just like vertebrate LaminB proteins (Stuurman et al., 1998). The only A-type lamin in Drosophila is LamC, which does not have the CAAX motif at the C-terminal end. Like vertebrate A-type lamin, its expression is developmentally regulated (Riemer et al., 1995). Drosophila lamC null mutants
displayed nuclear envelope disruption similar to cells from LMNA<sup>−/−</sup> mice (Schulze et al., 2009). Expression of LamC mutants modeling LaminA/C mutations found in AD-EDMD and DCM patients result in defective nuclear envelope structures (Schulze et al., 2009) and mislocalized NPCs similar to what was observed in muscle fibers isolated from patients (Dialynas et al., 2012). Flies expressing disease modeling LamC mutations develop phenotypes ranging from locomotor defects to lethality depending on the specific mutation and the target tissues (Dialynas et al., 2012; Schulze et al., 2009). These results indicate that Drosophila could be a potential model system to study laminopathies. Thus far, few Drosophila models have been made for progeria or progeroid syndrome, likely due to the lack of CAAX motif in LamC. However, as mentioned earlier, point mutations of LaminA/C can also cause premature aging.

In the second part of my thesis, I will characterize the aging-related phenotypes in Drosophila models carrying LamC mutations modeling human progeroid syndrome and demonstrate how defective nuclear envelope budding and mitochondrial dysfunction may contribute to these phenotypes.
Figure 1.1 megaRNP foci at the nuclear periphery (adapted from (Speese et al., 2012))

(A) LamC and DFz2C labeling (deconvolved) of muscle nucleus containing a DFz2C/LamC focus (box; enlarged in right panels) localized to the nuclear periphery (arrowhead in XZ plane). Arrows indicate DFz2C granule within the LamC framework-like structure.

(B) INM invaginations containing electron dense granules (g) from larval muscle. N: nucleus, C: cytoplasm, h: heterochromatin
Figure 1.2 Schematic illustration of the nuclear envelope budding pathway (adapted from (Speese et al., 2012))

Wg, when secreted by motorneurons, binds to DFz2 receptor on the muscle surface and triggers the internalization of the receptor. The C-terminus of DFz2 is cleaved and imported into the muscle nucleus, where it associates with mRNA. Atypical PKC phosphorylates LamC and dissociate the lamina, which allows megaRNPs to enter the perinuclear space through INM envelopment. The enveloped megaRNPs are then de-enveloped and released into the cytosol, presumably transported to the postsynaptic region for local translation.
CHAPTER II

Regulation of postsynaptic retrograde signaling by presynaptic exosome release
Summary

Retrograde signals from postsynaptic targets are critical during development and plasticity of synaptic connections. These signals serve to adjust the activity of presynaptic cells according to postsynaptic cell outputs and to maintain synaptic function within a dynamic range. Despite their importance, the mechanisms that trigger the release of retrograde signals and the role of presynaptic cells in this signaling event are unknown. Here we show that a retrograde signal mediated by Synaptotagmin 4 (Syt4) is transmitted to the postsynaptic cell through anterograde delivery of Syt4 via exosomes. Thus, by transferring an essential component of retrograde signaling through exosomes, presynaptic cells enable retrograde signaling.

Introduction

The Drosophila neuromuscular junction (NMJ) is a powerful system to investigate mechanisms underlying retrograde signaling (Keshishian and Kim, 2004). Spaced stimulation of Drosophila larval and embryonic NMJs results in potentiation of spontaneous (quantal) release (Ataman et al., 2008; Yoshihara et al., 2005) through a retrograde signaling mechanism requiring postsynaptic function of the vesicle protein, Synaptotagmin-4 (Syt4) (Barber et al., 2009; Yoshihara et al., 2005).
Synaptotagmins are a family of membrane trafficking proteins composed of an N-terminal transmembrane domain, a linker sequence, and two C-terminal C2 domains (Chapman, 2008). The most abundant isoform in the nervous system, Synaptotagmin 1, is associated with synaptic vesicles and has been proposed to function as a Ca\(^{2+}\) sensor for neurotransmitter release (Chapman, 2008). Among synaptotagmins Syt4 (Littleton et al., 1999; Vician et al., 1995) occupies an interesting, yet poorly understood position. Its expression is regulated by electrical activity (Babity et al., 1997; Vician et al., 1995), it is present in vesicles containing regulators of synaptic plasticity and growth, such as BDNF (Dean et al., 2009), it regulates learning and memory (Ferguson et al., 2001), and in humans the syt4 gene is localized to a locus linked to schizophrenia and bipolar disorder (Ferguson et al., 2001).

At the fly NMJ, spaced stimulation results not only in potentiation of spontaneous neurotransmitter release (Ataman et al., 2008; Yoshihara et al., 2005), but also in structural changes at presynaptic arbors, the rapid formation of ghost boutons, nascent boutons which have still not developed postsynaptic specializations or recruited postsynaptic proteins (Ataman et al., 2008). However, whether this activity-dependent bouton formation also requires Syt4-dependent retrograde signaling was unknown.
Here we demonstrate that retrograde Syt4 function in postsynaptic muscles is required for activity-dependent synaptic growth and that this function depends on exosomal release of Syt4 by presynaptic terminals.

Results and discussion

Postsynaptic depolarization is required for rapid activity-dependent synaptic growth

To determine whether, similar to the potentiation of spontaneous release (Barber et al., 2009; Yoshihara et al., 2005), the rapid formation of ghost boutons in response to spaced stimulation required retrograde signaling, we used an optogenetic approach to inhibit responses in the postsynaptic muscle cell. While body wall muscle preparations bathed in normal saline were stimulated after a spaced stimulation paradigm (Ataman et al., 2008), they were simultaneously hyperpolarized by activating the light-gated Cl⁻ channel Halorhodopsin (NpHR) (Zhang et al., 2007), which was expressed in muscles using the C57-Gal4 driver. Illuminating resting preparations expressing NpHR in muscle resulted in rapid hyperpolarization of the muscle membrane potential (Figure 2.1A). Using two electrode voltage clamp we found that the NpHR current peaked at +46±3.5 nA and decayed to +10.8±1.18 nA within 2 min (n=10). This was sufficient to induce a ~50% decrease in the amplitude of evoked excitatory junctional potentials.
(EJPs; Figure 2.1B-C; Figure 2.2A, recorded in 0.5mM Ca\(^{2+}\) saline), likely by shunting the depolarizing current induced by neurotransmitter release. This decrease in EJP amplitude was not due to a leaky UAS-NpHR transgene, as in the absence of Gal4 driver there was no significant change in EJP amplitude (Figure 2.1C; 2.2A). A similar result has been previously reported when expressing the EKO K\(^+\) channel in muscles (White et al., 2001).

Spaced stimulation of wild type NMJs (lacking NpHR) in the presence or absence of light elicited a 3- to 4-fold increase in the number of ghost boutons (Figure 2.1D), which were labeled by the presynaptic membrane marker, anti-HRP (Jan and Jan, 1982), but lacked postsynaptic Discs-Large (DLG) immunoreactivity (Figure 2.1E, F). Similarly, spaced stimulation of NMJs expressing NpHR in muscles in the absence of light resulted in a significant increase in the number of ghost boutons (Figure 2.1D). In contrast, light activation of NpHR in larval muscles completely blocked this effect (Figure 2.1D). Thus, postsynaptic depolarization is required for the formation of presynaptic ghost boutons in response to spaced stimulation, establishing that ghost bouton formation requires a retrograde signal.

Syt4 is required either pre- or postsynaptically for activity-induced ghost bouton formation and mEJP potentiation
To determine whether Syt4 was required for the retrograde signal, we conducted the above experiments in *syt4* null mutants over a deficiency of the *syt4* locus, which prevented the formation of ghost boutons upon spaced stimulation (Figure 2.1D). If Syt4 was part of a retrograde signaling mechanism that regulates nascent bouton formation, then expressing Syt4 in postsynaptic muscles in a *syt4* mutant background should rescue the block in ghost bouton formation upon spaced stimulation. We expressed a wild-type Syt4 transgene in either muscles or neurons using the Gal4 drivers Mhc (Myosin heavy chain)-Gal4 (for muscles) and elav-Gal4 (for neurons). Surprisingly, expressing Syt4 in either muscles or neurons completely rescued the ability of NMJs to respond to spaced stimulation by forming ghost boutons (Figure 2.1D).

Previous studies at the larval NMJ suggested that the potentiation of miniature EJP (mEJP) frequency upon spaced stimulation was due to a Syt4-mediated retrograde signal, based on the observation that postsynaptic expression of Syt4 in a *syt4* null mutant background, could rescue the lack of mEJP frequency potentiation upon stimulation (Barber et al., 2009). However, the ability of presynaptically expressed Syt4 to rescue this *syt4* mutant phenotype was not tested in this study. Given that *syt4* mutants were unable to form ghost boutons upon spaced stimulation and this phenotype could be rescued either by pre- or postsynaptic Syt4 expression, we determined if mEJP frequency potentiation could be rescued by expressing Syt4 in neurons and/or muscles of *syt4* mutants.
Recording from body wall muscles after spaced stimulation (Ataman et al., 2008) demonstrated an over 2-fold increase in mEJP frequency in wild-type larvae (Figure 2.1G). This response was significantly reduced in syt4 mutants (Figure 2.1G). Nevertheless, expressing Syt4 in either the neurons or muscles of syt4 mutants completely rescued this phenotype (Figure 2.1G). Consistent with a requirement for retrograde signaling, blocking activity in the postsynaptic muscle using NpHR also completely blocked this response (Figure 2.1G). Thus, Syt4 is required either pre- or postsynaptically for activity-dependent ghost bouton formation and mEJP frequency potentiation at the larval NMJ, raising questions about a purely retrograde role of Syt4.

**Syt4 is transferred trans-synaptically from presynaptic boutons to postsynaptic muscle compartments**

As previously reported (Adolfsen et al., 2004), Syt4 is localized both in pre- and postsynaptic compartments of wild-type NMJs, as determined by double labeling with anti-HRP antibodies, which is used as a neuronal membrane marker to determine the boundary between presynaptic boutons and postsynaptic muscles (Figure 2.1H). The Syt4 signal was specific, as it was virtually eliminated in syt4 null mutants (Figure 2.1I). Notably, expressing a Syt4 transgene exclusively in the neurons of syt4 null mutants rescued both the presynaptic and postsynaptic
localization of Syt4 (Figure 2.1J). This observation raises the possibility that presynaptic Syt4 might be transferred to the postsynaptic region, and that postsynaptic Syt4 might at least be partly derived from presynaptic boutons. Consistent with this, expressing a C-terminally Myc-tagged Syt4 (Syt4-Myc) transgene in wild-type motor neurons using the OK6-Gal4 driver mimicked the endogenous localization of Syt4 in both presynaptic boutons and the postsynaptic muscle region (Figure 2.1K). The same postsynaptic localization of Syt4-Myc was observed when expressing the transgene using either the neuronal Gal4 drivers elav-Gal4 or C380-Gal4 (Figure 2.2B-C). Like the wild type, untagged transgene, presynaptically expressed Syt4-Myc completely rescued the mutant syt4 mutant phenotype upon spaced stimulation (Figure 2.1N), suggesting that the tagged transgene is functional. These observations suggest that endogenous Syt4 might be transferred from synaptic boutons to muscles.

This was tested by downregulating endogenous presynaptic Syt4 by expressing Syt4-RNAi in neurons. In agreement with the above model, downregulating Syt4 in motor neurons resulted in near elimination of the Syt4 signal, not only from presynaptic boutons, but also from the postsynaptic muscle region (Figure 2.1L,O). Thus, the transfer of Syt4-Myc from neurons to muscles is not just the result of overexpressing the transgene in neurons, but is probably an endogenous process. Further, although Syt4-RNAi was highly efficient at decreasing the Syt4 signal from motorneurons and muscles when expressed in
motorneurons, expressing Syt4-RNAi in muscles, using the strong C57-Gal4 driver, did not decrease Syt4 levels in either the pre- or postsynaptic compartment (Figure 2.1M,O). These results support the idea that at least an important fraction, if not all postsynaptic Syt4 is derived from presynaptic neurons.

We also determined whether neurons and/or muscles contained syt4 transcripts. Reverse transcription (RT)-PCR using equal amounts of total RNA derived from either the nervous system or body wall muscles revealed the presence of a strong syt4 band in the nervous system (Figure 2.1P). However, virtually no syt4 transcript was in the muscles of wild-type controls or larvae expressing Syt4-RNAi in muscles (Figure 2.1P). This is again consistent with the possibility that muscle Syt4 might be exclusively derived from the transfer of neuronal Syt4 by synaptic boutons.

Syt4 traffics in a manner similar to Evi at the NMJ

Syt4 is a transmembrane protein (Littleton et al., 1999; Vician et al., 1995), and thus its transfer from pre- to postsynaptic cells is not possible through classical vesicle exocytosis. However, we have previously observed the intercellular transfer of a transmembrane protein through exosome-like vesicles at the NMJ (Koles et al., 2012; Korkut et al., 2009), a process also observed in the immune system (Thery et al., 2009). In particular, the release and extracellular trafficking
of hydrophobic Wnt-1 molecules at the NMJ appears to be mediated by Wnt binding to a multipass transmembrane protein, Evi/Wls, which is released to the extracellular space in the form of exosomes (Koles et al., 2012; Korkut et al., 2009). Exosomes are vesicles generated by the inward budding of endosomal limiting membrane into multivesicular bodies (MVBs). MVBs can either fuse with lysosomes to dispose of obsolete cellular material or with the plasma membrane to release vesicle-associated signaling components (Simons and Raposo, 2009). The similar transfer of transmembrane Evi and Syt4 across cells raised the possibility that like Evi, Syt4 could be secreted through exosomes, perhaps the same exosome. To address this possibility, we first determined the extent of Evi and Syt4 colocalization at the NMJ. Neuronally expressed Evi-GFP has a similar distribution pattern to that of endogenous Evi (Figure 2.3A-B), and the Evi-GFP transgene is functional, as it can rescue all mutant phenotypes in evi mutants (Korkut et al., 2009). Given that antibodies to Syt4 and Evi were raised in the same species, we expressed Evi-GFP in motorneurons and visualized the colocalization of the GFP label with endogenous Syt4. The colocalization of the GFP and Syt4 signal was not complete (Figure 2.3C). However, several of the postsynaptic GFP positive puncta also contained endogenous Syt4 signal (Figure 2.3C; arrows). Whether these puncta correspond to single exosomes, a group of exosomes, or exosomes that have fused to an intracellular compartment cannot be determined by confocal microscopy, as exosomes are 50-100 nm in diameter.
Nevertheless, we previously demonstrated that Rab11 is required for Evi-exosome release from presynaptic terminals (Koles et al., 2012). Thus, we expressed a dominant negative form of Rab11 (Rab11DN) in neurons and examined the levels of postsynaptic Syt4. We found that, as in the case of Evi (Koles et al., 2012), expression of Rab11DN in neurons drastically decreased the levels of endogenous postsynaptic Syt4 (Figure 2.3D-F). Most notably, interfering with Rab11 in neurons completely suppressed activity dependent ghost bouton formation (Figure 2.3G) and mEJP potentiation (Figure 2.3H). Thus, Syt4 transfer from neurons to muscles is likely to involve exosomes and these presynaptically derived exosomes are required for retrograde signaling.

In contrast to Rab11, Evi was not required for the release of Syt4, because in evi mutants, levels of postsynaptic syt4 were normal (Figure 2.4B). Similarly, Evi levels were normal at the postsynaptic compartment of syt4 null mutant (Figure 2.4A), suggesting that while Evi is (Koles et al., 2012), and Syt4 might be, an exosomal cargo, they are not required for exosomal release.

Interestingly, when both transgenic Syt4-Myc and Evi-GFP were overexpressed in neurons, both proteins became trapped in a compartment inside synaptic boutons, where they colocalized with the marker, hepatocyte growth factor (HGF)1-regulated tyrosine kinase substrate (HRS), which is often associated with endosomes (Komada et al., 1997) (Figure 2.5A, B). The mechanisms by which
both proteins become trapped at presynaptic terminals are unclear, but this might result from defects in trafficking when the proteins are overexpressed. Most importantly, labeling the NMJs of animals overexpressing both Syt4 and Evi, using Syt4 antibodies, which should label both endogenous and transgenically expressed Syt4, revealed that the entire Syt4 protein pool accumulated in HRS-positive compartments inside presynaptic boutons and that no detectable Syt4 signal was observed at the postsynaptic region (Figure 2.5C). Taken together, the observation that syt4 transcript is virtually absent in muscles, the ability of presynaptically Syt4-RNAi to eliminate Syt4 protein in postsynaptic muscles, and the finding that trapping Syt4 within presynaptic HRS positive compartments completely eliminates postsynaptic Syt4 immunoreactivity provide compelling evidence that Syt4 protein is synthesized in larval neurons and not in larval muscles. It also suggests a mechanism similar to the trans-synaptic trafficking of Evi, through the release of exosomes (Koles et al., 2012; Korkut et al., 2009).

The trapping of Evi and Syt4 in an intracellular neuronal compartment when the proteins were overexpressed raised the possibility that the proteins may form a biochemical complex during trafficking. This was tested by coexpressing Sy4-Myc and Evi-GFP in the neurons of larvae to immunoprecipitate Syt4-Myc from body wall muscle and CNS extracts using anti-Myc antibodies. We found that Myc antibodies specifically immunoprecipitated Evi-GFP in vivo (Figure 2.5D). In contrast, the vesicle protein neuronal Synaptobrevin (n-Syb) (DiAntonio et al.,
1993) did not co-precipitate with Evi-GFP and Evi-Myc (Figure 2.5D). We were also able to consistently co-precipitate Evi-GFP with endogenous Syt4 at the NMJ using a chicken Syt4 antibody (Figure 2.6A-C). However, the coprecipitation was weak (Figure 2.6C). Taken together with the lack of complete colocalization, this result suggests that an interaction between Syt4 and Evi might not be the dominant state of the proteins within the cell (also see below).

The trans-cellular transfer of Syt4 is through exosomes

To determine whether Syt4 could be found in the exosome fraction of S2 cells, we processed purified exosomes derived from a stable S2 cell line expressing Syt4-HA for immunoelectron microscopy. Since the HA tag is expected to be present within the lumen of exosomes, we developed a protocol for immunolabeling exosome luminal antigens. Briefly, purified exosomes were permeabilized with 0.05% saponin for 10 min, and after primary antibody incubation, a nanogold-conjugated secondary antibody was used, followed by silver intensification. Using this protocol, we detected either the GFP tag at the C-terminus of Evi inside exosomes derived from Evi-GFP S2 cells (Figure 2.7A) or the HA tag in exosomes derived from Syt4-HA S2 cells (Figure 2.7B; see Figure 2.8 for control), consistent with the model that Syt4 is present in exosomes. The gold label was observed either inside or at the outer edge of exosomes, which is
commensurate with the size of the primary/secondary antibody complex (20-30 nm).

Specific transfer of Evi-exosomes from cell to cell has been demonstrated between non-neuronal S2 cells (Koles et al., 2012; Korkut et al., 2009). To determine whether similar transfer of Syt4 could be observed, we separately transfected S2 cells with either Sy4-V5 or mCherry. Then, Syt4-V5 and mCherry S2 cells were co-incubated in the same culture dish. We observed that Syt4-V5 puncta were transferred to mCherry S2 cells (Figure 2.7C, D), consistent with our observations at the NMJ.

To determine whether some of the Evi and Syt4 could be sorted to the same exosome, S2 cells were cotransfected with tagged Evi and Syt4. Transfer of tagged Evi and Syt4 puncta into untransfected cells was observed (Figure 2.7E). However, most puncta contained either Syt4 alone (63.4±7.4% of transferred puncta) or Evi alone (23±6.3% of transferred puncta), and only in 13.2±1.9% of the transferred puncta Evi and Syt4 were found together (n=5 independent experiments, 2 experiments with Evi-V5 and Syt4-Dendra cotransfection and 3 with Evi-GFP and Syt4-Myc cotransfection; cotransfection efficiency= 69.4±8.1%). Thus, although Evi and Syt4 can be packaged together, most of the time they exist in independent puncta. This is also consistent with the observation that the
interaction between Evi and Syt4 is relatively weak or represents just a small portion of the entire Evi and Syt4 protein pool.

We also determined whether other cultured cell types were able to take up Syt4 exosomes. In particular, cultured myotubes derived from gastrula embryos (Bai et al., 2009) and a third instar neuronal cell line, CNS ML-DmBG1-c1 (Ui et al., 1994), were able to take up Syt4-containing exosomes purified from Syt4-HA-S2 cells (Figure 2.7F, G). Together with the observation that Syt4 is transferred from presynaptic compartments to postsynaptic muscle cells in vivo and that purified Syt4-containing exosomes are taken up by S2 cells as well as culture primary muscle cells and neurons, there results strongly suggest that Syt4-containing exosomes are transferred transcellularly. Nevertheless, the presence of other nonexosomal mechanisms of transcellular Syt4 transport, such as cytonemes (Roy et al., 2011) cannot be ruled out.

In conclusion, we show that Syt4 protein functions in postsynaptic muscles to mediate activity-dependent presynaptic growth and potentiation of quantal release. However, to mediate this function Syt4 needs to be transferred from presynaptic terminals to postsynaptic muscle sites. We present evidence that, most likely, the entire pool of postsynaptic Syt4 is derived from presynaptic cells. We also show that like the Wnt binding protein Evi, Syt4 is packaged in exosomes, which provides a mechanism for the unusual transfer of
transmembrane proteins across cells. Taken together, our studies support a significant mechanism for the presynaptic control of a retrograde signal, through the presynaptic release of exosomes containing Syt4.

Larval NMJs continuously generate new synaptic boutons and their corresponding postsynaptic specializations (Koon et al., 2011; Zito et al., 1999), ensuring constant synaptic efficacy despite the continuous growth of muscle cells (Li et al., 2002). This precise matching of pre-and postdynaptic compartments is regulated by electrical activity (Budnik et al., 1990), which induces a retrograde signal in specific nerve terminal-muscle cell pairs, each with a characteristic size. Given that most larval mechanism may also enable spatial coincidence to ensure the synaptic specificity of plasticity, making certain that only those activated synapses with a cell become structurally regulated (Yoshihara et al., 2005).
Materials and methods

Fly strains

The following fly strains were used: wild type (Canton-S); syt4BA1 (Adolfsen et al., 2004); m16 (deficiency of the Syt4 locus) (Agnel et al., 1989); UAS-Syt4 (Littleton et al., 1999); UAS-Syt4-RNAi (transformant ID 33317; Vienna Drosophila RNAi Center); UAS-EviEGFP (named Evi-GFP in this paper) and evi2 (Bartscherer et al., 2006); UAS-Syt4-Myc (see below); UAS-eNpHR3.0-EYFP (see below); UAS-Rab11DNN124I (Satoh et al., 2005) and the Gal4 drivers C155 (Lin and Goodman, 1994); C380 and C57 (Budnik et al., 1996); Mhc (Yoshihara et al., 2005); OK6 (Aberle et al., 2002).

Immunocytochemistry

Third instar larval body wall muscles were dissected in ice-cold Ca$^{++}$ free saline (Jan and Jan, 1976) and fixed using either 4% paraformaldehyde or nonalcoholic Bouin’s fixative (Budnik et al., 2006). The following primary antibodies were used: anti-Syt4 (1:1000, (Adolfsen et al., 2004)); anti-c-Myc (1:500, Roche); anti-GFP (1:200, Molecular Probes); anti-DLGPDZ (1:20,000, (Koh et al., 1999)); anti-Evi-Cin (1:100, (Korkut et al., 2009)); anti-FL-Hrs (1:1000, (Lloyd et al., 2002)); anti-V5 (1:500, Invitrogen); anti-HPRDyLight488 (1:400) and anti-HPR-Cy5 (1:200, Jackson ImmunoResearch); anti-HPRDyLight594 (1:400, Jackson ImmunoResearch); anti-HRP-TxRed (1:200, Sigma). The following fluorescent
secondary antibodies from Jackson ImmunoResearch were used: anti-rabbit-DyLight594 (1:400), anti-rabbit- FITC (1:200); anti-rat-DyLight488 (1:400); anti-rabbit-TxRed (1:200); anti-mouse-FITC (1:200); anti-mouse- TxRed (1:200); anti guinea pig-TxRed (1:200). We also used anti-mouse- Alexa647 (1:200, Molecular Probes); AlexaFluor-633- conjugated ConcanavalinA (ConA; 1:200; Molecular Probes), as well as Rhodamine-conjugated Phalloidin (1:500; Invitrogen).

Image Acquisition, Quantification and Morphometric Analysis

Confocal images were acquired using a Zeiss LSM5 Pascal confocal microscope with a Zeiss 63X Plan-Apochromat (1.4 numerical aperture) DIC oil immersion objective at 3X digital zoom. Fluorescence signal intensity was quantified by volumetric measurements of confocal stacks using Volocity 5 Software (Improvision) as described in (Korkut et al., 2009). Briefly, control and experimental samples were imaged at identical settings and the presynaptic intensity was measured by normalizing total intensity inside the presynaptic volume to the volume of the presynaptic terminals. Similarly, the postsynaptic intensity was measured by subtracting the presynaptic volume from the total and measuring the intensity in this subtracted region, followed by normalizing it to the presynaptic volume. To obtain normalized postsynaptic Syt4 or Evi levels in wild type controls, Rab11DN-pre, syt4 and evi mutants, total pre- or postsynaptic intensities were normalized to wild type values. Then, postsynaptic values were
divided by the mean presynaptic value within each genotype. For ghost bouton quantification, body wall muscle preparations were double stained with anti-HRP and anti-DLG, to identify HRP positive boutons devoid of DLG label. Measurements were taken from muscles 6 and 7, abdominal segment 3. The number of ghost boutons in the stimulated samples were then normalized to the mean number of ghost boutons in unstimulated samples from the same genotype.

**Statistical analysis**

Unpaired two-tailed Student’s t-tests were run for comparisons of experiments where a single experimental sample was processed in parallel with a wild type control, or where stimulated samples were compared to their unstimulated controls. In cases where multiple experimental groups were compared to a single control, a one-way ANOVA was performed, with Dunnet post-hoc test. Error bars in all graphs represent ±SEM.

**Spaced Stimulation**

Spaced K⁺ stimulation was performed as described previously (Ataman et al., 2008). Briefly, third instar larvae were dissected in ice-cold normal HL3 saline (70 nm NaCl, 5 nm KCl, 20 nm MgCl₂, 10 nm NaHCO₃, 5 nm trehalose, 5 nm HEPES, 115 nm sucrose) supplemented with 0.1 mM Ca⁺⁺, with the nervous system remaining intact. Osmotically balanced high K⁺ (90 mM K⁺) HL3 containing 1.5
mM Ca$^{++}$ was applied to the larval samples as 3X 2 min, 1X 4 min and 1X 6 min pulses each separated by 15 min incubation in normal HL3 saline. Control larvae were dissected and incubated the same way except with normal HL3 instead of high K$^+$ HL3. After the last incubation, the larvae were fixed with 4% paraformaldehyde fixative for 15 min followed by immunostaining with selected antibodies. For Halorhodopsin experiments, larvae expressing NpHR in muscle and control larvae were fed with 100 μM all trans-retinal (Sigma) and treated as described above with the exception of illuminating at 550-580 nm (Zeiss HBO 100 mercury lamp focused through a 10X Plan-Neofluor objective, and a Zeiss 3 BP565/30 Filter) on the larvae during the high K$^+$ HL3 stimulations. As a control experiment, NpHR expressing larvae underwent spaced K$^+$ stimulation in complete darkness.

**Electrophysiology**

For measurements of mEJP potentiation, spaced and sham stimulation were performed as above, and then samples were prepared for electrophysiology as in (Ashley et al., 2005). Briefly, larvae were bathed in 0.5 mM Ca$^{++}$ HL3 saline (Stewart et al., 1994), and impaled with a 15-20 MΩ electrode (filled with 3M potassium chloride). Only samples with resting potentials between -60 and -63 mV were used for quantification. The signal was collected through an Axoclamp2B (Molecular Devices) and digitally recorded with Pulse software.
Miniature EJP events were analyzed using Minianalysis software (Synaptosoft). All data was quantitated using Origin software (Originlab), or KaleidaGraph (Synergy). To determine the effect of NpHR stimulation on evoked EJPs, samples expressing NpHR in muscles or controls were dissected in 0.3 mM Ca$^{++}$ HL3 saline, and then perfused with 0.5 mM Ca$^{++}$ HL3 saline, and impaled with a 15-20 MΩ electrode as above. Evoked responses were triggered by 1 msec supra-threshold stimulations (<5 V) of the segmental nerve, which was drawn into a 10 μm suction electrode. After 2 minutes of evoked responses, the fluorescent shutter on the microscope was opened, exposing the preparation to 560 nm light. The sample was continually recorded throughout the 2-minute exposure to the 560 nm light pulse. Evoked signals were then analyzed using Minianalysis. Voltage clamp analysis of NpHR currents was performed as follows. Samples were dissected and perfused as above. Muscle 6 of segment A3 was then impaled by both a 15-20 MΩ electrode (filled with 3 M potassium chloride), and a 10 MΩ electrode (filled with a 3:1 mixture of 2.5 M potassium chloride : 2 M potassium citrate). Using an Axoclamp2A (Molecular Devices), the muscle was clamped at -80 mV, and the current required to maintain the clamp was recorded using Pulse software (HEKA instruments). After 40 sec the shutter was opened, exposing the sample to 560 nm light, and the shutter remained open for 2 min. The resulting positive current was analyzed using Pulse software (HEKA instruments), and the peak amplitude and amplitude
after 2 minutes were recorded and analyzed in Kaleidograph (Synergy). In each animal, muscle 6 on both sides was recorded from, with a total of 5 animals recorded.

S2 Cell Culture

*Drosophila* Schneider (S2) cells were cultured at 25 °C in SFX insect medium (HyClone) containing 10% (v/v) fetal bovine serum (FBS) (HyClone), penicillin (50 U/ml) and streptomycin (50 μg/ml) (Sigma). Cells were maintained in Nunclon TM Δ Surface T-flasks (Thermo Scientific). For immunocytochemistry, cells were plated on 6-well Nunclon plates (Thermo Scientific) and when cells reached 60-80% confluency, they were transfected with 0.5 μg DNA using Effectene transfection kit (Qiagen). The following plasmids were used: pAc-Evi-GFP (Bartscherer et al., 2006), pAc-Gal4, pUAST-Syt4 (Barber et al., 2009); pUAST-Syt4-Myc (see below); pAc-Syt4-V5 (see below); pAc-mCherry (Korkut et al., 2009). Cells were grown for 24-48 hours and then processed for immunocytochemistry. For exosome isolation and subsequent immunoelectron microscopy experiments, the following stably transfected S2 cell lines (cultured without FBS in the medium) with copper inducible promoters were used: Evi-GFP (in pMK33 vector) (Koles et al., 2012), Syt4-HA-sp11 (sp11 corresponds to amino acids 215-230 of GFP (Cabantous et al., 2005b); herein named Syt4-HA; in pMT-puro vector) (see below). The Evi-GFP stable cell line was maintained
under 0.5 mg/ml hygromycin (Invitrogen) selection and the Syt4-HA stable cell line was maintained under 10 µg/ml puromycin (Invitrogen) selection. Third instar larval CNS derived neuronal MLDmBG1-c1 cell line was from Drosophila Genomics Resource Center (DGRC, IN), cultured according to the DGRC guidelines in Shields and Sang medium (Sigma) containing 0.1% (w/v) yeast extract (Sigma), 0.25% (w/v) bactopeptone (Sigma), 10% (v/v) FBS (Hyclone) and 10 U/ml bovine insulin (Sigma).

Exosome Preparation

Exosomes were prepared as in (Lasser et al., 2012) with slight modifications. Shaking cultures of pMT-puro-Syt4-HA transfected stable S2 cells were cultured in the absence of FBS and induced for 24 hours with 0.7 mM CuSO$_4$ before exosome isolation. Cells (2 Liter; harvested at 3-5X10⁶ cells/ml density) were pelleted by centrifugation at 600 g for 10 min. The supernatant was then cleared of larger debris by centrifugation at 16,500 g for 20 min. The supernatant was passed through a 0.22 µm filter and exosomes were pelleted at 120,000 g for 75 min. Individual pellets were pooled, resuspended in maximal volume (~ 55 ml for Type 45 Ti rotor (Beckman)) of 0.1 M Tris and pelleted at 120,000 g for 60 min. The resulting exosome pellet was resuspended in minimal volume (~0.3-0.5 ml) of 0.1 M Tris pH 7.4 to yield a concentrated purified exosome fraction and kept at
-80 °C until further use or fixed overnight in 2% paraformaldehyde for subsequent immuno-electron microscopy.

**Immunoelectron Microscopy of Exosomes**

Exosomes were fixed in 2% paraformaldehyde at 4 °C overnight and 5 μl was spotted onto formvar coated Nickel grids (200 mesh). Exosomes were allowed to adhere to the grids for 20 min at room temperature. Next, grids were rinsed with 2X 3 min washes of 0.1M Tris (pH 7.4 throughout the procedure) and free aldehyde groups were quenched by 4X 3 min incubations in 50 mM glycine (in 0.1 M Tris). The grids were subsequently blocked in 0.1 M Tris pH 7.4 containing 5% BSA (w/v) with 0.05% (w/v) saponin for 10 min. Prior to antibody incubation, grids were rinsed once briefly in 0.1 M Tris containing 0.5% BSA (w/v). Rat anti-HA (1:400, Roche) was used in blocking buffer (0.1M Tris containing 5% BSA (w/v) and 0.2% (w/v) acetylated BSA-c (Aurion)) for both permeabilized and nonpermeabilized exosomes. After 1 hr of incubation at room temperature, grids were washed for 6X 3 min in wash buffer (0.1 M Tris containing 0.1% BSA (w/v) and 0.2% (w/v) acetylated BSA-c). After washing, the grids were blocked for 6X 3 min in blocking buffer (0.1 M Tris containing 0.5% BSA (w/v) and 0.2% (w/v) acetylated BSA-c). Secondary nanogold (1.4 nm) conjugated goat anti-rat (1:60, Nanoprobes) in blocking buffer (0.1 M Tris containing 0.5% BSA (w/v) and 0.2% (w/v) acetylated BSA-c) for 1 hr. Grids were then rinsed for 8X 2 min in 0.1 M Tris
and antibody complexes were crosslinked with 1% glutaraldehyde (in 0.1 M Tris) for 1 min. Grids were washed for additional 8X 2 min in water and the nanogold particles were silver enhanced for 8-10 min using the HQ Silver (Nanoprobes) silver enhancement kit. The grids were washed for 8X 2 min in water and negatively stained as described in (Koles et al., 2012).

**Primary cultures from gastrula embryos**

Primary cultures were prepared according to (Bai et al., 2009), with minor modifications. Briefly, embryos were collected on agar/molasses plates streaked with inactivated yeast paste at 25 °C for 1-2 hours just prior to the end of the 12 hour day cycle. Embryos were aged for 5-6 hours at 25 °C, collected using nylon sieves and dechorinated in 50% (v/v) bleach for 5 min. Dechorinated embryos were rinsed with sterile water, disinfected by rinsing in 70% ethanol, rinsed in sterile water again and incubated for 4 minutes in Shields and Sang (Sigma, S3652) medium containing penicillin (50 U/ml) and streptomycin (50 μg/ml) (Sigma) and 15% (v/v) heat inactivated FBS (Hyclone). Embryos were homogenized (at a ratio of 100 μl embryos in 8 ml medium) with 6 gentle strokes, and larger debris was removed by centrifugation at 50 g for 5 min. Subsequently, the supernatant was centrifuged at 350 g for 10 min to pellet the cells. This cell pellet was resuspended in 5 ml of medium additionally containing 10 μg/ml
bovine pancreas insulin (Sigma), and an aliquot taken to determine the viable cell density, while the remaining cells were pelleted again at 350 g for 10 min. This final cell pellet was resuspended in the appropriate volume of insulin-containing medium to yield 3-5X 10^6 cells/ml cell density. Cells (1 ml) were plated on sterilized cover slips in a 35 mm culture dish, and cultured at 22 °C in humidified Tupperware containers. After 2 days in culture, cells were incubated with a small volume (~5 µl) of purified, concentrated Syt4-HA exosome fraction was added to the culture medium and cells were incubated for 2 hours at room temperature. Cells were quickly rinsed 3X with PBS (to remove non-internalized exosomes) then fixed in 4% paraformaldehyde in PBS for 10 min. Next, cells were permeabilized in phosphate buffer containing 0.2% (v/v) TritonX-100 prior to labeling with anti-HA, and fluorescently conjugated ConA and phalloidin.

**Immunoprecipitation and Western Blotting**

For Syt4-Myc and Evi-GFP co-immunoprecipitation, 20 third instar larvae for each genotype were dissected in ice-cold Ca^{++} free saline and body wall muscles together with the nervous system were homogenized in lysis buffer (20 mM HEPES, 100 mM KCl, 0.5% TritonX-100, 5% Glycerol, 2.5 mM EDTA, 1 mM Dithiothreitol, with protease inhibitors). Lysates were then precleared with Protein G beads (GE life sciences) and then incubated with 2 µg anti-c-Myc (Roche)
together with Protein G beads for 2 hrs at 4°C. After incubation, beads were washed with ice-cold PBS with 0.5% TritonX-100, boiled with 2X SDS loading buffer for 5 minutes at 95°C and then separated by SDS-PAGE gel. Subsequently, they were transferred to a nitrocellulose membrane (Bio-Rad) and the blots probed with rabbit anti-GFP (1:10,000, Abcam Ab290, preabsorbed with whole fly powder); mouse anti-c-Myc (1:5000, Roche); rabbit anti-n-Syb (1:10,000; (Deitcher et al., 1998)) anti-rabbit-HRP light chain (1:2000, Jackson ImmunoResearch); anti-mouse-HRP (1:5000, Sigma). For endogenous Syt4 and Evi-GFP coimmunoprecipitation, 50 third instar larvae for each genotype were dissected and homogenized in lysis buffer containing 0.1% TrironX-100 as above. Lysates were precleared with Protein G beads and then incubated with ~5 μg chicken anti-Syt4 antibody overnight at 4°C. Then, samples were incubated with 6 μg mouse anti-chicken antibody (Abcam, Ab8922) for 2 hrs and then with Protein G beads for an additional 1hr at 4°C. After protein separation and membrane transfer as above, blots were probed with anti-GFP (1:5000, Abcam Ab290, preabsorbed with whole fly powder) (Harlow and Lane, 2006); chicken anti-Syt4 (1:2000, preabsorbed with whole S2 cell powder); anti-chicken- HRP (1:10,000, Jackson ImmunoResearch); anti-rabbit-HRP light chain (1:2000, Jackson ImmunoResearch).

Reverse transciption-PCR
Total RNA was extracted by homogenizing dissected larval body wall muscles (without CNS) or larval brains in Trizol (Invitrogen) at 4 °C using a BBX24B Bullet Blender Blue homogenizer (Next Advance Inc.) and then treating with DNase and extracted with the RNeasy Micro Kit (Qiagen). RNA concentrations were measured using a NanoDrop 2000c Spectrophotometer (Thermo scientific). cDNA was synthesized using a SuperScript III Kit (Invitrogen) from 500 ng of total RNA, primed with random hexamers. Reverse transcription reactions were then diluted to 1:12.5 and were amplified by PCR (35 cycles) with the following syt4 primers. Forward: ATCCCAGATGCCAGCGTCAT; reverse: AATCGGGGAGGTGGACTGGT. Both of these primers were designed to hybridize with exon junctions, to avoid false signals from genomic DNA. For the GAPDH control experiment, the reverse transcription reactions were diluted to 1:50 and were amplified using the forward ACTCGACTCACGGTGCTT and reverse GCCGAGATGATGACCTTCTT primers.

Molecular Biology
To generate pUAST-Syt4-Myc, Syt4 cDNA was PCR amplified from pUAST-Syt4 (Barber et al., 2009) using a forward primer containing an EcoRI site and a reverse primer containing a NotI site. The PCR product was then ligated into a pUAST-5Myc plasmid, which was constructed by PCR amplifying 5 tandem Myc tags (1xMyc repeat =
TCTGAGCAGAAGCTGATCTCCGAGGAGGACCTGAACGGA/SEQKLISEEDLN
G) with primers containing 5’ KpnI and a 3’ stop codon followed by an XbaI site. This 5Myc-stop PCR fragment was ligated into the KpnI-XbaI sites of pUAST yielding pUAST-5Myc-stop. To construct the pAc-Syt4-V5, Syt4 cDNA was PCR amplified from pUAST-Syt4 (Barber et al., 2009) using a forward primer containing an EcoRI site and a reverse primer containing an XhoI site. The PCR product was then ligated into a pAc5.1/V5-His plasmid (Invitrogen) to obtain a C-terminally V5 tagged Syt4. Syt4-HA-sp11 was PCR amplified from pUAST-attB-Syt4-HA-sp11 (see below) using a forward primer with an EcoRV site and a reverse primer with a NotI site. The PCR product was then ligated into pMT-puro plasmid (Addgene, ID: 17923). To make the original pUAST-attB-Syt4-HAsp11, sp11 was cloned in pUAST-attB plasmid (from Dr. Konrad Basler) using the KpnI and XbaI sites. Syt4 cDNA was then PCR amplified from pUAST-Syt4 (Barber et al., 2009) using a forward primer containing a NotI site and a reverse primer containing an AgeI site. The PCR product was then ligated into the pUAST-attB plasmid (from Dr. Konrad Basler, Institute of Molecular Life Science at University of Zurich). Subsequently, a synthetic oligonucleotide coding for three tandem HA tags- YPYDVPDYASGYPYDVPDYAGSYPYDVPDYAS (GS are linker amino acids) was ligated into the AgeI site of pUAST-attB-Syt4-sp11. To clone UAS-eNpHR3.0- EYFP, the pLenti-CaMKIIa-eNpHR3.0-EYFP vector (Zhang F, 2010) was obtained from Dr. Carl Deisseroth, and the eNPHR3.0-EYFP was PCR
amplified using the following primers: F-eNpHR3.0 (ATAATAGAATTCaacATGACAGAGACCCTGCCTCC) and REYFP (ATAATATCTAGATCA TTACACCTCGTTCTCGT). The resultant PCR product was cloned into pWalium10 (GU931386) (TRIP Facility, Harvard) using EcoRI and XbaI restriction enzymes. UAS-eNpHR3.0-EYFP flies were prepared by targeting the construct to the attP2 site on the third chromosome (Genetivision). To generate UASSyt4-Dendra, Syt4 was PCR amplified from pUAST-Syt4 (Barber et al., 2009) using a forward primer containing a EcoRI site and a reverse primer containing an NotI site. Dendra2 was PCR amplified from the pDendra2 (Evrogen), using a forward primer with NotI and a reverse primer with XbaI. The entire construct was cloned into pUAST. For S2 cell work, this construct was co-transfected with pAC-Gal4.
Figure 2.1 Retrograde control of synaptic growth and function by trans-synaptic Syt4 transfer
(A) Hyperpolarization of muscles (upper trace) upon 560 nm illumination (bottom trace), in a 3rd-instar larva expressing NpHR in muscle.

(B) Nerve-evoked EJPs in control and upon activating NpHR in muscles.

(C) Nerve-evoked EJP amplitude in wild-type, NpHR/+, and postsynaptic NpHR-expressing larvae. N (left to right)=5,5,5,5,7,7. Also see Figure 2.2A.

(D) Number of ghost boutons normalized to unstimulated controls induced after spaced stimulation of controls, animals expressing NpHR in muscles, and syt4 mutants. N (left to right)=14,15,11,12,27,20,16,15,28,25,15,15,13.

(E,F) NMJs from 3rd-instar larval muscles 6/7 (A3) labeled with anti-HRP and anti-DLG in wild-type (E) unstimulated and (F) after spaced stimulation, showing ghost bouton (arrows) induction after stimulation. Insets = high magnification of NMJ branches.

(G) mEJP frequency normalized to unstimulated controls. N (left to right)=27,30,8,9,24,26,21,23,21,8,8,6,7.

(H–M) 3rd-instar larval NMJ branches at muscles 6/7 (A3) shown at low (left two columns) or high (right two columns) magnification labeled with anti-HRP and (H–J, L,M) anti-Syt4 or (K) anti-Myc.(H) wild-type control; (I) syt4 null mutant; (J) syt4 null mutant expressing a wild-type Syt4 transgene in neurons;(K) a larva expressing Syt4-Myc in neurons; also see Figure 2.2B, C. (L) a larva expressing Syt4-RNAi in neurons; (M) a larva expressing Syt4-RNAi in muscles.
(N) Number of ghost boutons normalized to unstimulated controls in unstimulated and stimulated wild-type controls, as well as in unstimulated and stimulated syt4 mutants expressing the Syt4-Myc transgene in neurons. N (left to right)=15,14,21,15.

(O) Syt4 immunoreactivity levels normalized to control levels. N (left to right)=21,12,11,15.

(P) RT-PCR from larval CNS and muscles showing Syt4 mRNA in neurons but not in muscles, with GAPDH mRNA as control.

Calibration bar is 20µm for E,F and 10µm for insets; 6 µm for H–M left two columns; 2.5 µm for H–M right two columns.

***=p<0.001; **=p<0.01; *=p<0.05. Bars in plots represent mean±SEM.
Figure 2.2 Distribution of EJP amplitudes and Syt4-Myc transfer using different neuronal Gal4 drivers
(A) Percentile distribution of the mean peak EJP amplitude (in mV) in larvae expressing NpHR in muscles (NpHR-post) or animals bearing the UAS-NpHR transgene but lacking the muscle Gal4 driver, in the absence (black points) or presence (red points) of light. Recordings where done in 0.5 mM Ca++ HL3 saline. Points in the plot correspond to mean±SEM.

(B, C) Neuromuscular junctions double labeled with anti-Myc and anti-HRP antibodies in larvae expressing Syt4-Myc with (B) the pan-neuronal driver, elav-Gal4, and (C) the motorneuron driver C380-Gal4. Calibration bar is 5 μm.
Figure 2.3 Syt4 and Evi partially colocalize at the NMJ and interfering with Rab11 function in neurons inhibits Syt4 transfer from pre- to postsynaptic compartments as well as retrograde signaling
(A–E) 3rd-instar larval NMJs at muscles 6 or 7 (A3) in (A) wild-type, (B,C) larvae expressing Evi-GFP in neurons, (D) neuronal driver control, and (E) larvae expressing Rab11DN in neurons, labeled with (A) anti-Evi and anti-HRP; (B) anti-GFP and anti-Evi, (C) anti-GFP and anti-Syt4. Arrows=colocalization of transgenic Evi and endogenous Syt4; (D,E) anti-Syt4 and anti-HRP. Also see Figure 2.4.

Calibration Bar is 9 µm for A–C (left panels), 5µm for A–C (right panels) and D, E.

(F) Normalized postsynaptic Syt4 levels. N (left to right)=25,25.

(G) Number of ghost boutons normalized to unstimulated preparations in controls and animals expressing Rab11DN in neurons. N (left to right)=20,21,15,10,16,12.

(H) mEJP frequency normalized to unstimulated preparations in controls and larvae expressing Rab11DN in neurons. N (left to right)= 6,9,6,8.
Figure 2.4 Evi and Syt4 are not required for each other's transfer to postsynaptic muscles

(A) Normalized levels of endogenous Evi in postsynaptic versus presynaptic compartments, in wild type or syt4 mutants. N = 13 and 18 respectively.

(B) Normalized levels of endogenous Syt4 in postsynaptic versus presynaptic compartments, in wild type or evi mutants. N = 16 and 19 respectively.

Bars in plots correspond to mean±SEM.
Figure 2.5 Trapping Syt4 in presynaptic boutons reveals absence of endogenous Syt4 in postsynaptic muscles

(A–C) 3rd-instar larval NMJ branches at muscles 6 or 7 (A3) in larvae expressing both Evi-GFP and Syt4-Myc in neurons labeled with antibodies to (A) GFP, Myc
and HRP; (B) GFP, Myc and HRS; (C) GFP, Syt4 (labeling both endogenous and transgenic Syt4), and HRP. Calibration bar is 6.5 µm.

(D) Co-immunoprecipitation of Evi-GFP by Myc antibodies from body wall muscle and CNS extracts obtained from larvae expressing both Evi-GFP and Syt4-Myc in neurons. Numbers at the right= molecular weight x 10^3. IgG-HC= IgG heavy chain. Also see Figure 2-6.
Figure 2.6 Evi and Syt4 exist in a biochemical complex

(A) Western blot of body wall muscle extracts in wild type and syt4 mutant probed with a chicken Syt4 antibody, showing that the antibody recognizes the Syt4 band. *= an unspecific band also recognized by the antibody. (B) Immunoprecipitation of Syt4 from body wall muscle extracts prepared from larvae expressing Syt4-Myc in neurons using the chicken anti-Syt4 antibody and probed with an anti-Myc antibody, demonstrating the ability of the chicken Syt4 antibody to immunoprecipitate Syt4. (C) Coimmunoprecipitation of endogenous Syt4 and neuronally expressed Evi-GFP from body wall muscle extracts by using the chicken Syt4 antibody. Note that the Syt4 band is partially occluded by chicken IgY antibody heavy chain (IgY-HC). Numbers at the right of the blots correspond to molecular weight x 10^3.
Figure 2.7 Syt4 is present in purified S2 cell exosomes and purified exosomes from Syt4-HA S2 cells are taken up by S2 cells, primary myoblast cell cultures and a neuronal cell line

(A,B) Electron micrographs of purified, permeabilized, and negatively stained exosome fraction from the culture medium of (A) Evi-GFP-S2 cells labeled with anti-GFP, and (B) Syt4-HA-S2 cells labeled with anti-HA. Also see Figure SF4.
(C–E) S2 cells labeled with (C,D) anti-V5 and mCherry in co-cultures of Syt4-V5-S2 and mCherry-S2 cells. In (C) both a Syt4-V5 transfected and a mCherry transfected cell are observed. Note that V5 positive puncta are visualized within the mCherry cell, suggesting that Syt4-V5 is transferred transcellularly. In (D) a mCherry cell from the co-culture in (C) is shown, demonstrating the presence of transferred Syt4-V5 puncta. (E) Shows the transfer of Evi-GFP and/or Syt4 containing puncta to an untransfected cell, from S2 cells co-expressing Evi-GFP and untagged Syt4.

(F,G) Confocal image of (F) myotubes from gastrula embryos and (G) cells from a Drosophila neuronal cell line, incubated with purified exosome fraction from Syt4-HA-S2 cells, labeled with (F, G) fluorescently conjugated concanavalin A (ConA) to stain membranes, and anti-HA, as well as (F) fluorescent phalloidin to label myofibrils.

Calibration bar is 0.17µm for A,B; 12µm for C–E, 15µm for F and 8µm for G.
Figure 2.8 Control for exosome immuno-electron microscopy

Micrograph of purified and permeabilized exosomes from Evi-GFP S2 cell medium labeled with anti-HA primary antibody and a nanogold-conjugated secondary followed by silver intensification. Note the absence of extra-exosome signal in the entire field. Calibration bar is 250 nm.
Endnotes for Chapter II

Chapter II is adapted from the following publication:


Author contributions:

C.K. Figure 2.1D-F, H-P; 2.2B-C; 2.3A-C; 2.5A-C

Y. L. Figure 2.1D, N; 2.3D-G; 2.4; 2.5D; 2.6

K. K. Figure 2.7; 2.8

C. B. & J. A. Figure 2.1A-B,G; 2.2A; 2.3H

M. Y. Initial observation of trans-synaptic Syt4 transfer; intellectual discussion; generation of chicken anti-Syt4 antibody

V. B. Conceptualization, writing, visualization, supervision, funding acquisition
CHAPTER III

Lamin mutations accelerate aging via defective export of mitochondrial mRNAs through nuclear envelope budding
Summary

Defective RNA metabolism and transport are implicated in aging and degeneration (Chandris et al., 2010; Cookson, 2012), but the underlying mechanisms remain poorly understood. A prevalent feature of aging is mitochondrial deterioration (Dupuis, 2014). Here we link a novel mechanism for RNA export through nuclear envelope (NE) budding (Lee and Chen, 2010; Speese et al., 2012) that requires A-type Lamin, an inner nuclear membrane-associated protein, to accelerated aging observed in Drosophila LaminC (LamC) mutations. These LamC mutations were modeled after A-Lamin (LMNA) mutations causing progeroid syndromes (PS) in humans. We identified mitochondrial assembly regulatory factor (marf), a mitochondrial fusion factor (mitofusin), as well as other transcripts required for mitochondrial integrity and function, in a screen for RNAs that exit the nucleus through NE-budding. PS-modeled LamC mutations induced premature aging in adult flight muscles, including decreased levels of specific mitochondrial protein transcripts (RNA) and progressive mitochondrial degradation. PS-modeled LamC mutations also induced the accelerated appearance of other phenotypes associated with aging, including a progressive accumulation of poly-ubiquitin aggregates (Demontis and Perrimon, 2010; Rubinsztein, 2006) and myofibril disorganization (Demontis et al., 2013; Doran et al., 2009). Consistent with these observations, the mutants had progressive jumping and flight defects. Downregulating marf alone induced the
above aging defects. Nevertheless, restoring marf was insufficient for rescuing the aging phenotypes in PS-modeled LamC mutations, as other mitochondrial RNAs are affected by inhibition of NE-budding. Analysis of NE-budding in dominant and recessive PS-modeled LamC mutations suggests a mechanism by which abnormal lamina organization prevents the egress of these RNAs via NE-budding. These studies connect defects in RNA export through NE-budding to progressive loss of mitochondrial integrity and premature aging.

Results and discussion

Mitochondrial RNAs localize to NE-budding sites

NE-budding is a mechanism for the nuclear export of large ribonucleoprotein granules (megaRNPs) (Speese et al., 2012). This mechanism is akin to the nuclear egress of Herpes-type viruses (Mettenleiter et al., 2013) and involves megaRNP envelopment at the inner nuclear membrane (INM), and RNP de-envelopment at the outer nuclear membrane (ONM). Sites of NE-budding in Drosophila larval muscle nuclei are identified by using antibodies to the C-terminal fragment of DFrizzled2 (DFz2C), which associates with megaRNPs, and by the presence of locally thickened NE with brighter LamC immunoreactivity (DFz2C/LamC foci) (Jokhi et al., 2013; Speese et al., 2012). These DFz2C/LamC foci are enriched in RNAs encoding for several postsynaptic proteins (Packard et
al., 2015; Speese et al., 2012). Fluorescent in situ hybridization (FISH) demonstrated that a subset of LamC foci also contained marf RNA (Figure 3.1A; Figure 3.2A-B), implicating NE-budding in the export of mitochondria-related transcripts. RNA-immunoprecipitation (RIP) from larval muscles using anti-DFz2C established that marf RNA, but not the unrelated mad RNA (Speese et al., 2012), co-precipitated with DFz2C, consistent with its presence in megaRNPs (Figure 3.1B).

To determine if other mitochondria-associated RNAs existed in megaRNPs, we performed FISH to detect ATP-synthase (ATP-syn) subunits, the electron-transport chain component COX4, the kinase PINK1, and the mitochondrial fission factor Drp1. ATP-syn-β, -α (Bellwether/Blw), and -B RNAs were enriched at NE-LamC foci, suggesting their presence in megaRNPs (Figure 3.1C-E; Figure 3.2C-E). FISH signal for ATP-syn-γ, -C, and Drp1 was very low (not shown) and undetected for cox4 and pink1 (Figure 3.1F,G). Thus, only a subset of mitochondria-associated transcripts are present within megaRNPs.

**PS-modeled LamC mutations display age-dependent mitochondrial defects**

Marf, a GTPase of the mitofusin family, is involved in mitochondrial fusion, a process essential for maintaining mitochondrial integrity and respiratory function (Peterson et al., 2012). Similarly, ATP-synthase is required for the formation and maintenance of mitochondrial cristae (Davies et al., 2012). As aging and
neurodegenerative disorders have been linked to progressive mitochondrial disruption (Daum et al., 2013; Demontis et al., 2013), we examined mitochondria in flies expressing PS-modeled LamC mutations. These LamC variants contained point mutations in conserved amino acid codons that in human LMNA gave rise to PS (Figure 3.1I). We focused on LamC-E174K (E159K in LMNA) and LamC-R564C (R527C in LMNA; referred to as LamC-r564c to denote recessive inheritance) in the rod and tail domains of LamC (Figure 3.1I). We also generated flies expressing wild-type LamC (LamC-WT). In humans LMNA-E159K is an autosomal-dominant mutation causing Atypical-PS with a presentation similar to Hutchinson Gilford Progeria Syndrome (HGPS) (Garg et al., 2009). LMNA-r527c is an autosomal-recessive mutation inducing atypical-HGPS (Liang et al., 2009). Western-blots of body wall muscle proteins from lamC null mutants expressing LamC variants with MHC-Gal4 revealed that the transgenes were expressed in the same range as wild-type (Figure 3.1H). Since LMNA-E159K behaves as a dominant mutation in humans, LamC-E174K was expressed in a lamC/+ heterozygous background (genotype: LamC-E174K, lamC/+). Since LMNA-r527c is a recessive mutation in humans, LamC-r564c, was expressed in a lamC mutant homozygous background (genotype: LamC-r564c, lamC/lamC).

We focused on adult dorsal longitudinal muscles (DLM) of the indirect flight-muscles, since larval muscles are short-lived, precluding aging studies (Roy and VijayRaghavan, 1999). We examined mitochondria using antibodies against
mammalian ATP-syn-α, which cross-react with Blw (Li et al., 2014), in young (3-day post eclosion; 3d) and middle age (14d and 21d) adults.

In wild-type and LamC-WT, anti-ATP-syn-α labeled elongated mitochondria located above and below myofibrils at all stages (Figure 3.3A,D,G,B,E,H), which was similar to 3d LamC-E174K (Figure 3.3C). In 14d and 21d LamC-E174K, however, mitochondria were smaller, appeared rounded, and were sparse (Figure 3.3F,I). This was reflected by a significant reduction of mitochondrial volume (Figure 3.3J), suggesting an age-dependent decline in mitochondria production and/or maintenance. lamC null mutants did not survive to adulthood, precluding the examination of animals expressing LamC-r564c.

These mitochondrial phenotypes and the presence of marf RNA in megaRNPs raised the possibility that age-dependent mitochondrial deterioration could be partially attributed to defective Marf levels. Indeed, marf RNA was markedly depleted in thoracic muscles from 21d LamC-E174K, compared to 21d controls and LamC-WT (Figure 3.3K).

At the ultrastructural level, in 4d and 21d controls, mitochondria were packed between myofibrils, which was maintained even in 60d animals (Figure 3.4A; Figure 3.5A1,D1). This was similar to 4d and 21d LamC-WT (Figure 3.5B1,E1), and 4d LamC-E174K (Figure 3.5C1). Additionally, mitochondrial cristae appeared normal (Figure 3.4A; Figure 3.5A2,B2,D2,E2). Instead, 14d and 21d LamC-E174K animals exhibited swollen mitochondria (Figure 3.4B,C), with collapsing or
disintegrating cristae (Figure 3.4B,C). This was reflected in a significant reduction in mitochondria electron density (Figure 3.5F). Closer examination already revealed subtle abnormalities in 4d LamC-E174K animals (Figure 3.5C2). Expression of Marf-RNAi in DLMs also elicited dramatic mitochondrial aberrations (Figure 3.4D,E;Figure 3.5F). However, overexpressing Marf in the DLM of LamC-E174K, lamC/+ animals was insufficient to rescue mitochondrial defects (Figure 3.3J,L;Figure 3.4G;Figure 3.5F). Thus, Marf is not the only mitochondrial factor involved in maintaining mitochondrial integrity in PS-LamC mutations. Indeed, downregulating the megaRNP component, Blw, also elicited mitochondrial defects (Figure 3.4F;Figure 3.5F).

To determine if the mitochondrial phenotypes resulted from defective NE-budding and not NPC-mediated export (Muchir et al., 2003), we expressed DFz2-dominant-negative (DFz2-DN) (Mathew et al., 2005), a LamC-independent method to block NE-budding. This induced mitochondrial disruption, including sparse and collapsed cristae (Figure 3.4H; Figure 3.5F).

We also observed progressive defects in muscle fibers in LamC-E174K animals. In contrast to controls, LamC-WT, and 4d LamCE174K (Figure 3.6A-E), 21d LamCE174K animals exhibited thinner myofibrils, sarcomere disorganization, and vanishing Z-lines and A-bands (Figure 3.6F), consistent with the sarcopenia observed in very old wild-type flies. This phenotype was mimicked by expressing Marf-RNAi in DLMs (Figure 3.6G).
PS-modeled LamC mutations display additional signs of aging

An aging marker is a decline in protein quality control, resulting in accumulation of poly-ubiquitinylated protein aggregates (Demontis and Perrimon, 2010; Rubinsztein, 2006). In 3d, 14d, and 21d-day old wild-type flies we observed no poly-ubiquitin aggregates in DLMs (Figure 3.6H-I,K), but these were abundant in 60-day old flies (Figure 3.6J,K).

DLMs from 3d LamC-WT or LamC-E174K flies were devoid of poly-ubiquitin aggregates (Figure 3.4I,J,O). However, numerous poly-ubiquitin aggregates were observed in 14d LamC-E174K, but not LamC-WT flies (Figure 3.4K,L,P). Thus, LamC-E174K animals display accelerated defects in proteostasis, as in normal aging. Downregulating Marf in muscle also led to early appearance of poly-ubiquitin aggregates (Figure 3.4M,P), and overexpressing Marf in LamC-E174K DLMs partially rescued levels of poly-ubiquitin aggregates (Figure 3.4N,P). Given that expressing LamC-E174K leads to a reduction in Marf, and that downregulating Marf alone mimics LamC-E174K phenotypes, these results provide compelling evidence for a primary role of Marf in sustaining mitochondrial integrity, normal muscle mass and proteostasis. Lack of proper mitochondrial replenishment leads to reactive oxygen species (ROS) accumulation in mitochondria derived from oxidative metabolism, inducing cellular damage. Indeed, HGPS patients display an increase in ROS, altered energy generation,
and elevated genomic instability markers (Mateos et al., 2015). Our studies raise the possibility that at least some of the defects observed in PS are derived from abnormal transport of RNAs that are required to sustain mitochondrial replenishment.

Mitochondrial replenishment is based on equilibrium among mitochondrial fusion, fission and autophagy. Balanced mitochondrial fusion and fission restores damaged mitochondrial compartments, while excessive fission leads to fragmentation followed by autophagy to dispose of damaged mitochondria. Studies suggest that normal aging is accompanied by a shift towards fission and decreased fusion (Scheckhuber et al., 2007). Our studies indicate that a decrease in mitochondrial fusion might result from abnormal RNA export in PS-modeled mutations. Interestingly, we found that LamC-E174K induced a decrease in marf, accompanied by accelerated disruption of mitochondria. As marf RNA is a component of megaRNPs and LamC-E174K disrupts NE-budding (see below), these observations are consistent with a role of NE-budding in the export of mitochondrial factors required for normal aging.

MegaRNPs have been associated with developmental periods characterized by high protein synthesis demand, such as after fertilization of the mammalian oocyte and during the rapid expansion of the fly larval NMJ. Interestingly, marf RNA expression is moderate throughout development, with a peak near the end of embryogenesis, and a very large peak in the young adult, starting at late
pupariation and increasing into early adulthood (http://flybase.org/reports/FBgn0029870.html). The progressive deterioration of muscles and mitochondria observed in PS-modeled adults is consistent with a gradual run-down of mitochondrial components needed to be maintained at high levels in the adult. Flight-muscle function imposes great energy demands, making it likely that mitochondria in flight-muscles require continuous replenishment. It is thus conceivable that NE-budding serves to provide an early pool of the RNAs required to sustain this replenishment, and that blocking NE-budding dramatically decreases the size of this early pool. In turn, this leads to early aging, due at least in part to an inability to restore mitochondrial function, and thus the damaging effects of increased ROS.

**LamC-E174K expression disrupts flight behavior**

The abnormalities in middle-age LamC-E174K DLMs were reflected in flight and wing position defects. Unlike wild-type, LamC-WT and young LamC-E174K flies (Figure 3.7A), 21d LamC-E174K animals had a “wings-up” wing position (Figure 3.7B). Also, 21d LamC-E174K failed to jump or fly, as determined in a flight assay (Benzer, 1973) conducted within an oil-coated cylinder. Wild-type, LamC-WT and 3d LamC-E174K flies jump and fly when startled, becoming stuck at various heights of the cylinder walls (Figure 3.7C; Movie1,2). 21d LamC-E174K showed about a 50% decrease in landing height (Figure 3.7C), resulting from an
inability to jump and fly (Movie 2). Consistent with a role of Marf in the above
defect, Marf downregulation in muscles also elicited similar behavioral defects
(Figure 3.7C).

PS-modeled LamC mutations have defective NE-budding
The observations above raised the possibility that defects in NE-budding could
be linked to accelerated aging. Thus, we determined if PS-modeled LamC
mutations elicited NE-budding defects prior to the aging defects, using 3 read-
outs: the presence of nuclear DFz2C/LamC foci, megaRNP granules at the
perinuclear space, and the development of the larval NMJ, whose growth and
maturation require the export of certain postsynaptic RNAs through NE-budding
(Jokhi et al., 2013; Speese et al., 2012).

LamC-WT larval muscle nuclei contained DFz2C/LamC foci that were similar to
controls in appearance and number (Figure 3.7D,F,G; arrows). In contrast, nuclei
in LamC-E174K larvae displayed dramatic decrease in DFz2C/LamC foci (Figure
3.7D,H). Instead, the lamina exhibited numerous small blebs which, except for
rare occasions, were devoid of DFz2C (Figure 3.7H,L; arrowheads; Figure 3.8A).
Such blebs are observed in wild-type at a very low frequency (Figure 3.7F;
arrows). Consistent with LamC-r564c behaving as a recessive mutation,
LamC-r564c, lamC/+ heterozygotes did not display the above phenotypes
(Figure 3.7L). Nevertheless, when expressed in a lamC null background, LamC-
r564c showed a significant decrease in DFz2C/LamC foci (Figure 3.7E,K) and a copious increase in nuclear blebs (Figure 3.7K,M; Figure 3.8B-C). Blebs were not observed in controls, lamC nulls, or LamC-WT. (Figure 3.7I,J,M). Similar lamina blebs were observed in Drosophila Schneider-2 (S2) cells upon induction of LamC variant expression (Figure 3.8D-F). The disruption of DFz2C/LamC foci formation suggests defective NE-budding in PS-LamC mutations.

At the ultrastructural level, megaRNPs in control and LamC-WT cells/muscles were observed at the NE either as single (Figure 3.8G) or clustered electron dense granule(s) at the perinuclear space (Figure 3.7N) (Jokhi et al., 2013; Speese et al., 2012). These granules and associated NE represent the DFz2C/LamC foci observed by light microscopy (Speese et al., 2012). Upon expressing LamC-E174K or LamC-r564c several common abnormalities were observed. First, megaRNPs were localized within aberrant blebs of the NE (Figure 3.7O; white arrow; Figure 3.8H,M,N). These blebs were limited by INM and ONM, and beneath the INM there were areas of irregular and highly thickened lamina (Figure 3.7O,P; black arrows; Figure 3.8K). Thus, unlike the perinuclear space localization of normal megaRNPs, these granules were present in the nucleoplasm (Figure 3.8H,L). The thickened lamina was particularly prominent at the neck of the blebs (Figure 3.7O black arrowhead; Figure 3.8H,K), but also in networks surrounding small nucleoplasmic islands (Figure 3.7Q,R; Figure 3.8J; black arrows). Additionally, blebs devoid of
 megaRNPs were observed at high frequency (Figure 3.7P; white arrowheads). These blebs most likely correspond to the blebs observed at the light microscopy level. Thus, PS-modeled mutations in LamC induce defects in NE-budding. These blebs have been previously observed in HGPS cells (Kandert et al., 2007), but their significance was unknown. We propose that they represent sites of aborted NE-budding.

The above phenotype differed from the defects in NE-budding observed in torsin mutants (Jokhi et al., 2013). In torsin mutants megaRNP granules bud into the perinuclear space but remain tethered to the INM by a “neck”, as Torsin appears to be required in pinching off the INM-coated granule (Jokhi et al., 2013) (Figure 3.8L). In contrast, megaRNPs in PS-modeled LamC mutations were not surrounded by INM and remained naked in the nucleoplasmic side of the projection (Figure 3.8L). We propose that in PS-modeled mutations LamC forms a disrupted lamina network, preventing megaRNPs from accessing the INM to bud into the perinuclear space. Unlike torsin mutants where megaRNP granules accumulate at the perinuclear space, very few megaRNPs were observed in PS-modeled LamC mutations. As megaRNPs are tightly surrounded by INM in torsin mutants, it is possible that they are protected from degradation. Instead, megaRNPs in PS-modeled mutations remain in the nucleoplasm being susceptible to degradation. This is supported by the observation that marf RNA is
severely decreased in the LamC mutations and does not simply accumulate in the nucleus.

**PS-modeled LamC mutations impair NMJ development**

As another NE-budding read-out we examined the larval NMJ. Mutations in genes encoding components of NE budding result in the accumulation of immature synaptic boutons (ghost boutons) that fail to recruit postsynaptic proteins (Jokhi et al., 2013; Speese et al., 2012). This phenotype can be visualized by labeling NMJs with markers of the presynaptic (anti-HRP) and postsynaptic (anti-DLG) compartments, as ghost boutons lack DLG. We found that LamC-WT rescued the increase in ghost boutons in lamC mutants (Figure 3.8O-P,R). In contrast, neither LamC-E174K nor LamC-r564c rescued this defect (Figure 3.8Q,R). Thus, expression of PS-modeled LamC variants results in abnormal NMJ development and mimics the effects of inhibiting NE-budding. NE-budding blockade was observed in the larva, which is well before any signs of aging were detected, suggesting that NE-budding defects were unlikely to be caused by accelerated aging. We propose that certain transcripts required for mitochondrial renewal, are delivered from the nucleus through NE-budding. Mutations blocking NE-budding are initially normal, but fail to maintain this cellular state, resulting in accelerated aging.
HGPS patients do not display signs of intellectual disability (Nissan et al., 2012). However, PS patients commonly have decreased NMJ performance (Greising et al., 2012), defects in the enteric nervous system (Yang et al., 2015), low-frequency conductive hearing loss, and peripheral neuropathy (Goss et al., 2011). Thus, synaptic dysfunction in PS patients cannot be completely ruled out. Our studies reveal a new role for NE-budding in aging and provide a mechanism for premature aging in certain laminopathies.

Material and methods

Molecular Biology

*Drosophila* LamC cDNA was amplified from LamC cDNA clone LD31805 (Drosophila Genomics Resource Center, DGRC) using the following primers:

Forward 5′-3′: AAATTTACTAGTATGTCAGCACGCCGCGTC;

Reverse 5′-3′: AAATTTCTCGAGCTAGAAGAGCAGGGAGAAGGCTC and cloned into the pMT-puro plasmid between SpeI and XhoI sites. Mutagenesis of LamC was performed using the QuikChange II site-directed mutagenesis kit (Agilent Technologies) following the manufacturer's instructions. The mutagenesis primers used are listed as following:

E174K Forward 5′-3′: CGATCGCAAGAAGTTCAAGGATCAGGCCGCGTC

Reverse 5′-3′: CTCCTTGGCCTGATCCTTGAACTTCTTGCGATCG
R564C Forward 5’-3’: GGTGGCCAACCTCGATGTGTTCGGTGCTGGCCAATG
Reverse 5’-3’: CATTGGCCAGCACCGAACACATCGAGTTGGCCACC

For fly injection, wild type LamC (LamC-WT), and mutants LamC-E174K and LamC-R564C were further subcloned into pUAST-attB using EcoRI and XhoI. All LamC constructs were integrated at P2(3L)68A4. For bacterial expression, LamC-WT and mutants were subcloned into pET-30a plasmid using EcoRI and XhoI sites.

Fly strains

The following fly strains were used: Wild type (Canton-S), lamCEX296 and lamCEX265 (Schulze et al., 2005), Gal4 drivers: BG487 (Gorczyca et al., 2007), MHC-Gal4 (Schuster et al., 1996), UAS-LamC-WT, UAS-LamC-E174K, UAS-LamC-r564c (reported here), UAS-Marf-RNAi (ID:55189 Bloomington Drosophila Stock Center). UAS-Blw-RNAi (ID: 28059 Bloomington Drosophila Stock Center) UAS-Marf-HA (Kim et al., 2013)(Gift from Dr. Leo Pallanck); UAS-DFz2-DN (Mathew et al., 2005).

S2 cell culture

*Drosophila* Schneider (S2) cells were cultured at 25°C in SFX insect medium (HyClone) containing 10% (v/v) fetal bovine serum (FBS) (HyClone), penicillin (50 U/ml) and streptomycin (50 μg/ml) (Sigma) in NunclonTM Δ Surface T-flasks
Stable LamC cell lines were generated by transfection of pMT-puro constructs (see above) and 10 µg/ml puromycin (Invitrogen) was added to the culture medium 48 hours after transfection. Stabilized S2 cell lines were maintained in medium supplemented with 10 µg/ml puromycin (Invitrogen).

For S2 cell immunocytochemistry, LamC variant expression was induced by adding CuSO$_4$ (final concentration 0.7 mM) to the culture medium 24 hours before cells were seeded onto a glass coverslip. The cells were then washed with PBS and fixed for 10 min with 4% paraformaldehyde. Cells were permeabilized with 0.2% PBT (0.1 M phosphate buffer pH 7.2 supplemented with 0.2% Triton-X-100 (Sigma)) and immunostained with mouse anti-LamC.

**Western Blots**

Late third instar larva were dissected in ice-cold calcium free saline (128 mM NaCl, 2 mM KCl, 4 mM MgCl$_2$, 35.5 mM sucrose, 5 mM HEPES, 1mM EGTA). 5 larval body wall muscle preparations for each genotype were collected and homogenized in lysis buffer (phosphate buffered saline with 1% SDS, complete protease inhibitor (Roche), 25 µg/ml PMSF and 1 µg/ml pepstatin). Homogenized lysate was centrifuged at 20,817 g for 10 min at 4°C, supernatants collected and boiled with 5X SDS loading buffer (60 mM Tris-Cl, 2% SDS, 10% glycerol, 5% β-mercaptoethanol, 0.01% bromophenol blue) followed by SDS-PAGE separation and transfer to nitrocellulose membrane. The following
antibodies were used for immunoblotting: mouse anti-LamC (1:500, Developmental Studies Hybridoma Bank (DSHB); LC28.26), mouse anti-α-tubulin (1:10,000, Sigma; B-5-1-2) and goat anti-mouse-HRP (1:5000, Jackson Immunoresearch).

Immunochemistry

For larval body wall muscle preparation, late third instar larval body wall muscle preparations were dissected in ice cold calcium free saline (see above), followed by fixation with 4% paraformaldehyde. For adult indirect flight muscle preparations, as described in (Hunt and Demontis, 2013), thoraces of adult flies were isolated and pre-fixed in 4% paraformaldehyde for 20 minutes at room temperature, bisected using a double edged stainless steel razor blade (EMS) and further fixed in 4% paraformaldehyde for 15 minutes. Both larval body wall muscles and adult indirect flight muscles were washed and permeabilized with 0.2% PBT (see above). The following primary antibodies were used: rabbit anti-DLGPDZ (1:40,000 (Koh et al., 1999)), mouse anti-Ubiquitin-FK2 (1:200, Enzo Life Science), mouse anti-LamC (1:30, DSHB; LC28.26), mouse anti-ATP5A (1:100, Abcam; 15H4C4) (which cross-reacts with Drosophila ATPsyn-α/Blw.), rabbit anti-DFz2-C (1:500 (Mathew et al., 2005)). We used anti-HRP, anti-mouse or anti-rabbit (1:200, Jackson ImmunoResearch) secondary antibodies coupled
to either Dylight488 or Dylight594. Rhodamine- and Alexa647-conjugated Phalloidin (1:100, Molecular Probes) were used for labeling adult flight muscles.

**Fluorescent in situ hybridization (FISH)**

*In situ* hybridization was performed as described previously (Speese et al., 2012) with minor modifications. Briefly larval body wall muscles were dissected as described above and fixed with 4% paraformaldehyde for 30 min. Preparations were further fixed with ice-cold methanol for 10 min, and then washed three times, 10 min each, with 0.2% PBT. Samples were equilibrated with hybridization buffer (2XSSC, 10% dextran sulfate, 10 mM ribonucleoside-vanadyl complex (RVC, New England Biolabs), 50% formamide). Gene specific probes (equal volume of probes and blocking probes) were heated to 80°C for 5 min and chilled on ice immediately. Probes were then combined with equal volumes of 2X hybridization buffer (final concentration of 2.5 ng/µL gene specific probes). Samples were incubated with probes overnight at 37°C. From this point onward, the protocol was the same as in (Speese et al., 2012). To visualize nuclei, *Drosophila* samples were incubated with mouse anti-LamC (1:30, DSHB).

**Probe preparation**
Probe preparation was the same as described previously (Speese et al., 2012). Briefly, anti-sense probes were designed based on the cDNA sequences of target genes. The probes were produced by nick translation of the PCR product (Bionick; Invitrogen) with digoxigenin-11-dUTP (Roche) for 2.5 hrs at 18°C, and the reaction was inactivated by heating to 65°C for 10 min. Probes were precipitated and resuspended in formamide and stored at -80°C. Blocking probe against Shibire was prepared using the same method except dTTP was used instead of digoxigenin labeled dUTP.

Primers for probes

Marf Forward GAGGTGGAGGAGAAGGTGTCAAAG
Marf Reverse AGTTGGCCTTGTGTGCGAAGCAG
ATPsynβ Forward ATGTTCGCGTTACGTGCTGC
ATPsynβ Reverse CTAAGGCAGCTTCTTTGCCAG
ATPsynB Forward ATGTTCGAGGAGCGCTCTTC
ATPsynB Reverse TTAGGCAGACTTGACGCGCAG
Blw Forward CAACCAGGTCGCCTGCAAAG
Blw Reverse ATCACGGGAGACAGCTCAG
COX4 Forward CCTGCGACTACTCAACAGTG
COX4 Reverse GTCCCACTTTGGAGGTAATC
Pink1 Forward ATGTCTGTGAGACTGCTGACC
Pink1 Reverse GTACCGTCTCCTTGTACATGG
Image acquisition, quantification and morphometric analysis

Confocal images were acquired using either a Zeiss LSM700 confocal microscope with a Zeiss 63X Plan-Apochromat (1.4 numerical aperture) DIC oil immersion objective or an Improvision spinning disk confocal with a 40X (1.3 NA) oil immersion objective. Fluorescence signal intensity and volume was quantified by volumetric measurements of confocal stacks using Volocity 5 Software (Improvision). Control and experimental images were taken at the same settings. For ubiquitin and mitochondria (labeled with anti-ATP5A) studies in indirect flight muscles, images were collected from dorsal longitudinal muscle (DLM). Ubiquitin and mitochondrial volumes were determined based on ubiquitin and ATP5A signal respectively and normalized to muscle volume (phalloidin signal volume), for each image, and then normalized to wild type.

For bouton quantification, body wall muscle preparations were double labeled with anti-HRP and anti-DLG. Normal boutons were identified as those that were both HRP and DLG positive, while ghost boutons were identified as HRP positive boutons devoid of DLG label. Ghost bouton number was normalized to total number of boutons. All measurements were taken from muscles 6 and 7, abdominal segment 3.

The number of nuclear DFz2C/LamC foci, and percent of nuclei having more than 10 blebs, were determined from muscles 6 and 7 (A2, A3) of dissected
wandering third instar larvae. The number of foci per nucleus was normalized to wild-type controls.

**Electron Microscopy**

Transmission electron microscopy was carried out as described (Speese et al., 2012). Briefly, late third instar larval body wall muscles and adult thoraces were dissected and fixed with Trumps fix (50 mM cacodylate buffer pH:7.2, 4% EM grade paraformaldehyde, 2% EM grade glutaraldehyde, 1.25 mM MgCl₂, 2.5 mM CaCl₂) for 24 hours at 4°C. The samples were further fixed with 1% osmium in 50 mM cacodylate buffer for 45 minutes at room temperature dehydrated in ethanol, and then embedded in EM-BED 812 Resin (Electron Microscopy Sciences, EMS). To quantify mitochondrial density, gray scale EM images were first converted to 8 bit, inverted (white = 0, black = 255), the outer membrane was used to generate an region of interest and the mean gray value of each mitochondrion was measured using ImageJ.

**Flight Assay**

Flight assay was conducted as described (Benzer, 1973). Briefly, the inside of a 500 ml cylinder was coated with paraffin oil, and a group of 25-30 flies were dropped into the cylinder using a funnel. The landing height of each fly was
measured from the bottom of the cylinder, and the results from three independent experiments were pooled together.

RNA Immunoprecipitation

Twenty larvae of each genotype were dissected under calcium free saline (see above) and homogenized in RIP buffer (1 mM EDTA, 50 mM HEPES pH 7.5, 140 mM NaCl, complete protease inhibitor (Roche) and RNase inhibitor (ThermoFisher Scientific)) using 1 mm glass beads in a Bullet Blender (Next Advance). After homogenization, samples were spun down at 16,000 g, 4°C for 15 min. Supernatants were pre-cleared with washed Protein A/G Magnetic Beads (ThermoFisher), and incubated at 4°C for 1 hour. Beads were collected on a magnetic stand, and the supernatant was applied to a fresh set of washed beads with either 7.5 μL of rabbit anti-DFz2C antibody, or 7.5 μL of rabbit anti-mouse IGG. The samples were then incubated overnight at 4°C. The following day, the beads were magnetically separated, and washed 3X in RIP buffer. The RNA was then isolated off the beads as per the instructions of the Direct-zol™ RNA micro-Prep kit (Zymo), followed by complementary DNA synthesis using SuperScriptIII First-Strand kit (Life Technologies) for quantitative real-time PCR analysis.

Quantitative real-time PCR analysis
RNA was extracted from thoracic muscle isolated from 10 three-week old flies using Trizol (Life Technologies), and genomic contamination was removed using TURBO DNA-free kit (Ambion). Complementary DNA synthesis reactions were performed using SuperScriptIII First-Strand kit (Life Technologies) with random hexamer primers. Real-time PCR was performed in triplicate using gene specific primers, SYBR Green PCR master mix (Qiagen) and a StepOnePlus Real-Time PCR System (Applied Biosystems) as described previously (Ding et al., 2013). marf RNA levels were normalized to a reference gene, either Ef1α48D (elongation factor 1α48D), or RpL32 (Ribosomal protein L32) (Ling and Salvaterra, 2011) by the 2-ΔCt method. The RNA levels were then normalized to the control for each experiment, and then pooled for final analysis.

For RNA Immunoprecipitated samples, RNA levels were calculated by the 2^{-ΔCt} method, where ΔCt = (DFz2C IP Ct – control IP Ct) for either marf or mad. The RNA levels were then normalized to marf, to allow multiple experiments to be pooled together.

Primers for qPCR
Marf Forward CTGTCGTTGATCTCAGCTT
Marf Reverse CAGGTAGATGCAGCCGTAGA
Mad Forward CCACTGCAACAACAACTCCG
Mad Reverse CCTTGCCCTATATGACGGCGT
Statistics

One-way ANOVA with Tukey’s post hoc test was used. Error bars in the graphs represent SEM.
Figure 3.1 Association of *marf* mRNA with DFz2C and nuclear LamC foci

(A, C-G) FISH of a larval body wall muscle preparations using a (A) *marf* (C) ATP-syn-β, (D) *blw*, (E) ATP-syn-B, (F) *cox4*, (G) pink1 DNA probe and antibodies to LamC. Arrows denote foci containing mitochondrial transcripts.
(B) Real time PCR of marf and mad after RNA-immunoprecipitation with DFz2C antibody from larval body wall muscles.

(I) Schematic depiction of *Drosophila* LamC and human LMNA protein structure, indicating conserved (red vertical lines) and non-conserved (blue vertical lines) amino acid residues that when mutated cause progeroid syndromes in humans. Blue arrow indicates the mutation responsible for most HGPS cases.

(H) Western blot of body wall muscle protein extracts from animals of the indicated genotypes probed with antibodies to LamC (top) and tubulin (Tub; bottom). Numbers at the right represent molecular weight x 10^3. ***p<0.001 Error bars= SEM. Calibration bar in A, C-G is 6 µm.
Figure 3.2 marf RNA is found at nuclear LamC foci

(A-E) FISH of larval body wall muscle preparations using anti-LamC antibody and a (A-B) marf, (C) ATP-syn-β, (D) blw, and (E) ATP-syn-B DNA probe in (A) control and (B-E) RNaseA-treated samples. Calibration bar is 9µm. Arrows denote marf RNA positive puncta associated with LamC foci.
Figure 3.3 Age-dependent alterations in adult muscle mitochondria upon expressing LamC-E174K
(A-I, L) DLMs labeled with anti-ATP-syn-α and rhodamine-conjugated phalloidin from (A-C) 3- (D-F L) 14- and (G-I) 21-day old (A,D,G) MHC-Gal4 control; (B,E,H) LamC-WT, lamC/+; (C,F,I) LamC-E174K, lamC/+ and (L) LamC-E174K, lamC/+. Marf OE

(J) Quantification of mitochondrial volume normalized to muscle volume.

(K) Real time PCR of marf RNA levels in adult thoracic muscle from the indicated genotypes.

N (left-right)= J: 30,49,28; 35,33,31; 28,47,32; 36. ***p< 0.001 Error bars= SEM. Calibration scale is 9 µm.
Figure 3.4 Disruption of adult muscle mitochondria and progressive polyubiquitin aggregate accumulation upon expressing LamC-E174K

(A-H) EM micrographs of adult DLMs showing mitochondrial cristae structure in (A) 60-day, (B,D,G) 14- and (C,E,F,H) 21-day old flies of the following genotypes: (A) MHC-Gal4/+, (B,C) LamC-E174K, lamC/+, (D-E) Marf-RNAi-muscle, (F) Blw-RNAi-muscle, (G) lamC-E174K, lamC/+ Marf OE, (H) DFz2-DN OE muscle. Arrowheads denote collapsing cristae; arrows denote disintegrating cristae.
(I-N) DLMs double labeled anti-ubiquitin and Rhodamine-conjugated phalloidin in (I-J) 3-, (K-N) 14-day old adults from (I,K) LamC-WT, lamC/++; (J,L) LamC-E174K, lamC/++; (M) Marf-RNAi-muscle and (N) LamC-E174K, lamC/+ Marf OE.

(O-P) Quantification of ubiquitin aggregate volume normalized to muscle volume.
N (left to right)= O: 29,30,29; P:85,30,35,36,36.
*p<0.05  ***p<0.001; Error bars= SEM

Calibration bar is 0.5 µm for (A,B,H), 0.4 µm for (D,E,F,G), 1 µm for (C) and 16 µm for (I-N).
Figure 3.5 Ultrastructure of adult muscle mitochondria

(A-E) EM micrographs of adult DLMs showing (A1,B1,C1,D1,E1) mitochondria at lower magnification and (A2,B2,C2,D2,E2) mitochondrial cristae structure at high magnification in (A-C) 4- and (D-E) 21-day (A,D) MHC-Gal4 control, (B,E) LamC-WT, lamC/+ and (C) LamC-E174K, lamC/+ . (F) Quantification of normalized mitochondrial gray value.
N(left-right)=148; 65,426; 141,488; 56; 89,168; 84,85; 80

Calibration bar is 1.5 µm for A1-E1, and 0.5 µm for A2-E2.
Figure 3.6 Ultrastructure of adult muscle sarcomere and progressive polyubiquitin aggregates

(A-G) Lower magnification EM micrographs of adult DLMs showing myofibril organization of (A,C,E) 4-day and (B,D,F,G) 21-day old (A-B) MHC-Gal4 control, (C-D) LamC-WT; lamC/++; (E-F) LamC-E174K; lamC/++; (G) Marf-RNAi-muscle. z: Z-line; a: A-band.

(H-J) DLMs double labeled anti-ubiquitin and Rhodamine-conjugated phalloidin in (H) 3-day (I) 21-day and (J) 60-day old animals. Arrows denote ubiquitin aggregates.

(K) Quantification of ubiquitin aggregates volume normalized to muscle volume. N (left to right)= 29,47,32,24 ***p<0.001, Error bars= SEM; Calibration bar is 2 µm for A-G and 15 µm for H-J.
Figure 3.7 Expression of PS-modeled mutations disrupts NE morphology and ultrastructure
(A-B) Wing position in (A) control flies, showing normal wing position and (B) LamC-E174K, lamC/+ flies showing a “wings up” phenotype.

(C) Quantification of landing height in 4- and 21-day old adults.

(F-K) Larval body wall muscle nuclei labeled with anti-LamC or anti-LamDm0, and anti-DFz2C antibody from (F) BG487-Gal4/+ control; (G) LamC-WT, lamC/+; Arrows denote LamC/DFz2C foci; Arrowheads denote abnormal LamC blebs.

(H) LamC-E174K, lamC/+; (I) lamC null mutant; (J) LamC-WT, lamC; and (K) LamC-r564c, lamC. Arrows point to DFz2C/LamC foci, and arrowheads to blebs.

(D,E) Quantification of the number of DFz2C/LamC foci at body wall muscles normalized to BG487/+ controls in the indicated genotypes in a (D) lamC/+ or (E) lamC null mutant background.

(L,M) Quantification of the percentage of body wall muscle nuclei containing more than 10 blebs at the NE in the indicated genotypes (L) in a lamC/+ heterozygous background and (M) in a lamC null mutant background. Control is BG487/+ in both graphs.

(N-R) TEM of larval body wall muscle nuclei from (N) LamC-WT, lamC; (O-Q) LamC-E174K, lamC; and (R) LamC-r564c, lamC. White arrows denote megaRNP granules; White arrowheads denote empty blebs; Black arrows denote thickened lamina; Black arrowheads denote thickened lamina at the neck of blebs.

*p<0.05  **p<0.01  ***p<0.001 Error bars= SEM
N (left to right) = C: 81, 78, 83, 166, 83, 85, 89; D, E, L, M (number of hemisegments/number of nuclei) D: 21/1137, 21/1026, 24/1248; E: 17/958, 13/666, 17/951, 19/977; L: 20/1155, 20/1106, 20/1035, 12/647; M: 26/1500, 14/720, 23/1274, 19/1079, 16/829.

Calibration bar is 5.5 µm for F-K, 1.4 µm for P and 0.4 µm for N, O, Q, R
Figure 3.8 Nuclear foci at larval body wall muscles and S2 cells, and NMJ phenotypes

(A-C) Larval body wall muscle nuclei labeled with antibodies to LamC and DFz2C in (A) LamC-E174K, lamC/+ (B-C) LamC-r564c, lamC. Arrows denote DFz2C positive puncta associated with LamC blebs.
(D-F) S2 cells labeled with antibodies to LamC expressing (D) LamC-WT, (E) LamC-E174K or (F) LamC-r564c. Top panels represent single confocal slices and bottom panels are Z-projections. Arrowheads denote normal LamC foci; Arrows denote abnormal LamC blebs.

(G-K) Transmission electron micrographs (TEM) of S2 cell nuclei expressing (G) LamC-WT, and (H,K) LamC-r564c and (I,J) LamC-E174K. White arrows denote megaRNP granules; White arrowheads denote empty blebs; Black arrows denote thickened lamina; Black arrowheads denote thickened lamina at the neck of blebs.

(L) Schematic representation of NE-budding in wild type, PS-modeled LamC mutations and torsin mutations (see text for detailed description).

(M-N) Percentage of nuclei with (M) NE blebs and (N) normal NE foci.

(O-Q) Larval NMJs double labeled with antibodies to HRP and DLG in (O) lamC null; (P) LamC-WT, lamC; and (Q) LamC-r564c, lamC. Insets in the first column are shown at high magnification (O2-Q2). Arrows denote ghost boutons, devoid of postsynaptic DLG.

(R) Quantification of ghost bouton number normalized to total bouton number.

N (left to right)= M,N: 89, 45, 88; R:34, 16, 20, 17, 22.

*p<0.05; **p<0.01; ***p<0.001. Error bars= SEM. Calibration bar is 7.5 µm for A-C, 10 µm for D-F, 0.7 µm for G, 0.5 µm for H,J,K and 0.4 µm for I, 45 µm for O1-Q1, 7 µm for O2-Q2.
Endnotes for Chapter III

Chapter III is adapted from the following publication:


Author contributions:

Y.L. Figure 3.1C-G, H; 3.2C-E; 3.3A-I,J,L; 3.4I-P; 3.5F; 3.6H-K; 3.7A-M; 3.8A-F,M-R

L.H. & W.H. Figure 3.4A-H; 3.5A-E; 3.6A-G;

L.H. Figure 3.7N-R

T.T. Figure 3.1B

B.D. Figure 3.3K

J.A. Figure 3.1A; 3.2A-B; 3.8G-K

V.B. Figure 3.1I; 3.8L; Conceptualization, writing, visualization, supervision, funding acquisition
CHAPTER IV

Discussion
**Exosomes and *Drosophila* NMJ development**

Wg, a *Drosophila* Wnt protein, is secreted by motor neuron terminal in an activity-dependent manner, to serve bidirectional roles in controlling both the presynaptic and postsynaptic development of *Drosophila* larval NMJ (Ataman et al., 2008; Packard et al., 2002). A recent study from the Budnik lab demonstrated that the transfer of Wg at the larval NMJ is mediated by Evi, a multipass transmembrane protein (Korkut et al., 2009). Further study discovered that Evi, when expressed in S2 cells, is present in exosomes purified from the S2 cell culture (Koles et al., 2012). Immuno-EM verified that Evi is present in MVBs at presynaptic boutons, where the exosomes initially form (Koles et al., 2012). These studies together suggest that the transfer of Wg at the larval NMJ is likely though Evi-containing exosomes.

One question pertains to the identify of other exosomal proteins at the larval NMJ. In Chapter 2 of this thesis we demonstrate that another protein, Syt4, is transferred from pre- to the postsynapse. Knockdown of Syt4 presynaptically completely eliminates Syt4 signal from both the pre- and postsynapse, suggesting the postsynaptic Syt4 is exclusively derived from the presynaptic motor neuron. Presynaptic expression of Rab11DN, a dominant negative form of Rab11 able to block Evi-containing exosome release from both S2 cells and motor neurons (Koles et al., 2012), inhibits the secretion of Syt4 from presynaptic boutons. Immuno-EM further demonstrated the presence of Syt4 at exosomes.
purified from HA-tagged Syt4-ovexpressing S2 cells. These results further support the hypothesis that Syt4 is transferred from motor neuron to the muscle compartment likely through exosomes. In Chapter 2 of this thesis, the relationship between Sy4 and Evi was further investigated. Both exogenous and endogenous Syt4 co-immunoprecipitates with EviGFP, suggesting these two proteins form a biochemical complex. Immunofluorescence staining, however, demonstrated that Syt4 and Evi only partially colocalize with each other at the NMJ. The trans-synaptic transfer of Syt4 is not affected in evi mutant, while Evi transfer is not affected in syt4 null mutant, indicating Syt4 and Evi are not mutually required for their secretion. Moreover, our data showed that at most 13.2% of exosomes purified from S2 cells cotransfected with tagged Syt4 and Evi contain the signals of both proteins. These evidences strongly indicate that Syt4 and Evi, though both present in exosomes, are likely sorted into different exosome populations. Indeed, studies from other groups have illustrated the heterogeneity of exosome population (Colombo et al., 2014). Depending on the cell types and tissue origins, exosomes may contain different components. Our study raises the possibility that even a single cell type may secret exosomes containing different components. One interesting question in the field of exosomes is how exosomes are taken up by the recipient cells. So far there has been evidence supporting either their direct fusion with the plasma membrane of recipient cell or their endocytosis by
the recipient cells. In the latter case, the endocytosed exosomes are sorted into endosomes. Endosomes may eventually fuse with lysosomes resulting in degradation of the exosome, or the exosome can be fused with endosomal membrane and release its contents within the cell. Syt4 has a transmembrane domain at its N-terminus and two C2 domains at its C-terminus (Chapman, 2008). The C-terminus of Syt4 is presumably localized within the exosomal lumen. In Chapter 2 we demonstrated that the secreted Syt4 controls a retrograde signaling pathway required for nascent presynaptic bouton formation. An early study showed that the retrograde function of Syt4 depends on its C2 domains (Yoshihara et al., 2005). This indicates that the Syt4-containing exosomes have to fuse with either plasma membrane or endosomal membrane to allow the C2 domains to be exposed to the cytosol. There have been unpublished efforts in Budnik lab to establish the mechanisms by which exosomes release their contents using a GFP reconstitution method (Cabantous et al., 2005a; Feinberg et al., 2008; Pedelacq et al., 2006). Briefly, GFP is split into two fragments spGFP1-10 and spGFP11. Syt4 is tagged with spGFP11 at C-terminal and expressed in motor neuron, while the recipient muscle cells are labeled with spGFP1-10. Only when the C-terminal domain of Syt4 is exposed to the cytosol can spGFP11 and spGFP10 reconstitute fluorescent GFP. This method, however, suffered from high level of autofluorescence of spGFP1-10, which may mask the real reconstituted signal. The key to overcome this obstacle will be to increase
the real/noise signal ratio, which can be achieved by either lowering the noise background or amplifying the real signal.

The word exosome was continuously used in Chapter 2 to refer to the extracellular vesicles purified. However, with the improving understanding of the field, we now know that the vesicles we purified using ultracentrifugation were likely a heterogeneous population of different types of extracellular vesicles with possible contamination of protein aggregates. Currently, improved methods of exosome purification usually couple ultracentrifugation with sucrose gradient centrifugation to eliminate protein aggregates based on different density between protein and lipid. However, there is still no perfect method to isolate pure exosomes from a pool of extracellular vesicles with similar sizes.

Immune-EM of exosomes isolated from S2 cells expressing HA-tagged Syt4 demonstrated that Syt4 is sorted into exosomes (or exosome-like vesicles). Expression of Rab11DN in motor neuron, known to inhibit exosome release, largely reduced secretion of Syt4 from the presynaptic terminal suggesting the secretion of Syt4 is through exosomes. However, there is so far no direct evidence showing Syt4 is present in trans-synaptically travelling exosomes. The traditional EM can hardly preserve the vesical structure of exosome in situ due to the harsh fixation and dehydration conditions. High pressure freezing of the larval NMJ coupled with immune-EM might be able to catch Syt4-labeled exosomes at the synapse.
Syt4 dependent retrograde signal at NMJ development

It has been demonstrated by independent groups that Syt4 is required for retrograde signaling at *Drosophila* central nervous system and NMJ. (Barber et al., 2009; Yoshihara et al., 2005). In Chapter 2, we first showed that the alteration of presynaptic structure and function, induced by spaced stimulation, requires retrograde signaling. Inhibition of postsynaptic depolarization blocked activity-induced nascent bouton formation and miniature EJP potentiation. We then demonstrated that the activity-induced synaptic structural and functional enhancement requires Syt4. New bouton formation and miniature EJP potentiation upon spaced stimulation is largely abolished in the *syt4* null mutant. This defect is rescued by expressing Syt4 either postsynaptically or presynaptically, as postsynaptic Syt4 is exclusively derived from presynase. It would be interesting to see if blocking the postsynaptic depolarization in a *syt4* null mutant with presynaptic Syt4 rescue would have normal or abolished activity-induced synaptic alterations. If the activity-induced nascent bouton formation and miniature EJP potentiation are disrupted, it will be consistent with the idea that Syt4 is the mediator of retrograde signaling at the NMJ.

What could be the retrograde signal? One known retrograde signal at the larval NMJ is BMP signaling, in which Gbb is secreted by the muscle and activates Wit/
Tkv/Sax tetrameric receptor complex on motor neuron surface. This further triggers phosphorylation and nuclear translocalization of Mad/Medea complex (Keshishian and Kim, 2004), which promotes the transcription of Trio, a Rho GEF. Trio controls presynaptic bouton development via regulating synaptic actin cytoskeleton remodeling. Littleton group discovered that BMP signaling molecules are required for activity-dependent synaptic growth (Piccioli and Littleton, 2014). Spaced stimulation-induced nascent bouton formation is blocked in the wit mutant and in larvae expressing Gbb targeting dsRNA in the muscle (Piccioli and Littleton, 2014). It remains unclear whether BMP signaling could be the retrograde signaling downstream of Syt4. Spaced stimulation of syt4 null larvae with muscle overexpression of Gbb might help to address this possibility. If Gbb is the signaling molecule downstream of Syt4 function, overexpression of Gbb in muscle should at least partially restore the new bouton formation upon spaced stimulation.

**A *Drosophila* model of Progeroid Syndrome**

In Chapter 3, we described a transgenic fly model expressing LamC-E174K mutant, which corresponds to LaminA/C-E159K mutation identified from one progeroid syndrome (PS) patient. In this fly model, we focused on the adult indirect flight muscle (IFM), which is the biggest muscle in adult fly controlling
flight behavior and wing position. We observed accelerated accumulation of ubiquitin-positive protein aggregates, progressive mitochondrial and muscle degeneration and decline in flight ability. Are these phenotypes also observed in mouse models and human patients?

Several progeria mice lines have been reported displaying poor muscle development, muscle atrophy and locomotion defects. Mice carrying \textit{LMNA}^{L530P/L530P} mutation, which was initially generated to model AD-EDMD, displayed very similar phenotypes as HGPS patients including retarded growth, early death, loss of subcutaneous fat, decreased bone density, heart pathology. Hypoplasia and/or atrophy of cardiac muscle, as well as mild to moderate degeneration of skeletal muscle from several regions were observed in \textit{LMNA}^{L530P/L530P} mice (Mounkes et al., 2003). Zmpste24-deficient mice (\textit{Zmpste24}^{−/−}), a widely used model for HGPS since Zmpste24 is required for processing prelaminA to yield mature LaminA, exhibited muscle weakness, hind limb dragging and inability to hold onto a grid when placed upside down (Bergo et al., 2002). Tissue analysis done in an independently generated \textit{Zmpste24}^{−/−} line showed abnormal and dystrophic muscle fibers in paravertebral region, deltoid and quadriceps muscle (Pendas et al., 2002). Pronounced vascular smooth muscle loss, skeletal muscle atrophy and wasting are also commonly seen in HGPS patients (Goldman et al., 2004) and PS patients carrying LMNA point mutations (Garg et al., 2009; Liang et al., 2009).
Mitochondrial defects are also found in HGPS patients and progeria mouse models. Fibroblasts from HGPS patient showed defective ATP synthesis and downregulation of ETC (electron transport chain) components, which can be rescued by farnesyltransferase inhibitors (Rivera-Torres et al., 2013). Similar defects were also observed in progerin knockin mice and Zmpste24−/− mice (Rivera-Torres et al., 2013). Increased mitochondrial fragmentation and elevated ROS level were observed in HGPS fibroblasts as well as HGPS iPSC-derived smooth muscle cells (Xiong et al., 2016).

These phenotypic similarities among different organisms and systems suggest that our *Drosophila* model of progeroid syndrome can be useful in the study of this complicated premature aging disease. However, this fly model of human diseases may suffer from a lack of directly-comparable tissues and different developmental processes. For example, it would be difficult to study bone infarction, typically seem in HGPS patient, in *Drosophila* as insects evolved a very different skeletal system. Metamorphosis also introduces extra complexity in the study of developmental aspects of disease. Nevertheless, the simple genetics and powerful molecular genetic tools available make it easier to decipher the disease mechanisms.

**Nuclear envelope budding in mammalian systems**
The endogenous nuclear envelope (NE) budding pathway was discovered in *Drosophila* larval NMJ (Speese et al., 2012). In Chapter 2, we demonstrated PS-modeled LamC mutants result in defective NE-budding and premature aging related phenotypes. One obvious question is whether NE-budding pathway exists in human (and/or other mammals) and if so, how it is affected in progeria/progeroid patients.

*In situ* hybridization using an oligo-dT probe demonstrated the presence of polyadenylated mRNA-enriched LaminA/C foci at the periphery of control human fibroblast nuclei (Figure 4.1A Ding et al., in preparation) similar to what has been observed in larval muscle (Speese et al., 2012). The number of poly(A)/LaminA/C foci is significantly decreased in HGPS fibroblasts (Figure 4.1B,F Ding et al., in preparation). *In situ* hybridization using specific probes further demonstrated the enrichment of mitofusin1 (Mfn1) and mitofusion2 (Mfn2) transcripts in LaminA/C foci in human fibroblasts (Ding et al., in preparation), consistent with our discovery of *Marf* mRNA present at LamC foci in *Drosophila*.

In Chapter 3, we showed that *Marf* transcript levels significantly decrease in the PS-modeled LamC fly mutant. Consistent with this finding, both Mfn1 and Mfn2 transcripts levels are significantly decreased in HGPS fibroblasts compared to control fibroblasts (Figure 4.1G, Ding et al., in preparation). Similar electron dense granules present at perinuclear space have been observed by other groups long ago in mammalian systems including rabbit blastocyes and mouse
zygotes (Hadek and Swift, 1962; Szollosi and Szollosi, 1988) and by the Budnik group in human fibroblasts, Neuro2A, HEK293 and postnatal day 8 mouse brain (Ding et al., in preparation). These findings suggest the existence of nuclear envelope budding pathway in a variety of tissues in mammalian tissues.

**Nuclear envelope budding and progeroid syndrome**

In human, A-type lamins are encoded by a single gene *LMNA*. LaminA and LaminC, the two major A-type lamin proteins, are produced through alternative splicing. One of laminopathies with the most striking symptoms is progeroid syndrome (PS), a premature aging disorder. The most well characterized form of PS is Hutchinson-Gilford progeria syndrome (HGPS), caused by a single mutation (C1824-T, G608G) on *LMNA* gene that introduces an aberrant splicing site which leads to deletion of a proteolytic site. This cleavage site is essential for removal of the farnesylated C-terminus of prelaminA to form mature LaminA. In addition to the HGPS mutation, there are more than twenty point mutations throughout the *LMNA* gene which also result in progeroid syndromes. These point mutations affect both LaminA and LaminC proteins. In *Drosophila*, the only A-type lamin is LamC which lacks the C-terminal CAAX motif, thus precluding the possibility to study the G608G silent mutation in human LaminA in *Drosophila*. However, in Chapter 3, we generated transgenic flies expressing LamC
mutations modeling missense point mutations found in progeroid syndromes. Muscle nuclei from these LamC mutant flies showed nuclear envelope blebbing phenotypes at both light and EM level. EM analysis demonstrated that electron dense granules can be found within some of the nuclear envelope blebs, while blebs devoid of granules are present at high frequencies. This indicates that these blebs could be abolished nuclear envelope budding sites. Differently from the torsin mutant, which displays stuck granules in perinuclear space, PS-modeled LamC mutant megaRNP granules remain in the nucleoplasm. It was demonstrated previously that lamina needs to be dissociated before megaRNP or viral particle can reach INM and start the budding process (Marschall et al., 2011; Speese et al., 2012). A possible explanation is these PS-modeled LamC mutations affect the conformation of the lamin protein, which prevents the phosphorylation and/or dissociation of the lamina. MegaRNPs either get degraded when trapped in the blebs or they are not loaded into the blebs at the first place. Regarding the blebs, however, other interpretations cannot be ruled out. It is possible that these blebs result simply from the deformation of nuclear envelope due to structural defects of lamina caused by mutated LamC. Indeed, there are many aspects of the nuclear envelope budding process remain unclear: What controls the initial formation of the megaRNPs? What are the signals that trigger the dissociation of the lamina? Why would the blebs still form when megaRNPs cannot reach the INM? What controls the curving of the nuclear
envelope during bleb formation? Rigorous investigation of the budding machinery will be needed to answer these questions. Nevertheless, PS-modeled LamC mutant showed increased ghost bouton number and decreased numbers of normal LamC/DFz2C foci, which support the model that FNI/NE-budding pathway is compromised in PS-modeled LamC mutants.

As discussed earlier, it is likely the NE-budding pathway is present in mammalian system as well; one question is whether NE-budding is affected in human progeroid syndrome? As shown above, polyadenylated mRNA-enriched LaminA/C foci are observed in control human fibroblasts, and the number of these foci is significantly decreased in HGPS fibroblasts (Figure 4.1 A,B,F Ding et al., in preparation), suggesting defective NE-budding in HGPS fibroblasts. Moreover, similar nuclear envelope blebs and nucleoplasmic islands either partially or completely devoid of megaRNP are observed in HGPS fibroblasts at the EM level (Figure 4.1C-E, Ding et al., in preparation). These data indicate that NE-budding pathway is likely affected in human HGPS patients.

We demonstrated the NE-budding pathway is defective in the PS-modeled fly mutant, and the PS-modeled fly mutant has premature aging phenotypes. A pressing question is: does defective NE-budding contribute to these aging-related phenotypes? In situ hybridization data showed that several nuclear encoded mitochondrial transcripts including Marf and ATP synthase subunits are present at LamC foci. The enrichment of Marf mRNA at foci was further verified
with RNP immunoprecipitation using anti-DFz2C antibody followed by qRT-PCR. We also showed the level of Marf transcripts is significantly decreased in PS-modeled LamC mutant, consistent with defective NE-budding in the mutant. Marf, the *Drosophila* homolog of mitofusin, promotes mitochondrial fusion. PS-modeled LamC mutant fly exhibited highly fragmented mitochondria and collapsed cristae structure. Similar mitochondrial defects were also observed in fly with Marf-RNAi expressed in muscle. These data are consistent with the proposed model that defective NE-budding leads to loss of mitochondrial integrity through downregulation of cellular Marf levels. Interesting, restoring Marf levels in PS-modeled LamC mutant failed to rescue the mitochondrial defects, consistent with the finding that other mitochondrial transcripts like ATP synthase subunits are also localized at NE-budding sites.

However, it is unlikely that impaired NE-budding is the only explanation for mitochondrial defects observed. Lamin is involved in a diversity of cellular functions ranging from nuclear mechanical support, chromatin organization to DNA replication and transcriptional regulation. Studies using human fibroblast and mouse models have raised several possible underlying mechanisms of progeria and/or progeroid syndromes. It is possible that different LaminA/C mutations may contribute to the disease through different pathways and/or combination of these pathways. For example, nuclear pore complex (NPC) clustering has been observed in HGPS fibroblasts (Goldman et al., 2004).
However, no abnormal NPC morphology was observed in fibroblasts from progeroid patient carrying LMNA S143F mutation (Kandert et al., 2007). Whether the PS-modeled LamC mutations studied in Chapter 3 affect NPC functions, chromatin organizations and transcriptions, and whether these pathways may contribute to the mitochondrial and muscle phenotypes remain unclear.

In Chapter 3, in order to differentiate NE-budding and NPC on their contribution to the mitochondrial phenotypes, we expressed DFz2-DN in the muscle as an alternative way to disrupt FNI/NE-budding pathway. Expression of DFz2C-DN results in similar but milder loss of mitochondrial cristae structure as PS-modeled LamC mutant at comparable ages. This supports the idea that it is likely NE-budding instead of NPC that is involved in the mitochondrial phenotypes. However, we still cannot rule out the contribution of NPC in this process. More convincing evidences need to be provided to differentiate these two RNA export pathways. For example, does knockdown of Torsin, the AAA-ATPase that controls the release of megaRNP granules into perinuclear space, show the same mitochondrial defects? On the other hand, what if we block NPC export by knocking down components of NPC export pathway? Without these data, it is hard to conclude whether either one or both of NPC and NE-budding pathways are involved.
Another question is: why would a transcript “choose” NE-budding over NPC as export method? What is the specialty of NE-budding compared to NPC export? Ultrastructural analysis showed that the average size of megaRNP granules is 200nm, well beyond the diameter limit of NPC, suggesting megaRNP granules may contain extra-large quantity of mRNAs that cannot be exported by an NPC simultaneously (Speese et al., 2012). It has been shown previously that defective NE-budding leads to postsynaptic depletion of transcripts using NE-budding for export and increased ghost bouton number (Jokhi et al., 2013; Speese et al., 2012). These RNP granules likely traffic along filaments composed of F-actin and nuclear associated Nesprin1 (dNesp1) from nucleus to postsynaptic region presumably for local translation (Packard et al., 2015). During larval development, body wall muscle volume increases by 100-fold within four to five days (Griffith and Budnik, 2006). To compensate for this muscle growth, the NMJ must expand rapidly, which requires a large local supply of specific mRNAs and/or proteins. NE-budding likely plays a crucial role in NMJ development by providing a bolus of RNAs in large amount to be targeted to the postsynapse in order to meet the need of rapid postsynaptic development. This also suggests that NE-budding may be associated with developmental stages requiring high protein demands. Consistently, NE-budding has been found critical for Drosophila oocyte development (unpublished data, V. Jokhi). Similarly, the indirect flight muscle (IFM) is the largest muscle in the adult fly; it develops from myoblast
differentiation along larval thoracic muscle segments as template during pupal stage. The high energy consumption of flight muscle requires large numbers of mitochondria, which impose high demands on proper mitochondrial replenishment and maintenance. NE-budding might be responsible for the bulk supply of mitochondrial components. The progressive mitochondrial degeneration observed in PS-modeled LamC mutant could be caused by the quick run-down of the mitochondrial components due to the lack of supply via NE-budding.

Finally, it remains unclear whether a certain transcript uses exclusively one of the two export ways or both of them for nuclear export. Are these two export pathway independent of each other or complimentary to each other? To fully answer these questions, one can isolate the megaRNP granule contents and perform RNAseq to identify the RNAs present at NE-budding sites, followed by bioinformatics study identifying specific sequence motifs that allow the transcript get exported through NE-budding. On the other hand, one can block NPC using chemicals (or RNAi) inhibiting Crm1 or Nxf1 to block RNA export through NPC and examine whether a known NPC-exported RNA would switch to NE-budding for export.

Wnt, mitochondria and aging
In Chapter 3, we demonstrated that the nuclear envelope (NE) budding pathway is involved in export of nuclear genome encoded mitochondrial transcripts that are important for mitochondrial dynamics. NE-budding is regulated by the Wg/Frizzled nuclear import (FNI) pathway. Interestingly, several previous studies have shown the functions of Wnt family proteins in regulating mitochondrial functions. Wnt5a ligand alters Drp1 phosphorylation status through non-canonical Wnt/Ca^{2+} signaling pathway, allows the translocation of Drp1 to mitochondria and promotes mitochondrial fission without causing deleterious effects. This enhanced fission further promotes mitochondrial motility allowing translocation of mitochondria to dendritic spines in rat hippocampal neuron (Godoy et al., 2014). An RNAi screening done in mouse C2C12 muscle cell lines identified several canonical Wnt signaling components as regulators of mitochondrial biogenesis. Wnt3a treatment of C2C12 activates mitochondrial biogenesis and oxidative phosphorylation, which can be inhibited by applying the Wnt signaling antagonist Dkk1 (Yoon et al., 2010). Moreover, Wnt3a exhibits a neuroprotective function through inhibition of the mitochondrial permeability transition pore opening induced by Amyloidβ, preventing the mitochondrial membrane potential dissipation and integrity loss (Arrazola et al., 2015). In Chapter 3, we also showed that overexpression of DFz2-DN in muscle, which blocks Wg/Frizzled nuclear import pathway, led to defective mitochondrial cristae
structure. Taken together, these studies suggest that Wnts are involved in several aspects of mitochondrial dynamics and functional regulation.

Compared to numerous works on functions of Wnts during development, fewer studies have been done to understand the post-developmental functions of Wnt signaling. Nevertheless, several studies have linked Wnt signaling to the aging process. Finkel group uncovered enhanced Wnt activity in Klotho mice, a model deficient for Klotho which displays typical premature aging phenotypes including skin atrophy, infertility and reduced life span (Kuro-o et al., 1997). They demonstrated that Klotho interacts with Wnt3a in vitro and it functions as a Wnt signal antagonist. Loss of Klotho leads to increased Wnt activity. Continuous Wnt exposure, although initially enhancing cell proliferation initially, results in marked decrease in cell proliferation over time, leading to stem cell depletion (Liu et al., 2007). In the same year, another study reported increased myogenic to fibrogenic fate conversion of muscle stem cell in aged mice, which is associated with enhanced canonical Wnt signaling pathway in aged muscle and myogenic progenitor cells. Inhibition of canonical Wnt signaling results in reduced fibrosis in aged muscle (Brack et al., 2007). Together with a later report showing increased Wnt signaling in multiple tissues from aged mice, these studies suggest a pro-aging role of Wnt signaling. However, other evidence supporting an opposing model was also reported. Several studies have demonstrated that Wnt signaling promotes neurogenesis in the adult hippocampus through
regulation of neural stem cells proliferation and self-renewal (Kalani et al., 2008; Lie et al., 2005; Miranda et al., 2012). Downregulation of Wnt family members in astrocytes from aged animals was reported (Miranda et al., 2012). A study performed in *C. elegans* showed that different Wnt molecules can have differential effects on the longevity of the worm (Lezzerini and Budovskaya, 2014). These data suggest that the roles of Wnt signaling in aging can be complicated depending on the specific signaling pathway, the tissue and the organism.

*Drosophila* Wg is expressed in a variety of cell types such as germline cells, intestinal stem cells and epithelial cells in adult flies (Kuwamura et al., 2012; Song and Xie, 2003). However, the functions of Wg in adult fly remain largely unexplored. The study in Chapter 3 suggests a potential post-developmental role of Wg signaling in the overall maintenance of wellness and the aging process.
Figure 4.1 Nuclear envelope budding in human fibroblasts (Ding et al., in preparation)
(A-B) Human fibroblasts from (A) control (P23) and (B) a HGPS patient (P24) labeled with a FISH oligo-d(T) probe, antibodies to LMNA, and Hoechst. Arrows indicate oligo-d(T) positive lamin foci. Calibration bar is 3µm.

(C-E) TEM of human fibroblast nuclei from a HGPS patient. Black arrows point to areas of lamina-thickening; white arrows point to megaRNP granules; arrowheads point to the neck of NE protrusions. Asterisks indicate nucleoplasmic islands. Calibration bar is 0.4µm.

(F) Quantification of number of LMNA/poly(A) RNA foci in human control and HGPS fibroblasts. N=(left to right): 99, 90. Error bars =SEM. **p<0.01

(G) Quantitative Real-time PCR of human Mfn1 and Mfn2 RNA levels in control and HGPS fibroblasts.
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