The Presynaptic F-box Protein FSN-1 Regulates Synapse Development via Retrograde Insulin Signaling in *Caenorhabditis elegans*

by

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A thesis submitted in conformity with the requirements for the degree of Master's of Science

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Abstract

Synaptogenesis entails the development and establishment of functional synapses, which form the fundamental unit of communication in the nervous system. Initially identified in Caenorhabditis elegans (C. elegans), the FSN-1, F-box protein family has emerged as evolutionarily conserved binding partners of PHR family proteins, which regulate synaptogenesis. Previously, we have shown that FSN-1 and RPM-1 form a SCF$^{FSN-1/RPM-1}$ ubiquitin ligase complex that negatively regulates synapse growth by downregulating presynaptic targets, like the MAP kinase pathway.

For my master’s thesis, I used a combination of both candidate and forward genetic approaches to identify additional components of signaling pathways that are regulated by FSN-1 during synaptogenesis. Our studies are among the first to suggest diverging roles for these partners and provide the first evidence for a mechanism through which the F-box protein regulates synaptogenesis via retrograde insulin/IGF/FOXO signaling and glucosaminidase/O-GlcNAc modifications.
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Figure 1-4: Dorsal cord GFP images and all figures generated by Christine Hwang. Figure 3: adapted and reproduced with permission from Po, M. D., Hwang, C., and Zhen, M. (2010).

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Figure 7: Strains were generated by Dr. Edward Liao, Dr. Wesley Hung, and Christine Hwang. Quantifications and puncta data analysis were done by Dr. Hung, with the help of Christine Hwang. Dorsal cord GFP images were taken by Christine Hwang. Figure was made by Christine Hwang.

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1. Background

1.1. The Synapse and Synaptogenesis

The study of the synapse and nervous system has come a long way since the heated debates between the two founding fathers of modern neuroscience, Camillo Golgi (1843–1926) and Santiago Ramón y Cajal (1851–1934). The “reticular theory” of nerve cell communication, encouraged by Camillo Golgi, maintained that each nerve cell was connected to its neighbours by protoplasmic links, forming a continuous nerve cell network or *reticulum*. With the work of Ramón y Cajal, however, the reticular view fell from favour as Cajal argued persuasively that nerve cells are discrete entities that communicate by means of specialized contacts. Today, we know these specialized contacts are neuronal “synapses”.

With the advent of modern neuroscience techniques, our understanding of synapse development has greatly broadened. The formation of synapses hinges upon the precise migration of axonal processes to their targets and the coordinated switch between axon outgrowth and synaptogenesis. Synaptogenesis is the process during which a stable synaptic connection between the pre- and postsynaptic cell is established. Furthermore, given that the synapse is the basic unit of communication in the nervous system, knowing how they function and go awry is the key to understanding many neurological disorders.

While numerous factors are required for synapse development, the mechanisms through which they regulate synapse development have only begun to be understood (reviewed in Jennings and Burden, 1993; Jin and Garner, 2008). Various secreted factors and adhesive molecules have been proposed to regulate synapse formation. For example, Agrin, a large proteoglycan secreted by the growing end of motor neuron axons, potentiates the aggregation of
ACh receptors at developing neuromuscular junctions (NMJs) through its receptor, the muscle specific kinase (MuSK) (DeChiara et al., 1996; Ling et al., 2004). The activation of MuSK, a receptor tyrosine kinase, then induces intracellular signaling required for the formation of the NMJ (Wang et al., 2006). In addition to MuSK, agrin binds to other proteins on the surface of muscle, such as laminin (Denzer et al., 1997) and dystroglycan (Gesemann et al., 1998; Scotton et al., 2006). These findings and more recent studies suggest that agrin plays an essential role as a synapse maintenance factor to stabilize the NMJ (Flanagan-Steet et al., 2005, Misgeld et al., 2005). In addition to agrin, membrane-associated adhesive molecules neuroligins and their binding partners the neurexins are other proteins implicated in synapse maturation and function in the CNS (reviewed in Dean and Dresbach, 2006). During the development of excitatory glutamatergic and inhibitory GABAergic synapses in the mammalian brain, neuroligins and neurexins function as cell adhesion molecules to bridge the synaptic cleft. Each partner can trigger the formation of a hemisynapse: neuroligins trigger presynaptic differentiation and neurexins trigger postsynaptic differentiation (reviewed in Craig and Kang, 2007). Similarly, cadherins, a superfamily of adhesion molecules, are also implicated in synaptogenesis by recruiting presynaptic vesicles and factors in postsynaptic densities (reviewed in Takeichi, 2007).

Several signaling pathways that play key roles in development and morphogenesis have been shown to also regulate the maturation of the synapse. For instance, Wnt signaling regulates the accumulation of synaptic proteins during synaptogenesis at the vertebrate CNS (reviewed in Salinas, 2005). Likewise, Wingless/Wnt signaling at the Drosophila NMJ is essential for the formation of both pre- and postsynaptic structures (reviewed in Marques, 2005). At C. elegans cholinergic NMJs, Wnt signaling appears to provide positional cues for synaptic connections by
restricting synaptogenesis (Klassen and Shen, 2007). In addition, the canonical TGF-β signaling pathway has also been reported to regulate synaptic plasticity and neuronal survival (reviewed in Packard et al., 2003). Furthermore, it should be noted that synaptogenesis not only involves the activation of synapse formation, but also factors that prevent improper synapse formation to ensure proper synaptic connectivity between the designated partners. Thus, the formation of the synapse is the outcome of a complex integration of many different signaling components and pathways.

1.2. The structure and function of chemical synapses

The chemical synapse is a specialized junction through which neurons communicate with each other and other non-neuronal cells such as muscles or glands. The neuromuscular junction is a specific type of chemical synapse consisting of the presynaptic terminal from a neuron and the postsynaptic terminal from a muscle fibre (Figure 1).

The presynaptic terminal is comprised of three distinct functional domains: the active zone, the synaptic vesicle pool, and the periactive region. Firstly, the active zone is the site of synaptic vesicle docking and neurotransmitter release (Couteaux and Pecot-Dechavassine, 1970). Proteins such as UNC-10/RIM (Rab3 interacting molecule) (Koushika et al., 2000; Graf et al., 2009), UNC-18/Munc18 (Gengyo-Ando et al., 1993), CASK (CaMK/SH3/guanylate kinase domain protein) (Fenster et al., 2000), and SYD-2/liprin-α (Zhen and Jin, 1999) are among the many proteins that localize to the active zone and are required for its proper function. Secondly, the synaptic vesicle pool is where vesicles are loaded with neurotransmitter and await docking, priming and release (reviewed in Rizzoli and Betz, 2005). Lastly, the periactive zone, a loosely and ‘molecularly’ defined region surrounding the active zone and synaptic vesicle
pool, contains factors that regulate vesicle recycling and development of synaptic structures (Sone et al., 2000).

Upon the depolarization of the presynaptic terminal, vesicles dock at the active zone and fuse with the plasma membrane. The fusion of synaptic vesicles is mediated by synaptic vesicle proteins including the SNARE protein synaptobrevin, VAMP, the calcium sensor synaptotagmin, and a myriad of other vesicle trafficking proteins such as synaptogyrin, rabphilin, and synaptophysin (reviewed in Fernandez-Chacon and Sudhof, 1999). After vesicle fusion, neurotransmitters are released into the synaptic cleft which bind and activate receptors clustered at the postsynaptic density (PSD) of the postsynaptic terminal. The postsynaptic terminal contains not only the receptors for neurotransmitters, but also other ion channels, scaffolding proteins, and signaling molecules that respond to the release of neurotransmitter from the presynaptic terminal (reviewed in Montgomery et al., 2004). These synapses permit the transmission of information from pre- to postsynaptic cell and are the basis of neural networks which are crucial to biological functions.

1.3. **C. elegans** as a model organism to investigate mechanisms for synaptogenesis

1.3.1. The **C. elegans** nervous system

*C. elegans* is a free-living, transparent, microscopic nematode that lives in temperate soil environments. *C. elegans* is an excellent genetic model system due to its small size, short generation time, and readily available forward and reverse genetic tools (Brenner, 1974; reviewed in Antoshechkin and Sternberg, 2007; reviewed in Dixon et al., 2009). Its simple anatomy and well-documented invariant nervous system consisting of 302 neurons makes *C.
elegans an ideal model for studying nervous system development and function (White et al., 1986).

1.3.2. C. elegans GABAergic neuromuscular junctions

We have chosen the C. elegans GABAergic NMJ as the model system to study synapse formation (Figure 2). The GABAergic nervous system of C. elegans consists of 26 neurons: the 13 VD, 6DD, 4 RME, the RIS, AVL, and DVB neurons (McIntire et al., 1993). The DD and VD motoneurons innervate the body wall muscles and are required for the sinusoidal locomotion of the animal (Shan et al., 2005). The RME neurons innervate muscles in the head, which are necessary for foraging behaviour (Huang et al., 2004). The AVL and DVB neurons send processes to the enteric muscles that contract during the process of defaecation (McIntire et al., 1993). The RIS is an interneuron which sensory neurons synapse onto in the synapse-rich CNS-equivalent nerve ring. The RIS, in turn, synapses onto secondary interneurons such as the command interneuron AVD, which regulates motor output (White et al., 1986).

While both inhibitory and excitatory GABAergic synapses are present at the C. elegans nervous system, it is the inhibitory GABAergic synapses that are essential for locomotion (McIntire et al., 1993). The DD and VD motoneurons innervate the dorsal and ventral body wall muscles, respectively. Filopodial-like extensions from the muscle body, called muscle arms, form synapses with the DD and VD motoneurons along the length of their axons. These synapses are referred to as en passant synapses and are morphologically similar to the inhibitory GABAergic synapses found in the vertebrate CNS. In C. elegans, the activation of the DD and VD motoneurons causes the release of GABA from the presynaptic termini, which results in muscle relaxation (McIntire et al., 1993; Gao and Zhen, manuscript in preparation). Together
with the excitatory input, the coordinated relaxation of body wall muscles is necessary for the

### 1.3.3. Visualization of GABAergic NMJs using fluorescent synaptic markers

The identification of specific synaptic vesicle proteins such as synaptobrevin, synaptotagmin, and rabphilin has greatly facilitated the study of synapse structure and function in the live organism. The visualization of presynaptic termini has been accomplished by the development of green fluorescent protein (GFP) markers tagged to these vesicle-associated proteins (Nonet, 1999). Specifically, the construction of a synaptobrevin::GFP (SNB-1::GFP) fusion protein expressed in live *C. elegans* by M. Nonet, created the first genetic marker for directly visualizing the presynaptic termini in a live animal (Nonet, 1999). Under the expression of a GABAergic motoneuron specific promoter, *unc-25*, SNB-1::GFP signals exhibit an array of distinct fluorescent puncta accumulated in the individual presynaptic termini of GABAergic NMJs (Jin *et al.*, 1999). This *P_{unc-25}SNB-1::GFP*, also known as *juIs1* marker, has been used to screen for and isolate mutants with defective presynaptic morphology, such as *rpm-1* (Zhen and Jin 1999; Zhen *et al.*, 2000). Furthermore, it is the marker we have used to investigate novel regulators of synapse formation such as *fsn-1* (Figure 2).

Using similar principles, transgenic animals that express other fluorescently tagged pre- or postsynaptic proteins have been developed to allow the direct visualization of GABAergic NMJs. Most relevant to my studies, *P_{unc-25-UNC-10::GFP (hpIs3)}* allows the visualization of
active zones (Yeh et al., 2005), while the GFP-tagged GABA receptor (UNC-49::GFP) allows the visualization of postsynaptic termini of GABAergic NMJs (Bamber et al., 1999).

1.4. PHR proteins regulate the transition between axon outgrowth and synaptogenesis

(Adapted with permission from Po, M.D., Hwang, C., and Zhen, M. (2010) )

In the past decade, the PHR (human Pbm, mouse Phr1, zebrafish Esrom, Drosophila Highwire and C. elegans RPM-1) family proteins have emerged as key regulators of axon guidance, outgrowth, regeneration and synapse development (Figure 3). Averaging about 4000 amino acids, PHRs are large proteins with multiple conserved motifs: an RCC1-like domain with inferred guanine exchange activity, two PHR protein-specific repeats, a Myc-binding region, a B-box zinc finger, and a RING-H2 zinc finger, a hallmark motif for E3 ubiquitin ligase activity. These multiple motifs suggest their involvement in signaling, scaffolding, as well as ubiquitination-mediated protein degradation (reviewed in Jin and Garner, 2008; reviewed in Po et al., 2010).

The first indication that PHR proteins were essential regulators of synaptogenesis came from parallel studies of RPM-1 in C. elegans (Zhen et al., 2000; Schaefer et al., 2000) and Highwire in Drosophila (Wan et al., 2000) in genetic screens for defective axon and synapse morphology. In rpm-1 mutants, some motor neurons branch and overgrow while others show altered synaptic organization (Zhen et al., 2000). In Hiw mutants, however, while the establishment of synapses appears normal, the number of boutons and length of branches increases tremendously (Wan et al., 2000). Although invertebrate PHR mutants do not exhibit
systemic defects in axon guidance and outgrowth, recent studies suggest that RPM-1 activity can modify the phenotype of axon guidance mutants, and vice versa. For example, rpm-1 mutations partially suppress the ventral-dorsal guidance defects in motoneurons when the guidance cue (Netrin/UNC-6) is reduced. Reducing UNC-5/UNC-5 or Robo/SAX-3 guidance receptors partially suppresses the overextension of mechanosensory axons in rpm-1 mutants. The localization and expression of guidance receptors are moderately affected in rpm-1 mutants; RPM-1 may modulate axon guidance and outgrowth, at least in part, through affecting guidance receptors (Li et al., 2008). RPM-1 activity also affects axon regeneration. After axotomy, the regeneration of motoneuron axons is enhanced in rpm-1 mutants, whereas an over-expression of RPM-1 severely inhibits axon regeneration (Hammarlund et al., 2009). Therefore, while RPM-1 activity is not essential for axon guidance and outgrowth, it affects the sensitivity or responsiveness of developing axons to guidance cues and ability of injured axons to reinitiate outgrowth.

Moreover, studies on the vertebrate PHRs, zebrafish protein Esrom, and murine Phr1, have also uncovered PHR protein roles in axon outgrowth in early neural development (D’Souza et al., 2005 and Burgess et al., 2004). In esrom mutants, retinal axons fail to project to the posterior tectum and rather defasciculate at the anterior tectum (D’Souza et al., 2005). In Phr1 knockout mice, axon outgrowth defects have been observed in which the phrenic nerve contains fewer axons and fails to completely innervate the diaphragm (Bloom et al., 2007). Other studies show that microtubule remodelling is critical for Phr1/Esrom-mediated axon navigation (Hendricks et al., 2008, 2009). Thus, PHRs likely regulate the switch between axon guidance and synaptogenesis by promoting cytoskeleton rearrangements in response to extra- and intracellular signaling in a temporally and spatially regulated manner (Figure 4).
Interestingly, PHR proteins have also been shown to regulate pathways unrelated to axon outgrowth and synaptogenesis such as p53/CEP-1 dependent apoptosis in *C. elegans* (Gao *et al.*, 2008), and p73 in vertebrates (Peschiaroli *et al.*, 2009). Thus, future studies will reveal more biological processes and the mechanisms under PHR regulation.

### 1.5. PHR proteins negatively regulate MAPK signaling

Several studies have revealed that PHRs regulate multiple biological processes through negatively regulating MAPK signaling via selective ubiquitin-mediated degradation of components of the signaling pathway (DiAntonio *et al.*, 2001; Liao *et al.*, 2004; Wu *et al.*, 2007; and Saiga *et al.*, 2009). The p38 MAPK pathway was first identified as a target of RPM-1 in *C. elegans* (Nakata *et al.*, 2005). The p38 MAPK cascade consists of the dual-leucine zipper kinase MAPKKK DLK-1, the MAPKK MKK-4, and the p38 MAP kinase PMK-3. The inactivation of these MAPK components partially suppresses *rpm-1* defects, and their over-expression results in synaptic morphology defects resembling those of *rpm-1* mutants (Nakata *et al.*, 2005).

Furthermore, RPM-1 may specifically target the activated form of DLK-1 for degradation (Abrams *et al.*, 2008). Similarly, in *Drosophila*, the DLK- family MAPKKK *wallenda* was identified as a target and a partial genetic suppressor of *Highwire* (Collins *et al.*, 2006). Murine *Phr1* also appears to downregulate DLK and p38 MAP kinase signaling in developing embryonic motoneuron cultures (Lewcock *et al.*, 2007).

Nonetheless, there are additional unidentified targets for RPM-1, HIW and *Phr1*. Loss of function mutants in *dlk-1* and *wallenda* do not fully suppress the defects in *rpm-1* or *hiw* mutants. In *Phr1* knockout mice, no gross change in DLK levels is detected, and the CNS axon defects are not suppressed in *Phr1;Dlk* double mutants (Bloom *et al.*, 2007; Saiga *et al.*, 2009). Similarly, axon targeting and outgrowth defects in zebrafish *esrom* mutants are not associated
with aberrant p38 signaling (Hendricks et al., 2009). Therefore, in addition to DLK, PHR proteins regulate other cell type- or stage-specific targets and signaling pathways during development. Further investigation of the molecular mechanisms through which these PHRs regulate axon growth and synapse development will elucidate other key signaling pathways involved.

1.6. FSN-1 family of F-box proteins are partners of PHRs

Whether they affect synapse development or axon navigation, the function of PHR proteins involves the formation of an evolutionarily conserved E3 ubiquitin ligase complex with a family of F-box proteins, namely FSN-1 in C. elegans, DFsn in Drosophila, and Fbxo45 in vertebrates (Liao et al., 2004; Wu et al., 2007; Saiga et al., 2009). fsn-1 was first cloned in our lab from a genetic enhancer screen for mutants with similar developmental and synapse morphology defects as the rpm-1 mutant (Liao et al., 2004). A complete loss-of-function of either FSN-1 or RPM-1 results in abnormal clustering of synapses as well as areas devoid of synapses called ‘gaps’. These defects closely resemble those of fsn-1;rpm-1 double loss of function mutants, suggesting that fsn-1 and rpm-1 function in the same genetic pathway (Figure 5).

FSN-1 family proteins consist of a F-box and a SPRY domain (Liao et al., 2004) (Figure 6). F-box proteins are target recognition modules for the SCF (Skp/Cullin/F-box) E3 ubiquitin ligase complexes (Tyers and Jorgensen, 2000; Ko et al., 2002). Expressed in the nervous system, FSN-1 forms an SCF-like complex with RPM-1, scaffolding proteins Skp1, and Cullin at presynaptic periactive zones (Liao et al., 2004). Furthermore, the physical and functional interactions between the FSN-1 family proteins and PHRs are evolutionarily conserved. The
Drosophila F-box protein DFsn is present in the same protein complex with HIW (Wu et al., 2007). Furthermore, DFsn mutants exhibit synaptic overgrowth and defective synaptic transmission at glutamatergic NMJs, phenocopying hiw mutants. Knockout mice for the vertebrate homolog, Fbxo45, exhibit phenotypes reminiscent of the Phr1/Magellan mice, including the respiratory failure at birth and a reduction of axon tracks in the CNS (Burgess et al., 2004; Lewcock et al., 2007; Bloom et al., 2007; Saiga et al., 2009). Phr1 and Fbxo45 also form an E3 ligase complex that includes Skp1. However, instead of interacting through Cullin, Phr1 and Fbxo45 interact directly through their Myc-binding and SPRY domains (Saiga et al., 2009), respectively, diverging further from the classic SCF E3 ligase complexes.

Previously, we proposed a model whereby this SCF$^{RPM-1/FSN-1}$-like E3 ubiquitin ligase complex functions as a negative regulator of synapse formation by targeting specific components of signaling pathways for degradation (Liao et al., 2004). The identification of additional effectors or targets whose activities contribute to fsn-1 defects will facilitate our understanding of how FSN-1 restricts or regulates the maturation of synapses. Furthermore, by investigating the roles of these effectors or targets of fsn-1 in synapse formation, we will develop a greater understanding of the fundamental mechanisms for synaptogenesis.
**Figure 1: Structure of the chemical synapse.**

Key components of presynaptic and postsynaptic specializations of the synapse. The presynaptic terminal consists of three functionally distinct regions: the active zone, synaptic vesicle pool, and the periactive zone. The active zone is the region where vesicles, loaded with neurotransmitter, dock and fuse with the plasma membrane, releasing neurotransmitters into the synaptic cleft. The synaptic vesicle pool is where vesicles are loaded with neurotransmitter and await release. The periactive zone is the region where factors or genes that regulate synapse formation and development are located. The postsynaptic terminal opposes the presynaptic terminal and contains receptors that initiate and transduce intracellular signals upon binding to neurotransmitters.
Figure 2: Using the SNB-1::GFP marker to visualize presynaptic varicosities.

(A) The en passant GABAergic neuromuscular junctions of C. elegans are formed between projections of the body wall muscles (called muscles arms) and the axons of GABAergic motoneurons. (B) These individual presynaptic varicosities can be visualized in the live animal using the SNB-1::GFP marker expressed by a GABAergic neuron-specific promoter ($P_{unc-25}$).
Figure 3: PHR family proteins play important roles in the development of the nervous system.

(A) Conserved domains in the PHR family protein Pam, Phr1, Esrom, HIW and RPM-1. (B) PHRs interact with and modulate multiple signaling pathways to regulate axon growth and synaptogenesis. The RCC1 domain binds GLO-4 (RPM-1), KCC2 and adenylyl cyclases (Phr1/PAM), which either promotes or inhibits their activities. The RING-H2 finger motif binds TSC2, E2 conjugating enzymes (Phr1) and Medea (HIW). PHRs regulate mTOR (Phr1/PAM/Esrom), BMP (HIW) and Netrin/Slit (RPM-1) signaling. PHRs interact with FSN-1-family F-box proteins to down-regulate DLK level and activity (Phr1/HIW/RPM-1). PHR proteins also associate with microtubule- and F-actin-enriched cytoskeletal complexes (Phr1/PAM) and affect their dynamics (Phr1/Esrom). (C) Non-neural roles of PHR proteins: Esrom is required for pteridine synthesis. RPM-1 and FSN-1 are required to down-regulate phosphorylated CEP-1/p53, whereas p73 can directly bind and be ubiquitinated by Fboxo45. Black lines implicate genetic/functional interactions; grey lines indirect direct or indirect biochemical interactions. (Adapted with permission from Po, Hwang and Zhen, 2010).
Figure 4: PHR proteins regulate the switch between axon outgrowth and synaptogenesis. A model - PHR proteins integrate cellular signaling and cytoskeletal remodeling to mediate transitions between axon growth, termination and synapse formation. (A) PHRs initiate cytoskeletal remodeling in response to the intracellular signaling changes induced by extrinsic guidance cues. (B) PHRs directly (via RCC1 and other domains) and indirectly (via E3 ligase activity) regulate intracellular signaling cascades which alter microtubule-based vesicular transport, endosomal trafficking/lysosomal biogenesis, and local translation during axon growth or synapse formation. (C) Once the axon has reached its target, synaptogenesis is controlled by PHRs (through their ligase activity), which restrict the MAPK pathway to regulate synapse formation. (D) A schematic of how a wildtype axon switches from axon outgrowth (in response to guidance cues ▲) then finds its target and establishes a synapse (synaptogenesis cues ●). (E) PHR mutants show defects in axon outgrowth and synaptogenesis. In C. elegans rpm-1 mutants, mechanosensory neurons overshoot their targets, and often hook backwards to find their targets. Drosophila hiw mutants exhibit excessive synapse growth and branching at glutamatergic NMJs. The axons of Esrom and Phr-1 mutant animals stop short of their targets and often extend past them resulting in excessive branching.
Figure 5: The F-box protein, FSN-1, functions in the same genetic pathway as the PHR protein, RPM-1.

In *fsn-1* or *rpm-1* complete loss of function mutants, SNB-1::GFP (*juls1*) puncta are unevenly spaced with large gaps and huge clusters of puncta (denoted by arrows and asterisks, respectively). In the *fsn-1; rpm-1* double loss of function animal, the severity of such defects are the same as either single mutant alone. FSN-1 and RPM-1 function in the same genetic pathway (Figure adapted and reproduced with permission from Liao *et al.*, 2004).
Figure 6: FSN-1 encodes a conserved F-box SyNaptic protein that regulates synapse formation.

The FSN-1 family of F-box proteins consists of a F-box and a SPRY domain, which is conserved in *C. elegans, Drosophila, mouse*, and human. In wildtype *C. elegans*, we observe discretely round and evenly spaced presynaptic puncta in GABAergic neurons. *fsn-1* loss of function animals display abnormal presynaptic morphology similar to those of *rpm-1* mutant animals. These animals display large clusters of synapses as well as huge gaps or areas devoid of synapses. (Figure adapted with permission from Liao *et al.* 2004.)
2 FSN-1 regulates synapse formation through a retrograde insulin signaling mechanism

2.1 Abstract

The development of the functional synapse involves the interaction of many proteins and is an intricate process that hinges upon the proper communication between pre- and postsynaptic structures. The molecules required for synaptic formation are numerous, and elucidating the pathways they regulate is crucial to understanding their roles in synapse development. The F-box protein conserved in *C. elegans* (FSN-1), *Drosophila* (DFsn), and mammals (Fbxo45), plays an important role in the development of the nervous system. In *C. elegans* fsn-1 loss-of-function (lf) mutant animals, the absence of FSN-1 in the neurons results in abnormally spaced and clustered GABAergic neuromuscular junctions. Previously, we have shown that FSN-1 functions through its interaction with a known regulator of presynaptic morphology, RPM-1. FSN-1 and RPM-1 form a SCF-like E3 ubiquitin ligase complex which affects presynaptic development via selective ubiquitin-mediated protein degradation. Subsequently identified in mouse, *Drosophila*, and Human, this RING-finger and F-box protein complex have become known as evolutionarily conserved binding partners.

Here, we report that FSN-1 regulates synapse development, at least partially, through affecting a retrograde insulin/IGF pathway signaling in the postsynaptic cells. FSN-1 affects the processing of INS-18, an antagonistic insulin-like ligand that negatively regulates the activation of an insulin/IGF receptor (DAF-2) and the downstream signaling cascade in the postsynaptic muscle cells to modulate synapse development. Intriguingly, altered insulin/IGF signaling
activity does not affect the synaptic morphology of rpm-1 mutants, suggesting a potentially RPM-1-independent function of FSN-1. Although the DAF-2/insulin pathway has been shown to play roles in aging, stress response, and metabolism, whether insulin signaling is involved in regulating synapse development has not been fully explored and is poorly understood. Our studies provide the first evidence for and mechanism through which the F-box protein regulates synaptogenesis via retrograde insulin/IGF signaling.

2.2 Introduction

The chemical synapse is a specialized junction through which neurons communicate with each other and other postsynaptic targets. These synapses relay information from one neuron to the next allowing organisms to react and respond to their external environments. The proper development of chemical synapses hinges upon the interaction of various intra- and intercellular signaling molecules between presynaptic and postsynaptic cells.

Signals from the developing presynaptic termini to postsynaptic termini (anterograde signaling), and vice versa (retrograde signaling) are important for proper formation and maintenance of synaptic connections. In anterograde signaling, for example, morphogens like Wingless in Drosophila are secreted at presynaptic boutons of glutamatergic NMJs to stimulate the development of pre- and postsynaptic structures (Packard et al., 2002). At the developing mouse neuromuscular junction, the nerve-derived proteoglycan agrin binds to its MuSK receptor and increases postsynaptic expression of rapsyn, a cytoplasmic membrane-associated protein (Brockhausen et al., 2008). Agrin-induced rapsyn activity triggers ACh receptor (AChR)
aggregation and stabilizes these clusters by inhibiting the activity of calpain, a calcium
dependent protease that disperses AChRs (Chen et al., 2007; Brockhausen et al., 2008).

Various retrograde signals have also been shown to regulate synapse development in
mouse, Drosophila, and C. elegans. In the mouse cerebellum, Wnt-7a secreted by the
postsynaptic granule cells and purkinje cells prevents the growth of the presynaptic mossy fibre,
while promoting the accumulation of synaptic proteins and the maturation of synapses (reviewed
in Salinas, 2005). In Drosophila NMJs, the homophilic cell adhesion molecule Fasciclin II
(FasII) as well as the scaffolding protein Discs large (DLG) are crucial mediators of retrograde
signaling initiated by the postsynaptic activation of calcium/calmodulin-dependent protein
kinase II (CaMKII) (Kazama et al., 2007). Furthermore, in C. elegans, reduced activity of the
postsynaptic pro-hormone convertase aex-1 has been shown to alter the localization of the
presynaptic vesicle protein UNC-13 (Doi and Iwasaki, 2002). More recent studies show that
AEX-1 may interact with the syntaxin, SYN-1, to regulate vesicle exocytosis for retrograde
signaling from non-neuronal cells (Yamashita et al., 2009). Thus, cell adhesion molecules as
well as postsynaptically secreted proteins can contribute to the intricately regulated development
of presynaptic components.

During synaptogenesis, tightly controlled activation as well as inhibition of synaptic
components is crucial to the establishment of functional synapses. Various studies have
identified E3 ubiquitin ligases as critical regulators of synapse formation. The PHR family of
proteins (human Pam, mouse Phr1, zebrafish Esrom, Drosophila Highwire and C. elegans RPM-
1), large proteins with multiple motifs, have been identified as key regulators of synaptogenesis
as well as axon guidance, outgrowth, and regeneration (Guo et al., 1998; Schaefer et al., 2000;
Zhen et al., 2000; Wan et al., 2000; Burgess et al., 2004; D’Souza et al., 2005; reviewed in Po et
One of the motifs, the RING-H2 zinc finger, is a hallmark of E3 ubiquitin ligases, and many vertebrate and invertebrate PHR mutations affect the RING-H2 domain. hiw mutants exhibit genetic interactions with fat facets, a gene encoding a deubiquitinating enzyme in a dosage-dependent manner, providing the first evidence that HIW functions through ubiquitin-mediated proteasome degradation (DiAntonio et al., 2001). The identification of FSN-1 family F-box proteins as the functional partners for PHRs provides further evidence for their E3 ligase activity (Liao et al., 2004; Wu et al., 2007; Saiga et al., 2009). F-box proteins are target recognition modules for SCF (Skp/Cullin/F-box) E3 ubiquitin ligase complexes (Reviewed in Deshaies, 1999; Tyers and Jorgensen, 2000).

Previously, we identified the F-box protein, FSN-1, as a binding partner of the C. elegans PHR protein RPM-1 (Liao et al., 2004). Our in vivo studies showed that FSN-1 and RPM-1 form an SCF-like (Skp1, Cullin, F-box) E3 ubiquitin ligase complex that negatively regulates synapse promoting factors via an ubiquitin mediated process. In support of this model, the p38 MAPK signaling pathway, consisting of the MAPKKK DLK-1, MAPKK MKK-4 and MAPK PMK-3, was identified as a target of RPM-1 (Nakata et al., 2005). Similarly, in Drosophila, the MAPKKK wallenda was identified as a downstream target and a genetic suppressor of Highwire (Collins et al., 2006). Here we report that mutations in the p38 MAPK also suppress fsn-1 synaptic defects, supporting the notion that FSN-1 and RPM-1 function in the same genetic pathway.

In this study, we used a combination of both candidate and forward genetic approaches to identify components of signaling pathways that are regulated by FSN-1 during synaptogenesis. We report that mutations in the p38 MAPK also suppress fsn-1 synaptic defects, supporting the notion that FSN-1 and RPM-1 function in the same genetic pathway. In
addition, we report the identification of the DAF-2/insulin/IGF pathway as a regulator of synapse formation and negatively regulated target of FSN-1.

The *C. elegans* genome encodes 40 predicted insulin or IGF-1-like secreted peptides, but only a single insulin/IGF-like receptor tyrosine kinase, DAF-2. The canonical insulin signaling pathway in *C. elegans* involves the insulin/IGF receptor DAF-2, the PI-3 kinase AGE-1, the 3-phosphoinositide dependent kinase PDK-1, the Akt family of serine/threonine kinases AKT-1 and AKT-2, and the Forkhead transcription factor DAF-16 (Figure 7A). Activation of the pathway results in the inhibition of DAF-16, preventing its translocation into the nucleus to act as a transcriptional activator or represser of targets in various developmental processes, such as metabolism, lifespan, and stress response (Murphy *et al.*, 2006).

It is well known that insulin/IGF signaling is crucial for metabolism and development in both vertebrates and invertebrates (reviewed in Broughton and Partridge, 2009). More recent studies have revealed the importance of insulin/IGF signaling and its possible implications in neurodegenerative disorders such as Alzheimer’s, Rett’s Syndrome, and even Diabetes Mellitus (Liao and Xu, 2009; Tropea *et al.*, 2009; Pavlović and Pavlović, 2008). In the vertebrate nervous system, insulin signaling has also been implicated in synaptic plasticity. For instance, increased insulin signaling facilitates long-term potentiation (LTP) in the hippocampus, and also reverses the impairment of LTP due to beta-amyloids, a peptide that aggregates in the brains of Alzheimer’s patients (Gault and Hölscher, 2008). Moreover, reduced expression of insulin and insulin/IGF receptors in the frontal cortex are associated with Parkinson’s disease and dementia, suggesting that impairment of insulin/IGF signaling may also contribute to the pathologies of these patients (Tong *et al.*, 2009). Although these studies provide hope in treating neurodegenerative disorders through insulin administration, the lack of understanding of how insulin and the insulin receptor functions in the nervous system necessitates further dissection of
the role of insulin signaling. Furthermore, due to the lack of in vivo models and difficulty in defining the role of insulin/IGFs, there is hardly any direct evidence for insulin/IGF-mediated signaling in synapse development.

Here, we show that fsn-1 modulates the activity of ins-18, an insulin antagonist secreted from neurons that inhibits the activity of the postsynaptic DAF-2 receptor. This results in the subsequent hyperactivation of the daf-2/insulin pathway and inhibition of its downstream effector, the FOXO transcription factor DAF-16. We propose a model whereby, the inhibition of DAF-16 promotes retrograde signaling through synapse promoting/inhibiting factors that activate the presynaptic MAPK pathway, and thus, synapse growth. The activation of the MAPK pathway is also subject to the negative regulation by FSN-1 in the presynaptic terminal. Thus, FSN-1’s role in synaptogenesis is two-fold: it is not only a key negative regulator of synapse growth through its ligase activity, but also an activator of synapse growth via modulating insulin/IGF signaling.

2.3 Results

2.3.1. FSN-1 regulates pre- and postsynaptic marker morphology

FSN-1 was previously identified as a conserved presynaptic F-box protein that functions as a component of the SCF (Skp, Cullin, F-box) ubiquitin ligase complex which regulates presynaptic development in C. elegans (Liao et al., 2004). Using the presynaptic and postsynaptic markers, juls1 (SNB-1::GFP) and oxis22 (GABAR::GFP) respectively, we further investigated the synapse morphology of fsn-1(hp1) mutant and wildtype animals. In wildtype animals, there are evenly spaced and discretely round puncta across the length of the dorsal
nerve cord. In \textit{fsn-1} complete loss-of-function animals, however, there is abnormal clustering of puncta as well as gaps devoid of synapses due to the over- and underdifferentiation of pre- and postsynaptic structures.

In order to quantify these observations, we established methods to evaluate these phenotypes using two parameters that reflect the ‘bi-polar’ synapses of \textit{fsn-1} mutants. First, the total number of dorsal cord GFP puncta were counted and compared. In wildtype adult animals, there was on average 129.8 ± 5.0 SNB-1::GFP, and 116.7 ± 9.9 GABA\textsubscript{R}::GFP puncta per animal. In \textit{fsn-1} mutants, both the SNB-1::GFP and GABA\textsubscript{R}::GFP punctum numbers were significantly reduced due to regions devoid of synapses (to 78.3 ± 6.4 and 88.5 ± 7.7 puncta per animal, respectively, P<0.001 Figure 7D, F).

Secondly, using a program Punctanalyzer (developed by Dr. Taizo Kawano in our lab), we compared the distribution of the punctum widths between wildtype and \textit{fsn-1} animals. The density plot of the \textit{juIs1} punctum width in \textit{fsn-1} mutants showed a significantly broader distribution when compared to that of wildtype animals (P<0.001, Figure 7E). Qualitatively, the \textit{oxIs22} puncta also exhibited more uneven sizes (large and small) when compared to wildtype animals (Figure 7C). Thus, the loss of function of the \textit{fsn-1} gene results in similar defects in both pre- and postsynaptic punctum morphology and number indicating both over- and under-differentiation of GABAergic NMJs.

These structural defects in \textit{fsn-1} mutants have been confirmed by ultrastructural analyses. Like the panneuronal expression pattern of FSN-1, similar structural defects were also observed at cholinergic NMJs (by Edward Liao and Wesley Hung, data not shown). Thus, the defects we observed in \textit{fsn-1} loss of function mutants suggest that \textit{fsn-1} is an essential regulator for both pre- and postsynaptic development.
2.3.2. Reduced insulin/IGF signaling rescues the synaptic morphology defects of *fsn-1* animals

*fsn-1* is a key regulator of both pre- and postsynaptic development. The absence of FSN-1, a functional E3 ubiquitin ligase, should lead to an increased or prolonged activation of its protein targets, subsequently affecting the signaling pathways that they participate in. We hypothesize that the synaptic defects in *fsn-1* mutants may be in part due to the hyperactivation of specific signaling pathways. On that account, a simultaneous decrease of the activation of such signaling pathways in *fsn-1* mutants should result in at least a partial rescue of the *fsn-1* synaptic morphological defects.

Following this rationale, we screened among receptor tyrosine kinase mutants for *fsn-1* suppressors. The loss of function mutant for DAF-2, the sole insulin/IGF receptor in *C. elegans* strongly suppressed the synaptic morphology defects of *fsn-1* mutants (Hung et al., 2010, in preparation). As DAF-2 is required for viability, a partial reduction of the DAF-2 function allele, *daf-2(e1370ts)* was used for our studies. *daf-2(e1370ts)fsn-1* double loss of function mutants showed a significant increase in the number of dorsal cord, presynaptic SNB-1::GFP puncta when compared to *fsn-1* mutant animals alone (110.1 ± 5.3 versus 78.3 ± 6.4 punctum/animal, P<0.001; Figure 7B). In addition to the number of puncta, the synapse morphology of *daf-2 fsn-1* animals also showed a marked improvement when compared to *fsn-1* animals, as *daf-2 fsn-1* animals displayed more discrete and round GFP puncta (Figure 7D). A concomitant suppression of the postsynaptic marker number and morphology was also observed in *daf-2 fsn-1* animals (Figure 7C,F). Finally, we also confirmed the partial rescue of synapse morphology defects in *daf-2 fsn-1* animals by electron microscopy (EM) and serial reconstruction (Hung et al., 2010, in preparation, data not shown).
We further tested whether mutations in other downstream components of the insulin/IGF/DAF-2 signaling pathway also modify the synaptic defects of fsn-1 mutants. To test this, we analyzed the phenotype of age-1; fsn-1 and pdk-1;fsn-1 double mutants.

Insulin/IGF/DAF-2 signaling is activated upon the binding of an agonist to the receptor DAF-2. This binding activates the PI-3 kinase (AGE-1), which increases production of phosphatidyl inositol (PIP3) leading to the activation of phosphatidyl inositol dependent kinase, PDK-1. We observed that the loss of function mutations in both age-1 and pdk-1 partially rescued the fsn-1 synapse morphology defects, as well as the number of presynaptic SNB-1::GFP punctum (Figure 8A). Namely, age-1;fsn-1 and fsn-1;pdk-1 double loss of function mutants displayed 99.4 ± 5.3 and 105.9 ± 5.9 number of SNB-1::GFP puncta, respectively, when compared to fsn-1 mutant animals alone (78.3 ± 6.4) (P<0.001; Figure 8B). Using Punctanalyzer, we also confirmed that both age-1 and pdk-1 partially rescue the broad distribution of puncta widths seen in fsn-1 single mutant animals (P<0.001) (Figure 8C). Thus, this strongly suggests that an increased activation of the daf-2/insulin/IGF signaling pathway contributes to the synaptic morphology and development in fsn-1 mutants.

2.3.3. The FOXO/DAF-16 transcription factor is required for insulin/IGF signaling-mediated synapse development

A major effector of the InR/DAF-2, PI-3 kinase/AGE-1 and the phosphatidyl inositol-dependent kinase/PDK-1 signaling cascade is the FOXO family transcription factor, DAF-16. Insulin/IGF signaling ultimately phosphorylates DAF-16, which results in its cytoplasmic retention. Thus, Insulin/IGF/DAF-2 signaling negatively regulates FOXO/DAF-16 activity by
preventing it from translocating into the nucleus and act as a transcriptional activator or repressor (Lin et al., 2001; Henderson and Johnson, 2001) (Figure 7A).

In order to test whether daf-2-mediated suppression of fsn-1 hinges upon the signaling cascade through the FOXO/DAF-16 transcription factor, we compared the phenotypes between daf-16; daf-2 fsn-1 triple mutant and fsn-1, and daf-2 fsn-1 animals. Interestingly, daf-16; daf-2 fsn-1 triple mutants exhibited phenotypes that resembled fsn-1 mutant animals. While the number of presynaptic SNB-1::GFP puncta in daf-2 fsn-1 was rescued to 110.1 ± 5.3, in daf-16; daf-2 fsn-1 triple mutants the number SNB-1::GFP puncta decreased to 74.1 ± 5.1, similar to the number of puncta in non-rescued fsn-1 mutants (P<0.001) (Figure 7B, D). Moreover, the distribution of SNB-1::GFP punctum width using Punctanalyser in daf-16;daf-2fsn-1 became broadened, as in fsn-1 mutants. However, daf-16 mutants did not exhibit synapse defects on their own, or enhance the phenotype of fsn-1 null mutants (Figure 10A). Interestingly, although we did not detect a quantitative difference between daf-16;fsn-1 and fsn-1 mutants, qualitatively, however, fsn-1;daf-16 mutants consistently exhibit less variability of synaptic phenotype expression in response to environmental change, such as temperature (see Discussion later). These findings suggest that daf-2-mediated suppression of fsn-1 presynaptic morphology defects completely depends on presence of DAF-16.

We observed similar genetic interactions with the postsynaptic morphology. With a postsynaptic marker oxIs22, which expresses the GABA\textsubscript{R}::GFP in the body wall muscles, we compared the postsynaptic morphology of GABAergic NMJs in fsn-1, daf-2 fsn-1, and daf-16; daf-2 fsn-1 mutants. As described above, fsn-1 mutants exhibit clusters of oxIs22 puncta as well as more diffuse and dimmer puncta (Figure 7C). These defects were partially rescued in fsn-1 daf-2 double mutants. The GABA\textsubscript{R}::GFP punctum number was increased by loss of function of
Furthermore, the gaps and clustering of the oxIs22 puncta was also partially rescued in daf-2 fsn-1 mutants. However, in daf-16;daf-2 fsn-1 animals, the number of the GABA<sub>R</sub>::GFP punctum number (96.1 ± 5.9 per animal) was reduced to a similar level as those seen in fsn-1 mutants. daf-16; daf-2 fsn-1 animals also resembled fsn-1 mutants in postsynaptic morphology, with gaps and clustering of oxIs22 puncta.

Taken together, the DAF-16 transcription factor is thus the key downstream effector for insulin/IGF signaling to regulate synapse formation.

2.3.4. The DAF-2/FOXO/DAF-16 signaling cascade is required in postsynaptic cells to influence NMJ development

Most components of the DAF-2/DAF-16 signaling cascade are widely expressed in virtually all cell types in C. elegans. To determine whether the DAF-2/DAF-16 signaling cascade modulates fsn-1 synaptic defects from the pre- or postsynaptic cells, we expressed DAF-2 or DAF-16 using a pan-neural (P<sub>F25B3.3</sub>), or a muscle specific promoter (P<sub>myo-3</sub>) in daf-2fsn-1 or daf-16; daf-2 fsn-1 mutants, respectively, and examined their effect on the presynaptic SNB-1::GFP marker.

The neuronal expression of functional DAF-2 in fsn-1daf-2 mutants did not affect the rescuing effect of fsn-1 defects by daf-2 (Figure 9A, B). In contrast, the expression of DAF-2 in the body wall muscles resulted in a reversion of the daf-2 fsn-1 synaptic phenotype to that of fsn-1 mutant phenotypes (Figure 9A, B). The co-expression of DAF-2 from both neuron and muscle promoters resulted in a similar level of reversion of the daf-2 fsn-1 synaptic phenotype as when DAF-2 was expressed in muscles alone. Likewise, the panneuronal expression of DAF-16 in
*daf-16; daf-2 fsn-1* mutant animals showed no change in synapse morphology or total synapse number (Figure 9A, C). However, transgenic arrays expressing DAF-16 specifically in body wall muscles significantly improved synapse morphology and punctum number in *daf-16; daf-2 fsn-1* mutants, similar to that of *daf-2 fsn-1*. Thus, at the GABAergic NMJ, the DAF-2/DAF-16 insulin signaling cascade functions through the postsynaptic muscles to regulate synapse development.

### 2.3.5. Reducing insulin/IGF signaling modulates the synaptic defects of *fsn-1* but not *rpm-1* mutants

As previously shown, FSN-1 functions with its binding partner, RPM-1, to form an SCF E3 ubiquitin ligase complex (Liao *et al.*, 2004). We therefore addressed whether altering the activity of the insulin/IGF signaling also modifies the phenotype of *rpm-1* mutants. Intriguingly, while *daf-2* loss of function mutations robustly rescued the synaptic defects of *fsn-1* mutants, the synaptic defects of *rpm-1* mutants were unaffected by *daf-2* (Figure 10). Specifically, *daf-2; rpm-1* animals display large clusters of puncta and huge gaps devoid of puncta, identical to *rpm-1* single mutant animals. The total number of dorsal SNB-1::GFP puncta was also similar between *rpm-1* animals (60.3 ± 4.7/animal) and *daf-2; rpm-1* (57.5 ± 4.9/animal) (Figure 10C, E). Therefore, DAF-2/Insulin/IGF signaling through FOXO/DAF-16 may be interacting specifically with FSN-1 in regulating synapse formation. Alternatively, simply reducing insulin signaling may be insufficient to rescue RPM-1 defects since RPM-1 may be modulating the outcome of multiple signaling pathways.
2.3.6. DAF-16 is not required for MAPK-mediated suppression of synaptic defects of *fsn-1* and *rpm-1* mutants

It is well known that downstream components of the p38 MAPK kinase pathway (DLK-1, MKK-4, PMK-3) can suppress the synaptic defects of *rpm-1* mutants (Nakata *et al.*, 2005). Consistent with FSN-1 and RPM-1 functioning together to regulate the activity of MAPK signaling, mutations in these MAPK components also suppress *fsn-1* synaptic defects (Figure 10 B, D). We further investigated whether the MAPK and FOXO/DAF-16 signaling pathways modulate synapse development cooperatively, or independently of each other. In order to test this, we took advantage of the fact that *daf-2*-mediated suppression of *fsn-1* is completely dependent on the presence of DAF-16. We thus generated and compared the SNB-1::GFP phenotype of the following strains: *daf-16;rpm-1* versus *daf-16;rpm-1;mkk-4* and *daf-16;fsn-1* versus *daf-16;fsn-1;mkk-4*.

We found that, while loss of function of MKK-4 suppressed the synaptic defects of both *fsn-1* and *rpm-1* mutants, the loss of function in DAF-16 did not abolish *mkk-4* suppression of *fsn-1* or *rpm-1* synaptic defects. Thus, not only does the DAF-2/FOXO pathway appear to specifically affect *fsn-1*, the suppression of *fsn-1* by the MAPK pathway is not dependent on DAF-16 signaling (Figure 11). This may indicate that DAF-2/insulin signaling functions either independently, or genetically upstream, of the MAPK signaling pathway. These findings, along with the fact that the insulin pathway functions postsynaptically, suggests the presence of a retrograde insulin signaling mechanism through which FSN-1, alone, regulates synapse development. The retrograde signal likely feeds into the presynaptic MAPK signaling cascade that is also subjected to the regulation of RPM-1/FSN-1 during synapse development.
2.3.7. Identifying ligands/activators for the insulin/IGF receptor DAF-2 during synapse formation

Since FSN-1 and the DAF-2/DAF-16 signaling cascade are functionally required in pre- and postsynaptic cells of NMJs, respectively, FSN-1 must be regulating DAF-2 activity indirectly. In order to determine the mechanism through which FSN-1 regulates DAF-2/DAF-16 signaling, we tested the possibility that FSN-1 may be modulating the activity of the DAF-2/DAF-16 signaling cascade by affecting neuronally-expressed insulin/IGF ligands for DAF-2. We expected that the loss of function of an agonistic ligand that normally activates DAF-2 should exert a similar suppression effect on the synaptic defects of \( \text{fsn-1} \), as in \( \text{daf-2 fsn-1} \) loss of function animals. On the contrary, the over-expression of an antagonistic ligand for DAF-2 should mimic the effect of \( \text{daf-2} \) mutants.

We first tested the possibility that FSN-1 may be downregulating an unknown insulin agonist by generating double mutants between \( \text{fsn-1} \) and all available mutant alleles to date (called \( \text{ins} \)), and analyzed their synapse morphology with the presynaptic SNB-1::GFP marker. These include the loss of function deletion mutants for 29 out of the total 40 predicted insulin/IGF-like genes as well as \( \text{daf-28(tm2308)} \) and \( \text{daf-28(sa191)} \) (Figure 12A). Although none of the \( \text{fsn-1};\text{ins} \) double mutants showed rescue of \( \text{fsn-1} \) synapse defects, the gain-of-function allele of \( \text{daf-28(sa191)} \) rescued \( \text{fsn-1} \) synaptic defects. Interestingly, this \( \text{daf-28} \) allele, which behaves genetically as a dominant negative allele, disrupts a probable proteolytic processing site required for insulin maturation, and thus, specifically blocks the processing of all insulin/IGF-like ligands (Li et al., 2003). This suggests that multiple functionally-redundant insulin/IGF-like ligands likely activate DAF-2 to regulate synapse formation. It also indicates that FSN-1 is likely involved in the regulation of insulin/IGF processing.
2.3.8. **INS-18 is an antagonistic ligand for the insulin/IGF DAF-2 receptor during synapse formation**

We further tested for potential antagonistic insulin ligands by overexpressing all of the known insulin/IGF-like ligands (INS-1 to INS-40) in *fsn-1* mutants. As stated above, the overexpression of an agonistic ligand for DAF-2 should mimic the effect of *daf-2* loss of function mutants, therefore suppressing the synaptic defects in *fsn-1* mutants.

Fosmids or PCR-amplified fragments that harbor each of the 40 predicted insulin/IGF genes were injected into *fsn-1* mutant animals and analyzed for rescue of *fsn-1* synaptic defects. Among all 40 putative insulin/IGF-like ligands, only the overexpression of INS-18 robustly and consistently rescued the synaptic defects of *fsn-1* mutants (Figure 12B). In addition, the reporter construct with the INS-18 promoter showed exclusive and broad expression of the GFP reporter in the nervous system, including motoneurons, which is consistent with our hypothesis that insulin/IGF ligands are secreted from the presynaptic termini to modulate the activity of the DAF-2/DAF-16 signaling cascade in postsynaptic cells (data not shown). To further confirm that INS-18 is functionally required in neurons, we expressed INS-18 using either a panneural (*P_{F25B3.3}*) or muscle (*P_{myo-3}*) promoter in *fsn-1* mutants. Indeed, panneurally expressed INS-18, but not muscle-driven INS-18 expression, rescued both the morphology, as well as the number of GABAergic synapses in *fsn-1* mutants (Figure 13). Taken together, these results suggest that INS-18 is an antagonistic ligand that negatively regulates DAF-2/DAF-16 signaling. It further suggests that FSN-1 likely regulates DAF-2/DAF-16 retrograde signaling through modulating the activity of the antagonistic DAF-2 ligand during synapse formation.
2.3.9. *fsn-1* animals exhibit defects in INS-18::GFP processing

In mammals, insulin is initially synthesized in the cytoplasm as a pre-proinsulin precursor which consists of a signal peptide and 3 peptides linked in succession, B, C and A, respectively (Pierce *et al.*, 2001) (Figure 14A). Upon entering the endoplasmic reticulum, the signal peptide is cleaved and the pre-proinsulin is processed into a pro-insulin molecule. Ultimately, the C-peptide is removed resulting in the mature form of insulin in which the B and A peptides are linked by disulfide linkages (Figure 14A). Among the 40 known insulin/IGF-like ligands, INS-18 and INS-1 are the only two that are predicted to have a C-peptide (Pierce *et al.*, 2001). However, whether INS-18 or INS-1 indeed encodes the insulin-like peptide or undergoes processing of the C-peptide has never been experimentally determined.

We first investigated whether INS-18 undergoes insulin-like processing in wildtype animals. Due to the lack of specific antibodies against INS-18, we expressed INS-18::GFP, in wildtype animals, where GFP is fused to the C-terminal region of the A peptide under a panneural promoter. INS-18::GFP is indeed secreted in wildtype animals (Figure 14B). Moreover, the GFP fusion is unlikely to alter the function of INS-18, since overexpression of INS-18::GFP also rescued *fsn-1* synaptic defects as did the overexpression of INS-18 alone (data not shown).

We performed western blot analyses using an antibody against GFP using whole wildtype worm lysates, which were denatured with or without the reducing agent β-mercaptoethanol. Unlike the insulin precursors, where A and B peptides are covalently connected through the C-peptide, in the mature insulin, A and B peptides only associate with each through thioester bonds that are sensitive to reducing agents. If INS-18::GFP undergoes similar processing events as mammalian insulins, INS-18::GFP denatured in the absence of β-
mercaptoethanol should exhibit a slightly slower migration (A+B::GFP) than in the presence of β-mercaptoethanol (B::GFP only). This was indeed observed for both INS-18::GFP and INS-1::GFP, another close homologue of INS-18 (Figure 14B). It is unlikely that this shift in molecular weight is due to secondary structural changes in INS-18::GFP because we also consistently observed reduced migration of INS-18::GFP in a C. elegans loss of function mutants for EGL-3, a major neuronal proprotease convertase (data not shown).

The same INS-18::GFP array was crossed into fsn-1 mutants to examine its processing. Intriguingly, both processed and unprocessed forms of INS-18::GFP were detected, in both the presence and absence of β-mercaptoethanol. Moreover, multiple slow migrating (thus higher molecular weight) INS-18::GFP bands appeared in fsn-1 mutants (Figure 14B). Although the nature of these higher molecular weight bands of INS-18::GFP is unknown, fsn-1 animals clearly display INS-18::GFP processing defects. Using the same methods outline above, we also examined INS-1::GFP expression in fsn-1 animals, and however, observed no defects in INS-1::GFP processing (Figure 14B). Thus, FSN-1 specifically regulates INS-18 processing in C. elegans.

2.4. Discussion

To understand how synapse development is regulated by the F-box, FSN-1, we have utilized a combination of molecular genetics and biochemistry tools to investigate the signaling pathways regulated by FSN-1. Our studies have shown that loss of function of the insulin receptor, daf-2, partially rescues fsn-1 synaptic morphology defects. Thus, the postsynaptic insulin/IGF signaling pathway is a target or effector through which fsn-1 regulates synapse development. More specifically, our studies demonstrate that fsn-1 is required, directly or
indirectly, for the processing and maturation of INS-18, an antagonistic insulin-like ligand that negatively regulates the DAF-2/DAF-16 signaling cascade in postsynaptic muscle cells.

We propose a model whereby FSN-1 modulates insulin/IGF signaling through INS-18 modifications, which ultimately results in the inactivation of the FOXO/DAF-16 transcription factor. The FOXO/DAF-16 transcription factor regulates the expression of currently unidentified signaling molecules that may contribute to the activation of the MAPK signaling cascade in presynaptic cells, which is also subject to negative regulation by the E3 ubiquitin ligase complex formed by FSN-1/RPM-1. This auto-regulatory loop, consisting of both anterograde and retrograde signaling, regulates synapse development and maturation.

**Loss of function mutations in the DAF-2/insulin pathway rescue fsn-1 defects, yet do not result in significant changes in synapse morphology**

Although loss of function mutants in the DAF-2 pathway can suppress fsn-1 synaptic morphology defects, the daf-2(ts) loss of function mutants we utilized in our study did not have any obvious synaptic defects on their own. Synaptic defects may not have been observed since the daf-2 allele is a temperature-sensitive and only a partial loss of function (not null) allele. In this case, residual DAF-2 function and the presence of many redundant pathways regulating synaptogenesis may result in the normal synapse morphology observed in daf-2 animals. Furthermore, although downstream components of the DAF-2 signaling pathway, namely age-1 and pdk-1, can partially rescue fsn-1 synapse defects, neither of these mutants showed complete rescue of fsn-1. This may be also due to variations in strength of the loss of function alleles as in the case with DAF-2. Unfortunately, the use of stronger or complete null alleles of the DAF-2 signaling pathway would induce a constitutive dauer phenotype and/or embryonic sterility and lethality, thus, inability to conduct studies of this nature (Paradis and Ruvkun, 1998).
Furthermore, it is possible that loss of function of the DAF-2/insulin/IGF signaling pathway results in only a partial rescue of fsn-1 synaptic defects because there are many redundant pathways regulating synapse development. Moreover, the canonical DAF-2/AKT/FOXO cascade may be only one of the effectors triggered upon InR and PI-3K activation (Kahn and White, 1988; Vanhaesebroeck et al., 2001). This is indeed the case, as InR and PI-3K activation has also been shown to modulate several cellular functions including the activity of GSK-3β to regulate apoptosis and cell survival (Bijur and Jope, 2001), as well as the Ras/MAPK/ERK1/2 pathway and PKC to regulate gene transcription necessary for learning and memory formation (Sweatt, 2004; Bonini et al., 2007).

Consequently, the partial rescue of fsn-1 synaptic defects by the canonical insulin/IGF signaling components suggests that fsn-1 is likely regulating synapse development via modulating multiple different processes and signaling pathways.

**Agonizing over potential insulin/insulin-like agonists and antagonists**

The sole insulin receptor in *C. elegans*, DAF-2, is likely activated by various insulin and/or insulin-like growth factors. In the *C. elegans* genome, there are 40 known insulin-like molecules which are putative ligands for the DAF-2 receptor. We tested the possibility of these insulins as potential agonists and antagonists by generating *fsn-1;insulin* double mutants as well as overexpressing all of the available INS into *fsn-1* mutant animals. Although, none of the *ins* mutants, except the dominant-negative *daf-28 (sa191)* allele, rescued the *fsn-1* synaptic morphology defects, this does not rule out the possibility of INS proteins functioning as agonists for DAF-2. It is very likely that multiple ligands are required in order to activate the INS receptor, indicating a functional redundancy among the 40 INS proteins in regulating synapse formation.
Various structural predictions and possible C-peptide cleavage sites typical of mammalian insulins suggest that INS-1 and INS-18 are most closely related to human insulin (Kawano et al., 2000; Pierce et al., 2001). INS-1 and INS-18, two of the 40 insulin/IGF-like peptides, are the only *C. elegans* insulins that contain a C-peptide, characteristic of mammalian insulins (Pierce et al., 2001). However, we discovered through genetics and biochemical assays that INS-18, not INS-1, interacts with *fsn-1*, suggesting that INS-18, specifically, antagonizes DAF-2/insulin/IGF-like signaling in synaptogenesis. Interestingly, overexpression of INS-1 and human insulin has been reported to enhance dauer arrest in *daf-2* mutants, suggesting that INS-1 antagonizes development-specific DAF-2 signaling (Pierce et al., 2001). Together, these findings suggest a functional diversity within the INS family, and provide the first steps towards dissecting the roles of the divergent insulin superfamily members.

**Neuronally expressed INS-18 antagonizes DAF-2/insulin/IGF signaling in muscle**

In mammals, although insulin is primarily synthesized by the β-cells of the pancreatic islets of Langerhans, the existence of preproinsulin I and II mRNA and insulin immunoreactivity in neurons suggest that insulin can be synthesized *de novo* in the brain (Schechter et al., 1996; Zhao et al., 1999). In our study, we showed that the neuronal overexpression of the insulin-like peptide, INS-18, specifically rescues *fsn-1* synaptic defects. Consistent with the above studies, our findings not only suggest that insulin-like peptides can be secreted from neurons, but also that they may undergo processing at the nerve terminal, which is regulated by the ubiquitin ligase, FSN-1. We speculate that FSN-1 may be regulating INS-18 processing to modulate synapse development in several ways. FSN-1 regulates the processing of the INS-18 antagonist, whose inactivation results in the increase in activity of the DAF-2/insulin pathway to stimulate synapse growth. More specifically, we speculate that FSN-1 may be involved in regulating the
cleavage and/or activity of the C-peptide of INS-18. In the last decade, C-peptides have emerged as much more than just a byproduct of insulin biosynthesis; they have become therapeutic agents of neurodegenerative disease as studies have shown that C-peptide administration can effectively prevent and even reverse cardiovascular disease and nerve damage in diabetic patients (Ido et al., 1997). In animal models, the C-peptide has also been shown to promote nerve fiber regeneration and prevent apoptosis of central and peripheral nerve cell components (Sima et al., 2004). More recent studies have uncovered the molecular and cellular mechanisms of C-peptide function, which include the activation of protein kinase C, Na+,K+- ATP-ase, nitric oxide synthase, MAP and ERK 1/2 kinases (Zhong et al., 2004; Kamiya et al., 2009). Interestingly, the MAPK/ERK pathway is also required for IGF-1-stimulated proliferation of nerve cells, suggesting that C-peptides and IGFs may function in concert to enhance nervous system development (Aberg et al., 2006). On that account, an intriguing possibility is that INS-18/C-peptide activity is tightly regulated by FSN-1 in order to achieve proper DAF-2/insulin/IGF signaling levels required for synapse maturation. Specifically, FSN-1 likely regulates the levels of processed INS-18/C-peptide in order to fine-tune the inhibition/activation of DAF-2/insulin signaling levels, respectively. Furthermore, the resulting C-peptide secreted from neurons may behave like IGFs by stimulating PKC and MAPK/ERK cascades to further confer an environment appropriate for synapse development.

**FSN-1 requires FOXO/DAF-16 signaling to fine-tune a DAF-2/insulin/IGF retrograde signaling cascade**

Our studies show that FSN-1-regulated DAF-2/insulin/IGF signaling depends on the presence of the FOXO transcription factor, DAF-16. Furthermore, the inhibition of the FOXO transcription factor can result in the activation or inactivation of different genes in various
development processes regulating dauer formation, longevity, stress, and metabolism (Murphy et al., 2003). Thus, we predict that DAF-16 likely inhibits the activity of an unknown synapse promoting factor(s), either directly via transcriptional repression, or indirectly through transcriptional activation of an inhibitor of this factor. This hypothesis is supported by our findings that the DAF-2 and DAF-16 signaling is required postsynaptically to alter presynaptic morphology, as well as the fact that the suppression of fsn-1 by p38 MAPK components does not require FOXO signaling. These findings are consistent with the regulation of presynaptic growth through a retrograde signaling mechanism involving synapse promoting factors secreted from postsynaptic cells.

The finding that FSN-1-mediated retrograde insulin signaling functions through the FOXO/DAF-16 transcription factor is intriguing. FOXO activity is a critical integration point of multiple signaling pathways, and thus, tightly controlled regulation of FOXOs by multiple mechanisms is necessary. In response to different inter- and intracellular signals and stimuli, FOXOs have been shown to undergo inhibitory phosphorylation by protein kinases such as Akt, SGK, IKK, CDK2 (Reviewed in Huang and Tindall, 2007). Under conditions of stress, FOXO activity can also be upregulated by upstream modulators such as the JNK pathway, MST1, and PTEN/DAF-18 phosphatase (Huang and Tindall, 2007). Effectors of FOXO signaling include the nutrient-seeking TOR pathway regulator, DAF-15, as well as the detoxification genes Nrf/SKN-1, the ephrin receptor VAB-1, and the superoxide dismutase, SOD-3, suggesting that dysfunction of FOXO signaling can lead to grave pathologies such as cancer (Murphy, 2006). Interestingly, during our studies, we also observed that daf-16;fsn-1 mutants consistently exhibit less variability of synaptic phenotype expression than fsn-1 in response to environmental changes. Thus, under different cellular contexts, DAF-16 not only regulates state-dependent effects, but may also fine-tune insulin/IGF signaling during synapse development.
Our findings signify that additional downstream effectors of FOXO/DAF-16, outside the realm of longevity and detoxification, likely exist. Specifically, FOXO/DAF-16 likely regulates the expression of unknown synapse promoting agonists or retrograde signals that activate the presynaptic MAP kinase pathway, and thus, synapse development. Our studies also suggest that the MAP kinase pathway may function genetically downstream of the DAF-2/insulin/FOXO signaling cascade and other signaling pathways to regulate synapse formation and function. Most importantly, our findings suggest that FSN-1 functions as a key modulator of an auto-regulatory loop between the insulin/IGF and MAPK signaling cascades in order to fine-tune synapse maturation and function.

**In search of potential retrograde signaling agonists and therapeutic agents**

Since FOXO/DAF-16 is a transcription factor, there have been many attempts to identify DAF-16 targets using expression microarrays (Murphy *et al.*, 2003). Chromatin immunoprecipitation (ChIP) have been also used to identify downstream targets of the DAF-2/insulin/FOXO pathway (Oh *et al.*, 2006). Although these approaches have identified genes that are regulated by FOXO, they are only the first step towards finding physiologically-relevant retrograde signaling agonists for DAF-16 in synapse development. We have shown that the SCF-FSN-1 ubiquitin ligase is a regulator of the DAF-2/insulin signaling pathway, and that this pathway requires DAF-16 signaling to regulate synapse formation. Future studies will involve testing candidate genes to identify those that are regulated by DAF-16 in a FSN-1-dependent manner. Using RNAi, for example, we can knock down the expression of candidate DAF-16 targets in a fsn-1 sensitized background in order to identify the retrograde signals that mediate synapse formation. The identification of FOXO targets will augment our understanding of the retrograde signaling mechanism necessary for FSN-1-mediated synapse development. More
importantly, it will enhance our knowledge of and elucidate the alternative mechanisms we can use to treat neurodegenerative disease.

2.5. Materials and Methods

2.5.1. Strains

All *C. elegans* strains were cultured and maintained at 22°C on standard NGM plates as described in Brenner (1974), with OP50 *Escherichia coli* as the food source.

*juIs1* (*P* _unc-25-snb-1::gfp*) is a presynaptic marker that facilitates the visualization of presynaptic termini of GABAergic NMJs, was kindly provided by Dr. Y. Jin (Zhen and Jin, 1999).

*oxIs22* (*P* _unc-25-unc-49::gfp*) is a GFP-tagged GABA receptor (UNC-49::GFP) marker which allows the visualization of postsynaptic termini of GABAergic NMJs, and was kindly provided by Dr. B. Bamber (Bamber et al., 1999).

*fsn-1(hp1)* was isolated from an enhancer screen using EMS-mutagenesis of CZ465 _syd-2(ju37)_ animals (Zhen and Jin, 1999). Severely uncoordinated animals were picked from the F2 generation, and the *fsn-1* mutation was outcrossed and separated from the _syd-2_ mutation. _hp1_ is a genetic and protein null allele of *fsn-1* (Liao et al., 2004).

To determine the homozygosity of strains, single worm lysis and subsequent PCR analyses were performed to confirm the genotype of strains. The primers used in this study are outlined in Appendices (3. Oligonucleotides).

For a complete list of strains used in this study, refer to the Appendices (2. Strains).
2.5.2. Plasmids used in this study

- **pJH616**
  - \( P_{\text{myo-3}} \text{ daf-2} \)
  - muscle specific \( \text{daf-2} \)
- **pJH665**
  - \( P_{F25B3.3} \text{ daf-2} \)
  - pan-neural \( \text{daf-2} \)
- **pJH1170**
  - \( P_{F25B3.3} \text{ daf-16} \)
  - pan-neural \( \text{daf-16} \)
- **pJH1171**
  - \( P_{\text{myo-3}} \text{ daf-16} \)
  - muscle specific \( \text{daf-16} \)
- **pJH1464**
  - \( P_{\text{myo-3}} \text{ ins-18::RFP} \)
  - muscle specific \( \text{ins-18} \)
- **pJH1477**
  - \( P_{F25B3.3} \text{ ins-18::RFP} \)
  - pan-neural \( \text{ins-18} \)
- **pJH1498**
  - \( P_{F25B3.3} \text{ ins-18::GFP} \)
  - pan-neural \( \text{ins-18} \)
- **pJH1922**
  - \( P_{F25B3.3} \text{ ins-1::GFP} \)
  - pan-neural \( \text{ins-1} \)

2.5.3. Fluorescent marker analyses

*Caenorhabditis elegans* strains with SNB-1::GFP (*juIs1*) or UNC-49::GFP (*oxIs22*) markers were observed on glass slides with 3% agarose pads and M9 buffer to immobilize the animals. The number of SNB-1::GFP or UNC-49::GFP puncta along the dorsal cord were counted manually using a Zeiss Axioskop 2 plus fluorescent microscope at 63x magnification. Images of the SNB-1::GFP and UNC-49::GFP markers in live animals were taken with the Improvision Openlab system (Quorum Technologies Inc.). Distribution plots of puncta widths were generated using Matlab in conjunction with a Punctaanalyzer program developed by Dr. Taizo Kawano. Images taken at a 63x magnification were processed using ImageJ and inputted into Punctaanalyzer. Punctaanalyzer generated the following parameters of puncta: count, linear density, distance, width, gap, intensity and volume. For my thesis, although all of the parameters were tested, the
puncta width was the parameter that was most significantly defective in \textit{fsn-1} mutants, and thus, was used for subsequent analyses.

2.5.4. Western blot analyses

\textit{C. elegans} strains were grown on large plates, and whole worm lysates were prepared by washing the animals off the plates using M9 buffer. Equal amounts of total protein from each lysate were used for Western blot analysis as previously described in Liao \textit{et al.}, (2004). The western blots were performed using anti-GFP and anti-tubulin antibodies.

For reducing conditions, 20\(\mu\)l/ml of \(\beta\)-mercaptoethanol was added to a 2X SDS page sample buffer prior to adding the SDS buffer to the lysates.
Figure 7: FSN-1 regulates both pre- and postsynaptic marker morphology.

(A) A schematic drawing of the daf-2/insulin/IGF pathway, and how FSN-1 may be regulating insulin signaling. (B)(C) Comparison of presynaptic (SNB-1::GFP) and postsynaptic (UNC-49::GFP) marker morphology of wildtype, fsn-1, daf-2 fsn-1, and daf-16;daf-2 fsn-1 mutant animals. Using both markers, wildtype animals display round, discrete, and evenly spaced puncta. fsn-1 animals display large clusters of puncta and gaps between puncta (indicated by asterisks and arrows, respectively). This defect is rescued by a loss of function of daf-2. Loss of function of daf-16 reverts the daf-2 fsn-1 rescue back to fsn-1, thus, DAF-16 is required for daf-2 suppression of fsn-1. (D)(F) The quantifications of the SNB-1::GFP and UNC-49::GFP puncta numbers, respectively. (E) A plot of the distribution of puncta widths of each animal using Punctanalyser. fsn-1 animals display the broadest range of puncta width, and the loss of daf-2 in daf-2 fsn-1 animals rescues puncta width to wildtype, which is consistent with our observations. Density on the y-axis represents the percentage of total puncta that are of a certain puncta width size. Scale bar, 5µm. **p<0.001, N=15.
Figure 8: *fsn-1* synaptic defects can be suppressed by downstream components of the *daf-2* signaling pathway.

(A) SNB-1::GFP expression of wildtype, *fsn-1*, *age-1;fsn-1*, and *fsn-1;pdk-1* animals. *fsn-1* animals display abnormal clusters of puncta and gaps devoid of puncta (indicated by asterisks and arrows, respectively). (B) Quantification of SNB-1::GFP puncta displaying the partial rescues of *fsn-1* in *age-1;fsn-1*, and *fsn-1;pdk-1* animals compared to wildtype and *daf-2 fsn-1*. (C) Density plot of puncta widths of wildtype, *fsn-1*, *age-1;fsn-1*, and *fsn-1;pdk-1* animals using Punctanalyser. Both *age-1;fsn-1*, and *fsn-1;pdk-1* animals show partial rescue of *fsn-1* in puncta width distribution. Scale bar, 5µm. **P<0.001, N=15.
Figure 9: The DAF-2/FOXO/DAF-16 signaling cascade is required in postsynaptic cells to influence NMJ development.

(A) SNB-1::GFP expression of wildtype, fsn-1, daf-2fsn-1, and daf-16;daf-2fsn-1 animals. fsn-1 animals display abnormal clusters of puncta and gaps devoid of puncta (indicated by asterisks and arrows, respectively). While daf-2(lf) rescues fsn-1 synaptic morphology defects, it requires daf-16 for suppression of fsn-1. (B) Pan-neural expression of DAF-2 has no effect on the rescue of daf-2fsn-1 animals by daf-2, while muscle-specific expression reverts daf-2fsn-1 back to fsn-1. (C) Likewise, muscle-specific expression of DAF-16 reverts daf-16;daf-2 fsn-1 animals back to the daf-2fsn-1 rescued synapse morphology. Scale bar, 5µm. **P<0.001, N=15.
Figure 10: The DAF-2 pathway specifically rescues *fsn-1*, not *rpm-1*, synaptic defects.

(A) Comparison of SNB-1::GFP (*juIs1*) expression in wildtype, *mkk-4*, and *daf-2* single mutant animals, and (B) *fsn-1*, *fsn-1;mkk-4*, *daf-2 fsn-1*, and (C) *rpm-1*, *rpm-1;mkk-4*, and *daf-2;rpm-1*. (D) Quantification of total dorsal cord SNB-1::GFP puncta in wildtype, *fsn-1*, *fsn-1;mkk-4*, and *daf-2;fsn-1*. (E) Quantification of total SNB-1::GFP puncta in wildtype, *rpm-1*, *rpm-1;mkk-4*, and *daf-2;rpm-1*. Both *mkk-4* and *daf-2* show partial rescue of *fsn-1* synaptic defects, however, *daf-2* does not suppress *rpm-1* synaptic defects, suggesting that the DAF-2/insulin/IGF pathway specifically rescues *fsn-1*. Scale bar, 5µm. **P<0.001, *P<0.05, N=15.**
Figure 11: MAPK suppression of *fsn-1* or *rpm-1* does not require DAF-16/FOXO signaling.

Comparison of SNB-1::GFP (juIs1) expression in: (Ai, ii, iii) wildtype, *mkk-4*, *daf-16*, (Bi, ii, iii) *fsn-1*, *fsn-1; mkk-4*, and *daf-16; fsn-1; mkk-4*, (Ci, ii, iii) *rpm-1*, *rpm-1; mkk-4*, and *daf-16; rpm-1; mkk-4*. (D) Quantification of total dorsal cord SNB-1::GFP puncta in wildtype, *fsn-1*, *fsn-1; mkk-4*, and *daf-16; fsn-1; mkk-4*. There is no significant difference between *fsn-1; mkk-4* and *daf-16; fsn-1; mkk-4* animals, suggesting that DAF-16 signaling is not required for *mkk-4* suppression of *fsn-1*. (E) Quantification of total SNB-1::GFP puncta in wildtype, *rpm-1*, *rpm-1; mkk-4*, and *daf-16; rpm-1; mkk-4*. There is no significant difference between *rpm-1; mkk-4* and *daf-16; rpm-1; mkk-4* mutant animals, thus, DAF-16 is not required for MAPK suppression of *fsn-1* or *rpm-1* synaptic defects. Scale bar, 5µm. *P<0.001, N=15.
### fsn-1; ins double mutants

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</tr>
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</tr>
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<td>tm2632</td>
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<td>n/a</td>
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<tr>
<td>daf-28</td>
<td>tm2308</td>
<td>no rescue</td>
</tr>
<tr>
<td>daf-28</td>
<td>sa191</td>
<td>rescue</td>
</tr>
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</table>

Summary of ins and fsn-1 double mutant animals lines.

n/a: mutant not available

### Overexpression of insulins

<table>
<thead>
<tr>
<th>Insulin</th>
<th>Fosmid (WRM)</th>
<th>Rescuing lines</th>
</tr>
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<tr>
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<td>0618bG05</td>
<td>0/3</td>
</tr>
<tr>
<td>ins-2 to ins-6</td>
<td>0614ah11</td>
<td>0/2</td>
</tr>
<tr>
<td>ins-7 &amp; ins-8</td>
<td>0612aA09</td>
<td>0/3</td>
</tr>
<tr>
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<td>0618ba10</td>
<td>0/2</td>
</tr>
<tr>
<td>ins-10</td>
<td>0611ba09</td>
<td>0/1</td>
</tr>
<tr>
<td>ins-11 &amp; ins-12</td>
<td>0618dd07</td>
<td>0/1</td>
</tr>
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<td>0/1</td>
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<td>ins-18</td>
<td>065aF10</td>
<td>5/5</td>
</tr>
<tr>
<td>ins-19 &amp; ins-31</td>
<td>0639db04</td>
<td>0/1</td>
</tr>
<tr>
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<td>068cg09</td>
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<td>0619cg07</td>
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</tr>
</tbody>
</table>

Summary of transgenic fsn-1 animals with ins overexpression by fosmid injection.

*PCR products were generated and injected for insulins with no available fosmids.

---

**Figure 12: Genetic interactions between fsn-1 and insulin/IGF mutants.**

(A) fsn-1; ins double mutants generated. The dominant negative allele of daf-28 (sa191) showed rescue of fsn-1 synaptic defects. (B) Overexpression of insulins. The overexpression of ins-18 displayed rescue of fsn-1 synaptic morphology defects (see Figure 13).
Figure 13: Overexpression of INS-18 in presynaptic neurons rescues fsn-1 synaptic defects.

(A)(B) Comparison of wildtype and fsn-1 SNB-1::GFP presynaptic morphology. fsn-1 animals display abnormal clusters of puncta and gaps devoid of puncta (indicated by asterisks and arrows, respectively). (C) The overexpression of ins-1 does not affect fsn-1 synaptic defects. (D) Overexpression of ins-18 rescues fsn-1 defects, and (E)(F) this rescue is neuron-specific. (G)(H) Quantifications of total number of dorsal cord puncta in ins-1 and ins-18 rescue experiments.
Figure 14: \textit{fsn-1} animals exhibit INS-18::GFP processing defects.

(A) The stages of insulin maturation from pre-pro-insulin to mature insulin. In the cytosol, insulin is initially formed as a pre-pro-insulin, which contains a signal peptide (\textcolor{red}{red}), B-peptide (\textcolor{green}{green}), C-peptide (\textcolor{pink}{pink}), and A-peptide (\textcolor{blue}{blue}). Upon cleavage of the signal peptide in the ER and Golgi, the pre-pro-insulin becomes a pro-insulin molecule that enters a secretory vesicle. Here, mammalian prohormone convertases PC1/3 and PC2 cleave the pro-insulin to release the C-peptide, resulting in mature insulin. (B) Western blot analyses for INS-18::GFP in N2 control (without GFP), wildtype, \textit{fsn-1}, and \textit{rpm-1} mutant animals. Wildtype animals display a shift in the molecular weight of INS-18::GFP from 34 kD (B+A::GFP) to 26kD (A::GFP) under reducing conditions, likely as a result of the cleavage of the C-peptide during insulin maturation. \textit{rpm-1} animals exhibit the same shift as wildtype animals. \textit{fsn-1} mutants do not display this shift in INS-18::GFP. However, \textit{fsn-1} animals exhibit normal processing of INS-1::GFP, suggesting that \textit{fsn-1} specifically regulates INS-18::GFP processing.
An RNAi Screen for Components of the Insulin-mediated Retrograde Signaling Cascade in Synapse Formation

3.1. Abstract

The insulin/IGF/FOXO signaling pathway is remarkably conserved in *C. elegans*, *Drosophila*, and mammals. The Forkhead transcription factor, FOXO, has emerged as a family of versatile regulators of processes mediating lifespan, cellular metabolism, and development. However, direct evidence for the involvement of FOXO in synapse formation and function remain scarce. We have shown that, in *C. elegans*, the insulin/IGF signaling pathway is regulated by the SCFFSN-1 ubiquitin ligase, and the insulin/FOXO signaling cascade in postsynaptic cells likely activates a retrograde signaling pathway to modulate synapse development (Chapter 2; Hung et al., in preparation). Here, we systematically tested *C. elegans* genes whose expression is regulated by the DAF-16/FOXO transcription factor using RNA interference (RNAi), and identified 30 candidate DAF-16 targets that may regulate synapse formation via a DAF-16-dependent mechanism. Specifically, we have validated that the loss of function mutants in the *C. elegans* glucosaminidase, OGA-1, display a genetic interaction with *fsn-1* mutants, making it a plausible component of the insulin/IGF activated retrograde signaling cascade that regulates synapse development. We propose that OGA-1 is a component of the retrograde signaling cascade that activates the presynaptic MAPK pathway, whose activity is also under the regulation of the presynaptic ubiquitin ligase, SCFFSN-1/RPM-1.
3.2. Introduction

In the last two decades, the insulin/IGF signaling cascade has emerged as more than just a regulator of energy flux and nutrient metabolism; It has become a key mediator of immune and oxidative stress responses that not only affect the life spans of mammals as well as invertebrates (Reviewed in Barbieri et al., 2003). Although the physiological effects of the insulin/IGF signaling cascades may vary between organisms, the components of the insulin/IGF pathway and their interactions are largely conserved (Reviewed in Broughton and Patridge, 2009). Thus, understanding how this conserved pathway regulates a myriad of biological processes specific to different animals is of intense interest.

In C. elegans, the insulin/IGF-1 signaling pathway controls many biological processes such as life span, fat storage, dauer formation, stress response and reproduction. (Reviewed in Braeckman et al., 2001). The insulin/IGF pathway is activated by the binding of insulin/IGF peptides to the sole insulin receptor InR/DAF-2. DAF-2 activates AGE-1, a phosphoinositide-3-kinase (PI-3K) PDK-1, which in turn activates downstream AKT serine/threonine kinases. Activated AKT can directly inactivate the FOXO/DAF-16 transcription factor through phosphorylation, and phosphorylated FOXO binds to the small regulatory proteins 14-3-3, which results in its cytoplasmic retention (Lin et al., 2001; Cahill et al., 2001). In the absence of DAF-2/insulin signaling, dephosphorylated FOXO/DAF-16 translocates into the nucleus and regulates transcription of its many targets (Ogg et al., 1997).

The identification of these FOXO targets genes have been of great interest as dysfunction in FOXO in mammals has been implicated in aging and various metabolic diseases. For instance, the downregulation of mammalian FOXO3a has been linked to cell aging and senescence (Kyoung Kim et al., 2005). Similarly, Akt activity has been shown to increase
cellular senescence in primary cultured human endothelial cells, and inhibition of FOXO3a by Akt is crucial for initiating this arrest in growth (Miyauchi et al., 2004). Interestingly, more recent studies are beginning to reveal how Akt/FOXO signaling regulates the expression of atrophy-related genes, such as the ubiquitin ligase atrogin-1 (MAFbx) (Shurk et al., 2005). In mice, atrogin-1 has been shown to inhibit Akt-dependent cardiac hypertrophy, a major cause of human morbidity, via a concomitant ubiquitin-dependent coactivation of FOXO proteins (Li et al., 2007). Moreover, mechanisms of regulation of the FOXO proteins are receiving increasing attention in cancer research, as dysfunction of FOXO is now more frequently found to be associated with human cancers (Reviewed in Zanella et al., 2010). Under conditions where the PI3K/Akt pathway is downregulated due to starvation or stress, FOXOs promote the transcription of pro-apoptotic molecules like FasL and Bim, and thus, have been established as tumor suppressors (Yang et al., 2006). Although these studies suggest the importance of FOXO signaling in metabolism, aging, and disease, investigating the FOXO targets and downstream effectors will further elucidate the mechanisms through which FOXOs regulate the normal and pathological states of development.

Thus far, the most comprehensive list for potential transcriptional targets of the FOXO transcription factor has resulted from studies in *C. elegans*. Since DAF-16 is a transcription factor, extensive microarray analyses have been carried out to determine the expression profile changes in mutants of the DAF-2/insulin/FOXO signaling pathway (Murphy et al., 2003; Yu et al., 2008). One of the most important steps to identifying DAF-16 targets was the discovery of the FOXO binding sequence, TTGTTTAC, also known as the DAF-16 binding element or DBE (Furuyama et al., 2000). Using a combination of microarrays, chromatin-IP analyses, and bioinformatic predictions, a few hundred putative DAF-16 targets have been identified in *C. elegans*. Moreover, due to the completely sequenced *C. elegans* genome, genome-wide genetic
analyses of all putative targets and validation of their functions in DAF-16-mediated processes have become feasible (C. elegans Genome Sequence Consortium, 1998). Examples for some of the confirmed DAF-16 targets are the C. elegans superoxide dismutase gene, sod-3 (Honda and Honda, 1999), a regulator of longevity, and the C. elegans homolog of mammalian Raptor, daf-15, a regulator of the TOR pathway in metabolism (Jia et al., 2004). Furthermore, using the above assays as well as RNAi against putative targets, Lee et al. (2003) identified 17 DAF-16 target genes that affect lifespan. Similarly, the SCP-like secretory protein SCL-1 was identified as a DAF-16 target that affects longevity, fat storage, and stress response (Ookuma et al., 2003).

An important discovery made from these studies was that DAF-16 regulates both overlapping and non-overlapping sets of targets in different tissues. Furthermore, like DAF-2, DAF-16 is widely expressed in virtually all cell types in C. elegans (Ogg et al., 1997; Henderson and Johnson, 2001). Thus, DAF-16 must integrate both environmental and developmental signals to mediate specific processes in different tissues. For instance, DAF-16 activity in the intestine, which is C. elegans’ adipose tissue, is critically required for its role in the aging process, whereas DAF-16 in neurons or muscles has only moderate, or no effect at all, respectively (Libina et al., 2003). Another example of the tissue-specific function of DAF-16 is dauer formation. Under stressful conditions such as starvation, animals enter into a diapause-like state, or “dauer” until more favourable environmental conditions resume. DAF-16 expression in the nervous system increases during dauer formation (Lin et al., 2001), and neuronal DAF-16 appears to be sufficient to induce dauer formation (Libina et al., 2003). Thus, DAF-16 expression can influence different developmental processes in a cell non-autonomous fashion and acts on many tissues of the animal.

While these studies have greatly facilitated our understanding of longevity and thus, age-related diseases, very few studies have focused on the implications of FOXO signaling during
development of somatic tissues such as neurons and muscles. Previously, we have shown that the SCF^{FSN-1} ubiquitin ligase is a regulator of the DAF-2/insulin signaling pathway, and that this pathway requires DAF-16 signaling in the postsynaptic muscle to regulate presynaptic development. Most importantly, our findings demonstrate a strict postsynaptic requirement of the DAF-2/DAF-16 signaling cascade at the NMJ, which necessitates further dissection of the targets of DAF-16 during synapse development.

In this study, we chose 526 candidate DAF-16 target genes based on the previous microarray studies, and examined their potential involvement in synapse development. As mutations in the insulin/IGF/DAF-2/DAF-16 signaling cascade modify the synapse morphology defects in \( fsn-1 \), we sought out to identify genes regulated by DAF-16 that exhibit similar genetic modifying effects of \( fsn-1 \). Using RNAi, we knocked down the expression of these genes in a \( fsn-1 \) sensitized background in order to identify DAF-16 targets that mediate synapse formation.

### 3.3. Results

#### 3.3.1. Designing an RNAi screen for DAF-16 targets in the insulin/IGF-mediated retrograde signaling pathway

The objective of our studies was to discover transcriptional targets of DAF-16 that mediate FSN-1-regulated retrograde signaling during synapse formation. Since loss of function mutations in InR/DAF-2 suppress the synaptic defects of \( fsn-1 \) mutants in a DAF-16-dependent fashion, we speculate that at least some of the DAF-16 targets would also behave similarly, as a suppressor of \( fsn-1 \) defects when mutated. Using RNAi, we can test the contribution of all
predicted DAF-16 targets, through their effects on modifying the synaptic defects in a *fsn-1* mutant background.

Although *fsn-1* mutant animals exhibit severe synapse morphology defects, they do not exhibit obvious locomotion defects. In order to perform an effectively objective and systematic screen, we utilized the fact that *fsn-1* loss of function animals display severe locomotion defects in the absence of another unrelated regulator of presynaptic morphology, a serine/threonine kinase, *sad-1* (Crump *et al.*, 2001) that is easily discernable from wildtype animals. We generated *fsn-1;sad-1* double mutants and *daf-16;fsn-1;sad-1* triple mutants (Figure 15) and knocked down the expression of candidate DAF-16 targets in these animals using RNAi. Our rationale was that if the loss of function of a gene suppresses both *fsn-1;sad-1* and *daf-16;fsn-1;sad-1* animals, then that gene function is likely regulated by *fsn-1*, and likely functions genetically downstream of *daf-16*. Alternatively, if the loss of function of a gene suppresses *fsn-1;sad-1* but not *daf-16;fsn-1;sad-1*, this gene is likely regulated by *fsn-1*, but functions genetically upstream of *daf-16*, or in a *daf-16* independent manner. We applied this strategy to 526 candidate genes whose expression profile was reported to be altered in *daf-16* mutants. Further analyses are required to validate the potential downstream targets of DAF-16 that are regulated by insulin/IGF-mediated retrograde signaling during synapse development.

### 3.3.2. *daf-16;fsn-1;sad-1* and *fsn-1;sad-1* mutant animals are severely uncoordinated

While neither *fsn-1*, *sad-1* or *daf-16* single mutants exhibit any obvious locomotion defects, both *fsn-1;sad-1* and *daf-16;fsn-1;sad-1* double or triple mutant animals are severely uncoordinated. These animals are much shorter in body length and appear overall smaller
compared to wildtype animals. Furthermore, both mutant strains cannot propagate the signature sinusoidal wave-like movements of C. elegans. Less active than wildtype, these mutants exhibit occasional and brief spontaneous movements. When stimulated, the mutant animals do move, but after a few moments, they come to a halt. Suppression of this easily visible behavioural phenotype is much easier to score than the suppression of synapse morphology, and facilitates the large-scale RNAi screen for genes that function genetically downstream of fsn-1 or daf-16.

In order to validate our approach of using the behavioural defects associated with fsn-1;sad-1 and daf-16;fsn-1;sad-1 mutants as a readout for the synapse defects of fsn-1 mutants, we first examined the effect of loss of function mutants in the p38 MAPK signaling pathway on fsn-1;sad-1 and daf-16;fsn-1;sad-1. Previously, we have established that the MAPK signaling pathway suppresses fsn-1 synaptic defects and likely functions genetically downstream of the DAF-2/insulin/IGF signaling cascade (Chapter 2; Hung et al., in preparation). Thus, loss of function mutations in the MAPK components should in theory rescue fsn-1;sad-1 behavioural defects, as well as daf-16;fsn-1;sad-1. To test this, we constructed mapk;fsn-1;sad-1 and mapk;daf-16;fsn-1;sad-1 mutant animals and looked at their behavioural suppression. Specifically, we generated triple (fsn-1;sad-1;mapk) and quadruple (daf-16;fsn-1;sad-1;mapk) mutant strains with mapkkk/dlk-1(hp251) and mapk/pmk-3(hp182). The dlk(hp251) and pmk-3(hp182) alleles were obtained from a previous EMS mutagenesis screen for FSN-1 suppressors by previous members of the lab, and then cloned by myself and Wesley Hung, respectively. As expected, both hp251;fsn-1;sad-1 and fsn-1;hp182;sad-1 mutant strains were behaviourally suppressed and exhibited sinusoidal body bends during locomotion (data not shown). Furthermore, daf-16 hp251;fsn-1;sad-1 and daf-16;fsn-1;hp182;sad-1 mutant animals also remain behaviourally suppressed and display sinusoidal locomotion. This validated our
behavioural approach to identifying genes that are regulated by *fsn-1* and function genetically downstream, or independent of *daf-16*.

### 3.3.3. Identification of potential retrograde signaling components by a RNAi screen

Using the easily visible behavioural phenotype of *daf-16;fsn-1;sad-1* mutant animals, we performed a small-scale RNAi screen to identify targets of *daf-16* that function in a FSN-1-dependent retrograde signaling pathway. We tested 526 candidate genes that have been shown to be transcriptionally regulated by DAF-16 (Lee *et al.*, 2003; Murphy *et al.*, 2003; Mcelwee *et al.*, 2003; Oh *et al.*, 2006). We screened for any improvement in body length or behaviour when these genes were inactivated by RNAi in *daf-16;fsn-1;sad-1* and *fsn-1;sad-1* mutant backgrounds. Out of these 526 genes, RNAi against 30 of them led to partial suppression, or modest improvement, of the locomotion defects of the *fsn-1;sad-1* and *fsn-1;sad-1;daf-16* mutants (Figure 16).

### 3.3.4. The glucosaminidase OGA-1 is a candidate component of DAF-16 dependent retrograde signaling pathway

As RNAi often leads to variable and incomplete inaction of gene expression, among the 30 candidates identified to modify *fsn-1;sad-1* behavioural defects by RNAi, we further tested a few of them by genetic analyses. We obtained deletion mutants for some, specifically, the ubiquitin-associated protein SKP-5, and OGA-1, the *C. elegans* ortholog of the mammalian O-linked N-acetylglucosamine (O-GlcNAc)-selective N-acetyl-beta-D-glucosaminidase (O-
GlcNAcase), and tested their ability to modify the synapse morphology defects of *fsn-1*. *skp-5;fsn-1* mutants showed similar synaptic morphological defects as *fsn-1* mutants (data not shown). On the other hand, the loss of function of *oga-1*, showed significant rescue of the *fsn-1* loss of function defects (Figure 17). Thus, OGA-1 may be a target of DAF-16 that participates in FSN-1-regulated synapse development.

The fact that we observed rescuing effect of OGA-1 RNAi in both *fsn-1;sad-1* and *daf-16;fsn-1;sad-1* mutants suggest that OGA-1 functions downstream of DAF-16. However, future studies should closely examine whether OGA-1 functions either genetically downstream, or independent of DAF-16 during synapse formation. Like the loss of function of the components of the MAPK pathway, the suppression of *fsn-1* synapse defects by *oga-1* should not be affected by the presence or absence of DAF-16. Nonetheless, OGA-1 is the first plausible component of the insulin/IGF/FOXO mediated retrograde signaling cascade during synapse formation.

**3.4. Discussion**

Although the FOXO family of transcription factors have been implicated as key regulators of lifespan and metabolism, very few studies address a direct role for FOXO proteins in nervous system development. Our studies provide the first evidence of FOXO/DAF-16 signaling regulating synapse development. Previously, we identified a mechanism through which FSN-1 governs synaptogenesis; FSN-1 regulates an insulin/IGF-1-mediated retrograde signaling cascade in postsynaptic cells, which signals through the DAF-16/FOXO transcription factor in *C. elegans*. The activation of insulin signaling by FSN-1 results in the inactivation of DAF-16. Our studies suggest that OGA-1 may be a target of DAF-16 in the postsynaptic cells. Under conditions where the DAF-2/insulin signaling pathway is activated, DAF-16 activity is
repressed; OGA-1, whose expression or inhibition requires DAF-16 may affect the retrograde signal(s) that promotes synapse formation. Since the presynaptic MAPK pathway is a key target of SCF<sup>FSN-1</sup> E3 ligase complex at the presynaptic termini during synapse formation, we speculate that OGA-1 is a component of the retrograde signaling cascade that activates the MAPK pathway at the developing presynaptic termini.

The identification of OGA-1, an O-GlcNAcase, as a component of insulin/DAF-16 mediated retrograde signaling is intriguing. O-linked N-acetylglucosamine (O-GlcNAc) is an evolutionarily conserved carbohydrate modification of a variety of proteins, including nuclear pore proteins, signaling kinases, and transcription factors. Many studies suggest that the addition and removal of O-linked N-acetylglucosamine (O-GlcNAc) at serine and threonine residues is emerging as key regulators of nuclear and cytoplasmic protein activity (reviewed in Love and Hanover, 2005). Furthermore, O-GlcNAc addition and removal have been implicated in histone remodeling, transcription, proliferation, apoptosis, and proteasomal degradation (reviewed in Love and Hanover, 2005).

In addition, glycosylation has been shown to modulate many important cellular pathways, including the insulin signaling cascade in animals. For instance, blood glucose studies in mice have shown that overexpression of O-GlcNAcase in the livers of euglycemic mice significantly increased Akt activity, suggesting that O-GlcNAc regulates Akt signaling in hepatic models under euglycemic conditions (Soesanto et al., 2008). O-GlcNac regulation has also been shown to be critical in diabetes mellitus and neurodegenerative disorders. For instance, a single nucleotide polymorphism in the human O-GlcNAcase gene is linked to type 2 diabetes (Lehman et al., 2005; Forsythe et al., 2006). In <i>C. elegans</i>, oga-1 mutant animals form dauers even under nutritional conditions, a phenotype that indicates reduced insulin signaling and is shared by daf-2/InR loss of function mutants (Forsythe et al., 2006). More recent studies
suggest that O-GlcNAcylation may affect dauer formation through cytoskeletal modifications and protein turnover (Lee et al., 2010). Thus, O-GlcNAc modifications may be essential for fine-tuning insulin-like signaling in response to DAF-16 in both vertebrates and invertebrates.

Future studies should address the precise mechanism through which O-GlcNAc modifications affect insulin signaling and synapse growth. Although OGA-1 has been established as a transcriptional target of DAF-16 (Lee et al., 2003) and a regulator of insulin/IGF signaling (Forsythe et al., 2006), whether OGA-1 is activated or repressed by DAF-16 during synapse development still remains to be discovered. Also, we have so far only examined two of the thirty candidate DAF-16 targets for their genetic interactions with \( fsn-1 \) mutants. Other candidates should be examined with similar genetic analysis to test their interactions with \( fsn-1 \). The investigation of these candidates will further elucidate how neuromuscular junctions are formed under the regulation of FSN-1 in \( C. elegans \).

### 3.5. Materials and Methods

#### 3.5.1. RNA Interference

RNAi was performed by feeding as previously described (Fraser et al., 2000; Kamath et al., 2001) with empty pL4440 as the control. Each RNAi bacterial clone was inoculated in LB plus 100 µg/ml tetracycline and 100 µg/ml carbenicillin and grown overnight at 37°C. The overnight culture was seeded onto normal growth medium-carbenicillin large plates with 0.4 M isopropyl-\( \beta \)-D-thiogalactoside (IPTG), and left to grow and induce overnight at room temperature. Two young L4 worms were placed onto each plate, fed RNAi bacteria, and allowed to lay eggs. After 3 days, we scored the progeny of each plate for any change in behaviour or body size.
3.5.2. Strains

We used the following strains in the study: wildtype (N2), \( fn-1(hp1); sad-1(ky289) \), \( daf-16(mu86); fn-1(hp1); sad-1(ky289) \), \( skr-5(ok3068); fn-1(hp1); skr-5(ok3068) \), \( oga-1(1207); fn-1(hp1); oga-1(1207) \), \( dlk-1(hp251); fn-1(hp1); sad-1(ky289) \), \( fn-1(hp1); pmk-3(hp182); sad-1(ky289) \), \( daf-16(mu86); dlk-1(hp251); fn-1(hp1); sad-1(ky289) \), and \( daf-16(mu86); fn-1(hp1); pmk-3(hp182); sad-1(ky289) \).

We obtained the \( oga-1(1207) \) and \( skr-5(ok3068) \) strains from the \textit{C. elegans} Genome Sequence Consortium, and constructed the \( fn-1(hp1); skr-5(ok3068) \), and \( fn-1(hp1); oga-1(1207) \) double mutants.

The \( dlk-1(hp251) \) and \( pmk-3(hp182) \) alleles were obtained from a previous EMS mutagenesis screen for FSN-1 suppressors by previous members of the lab (Dr. Wesley Hung, Dr. Edward Liao and Maja Salibegovic), and then cloned by myself and Dr. Hung, respectively. From the \( hp251; fn-1; sad-1 \) and \( fn-1; hp182; sad-1 \) triple mutant strains, we generated the \( daf-16 hp251; fn-1; sad-1 \) and \( daf-16; fn-1; hp182; sad-1 \) mutant strains.

Strains were maintained at 22°C at described in Brenner, 1974.
A  **Strain Construction:**

\[
\begin{align*}
\text{fsn-1(hp1)Ill; juls1 IV} & \quad \times \quad \text{daf-16(mu86); fsn-1(hp1)Ill; juls1 IV} \\
\text{daf-16; fsn-1; juls1} & \quad \times \quad \text{fsn-1(hp1)Ill; sad-1(ky289) X} \\
\text{P₀:} & \quad \text{daf-16; fsn-1; sad-1} \\
& \quad \text{Pick heterozygous progeny} \\
& \quad \text{These animals may be daf-16/+ or +/+} \\
& \quad \text{PCR for daf-16/+} \\
\text{F₁:} & \quad \text{fsn-1; sad-1 animals are short and unc} \\
& \quad \text{Pick fsn-1; sad-1} \\
& \quad \text{From daf-16/+ P₀ plates} \\
\text{F₂:} & \quad \text{daf-16; fsn-1; sad-1} \\
& \quad \text{Homozygose daf-16} \\
\text{F₃:} & \quad \text{PCR to confirm daf-16/daf-16 homozygosity}
\end{align*}
\]

B  **Figure 15: Generation of daf-16;fsn-1;sad-1 mutant animals.**

(A) Construction of daf-16;fsn-1;sad-1 triple mutant strain from daf-16;fsn-1 and fsn-1;sad-1 double mutants. daf-16 (mu86) must be identified by PCR analysis (See Appendices 3). The presence of the sad-1 mutation is confirmed by the fsn-1;sad-1 behaviourally uncoordinated phenotype. (B) Wildtype animals present body bends and move in a sinusoidal manner, while daf-16; fsn-1; sad-1 (like fsn-1;sad-1) animals are severely uncoordinated and short in body length. This easily discernable behavioural phenotype facilitates a behavioural screen.
### Putative Retrograde Signaling Components

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<td>Galactosyltransferases</td>
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<td>Beta-Glucuronidase USB (glycosylhydrolase superfamily 2)</td>
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<td>T20B5.3 (oga-1)</td>
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<td>O-linked N-acetylglucosamine (O-GlcNAc)-selective N-acetyl-beta-D-glucosaminidase (O-GlcNAcase)</td>
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Figure 16: Identification of putative retrograde signaling components.

Summary of the 30 candidate genes that display an interaction with daf-16;fsn-1;sad-1 and fsn-1;sad-1. We tested skr-5 and oga-1 mutants with further genetic analysis, and found that oga-1 mutants suppress fsn-1 synaptic defects (Figure 17).
Figure 17: The glucosaminidase OGA-1 is a candidate component of DAF-16 dependent, retrograde signaling pathway.

(A) Wildtype animals display evenly spaced and discretely round SNB-1::GFP puncta across the dorsal cord. (B) *fsn-1* mutant animals have abnormal presynaptic morphology with large clusters of puncta and gaps between puncta. (C) Loss of function of *oga-1*, a downstream target of *daf-16*, partially suppresses *fsn-1* synaptic defects. *oga-1; fsn-1* double mutants display less gaps and clustering of SNB-1::GFP puncta. Scale bar, 5µm, *P<0.001, N=15.
**Figure 18: Model of fsn-1 regulated retrograde insulin signaling.**

During synaptogenesis, the SCF<sup>FSN-1/RPM-1</sup> E3 ubiquitin ligase complex negatively regulates the p38 MAP kinase pathway to stabilize synapse development. Concomitantly, FSN-1 also positively regulates the activity of an insulin/IGF signaling in the postsynaptic muscle, via downregulating the antagonistic insulin ligand, INS-18. The activation of the insulin/IGF cascade results in the phosphorylation of the downstream DAF-16/FOXO transcription factor, which regulates the activity of an unknown synapse promoting factor (or retrograde signaling agonist) that involves the O-GlcNAcase, OGA-1. We speculate that this retrograde signaling cascade converges on the presynaptic p38 MAP kinase pathway, forming a regulatory loop in which FSN-1 regulates synaptic development.
4. Conclusions and Future Directions

The development of complex neural networks hinges upon synaptogenesis: the proper targeting and maturation of functional synapses. Although synaptogenesis involves a myriad of signaling molecules and pathways, the simple and accessible neuromuscular junction in *C. elegans* has enabled our investigation of evolutionarily conserved mechanisms that govern nervous system development.

Our studies have elucidated the mechanisms through which a conserved family of F-box proteins, FSN-1, regulates synapse development. In addition to forming a presynaptic ubiquitin ligase with its binding partner, RPM-1, our studies reveal an intricate interaction between FSN-1 and the postsynaptic insulin/IGF/InR(DAF-2)/FOXO(DAF-16) signaling pathway during synapse development. We propose a model where FSN-1 functions as a key modulator of an auto-regulatory loop between the presynaptic MAPK signaling and the postsynaptic insulin/IGF signaling pathways (Figure 18):

During synaptogenesis, the p38 MAP kinase pathway is negatively regulated by the SCF<sup>FSN-1/RPM-1</sup> E3 ligase complex to stabilize synapse development. Concomitantly, the SCF<sup>FSN-1</sup> restricts synapse growth by positively regulating the activation of an insulin/IGF signaling in the postsynaptic muscle, via downregulating the antagonistic insulin ligand, INS-18. The insulin/IGF signaling, through the FOXO/DAF-16 transcription factor, regulates the activity of a retrograde signal that involves the O-GlcNAcase, OGA-1. We speculate that this retrograde signaling cascade converges on the presynaptic p38 MAP kinase pathway, forming a regulatory loop in which FSN-1 regulates synaptic development.

Future studies should address the precise mechanism through which FSN-1 is modifying the insulin antagonist, INS-18. Currently, we are performing mass spectrometry analyses of
INS-18::GFP in wildtype and *fsn-1* mutant animals to further confirm that INS-18 processing is
defective in *fsn-1* mutants. Our preliminary data suggest that *fsn-1* animals cannot fully process
INS-18, and thus retain the immature pre-proinsulin containing the C-peptide. Furthermore, we
need to test whether any agonistic insulin/IGF molecules function redundantly to activate the
DAF-2/DAF-16 signaling cascade. It would also be of interest to examine if FSN-1 regulates
the processing of insulin-like molecules in a developmental-specific manner, and thus, to
determine whether insulin/IGF signaling is crucial for early synapse formation (coinciding with
the L1/L2 larval stages), and/or the growth and maintenance of synaptic connections in
(L4/adults).

Future studies should also investigate the molecular mechanisms through which O-
GlcNAc modifications affect insulin signaling and synapse development. First we need to
confirm whether OGA-1 function is required in postsynaptic cells, and if so, whether it functions
geneitically in the DAF-2/DAF-16 signaling cascade. Like DAF-16, O-GlyNAc is expressed
ubiquitously (reviewed Hart *et al.*, 1996), suggesting that O-GlyNAcylation may also function to
regulate multiple signaling pathways in a context-dependent manner. One intriguing, yet
speculative possibility is that O-GlcNAc modifications affect the activity of yet to be identified
adhesive and signaling surface proteins or secreted molecules that promote synapse
development. It will be interesting to discover how OGA-1, in response to DAF-2/insulin
signaling, regulates the activity of secreted proteins from the postsynaptic muscle to activate
synapse formation, and if this involves the activation of the presynaptic MAP kinase pathway.

Lastly, the remaining putative DAF-16 targets should be examined with similar genetic
analysis with *fsn-1* to determine their role in synapse development. We hope that the
characterization of additional putative retrograde signaling agonists will facilitate our
understanding of how neuromuscular junctions are formed in not only \textit{C. elegans}, but in vertebrates and humans.
References


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Endocrinol Metab* 295, E974-80.

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K., Nabeshima, Y. and Hama, C.** (2000). Synaptic development is controlled in the periactive


## Appendices

### 1. Abbreviations

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<th>Full Form</th>
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<tr>
<td>ACh</td>
<td>Acetylcholine</td>
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<tr>
<td>AChR</td>
<td>Acetylcholine receptor</td>
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<td>Aboc EXpulsion defective</td>
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<td>AKT kinase family</td>
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<td>β-ME</td>
<td>β-Mercaptoethanol</td>
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<tr>
<td>Bim</td>
<td>Bcl-2-Interacting Mediator of cell death</td>
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<td>BMP</td>
<td>Bone Morphogen Protein</td>
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<td>CAM</td>
<td>Cellular Adhesion Molecule</td>
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Munc-18  Mammalian-uncoordinated
MuSK    Muscle Specific Kinase
MST1    Mammalian Ste20-like protein kinase
NMJ     Neuromuscular junction
Nrf     NF-E2-Related Factors
OGA-1   O-GlcNAc selective N-Acetyl-beta-D-glucosaminidase (O-GlcNAcase)
O-GlcNAc O-linked N-acetylglucosamine
PAM     Protein Associated with Myc
PDK-1   Phosphoinositide Dependent Kinase class protein kinase
PHR     Pam, Highwire, RPM-1
PI-3K   Phosphoinositide-3 Kinase
PKC     Protein Kinase C
PMK-3   p38 Map Kinase family
PSD     Postsynaptic Density
PTEN    Phosphatase and Tensin
RCC1    Regulator of Chromatin Condensation 1
RFP     Red Fluorescent Protein
RIM     Rab3 Interacting Molecule
RING    Really Interesting New Gene
RNAi    Ribonucleic Acid interference
Robo    Roundabout
RPM-1   Regulator of Presynaptic Morphology
sad-1   Synapses of the Amphids Defective
SAX-3   Sensory Axon guidance
SCF  Skp1, Cullin, F-box
SCL-1  SCP-Like extracellular protein
SGK  Serine/threonine protein Kinase, Sgk1
SH3  Src Homology 3 domain
SKN-1  SKiNhead
skr-5  Skp1 related (ubiquitin ligase complex component)
SNARE  Soluble N-ethylmaleimide-sensitive fusion (NSF) attachment protein receptor
SNB-1  Synaptobrevin
SOD-3  Superoxide Dismutase 3
SPRY  Sp1a and Ryanodine receptor
SYD-2  Synapse Defective
SYN-1  Syntaxin
TGF-β  Transforming Growth Factor β
ts  Temperature Sensitive
UNC  Uncoordinated
VAB-1  Variable ABnormal morphology-1
VAMP  Vesicle Associated Membrane Protein
VD  Ventral D-type
Wnt  Wingless-Int
Wt  Wildtype
2. Strains

*juIs1* strains

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ZM4863  fsn-1(hp1) III; ins-8(tm4144) juIs1 IV

oxIs22 strains
ZM4763  daf-16(mu86) I; oxIs22 II; daf-2(e1370) fsn-1(hp1) III
ZM4765  oxIs22 II; daf-2(e1370) fsn-1(hp1) III
ZM4766  daf-16(mu86) I; oxIs22 II
ZM4864  oxIs22 II; daf-2(e1370)

Ex strains (transgenic lines)
ZM2530  daf-2(e1370) fsn-1(hp1) III; hpEx791 (muscle daf-2)
ZM2532  daf-2(e1370) fsn-1(hp1) III; hpEx793 (neuron daf-2)
ZM3783  fsn-1(hp1) III; hpEx1447 (ins-1::GFP)
ZM4895  fsn-1(hp1) III; hpEx1956 (ins-1::GFP)
ZM4896  hpEx1957 (ins-1::GFP)
ZM3819  daf-16(mu86) I; daf-2(e1370) fsn-1(hp1) III; hpEx1465 (neuron daf-16)
ZM3828  daf-16(mu86) I; daf-2(e1370) fsn-1(hp1) III; hpEx1459 (muscle daf-16)

Is strains (stable integrated transgenes)
ZM4468  fsn-1(hp1) III; hpIs164
ZM4602  egl-3(gk238) V; hpIs164
ZM4817  rpm-1(ju44) V; hpIs164
ZM5006  hpIs164 (ins-18::GFP)
### 3. Oligonucleotides

#### PCR primers for genotyping *C. elegans* mutations

<table>
<thead>
<tr>
<th>Gene and allele</th>
<th>PCR Primer Pair</th>
<th>Wildtype Fragment</th>
<th>Mutant Fragment</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>daf-16</em> (mu86)</td>
<td>OZM1152/1153</td>
<td>n/a</td>
<td>1.3kb</td>
</tr>
<tr>
<td><em>fsn-1</em> (hp1)</td>
<td>OZM510/511</td>
<td>900bps</td>
<td>500bps, 400bps</td>
</tr>
<tr>
<td></td>
<td>+ BspH1 digest</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>mkk-4</em> (ok1545)</td>
<td>OZM799/441</td>
<td>1.9kb</td>
<td>1kb</td>
</tr>
<tr>
<td><em>oga-1</em> (1207)</td>
<td>OZM2491/2492</td>
<td>n/a</td>
<td>1.7kb</td>
</tr>
<tr>
<td><em>rpm-1</em> (ju44)</td>
<td>OZM1892/1893</td>
<td>535bps</td>
<td>324bps, 211bps</td>
</tr>
<tr>
<td></td>
<td>+ Accl digest</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>skr-5</em> (3068)</td>
<td>OZM2489/2490</td>
<td>n/a</td>
<td>800bps</td>
</tr>
</tbody>
</table>

*n/a: wildtype fragment is too large to be detected by regular PCR*
4. Plasmids

pJH616 $P_{\text{myo-3}}$ daf-2 muscle specific daf-2

pJH665 $P_{F25B3.3}$ daf-2 pan-neural daf-2

pJH1170 $P_{F25B3.3}$ daf-16 pan-neural daf-16

pJH1171 $P_{\text{myo-3}}$ daf-16 muscle specific daf-16

pJH1464 $P_{\text{myo-3}}$ ins-18::RFP muscle specific ins-18 (mCherry)

pJH1477 $P_{F25B3.3}$ ins-18::RFP pan-neural ins-18 (mCherry)

pJH1498 $P_{F25B3.3}$ ins-18::GFP pan-neural ins-18

pJH1922 $P_{F25B3.3}$ ins-1::GFP pan-neural ins-1