The F-box Protein FSN-1 Governs Presynaptic Development 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

Synapses are specialized sub-cellular junctions that transmit signals between neurons and their targets. In *Caenorhabditis elegans* (*C. elegans*) the F-box protein FSN-1 and the PHR family member RPM-1 form the SCF$_{FSN-1}$ E3 ubiquitin ligase, which plays an important role in regulating synaptic growth factors. This SCF complex is evolutionarily conserved across species, and regulates many cellular processes including axon outgrowth, apoptosis and synaptogenesis.

This thesis focuses on identifying targets of SCF$_{FSN-1}$ that contribute to synaptogenesis. Forward genetics was employed to screens and isolate mutants that exhibit genetic interactions with *fsn-1*. I have identified an allele of the MAPK *pmk-3(hp246)* and three alleles of the MAPKKK *dlk-1(hp180, hp192, hp195)* that suppress *fsn-1* defects. In addition, I have isolated five *fsn-1* suppressing alleles and evidence suggests that these suppressors are likely novel *fsn-1* suppressors.
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Specific Contributions

This project was made possible by the work of many individuals. My work benefitted from the guidance and experience of my supervisor Dr. Mei Zhen and the contribution of the following individuals:

Dr. Wesley Hung, Dr. Edward Liao and Maja Salihbegovic performed the $fsn-1;sad-1$ suppressor screen and isolated the mutants $hp246$, $hp180$, $hp192$ and $hp195$.

Dr. Mei Zhen and Tetyana Pekhar performed the $daf-16;fsn-1;sad-1$ screen and isolated the mutants in Table 1 and Table 2.
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1 Introduction

1.1 The Synapse

Neurons send or relay electrical and chemical signals to effector cells such as muscle and gland cells or to other neurons. Neurons connect to effector cells via sub-cellular junctions called synapses, which are essential for neuronal communication. In the late 19th century the underlying mechanism for the connectivity of the nervous system was heavily debated by physiologists, some believing the reticular theory which directly challenged the neuron doctrine as well as the cell theory in general. According to the reticularist theory, the nervous system is one continuous nerve process and serves as a conductor, like an electrical wire, for the current flow (Peters et al., 1976). The debate was understandable given the inability, at the time, to visualize cell membranes, let alone the imagined contact points between cells proposed by the neuron theory (Ramon y Cajal, 1937). These previously imagined contact points are now termed synapses, a word derived from ‘synapetin’, which was originally coined by Sir Charles Scott Sherrington. The word is derived from the Greek ‘syn’ (meaning ‘together’) and ‘hapetin’ (meaning ‘to clasp’). Synapses create neuronal circuits that underlie cognitive functions such as perception and thought. They allow our minds to control the body’s functions. Nowadays, electron microscopes and biochemical analysis has revealed that the synapse is highly structured and vastly complex with a myriad of molecular components. Although many proteins have been defined as synaptic components, many questions concerning what are involved in making and maintaining a synapse remain. The goal of my project has been to identify regulators of synapse development using forward genetics as the main tool for discovery.

There are two types of synapse in the nervous system that differ in function and morphology; yet both provide the means through which neurons communicate among themselves, and to other systems. Electrical synapses are mediated by specialized structures called gap junctions that form between two closely opposed neurons. Each cell membrane harbours what is called a hemi-channel. When two align, they form a gap junction and create a channel or pore connecting the cytoplasm of the two cells. This inter-cellular pathway provides a metabolic and electrical link between coupled cells that are 2-4nm apart (Hormuzdi et al., 2004; Palay, 1958; Revel and Karnovsky, 1967). The direct passage of molecules makes electrical synapses the most rapid form of communication between two cells. The bi-directionality of electrical
synapses allows neuronal groups to synchronize their activity (Connors and Long, 2004; Hormuzdi et al., 2004).

The chemical synapse is the main interest of this study; its general structure is outlined in Figure 1. It consists of a presynaptic membrane directly opposed to a postsynaptic membrane, separated by an extracellular space called the synaptic cleft (Palay, 1958). The presynaptic terminal stores organelles called synaptic vesicles that contain neurotransmitters. When released, neurotransmitters diffuse into the synaptic cleft and interact with their receptors that are present on the membrane of the postsynaptic terminal. There are many different types of neurotransmitters and the type present at each synapse defines the sub-class of chemical synapse for example the GABAergic, cholinergic, serotoninergic and glutamatergic synapses (Dale, 1933).
Figure 1. Structure of the chemical synapse. Chemical synapses are sub-cellular structures that mediate the transmission of signals in the form of neurotransmitter. They serve to connect neurons with other neurons as well as effector cells such as muscle or gland cells. The presynaptic terminal contains three discrete functional regions; the active zone, the synaptic vesicle pool and the periactive zone. In the periactive zone some factors or proteins that regulate synapse formation and development are present. Synaptic vesicles are loaded with neurotransmitters. At the active zone these synaptic vesicles dock and fuse with the plasma membrane and release neurotransmitters into the synaptic cleft. Neurotransmitters then diffuse across the synaptic cleft and activate their receptors clustered at the region of the postsynaptic membrane called the postsynaptic density.
1.2 An overview of synaptic structure

In mature chemical synapses, the presynaptic terminal can be subdivided into three distinct functional regions. The active zone (AZ) is a specialized region of the plasma membrane where synaptic vesicles dock and fuse, and neurotransmitter release occurs (Burns and Augustine, 1995; Couteaux and Pecot-Dechavassine, 1970). Neurotransmitters await release in the region termed the synaptic vesicle pool (von Schwarzenfeld, 1979). Finally the periactive zone surrounds the synaptic vesicle pool and the active zone; it contains many factors for synaptic vesicle retrieval, synapse growth and function (Sone et al., 2000).

1.2.1 Components and functions of the SNARE complex

Three main steps are necessary for releasing neurotransmitters from the presynaptic terminal. First synaptic vesicles must dock at the plasma membrane at the active zone. Next the synaptic vesicles undergo priming. Finally once competent for the final step, the synaptic vesicle fuses to the plasma membrane (Becherer and Rettig, 2006). Presynaptic Ca2+ channel opening is triggered by an action potential. The Ca2+ influx induces the activation of the SNARE (Soluble N-ethylmaleimide-sensitive component attachment protein receptor) complex, which mediates synaptic vesicle exocytosis by initiating the fusion of the synaptic vesicles with the plasma membrane (Chen et al., 1999; Weber et al., 1998).

Mainly involved in exocytosis, the SNARE complex is composed of a vesicle protein synaptobrevin and the plasma membrane proteins Syntaxin-1 and SNAP-25 which were elucidated as targets of the clostridial neurotoxins, as well as through genetic experiments (Jahn and Scheller, 2006; Montecucco et al., 2005; Sudhof, 2004). They form a stable complex consisting of four parallel alpha helix bundle, contributed by the SNARE motifs common to the three proteins (Poirier et al., 1998; Sutton et al., 1998). The SNARE complex assembles in a N-to C-term direction (Sudhof, 2004). The SNARE complex is formed during vesicle priming, when the vesicle SNARE domain interacts with the target SNARE domain that results in the formation of an unstable intermediate bringing the synaptic vesicle in close proximity to the plasma membrane (Jahn et al., 2003; Rizo, 2003; Sudhof, 2004). The fusion pore is formed when the SNARE intermediate is stabilized by a Ca2+ influx, sensed by synaptotagmin (Chapman et al., 1995). The SNARE complex assembly appears sufficient to mediate membrane fusion (Weber et al., 1998). Current research is being directed at understanding how
other factors regulate the SNARE complex formation during synaptic vesicle docking, priming and fusion (reviewed in (Rizo and Rosenmund, 2008).

1.2.2 The active zone

The active zone (AZ) is the site of synaptic vesicle clustering, docking and fusing, and therefore is the site of neurotransmitter release (Couteaux and Pecot-Dechavassine, 1970). It is closely and precisely aligned with the area of the post synaptic cell called the post synaptic density (PSD), which is the area of the membrane harboring the neurotransmitter receptors. The AZ can be further subdivided into three morphologically and functionally distinct components (Zhai and Bellen, 2004; Zhen and Jin, 2004; Ziv and Garner, 2004): (1) The plasma membrane region, which is directly juxtaposed to the post synaptic density and mediates neurotransmitter release; (2) the adjacent intracellular membrane component called the cytomatrix, where synaptic vesicles dock; and (3) the projections extending from the cytomatrix that tether synaptic vesicles. The second and third components comprise what is known as the cytomatrix at the active zone (CAZ).

The plasma membrane at the active zone controls the organization of voltage-gated Ca2+ channels and the SNARE complex necessary for synaptic vesicle fusion at the membrane (Zhai and Bellen, 2004). The membrane contains both N (Leveque et al., 1992; Yoshida and Plant, 1992) and/or P/Q type Ca2+ channels (Lopez et al., 2001) that regulate Ca2+ influx at the presynaptic fusion site. It also contains core components of the SNARE complex SNAP-25 and syntaxin (Bennett and Scheller, 1994; Sollner et al., 1993; Sudhof et al., 1993). It is hypothesized that in order to form and maintain a tight connection with the PSD, the immediate or flanking plasma membrane regions also contain cell adhesion molecules (CAMs) (Ziv and Garner, 2004). Integrins (Chavis and Westbrook, 2001), nectins (Mizoguchi et al., 2002; Takai et al., 2003), cadherins (Shapiro and Colman, 1999; Yagi and Takeichi, 2000), neural CAM (NCAM) (Rougon and Hobert, 2003), synaptic CAMs (SynCAMs) (Biederer et al., 2002) and neurexins comprise the many classes of such CAMs (Missler and Sudhof, 1998).

Synaptic vesicle priming, translocation to the active zone, and exocytosis is thought to be regulated by the CAZ (reviewed in (Dresbach et al., 2001). The CAZ contains many well
studied proteins including those that interact with the SNARE complex, Munc13, RIM (Rab3 interacting molecule) and Munc18 (Hata et al., 1993; Rizzoli and Betz, 2005; Verhage et al., 2000). The CAZ also contains an abundance of additional proteins including Bassoon (tom Dieck et al., 1998), Piccolo (Cases-Langhoff et al., 1996) and ELKS/CAST/ERC (glutamate(E) leucine(L), lysine(K) and serine(S) rich proteins/CAZ associated structural proteins/ELKS Rab-6 interacting protein 2 and CAST) (Ohtsuka et al., 2002).

The Munc13s are essential in the priming of synaptic vesicles (Aravamudan et al., 1999; Augustin et al., 1999; Richmond et al., 1999; Varoqueaux et al., 2002). RIMs are a family of multidomain scaffolding proteins that interact with many of the active zone proteins; they are known to play a role in synaptic vesicle release as well as short and long term plasticity (Mittelstaedt et al.; Wang et al., 1997). The ELKS/CAST/ERC proteins are a more recently identified and abundant protein of the AZ. They have been found to bind directly to AZ proteins RIM1, Piccolo, and Bassoon and indirectly with Munc13-1 through RIM1 (Lu et al., 2005; Ohtsuka et al., 2002; Takao-Rikitsu et al., 2004; Wang et al., 2002). CAST interaction with RIM1 and Bassoon appears to be involved in neurotransmitter release, whereas ELKS is important in the assembly and function of the AZ. Piccolo and Bassoon are large scaffold components of the AZ cytomatrix at 420 and 530 kDa respectively (Cases-Langhoff et al., 1996; tom Dieck et al., 1998). They are related, possibly functionally redundant, multi-domain proteins that play roles in synaptic vesicle clustering (Mukherjee et al.). Liprins interact directly with other AZ proteins, RIM and ELKS and indirectly with Piccolo (Kim et al., 2003; Ko et al., 2003a; Ko et al., 2003b; Schoch et al., 2002). Liprins-α are not only found at the AZ. In both Drosophila and C. elegans active zone formation and maintenance also involves liprins-α (Kaufmann et al., 2002; Zhen and Jin, 1999). The AZ proteins mentioned above all have roles in synaptic function and formation, which are relatively well defined. Many more AZ proteins exist but their roles are less understood.

1.2.3 The synaptic vesicle pool

The synaptic vesicle pool is one of the defining features of the chemical synapse. Synaptic vesicles store neurotransmitters that are released upon Ca2+ influx at the active zone plasma membrane. The neurotransmitters can be separated into 3 functionally distinct groups (Elmqvist
and Quastel, 1965; Liley and North, 1953; Zucker and Regehr, 2002). Although these groups have been assigned many different titles they are generally termed the ready releasable pool (RRP), the recycling pool, and the reserve pool (Rizzoli and Betz, 2005). The RRP is comprised of the synaptic vesicles that are instantly available upon stimulation. Just milliseconds of depolarization (Mennerick and Matthews, 1996; Neves and Lagnado, 1999) will rapidly deplete the RRP. The recycling pool is clustered between the RRP and the reserve pool; some of its synaptic vesicles are likely tethered to the active zone through a matrix of filaments (Brodin et al., 1997; Landis, 1988; Pieribone et al., 1995). Upon physiological frequencies of stimulation it is refilled by recently recycled vesicles (de Lange et al., 2003; Harata et al., 2001). The reserve pool contains the majority of the synaptic vesicles at the presynaptic terminal that are distal to the AZ. These vesicles are only released under intense stimulation. The frog NMJ (neuromuscular junction) requires stimulation frequencies of 10-15 Hz (Delgado et al., 2000; Heuser and Reese, 1973; Richards et al., 2000), and the *Drosophila* NMJ requires 30 Hz to stimulate the release of the reserve pool vesicles (Kuromi and Kidokoro, 2000).

### 1.2.4 The periactive zone

The periactive zone is defined by molecular components that are localized to the region surrounding the active zone. It generally contains plasma membrane regions, as well as factors that regulate synaptic vesicle recycling and synaptic growth (Jin, 2002; Sone et al., 2000). The *Drosophila* dynamin *shibrie* regulates endocytosis and localizes to the periactive zone (Sone et al., 2000). Localized to the *C. elegans* periactive zone is RPM-1, a component of an E3 ubiquitin ligase complex SCF^{FSN-1} (Liao et al., 2004; Wan et al., 2000; Zhen et al., 2000). SCF^{FSN-1} will be discussed in more detail in separate sections as it is the main focus of this thesis.

### 1.2.5 The post synaptic terminal

At the postsynaptic terminal, there are neurotransmitter receptors, ion channels and scaffolding and signaling molecules all waiting to respond to the presynaptic release of neurotransmitters in the region termed the postsynaptic density (PSD). Some well established scaffolding proteins
including PSD-95, Shank, Liprins and MAGUKs (membrane associated guanylate kinases) assemble neurotransmitter receptors, adhesion molecules, as well as signal transducers (Cho et al., 1992; Kim and Sheng, 2004; Montgomery et al., 2004; Naisbitt et al., 1999; Serra-Pages et al., 1995).

1.3 Synapse formation

1.3.1 Nature of synapse formation

During synapse formation neurons must recognize their proper synaptic partners. *in vitro* synaptic factors such as neuroligins can cause synaptic formation in nonneuronal cells (Scheiffele et al., 2000). Therefore, among so many potential contacts, selecting a proper synaptic partner is critical to developing a functional neuronal circuit (Yamagata et al., 2003). There are two general manners in which synapse formation occurs: *en passant*, which describes synapses forming along the axon shaft and *terminaux*, which describes synapses forming at the termini of axon branches.

1.3.2 Active zone assembly

Major aspects of presynaptic assemble include: formation of a presynaptic density juxtaposed to a post synaptic cell, establishment of the active zone, and the aggregation of synaptic vesicles (Murthy and De Camilli, 2003). Synaptic vesicles are docked in the active zone which appears as an electron dense “presynaptic grid” presumably due to scaffolding proteins (Akert, 1972; Pfenninger et al., 1969).

Loss-of-function mutations in the *C. elegans* liprin-α/syd-2 gene (for synapse defective-2) cause diffuse localization of presynaptic proteins and less electron-dense active zones (Zhen and Jin, 1999). Liprins are LAR-interacting proteins that interact with LAR-type (for leukocyte common antigen related) receptor proteins with tyrosine phosphatase activity (RPTPs) (Serra-Pages et al., 1998). Liprin-α proteins have been shown to bind to other active zone proteins RIM and CAST/ERC (Ko et al., 2003b). The localization of the presynaptic RIM/UNC-10 is also altered
in *syd-2* mutants (Ackley et al., 2005). SYD-2 localizes to synapses independent of synaptic vesicles (Zhen and Jin, 1999) and SYD-2 is found directly in the presynaptic density using immunoelectron microscopy (Yeh et al., 2005). Liprins can physically interact with the leukocyte common antigen-related protein (LAR) – receptor tyrosine phosphatases (RPTPs) (Serra-Pages et al., 1998), and the LAR-liprin complex clusters to focal adhesions (Serra-Pages et al., 1995). Moreover, studies have shown that *Drosophila* LAR binds to the extracellular matrix (ECM) molecules Syndecan and Dallylike (Johnson et al., 2006). A *C. elegans* LAR long isoform PTP-3A also localizes to synapses and mutations specifically for *ptp-3a* cause synapse morphology defects (Ackley et al., 2005). LAR has been shown to bind Nidogen-laminin in mammalian cultured cells (O'Grady et al., 1998) and *C. elegans* Nidogen/nid-1 (nidogen) mutants show similar synaptic defects as *syd-2* mutants (Ackley et al., 2003). In *Drosophila*, Dlar and Dliprin also regulate synaptic bouton size and shape (Kaufmann et al., 2002), and vertebrate liprins and LAR have been shown to regulate excitatory synapse morphology (Dunah et al., 2005). It is hypothesized that liprin-α, nidogen and LAR-RPTP interact to organize the presynaptic assembly (Ackley et al., 2005).

### 1.3.3 Vesicle clustering

A kinesin-like motor protein UNC-104 is required for synaptic vesicle transport from the cell body to the presynaptic terminal (Hall and Hedgecock, 1991). Another motor protein kinesin-1, consisting of UNC-116 (the kinesin-1 heavy chain) and KLC-2 (the kinesin-1 light chain), forms a tetramer (Patel et al., 1993; Sakamoto et al., 2005). Mutations in either chain also affect synaptic vesicle trafficking (Byrd et al., 2001; Sakamoto et al., 2005).

A link between synaptic vesicle recycling and anterograde transport of synaptic vesicles remains unclear; however, the presence of clathrin coated vesicles at the presynaptic terminal indicates that vesicles can be assembled *de novo* at synapses (Murthy and De Camilli, 2003). *C. elegans* mutants identified by behavioral defects or through aldicarb resistance screens (Nguyen et al., 1995) have led to the identification of the key regulators of synaptic vesicle endocytosis proteins, Synaptojanin/UNC-26 (Harris et al., 2000), Endophilin/UNC-57 (Schuske et al., 2003), and AP180/UNC-11 (Nonet et al., 1999). Mutations of these genes cause a drastic reduction of
synaptic vesicles at the presynaptic terminal, as well as the presence of immature synaptic vesicle intermediates.

SAD-1 is a novel serine/threonine kinase expressed in the nervous system and related to PAR-1, a kinase that regulates cell polarity during asymmetric cell division (Guo and Kemphues 1995). SAD-1 may also have a role in organizing synaptic vesicles as sad-1 mutants exhibit a broader distribution of synaptic vesicles than that of wild type animal at NMJs (Crump 2001). Moreover an overexpression of SAD-1 caused ectopic clustering of synaptic vesicle markers at dendritic regions (Crump 2001).

1.4 *C. elegans* as a model organism

1.4.1 *C. elegans* as a genetic model

The free-living nematode *C. elegans* is an excellent model organism for genetic studies. *C. elegans* shares a high degree of conservation with higher organisms in respect to cellular structures and molecular pathways that govern nervous system development and function (reviewed in Jin 2002, Chisholm and Jin 2005). *C. elegans* offers the advantage of a transparent body that benefits cell biology studies, hermaphroditic and sexual means of replication, fast life cycle for easy genetics, and well-described, invariable cell lineage (Brenner 1974; Antoshechkin and Sternberg, 2007).

A major advantage of the *C. elegans* model came in 1998 when it became the first multicellular organism to have its genome completely sequenced (1998). This breakthrough led to excellent genome annotation and allowed the application of various bioinformatic tools. In addition, the complete sequencing of a related nematode species, *C. briggsae*, makes them an excellent model for comparative genomics (Stein et al., 2003). Researchers chose to sequence the genome of these nematodes because they were already heavily used as model organisms.
1.4.2 *C. elegans* nervous system

Particular model organisms often lend themselves in varying degrees to different areas of biological studies. *C. elegans* is a choice organism for the study of nervous system development due to their small and invariant number of neurons. As a result, researchers have been able to determine the precise identity, connectivity and cell lineage of each of the hermaphrodite’s 302 neurons by electromicroscopic reconstruction (Sulston, 1983; White et al., 1976, 1986).

1.4.3 *C. elegans* neuromuscular junctions as a model for synapse development and function

The *C. elegans* nervous system contains about 8000 chemical synapses; ~2000 of these connections are neuromuscular junctions (NMJs) (White et al., 1976, 1986). NMJs are specialized synapses in which the presynaptic cell is a neuron and the postsynaptic cell is a muscle. In *C. elegans*, synapses form between neurons or between neurons and muscle arms in an *en passant* manner (White et al., 1986). Muscle arms are filopodial-like processes that extend from the muscle cells towards the motorneuron processes that run along the dorsal and ventral nerve cord of the animal. In general, acetylcholine (ACh) is released at excitatory NMJs leading to muscle contraction, while γ-aminobutyric acid (GABA) is released at inhibitory NMJs to cause muscle relaxation (although excitatory GABAergic synapse have been described) (Gao and Zhen, in press; McIntire et al., 1993). The sinusoidal body bends of the *C. elegans* movement are proposed to be generated via a contra-lateral activation model of the locomotion circuit; muscle contraction on one side of the body triggered by excitatory synapses is paired with simultaneous relaxation of contralateral muscles. The GABAergic motorneurons responsible for muscle relaxation are the DD and the VD motorneurons (McIntire et al., 1993). *C. elegans* mutants defective in synaptic transmission often exhibit a locomotion phenotype resulting from disrupted signaling at NMJs. Recent advances in the development of live fluorescent synaptic markers, as well as electrophysiology have allowed researchers to more closely study *C. elegans* NMJs. A disruption in the development and synaptic transmission at NMJs often disrupts smooth sinusoidal locomotion (Francis et al., 2003; Richmond et al., 1999; Richmond and Jorgensen, 1999).
1.4.4 SNB-1::GFP as a synaptic marker

In *C. elegans* the Synaptobrevin::GFP fusion reporter has become a common tool to visualize synapses *in vivo* (Hallam and Jin, 1998; Nonet, 1999). Synaptobrevin (SNB-1) is a synaptic vesicle membrane protein (Rand, J. B. & Nonet, M. Synaptic transmission, *C. elegans* II). The fusion protein SNB-1::GFP is constructed with the GFP fused to the C-terminus of SNB-1. The efficacy of this marker has been tested to prove it is indeed labeling synaptic vesicles. Firstly the SNB-1::GFP co-localizes with endogenous synaptic vesicle proteins and has the same staining pattern as the synaptic vesicle protein SNT-1, suggesting the GFP tag is not altering the localization of the protein (Nonet, 1999). Secondly when fused to an engineered SNB-1 protein missing the transmembrane domain, the synaptic restriction of the GFP is lost (Nonet, 1999). Lastly in a kinesin/unc-104 mutant background, where synaptic vesicles fail to be transported out of the soma, the GFP signal is seen mainly in the cell bodies (Hall and Hedgecock, 1991; Jin, 2002; Otsuka et al., 1991). Expressing the SNB-1::GFP marker in interneurons has further revealed that it is directly opposed to post-synaptic markers GLR-1 and LIN-10 (Rongo et al., 1998; Shen and Bargmann, 2003). Several groups have generated multiple GFP-based synaptic markers allowing the visualization of various types of synapses (Francis et al., 2005; Sieburth et al., 2005; Yeh et al., 2005).

I have utilized a version of the SNB-1::GFP marker called *juIs1*. It is driven by the *unc-25* promoter which limits the expression to the GABAergic VD and DD motorneurons (Hallam and Jin, 1998; Jin et al., 1999; Zhen and Jin, 1999). The *unc-25* gene encodes for a biosynthetic enzyme, glutamic acid decarboxylase (GAD) which is necessary for GABA synthesis (Jin et al., 1999). In wild type animal SNB-1::GFP fluoresces in the presynaptic terminals of the 13 VD and 6 DD neurons, and appears as small puncta with relatively even spacing and size (Figure 2a). This *juIs1* pattern corresponds with the known synaptic pattern (White et al., 1986). The consistent expression pattern of *juIs1* allows us to examine and search for synaptic mutants. Our laboratory members have successfully preformed forward genetic screens using *juIs1* to identify genes involved in synaptic development (Zhen and Jin, 1999, Liao et al., 2004).
Figure 2. *fsn-1* mutants display synaptic morphology defects.
Vesicle marker *juls1* in GABAergic motoneurons in wild-type (a) and *fsn-1* (b) animals. Arrows indicate clustered puncta; arrowheads indicate gaps. (c) Quantification of the total number of *juls1* puncta by DD motorneurons; n = 30. Asterisk, P < 0.001 compared with wild type (Kruskal–Wallis test). Error bars show standard deviation. Scale bar, 5 mm. (adapted with permission from Liao et al. 2004)
1.5 Identifying genetic interactors

1.5.1 Genetic Suppressor screens

Genetic suppressor screens are a specific type of a simple forward genetics and are an extremely effective genetic tool and particularly suited to *C. elegans* research. It is not cumbersome to deal with a very large population of *C. elegans*; therefore even rare mutational events can be recovered by EMS mutagenesis. In addition *C. elegans* hermaphrodism allows for easy recovery and maintenance of both recessive and dominant suppressors. Finally, very strong selection can be applied; for example, among a population of paralyzed animals a suppressor restoring movement is easily found. Therefore detecting a single mutation within a vast amount of events becomes relatively easy.

Generating genetic suppressors is one of the most powerful tools a geneticist has. Often it allows researchers to identify genes that may have no obvious phenotype on their own, but are able to suppress the phenotype of another mutation. Suppressors can lie in the same gene as the initial mutation or in a different gene and are termed intragenic or extragenic respectively.

Intragenic suppression can come in a variety of forms but general types do occur. The simplest and least informative type of intragenic suppression is a reversion which can occur by mutating the DNA sequence back to wild type. Intra-codonic suppression can also occur where a functional product is created by a mutation to a different base. An example would be the collagen-processing protease *dpy-31* Gln-to-Pro mutation that can be suppressed by either a back mutation Pro-to-Gln or by the different mutation Pro-to-Ser (Novelli et al., 2004). A rarer occurrence is a compensatory second site mutation that restores function to a non-functional protein or knocks-out the function of a dominant allele. The recessive temperature-sensitive *glp-1*, a cell interaction protein, is a good example because it has been suppressed by second site mutations that are able to correct the original defect (Lissemore et al., 1993). Finally second site mutations can occur that lead to the omission of the original mutation through exon skipping. *unc-52* is an example where a G-to-A change of an acceptor site caused the loss of an exon containing a deleterious mutation and leads to an in-frame and functional protein (Rogalski et al., 1995).
An extragenic mutation is often desired to gain insight into genes related to the gene of interest. Extragenic suppression can occur in an information sense affecting the mechanisms of translation, alternative splicing or nonsense-mediated decay (Hodgkin et al., 1989; Waterston and Brenner, 1978; Zahler et al., 2004). Additionally, extragenic suppressors have been invaluable in unveiling entire signaling pathways. The epistasis relationships in linear genetic pathways can be determined by perturbing and/or activation different members of the pathways and using a phenotype that is caused by altered pathway activity (Avery and Wasserman, 1992).

In this study I use genetic suppression to search for targets of a known SCF ubiquitin ligase.

1.5.2 Alternative to Forward Genetics: Reverse Genetics to identify novel interactions

Suppressor screens allow for specific gene interactions to be elucidated. As this thesis is concerned mainly with discovering novel genes that interact with \textit{fsn-1}, it is important to discuss additional methods for identifying unknown genes.

Reverse genetics as implied is opposite in principle to forward genetics in that an investigation begins with a known gene, and an unknown mutant phenotype. Reverse genetics is a powerful and complementary approach to forward genetics. For example, with the discovery of a gene using forward genetics, reverse genetics approaches can be used to investigate functions of genes with homologous sequence in the same or another organism. With the genomic sequence now known for most model organisms, a reverse genetics approach can be applied to explore the function of almost every gene.

Most notably two types of reverse genetics approaches are used: 1) RNA interference (RNAi) to knockdown a gene’s expression hence its functions; and 2) deletions that permanently remove the gene from the genome, and thus its functions along with it. Which method to use can depend on many factors, but generally RNAi is most convenient for a global survey of gen function, whereas when the focus is on only a few genes, the extra time investment in generating genetic mutants is favoured.
1.6 The ubiquitin pathway regulates protein levels

The cell controls protein levels in two opposing manners; the *de novo* creation of new proteins, and the degradation of proteins when levels are too high, or the protein is no longer required. The ubiquitin pathway is one of the mechanisms responsible for negative regulation of protein levels both temporally and spatially (Hershko and Ciechanover, 1992). Many different molecular pathways are involved in the formation of synapses, including the ubiquitin pathway (Liao et al., 2004; Zhen et al., 2000). This pathway is involved in the degradation, internalization, trafficking and sequestering of proteins in a number of processes including endocytosis, signal transduction, cell-cycle progression, transcriptional regulation and receptor down-regulation (Hershko and Ciechanover, 1998).

Throughout the cell, specific proteins are targeted for degradation by the ubiquitin proteosome pathway (UPS). Upon the targeted proteins ‘tags’ are deposited in the form of a small 76 amino acid protein called ubiquitin (Ub). The ubiquitin protein is passed by a cascade of three enzymes to the eventual target. Briefly, Ub is specifically activated in an ATP-dependent manner by the E1 activating enzyme. Next the activated Ub is transferred to a carrier protein called the E2 or the conjugating enzyme. Finally, it is catalyzed by the E3 ubiquitin ligase, which transfers the Ub to a specific substrate recruited by the E3 ligase (Ciechanover, 1994; Hershko, 1983; Hershko and Ciechanover, 1992).

1.6.1 SCF complexes are E3 ligases

Among the superfamily of E3 ligases the SCF complexes have been implicated in all types of cellular processes including synaptic development (Cardozo and Pagano, 2004; Liao et al., 2004; Zhen et al., 2000). The typical SCF complex is composed of a F-box protein, a RING domain containing protein, and adaptor proteins Skp1 and Cul1 (Figure 3A). The RING domain protein, usually Rbx1/Roc1/Hrt1, binds to the C-terminus of Cul1. The Ub-loaded E2 is contacted by the RING domain protein. Over 70 F-box proteins have been identified in humans. They may recruit specific substrates to the SCF complex (Patton et al., 1998; Skowyra et al., 1997). Its F-box domain, being the hallmark feature of the E3 ligase class binds to the adaptor protein Skp1. F-box proteins also contain substrate recognition domains, often being a WD or LRR repeat
domain among many others. A major focus of my thesis is the *C. elegans* neuronal-specific SCF^{FSN-1}. This complex contains two invariable members: the adaptor protein SKP/SKR-1 and the scaffolding protein Cul1/CUL-1. The variable members are RPM-1, a large protein containing a RING-H2 zinc finger motif, and the F-box protein FSN-1 (Liao et al., 2004; Zhen et al., 2000). The murine orthologues of FSN-1 and RPM-1, Fbxo45 and PAM respectively, have been shown to form an a-typical SCF complex with the exclusion of Cul (Figure 3B) (Saiga et al., 2009), it is currently unclear exactly how the *C. elegans* SCF^{FSN-1} is forming.
Figure 3: Model for formation of a SCF complex. A typical SCF complex (A) and an Fbxo45-PAM complex (B). In typical F-box proteins, the F-box domain interacts not only with Skp1 but also with Cul1, resulting in the formation of an SCF complex. In the SCF^{Fbxo45}, the SPRY domain of Fbxo45 binds directly to PAM, resulting in the formation of an Fbxo45-PAM complex that excludes Cul1. Ub, ubiquitin (adapted from Saiga et. al. 2009).
1.7 The role of PHR family proteins

1.7.1 PHR family of proteins are negative regulators of axon and synapse development

The PHR (Pam, Highwire, RPM-1) proteins are large multi-domain proteins now implicated in many biological processes. PHRs have the following conserved motifs: the RING-H2 zinc fingers, a hallmark feature of an E3 ubiquitin ligase, a B-box zinc finger, a putative Myc binding region, two PHR-family protein unique repeats and a RCC1 like domain with putative guanine exchange activity (Guo et al., 1998).

Pam (Protein associated with Myc) was the first PHR to be identified as an interactor with Myc in the human Akata Burkitt’s lymphoma cell line (Guo et al., 1998). RPM-1 is the C. elegans member of the PHR family of proteins. rpm-1 mutants were found in C. elegans forward genetic screens for mutants with presynaptic morphology defects in the VD and DD neuromuscular junctions, as well as in mechanosensory neurons (Schaefer et al., 2000; Zhen et al., 2000). The Drosophila homologue Highwire (Hiw), exhibited overgrowth of their NMJs (Wan et al., 2000). Both the worm and fly mutants suggest that RPM-1 and Highwire are regulators of synapse formation. Later studies in the zebrafish PHR member Esrom and the mouse PHR Phr1, however has unveiled a role for PHRs in axon navigation in neural development (Burgess et al., 2004; D'Souza et al., 2005; Karlstrom et al., 1996).

1.7.2 rpm-1 mutants have nervous system defects

rpm-1 mutants exhibit an over-development of some synapses and an under-development of others in the GABAergic system; cholinergic and glutamatergic synapses are also affected (Park et al., 2009; Zhen et al., 2000). Observed both by electron microscopy and with antibody staining of the active zone and the synaptic vesicle pool, the enlarged synapses were found to contain multiple active zones (Zhen et al., 2000). At under-developed synapses there is an accumulation of electron dense material and almost an absence of synaptic vesicles (Zhen et al., 2000). In addition to the synaptic defects, rpm-1 mutants also exhibit excessive axon branching of the PLM mechanosensory neuron (Schaefer et al., 2000). However in C. elegans, rpm-1 mutants do not display any obvious locomotion phenotype; animals are slightly shorter but are
otherwise normal looking and active (Schaefer et al., 2000; Zhen et al., 2000). Double mutants between \textit{rpm-1} and mutations in other synaptic genes, \textit{syd-2} and \textit{sad-1}, have the useful phenotype of being severely uncoordinated.

1.7.3 FSN-1 and RPM-1 participate in a neural specific SCF E3 ubiquitin complex

The F-box protein FSN-1 was discovered in our lab in a genetic screen for mutants with a similar phenotype to \textit{rpm-1}. Similar to \textit{rpm-1}, \textit{fsn-1} mutants have an over-development of some synapses, and under-development of others (Figure 2b,c) (Liao et al., 2004). The double mutants \textit{fsn-1;sad-1} or \textit{fsn-1;syd-2} also exhibit the same uncoordinated movement as the \textit{rpm-1} double mutants, and the \textit{fsn-1} mutants alone appear morphological and behaviorally normal (Liao et al., 2004). Moreover the synapse morphology defects are not enhanced in \textit{fsn-1;rpm-1} double mutants, suggesting that FSN-1 and RPM-1 function together (Liao et al., 2004).

FSN-1 forms a SCF-like complex with RPM-1, CUL-1 and SKR-1. Similar to RPM-1, FSN-1 is expressed exclusively in the nervous system, although its localization is not limited to perisynaptic regions (Liao et al., 2004). In addition to the F-box domain, it contains a SPRY domain that is thought to act as the target recognition domain in lieu of the typical WD or LRR domain. Importantly, FSN-1, as well as its interaction with RPM-1, is conserved throughout evolution. The \textit{Drosophila} FSN-1 homologue DFsn belongs to the same SCF complex along with Hiw (Wu et al., 2007). In mouse the loss of function mutants of its homologue Fbxo45 and the RPM-1 homologue Phr1 exhibit the same respiratory failure at birth and axon defects in the CNS (Bloom et al., 2007; Burgess et al., 2004; Lewcock et al., 2007; Saiga et al., 2009). Interestingly the murine SCF\textsuperscript{Fbxo45} complex has been found to involve Phr1, Fbxo45 and Skp1, but the scaffolding protein Cullin is excluded (Saiga et al., 2009).
1.8 Targets of SCF^{FSN-1}

1.8.1 fsn-1 genetically interacts with scd-2

Extensive work has gone into searching for the targets of the SCF^{FSN-1} complex. The most obvious and thus far most effective method has been to conduct suppressor screens of the fsn-1 or rpm-1 mutant phenotypes. The *C. elegans* receptor tyrosine kinase homologous to a mammalian proto-oncogene anaplastic lymphoma kinase (ALK), also known as SCD-2 was found to partially suppress the synaptic morphology defects of *fsn-1* animals. SCD-2 levels were increased in both *fsn-1* and *rpm-1* animals (Liao et al., 2004). However whether SCD-2 is directly targeted by SCF^{FSN-1} for ubiquitination or just FSN-1 to affect synaptic development was not examined.

1.8.2 The MAP kinase signaling pathway is negatively regulated by SCF^{FSN-1}

Nakata et al. found that a p38 MAPK (Mitogen Activated Protein Kinase) signaling pathway is negatively regulated by RPM-1 (Nakata et al., 2005). This signaling cascade is composed of DLK-1, a duel leucine zipper MAPKKK, MKK-4, a MAKK and PMK-3, the p38 MAPK. The MAPK p38 has been extensively studied and found to have roles in synaptic plasticity, long-term depression and axon regeneration (Bolshakov et al., 2000; Guan et al., 2003; Krapivinsky et al., 2004; Thomas and Huganir, 2004; Zhu et al., 2002). The synaptic defects of *rpm-1* animals are potently suppressed by an inactivation of any of the MAPK pathway members, but most sensitively to DLK (Nakata et al., 2005). Moreover, the over-expression of a genetically engineered and constitutively activated form of MKK-4 partially phenocopies *rpm-1*. Expressing the active MKK-4 and the synaptic morphology defect as an indicator, the order of the kinase cascade was determined (Nakata et al., 2005). Finally DLK-1 was determined to be the direct target of the SCF^{FSN-1} through an *in vitro* ubiquitination of DLK-1 by RPM-1, and an increased level of DLK-1 is observed in the absence of RPM-1 (Nakata et al., 2005). Importantly it seems that activated DLK-1 is specifically targeted for degradation by RPM-1 (Abrams et al., 2008). In accordance with the evolutionary conservation of this pathway, the *Drosophila* homologue of DLK-1, Wallenda, is also a partial genetic suppressor of Highwire (Collins et al., 2006). In embryonic motorneurons cultures the localization of murine DLK-
1(DLK) and p38 MAPK also appear to be negatively regulated by murine RPM-1(Phr1). DLK localizes to growth cones and Phr1 to axon shafts however in Phr1 mutants DLK was detected in distal axons and P38MAPK inhibition reversed axon defects of Phr1 mutants (Lewcock et al., 2007).

1.8.3 There are unidentified targets of RPM-1 and FSN-1

Many lines of evidence suggest that additional targets of the SCF^{FSN-1} complex remain to be uncovered. Loss of function mutations in dlk-1/wallenda do not fully restore the synaptic defects of rpm-1/Highwire. In zebrafish, Esrom mutants’ defects in axon outgrowth and targeting are not associated with abnormal p38 signaling (Hendricks and Jesuthasan, 2009). Moreover, the CNS defects of the Phr1 knockout mouse are not suppressed by Dlk and there is no detectable changes in Dlk levels (Bloom et al., 2007; Saiga et al., 2009). Therefore the MAPK pathway cannot be the only target of the SCF^{FSN-1} complex.

1.8.4 The insulin/IGF-like pathway is involved in synapse development

fsn-1 synaptic defects are suppressed by mutation in the MAPK pathway members DLK-1, MKK-4 and PMK-3. In addition we have found that mutations in an insulin/IGF receptor DAF-2 can also partially suppress the defects of fsn-1 mutants. daf-2 mutants, however, do not significantly rescue rpm-1, suggesting that although FSN-1 complexes with RPM-1, its biological functions are not exclusive to this relationship.

The C. elegans genome encodes 40 different insulin and IGF-like molecules, however DAF-2 represents the lone insulin/IGF-like receptor tyrosine kinase. In C. elegans, DAF-2 is auto-phosphorylated in response to the binding of an insulin-like ligand, the PI-3 kinase AGE-1 is then recruited and activates a cascade of phosphorylation events that include PKD-1 the 3-phosphoinositide dependent kinase, and ATK-1 and AKT-2 serine/threonine kinases (Mukhopadhyay et al., 2006). Finally a forkhead transcription factor DAF-16 is phosphorylated and sequestered in the cytoplasm, preventing it from its function as both an activator and repressor of gene transcription (McElwee et al., 2006; Murphy et al., 2003). Components of
Insulin/IGF signaling pathway are expressed ubiquitously, and the signaling pathway has been implicated in a myriad of biological processes including oxidative-stress resistance, apoptosis, cell-cycle progression and longevity (Murphy, 2006).

The role that insulin and insulin-like growth factors play in the nervous system has been under investigation for many years. For instance it is known that cognitive defects are associated with diabetic patients and that a treatment with insulin for both diabetic and unaffected individuals can enhance their short-term memory and attention (Hoyer, 2004; Stockhorst et al., 2004; Strachan, 2005). Furthermore a glucose dependent insulinotropic polypeptide (GIP) that is known to enhance insulin secretion has been shown to reverse the impairment of long-term potentiation (LTP) induced by beta-amyloid, a peptide implicated in Alzheimers patients (Gault and Holscher, 2008). Insulin signaling has been implicated in Parkinson’s disease, where decreased expression of both insulin and insulin/IGF receptors in white matter and the amygdale of human postmortem brain tissue were found (Tong et al., 2009). These observations seem to be building towards the idea of insulin as a therapy for neurodegenerative disease, or at least a need for proper insulin signaling in neurological functions. However we know very little as to how insulin signaling is affecting synaptic function at the physiological level. The identification of the genetic interaction between daf-2 and fsn-1 allows us to begin to examine the connection between insulin signaling and the nervous system.

1.9 Goals of this Project

1.9.1 Investigating targets of SCF^{FSN-1} as new synaptic regulators

The aim of this thesis is to identify genetic suppressors of fsn-1 synaptic morphology defect. Through these suppressors, I expect to identify components of various signaling pathways involved in synaptic development and regulated by FSN-1. I began with the mapping and characterization of such a suppressor hp246. I describe the discovery that hp246 is a novel allele of pmk-3, a member of the MAPK pathway and a known suppressor of rpm-1. This is followed by the identification and characterization of additional suppressors of fsn-1 that represent new alleles of MAPK pathway members, as well as those that do not map to, or are able to complement alleles of the known MAPK members.
2 Materials and Methods

2.1 C. elegans Culture

C. elegans strains were cultured and maintained on NGM plates seeded with OP50 Escherichia coli, and kept at 20°C or 15°C incubators (Brenner, 1974).

2.1.1 NGM plates

In order to create 3 Liters of NGM Agar the following steps were taken:

Using sterile techniques, the materials below were combined:

- 9g NaCl
- 51g Agar
- 7.5g Peptone
- 1.5mL Cholesterol [5mg/mL]
- ddH2O up to 3L

These materials were autoclaved for 45 min, and then allowed to cool at 4 °C for 25 minutes. After the solution was cooled the following materials were then added:

- 3mL 1M CaCl2
- 3mL 1M MgSO4
- 1mL 1M KPO4 pH 6 (1M KPO4 pH 6 stock solution: 136.1 g KH2PO4 (NB Monobasic) 17.9 g KOH ddH2O to 1 L, pH should be ~6).

After each addition the solution was mixed.

2.1.2 Plating E. coli OP50

The NGM plates were seeded with OP50 once they have solidified. E. coli OP50 strain’s growth is limited on NGM agar plates because it is auxotrophic for uracil. OP50 was grown overnight in
B broth at 37°C until the solution was turbid. Using sterile techniques, OP50 was seeded onto NGM plates. The following steps were included in this process:

- using a glass pipette a small amount was spread onto the center of the NGM plates;
- care was taken not to disturb the agar surface or extend the OP50 liquid to the edge of the dish;
- the seeded NGM plates were grown at room temperature overnight;
- the seeded NGM plates were then stored upside-down at 4°C until ready to use;
- OP50 was ordered from the CGC.

2.1.3 B Broth for OP50 *E. coli* culture

B broth is used to culture the *C. elegans* food, the OP50 *E. Coli*. To prepare the B Broth 1 Liter of the following materials were autoclaved before they were used:

- 10g Bacto tryptone
- 5g NaCl
- The solution is then filled with ddH20 to 1L

2.1.4 Freezing *C. elegans* Strains

All strains generated are frozen in duplicate for long-term storage. In order to freeze the *C. elegans* strains the following steps were taken:

- medium size plates almost fully populated with starved L1/L2 stage *C. elegans* were used;
- both plates were washed with the same 2mL freezing solution which was transferred between plates using a glass pipette;
- The plates were stored in cryogen tubes at -80°C within 10 minutes of the washing.

Freezing Solution Formula:
The solution was autoclaved in 100mL aliquots. The following ingredients were used to make 1L of freezing solution:

- 5.89g NaCl (final concentration 0.1M)
- 6.8g KH2PO4 (final concentration 0.05M)
- 300g Glycerol (To final concentration of 30%)
- 5.6ml 1M NaOH
- ddH2O to 1L.
- 30 μL of 1M MgSO4 per was added to each 100ml before use (Mg2+ final concentration of 0.3mM).

2.1.5 M9 Buffer

In order to create the M9 Buffer the following materials were combined and autoclaved for 30 minutes:

- 5.8g Na2HPO4 (10.94g Na2HPO4·7H2O)
- 3.0g KH2PO4
- 0.5g NaCl
- 1.0g NH4Cl
- ddH2O to 1 L

2.1.6 Transgenic C. elegans lines

C. elegans transgenic strains carrying an extra-chromosomal array over-expressed any given transgene as well as a selection marker. The expression vectors are injected directly into the gonads of adult C. elegans. The co-injection marker used in my studies was Podr-1::GFP, or PRF4 rol-6 when animals were to be examined using additional florescent markers. Transgenic animals were isolated based on the phenotype caused by the marker; ODR-1::GFP caused bright
florescence in the nerve ring of the animals and rol-6 caused the animals to roll (Kramer et al., 1990; Mello et al., 1991). The pmk-3(hp246) phenotype was rescued using the cosmid WRM064cD07. This cosmid encompasses pmk-3 and 5kb upstream. The constitutively active MKK-4 construct used in section 3.5 was generated by Dr. Li Hang.

2.2 DNA protocols

2.2.1 PCR amplification of DNA fragments

The PCR protocol outlined below was used for all SNP mapping and genotyping reactions. PCR for sequencing reactions used high fidelity Taq and extension time lengthened for longer fragment (~1min/kb).

In a 20 μL reaction:

- 2μL 10xPCR buffer with MgSO4 [200mM Tris-HCl (pH8.8 at 25oC), 100mM (NH4)2SO4, 100mM KCl, 1% Triton X-100, 1mg/ml BSA, 20mM MgSO4 ]

- 1μL Pfu Taq polymerase

- 1μL 2.5mM dNTPs

- 1μL each Primer

- 1μL DNA template

- 13μL ddH20

PCR Program: 95oC 5min, 35 cycles of (95oC 30s, 55oC 1min, 72oC 1min), 72oC 5min, 4oC 1min
2.2.2 Purification of DNA from gel slices for sequencing

DNA fragments were purified via 0.5-2% agarose gel electrophoresis followed by extraction with UltraClean 15 (Mo Bio Laboratories Inc.). Gel slices of 0.1-0.3 g were purified using silica beads according to Mo Bio Laboratories protocol.

2.2.3 Purification of plasmid or fosmid DNA

The following procedure was followed:

- To amplify the plasmids grow the *E. coli* (DH5α) in liquid culture (LB and appropriate antibiotic) overnight.
- Pellet 2mL of culture in centrifuge at 3000rpm.
- Resuspend pellet in TE buffer – vortex vigorously.
- Add 400 μL lysis solution and gently invert tube ~3 times, the mix should turn clear.
- Leave on ice 2-3 minutes.
- Add 300μL 7.5M NH4OAc, invert the tube ~3 times and leave on ice for 5-10 minutes.
- Centrifuge at 14,000 rpm for 8 minutes and transfer top 750μL to a new tube. Do not touch the lower part of the liquid.
- Centrifuge supernatant at 14,000 rpm for 5 minutes and keep top 700μL.
- Mix with 420μL isopropanol and incubate at room temperature for 5 minutes.
- Centrifuge for 10 minutes at 14,000rpm.
- Pour out supernatant and wash pellet with 70% EtOH, vacuum dry until completely dry.
- Dissolve the DNA pellet in 30-50μL ddH2O.
- For fosmid preparation increase overnight culture volume to 3mL.
TE buffer: 400μL 1M Tris pH 7.5, 80μL 0.5M EDTA, 35mL ddH2O

Lysis solution: 40 μL 10%SDS, 8 μL 10N NaOH, 352 μL ddH2O

**2.3 SNB-1::GFP florescent marker analysis**

*C. elegans* strains carrying the SNB-1::GFP (*juls1*) marker were observed on glass slides with 3% agarose pads and a drop of M9 buffer. A glass cover slip was placed on top of the preparation to immobilize the animals. Using a Zeiss Axioskop 2 plus fluorescent microscope at 63x magnification, the SNB-1::GFP puncta along the dorsal cord were counted manually. The Improvision Openlab system (Quorum Technologies Inc.) was used to capture the live images of the animals.

The distribution plots of puncta gaps were generated using an in-house developed Matlab program Punctaanalyzer (by Calvin Mok and Dr. Taizo Kawano). Images were processed using Image J 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 gap was the parameter that was most significantly defective in *fsn-1* mutants, and thus, was used for subsequent analyses.

**2.4 Genotyping C. elegans mutations**

To verify the genotype of strains worms were subjected to lysis and PCR reactions (Section 2.3.1).

<table>
<thead>
<tr>
<th>Gene and Allele</th>
<th>Primers (Enzyme when necessary)</th>
<th>wild type fragment size</th>
<th>mutants fragment size</th>
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<td>1kb</td>
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<tr>
<td><em>fsn-1(hp1)</em></td>
<td>OZM510/511</td>
<td>900bps</td>
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</table>
2.4.1 Single Nucleotide Polymorphism Mapping

All mutant strains have the overall genetic background of the N2/Bristol strain. In addition, the alleles *hp180*, *hp192*, *hp195* and *hp246* were isolated and mapped in the *fsn-1;sad-1* genetic background. The dominant alleles mapped were isolated in the *daf-16;fsn-1;sad-1* mutant background. All mutants were mapped based on their suppression of the synthetic *fsn-1;sad-1* interaction that causes the animals to be paralyzed. The mutant strain was crossed with the mapping strain, CB4856 (Hawaiian). In the F1 generation, heterozygous animals were isolated at the L4 stage and separated at 1-2 animal(s)/plate. In the F2 generation, animals displaying the *fsn-1;sad-1* phenotype were isolated and separated 1 animal/plate. In the F3 generation 1 animal/plate that was moving like the wild type was isolated, allowed to lay eggs, then lysed, and the DNA was pooled along with the other suppressing lines. The plates that have absolutely no wild-type animals were also used as lines that had lost the suppressor. From each of these lines one F3 animal was lysed and the DNA was pooled.

DNA from each pool was amplified using each set of primers that distinguish CB and N2 SNPs across the entire chromosomes. 8 primer sets/chromosome, 48 primers in total were used for my mapping. All PCR reactions were digested with DraI and compared to N2 and Hawaiian controls (Figure 4). The suppressing lines should contain the suppressing mutations that gave a N2/Bristol genotype at the loci of the suppressor. Details of SNP genotyping are available in (Davis et al., 2005).
Figure 4. Chromosome mapping procedure. Typically 15 suppressing animals (homozygous Bristol DNA surrounding the mutation) and 15 fsn-1;sad-1 animals (homozygous Hawaiian DNA surrounding the mutation loci) are lysed in 20μL lysis buffer. The non-suppressed F3 animals were picked from plates that had absolutely no suppressed F3 animals. The lysate is then added to a PCR mix lacking primers, and the mix is aliquoted into a 96 well plate. Primers are added from pre mixed primer sets for each snip-SNP site. When mapping a recessive mutant in the Bristol background against the Hawaiian strain, unlinked SNPs will display a 50-50 mix of Bristol bands and Hawaiian bands in both mutant and non-mutant lanes. Linked SNPs will display an enrichment of Bristol bands in the mutant lane, approaching 100% Bristol for tight linkage. The non-mutant lane will display an enrichment of Hawaiian bands again approaching 100% near the mutation loci (figure adapted from Davis et al. 2005)(Wicks et al., 2001).
3 Results

3.1 fsn-1 alters synaptic morphology

I have used *juIs1*, the *Punc*-25 driven presynaptic marker (SNB-1::GFP) to visualize the GABAergic NMJ morphology of wild type animals and the *fsn-1* mutants (Hallam and Jin, 1998). Each SNB-1::GFP punctum represents a presynaptic terminal of a GABAergic NMJ in wild type animals (Jorgensen et al., 1995). Wild type animals have round and discrete puncta along the DD and VD neuron axons that run the entire length of the dorsal and ventral nerve cords (Figure 2a). These puncta are uniform both in their spacing and size, and are evenly distributed along the dorsal cord. *fsn-1* loss-of-function (lf) mutants exhibit a reduced number of SNB-1::GFP puncta with irregular shapes and sizes. They display large clusters of over-differentiated synapses, along with gaps devoid of the synapses (Figure 2b,c) (Liao et al., 2004).

To quantify the synaptic morphology phenotype, I first counted the total number of puncta along the dorsal cord of the animal. The under-differentiated synaptic pools or gaps along the dorsal cord in *fsn-1* animals led to a lower number of puncta. In wild type adult animals I find an average of 130 +/- 2.1 SNB-1::GFP puncta per animal. In *fsn-1* mutants this number is significantly reduced to 101 +/- 3.5 SNB-1::GFP puncta per animal (p<0.001) (Figure 5).

To precisely characterize the synaptic phenotype I further compared different parameters of the synaptic morphology between wild-type animals and *fsn-1* mutants. Using a program called Puntanalyzer (Developed by Calvin Mok and Dr. Taizo Kawana), I compared the gaps in between the puncta, then plotted a distribution of the gap sizes and compared between genotypes (Figure 5c,d). Wild type animals exhibit relatively even spacing and size of their synapses, thus the distribution of the gap sizes between puncta has a small range and a sharp peak. The *fsn-1* and *rpm-1* mutants both have fewer puncta with a more variable range in the distance between them, plotting these distance gives a curve with a wide base and a dull peak (Figure 5c,d).
Figure 5. *fsn-1*(hp1) mutants have synaptic defects that are suppressed by *hp246*. A) A synaptic vesicle marker *juls1* allows the visualization of GABAergic NMJs in wild-type, *fsn-1*(hp1), *hp246* and *hp246;fsn-1*(hp1) animals. Arrowheads indicate clustered puncta; arrows indicate gaps. Scale bar 5 µm. B) Quantification of the total number of *juls1* puncta in the DD motorneurons: n=15. Error bars show standard deviation. C) Density plot of punctum widths of wild type, *fsn-1*, *hp246* and *hp246;fsn-1* animals using Punctanalyser. *hp246;fsn-1* animals show partial rescue of *fsn-1* in the distribution of gap size between puncta n=15. D) Density plot of punctum widths of wild type, *rpm-1*, *hp246* and *hp246;rpm-1* animals using Punctanalyser. *hp246;rpm-1* animals show partial rescue of *rpm-1* in the distribution of gap size between puncta. n=15. Asterisk, P<0.001 compared to wild type (One way ANOVA).
3.2 sad-1 mutations enhance synaptic and locomotion defects of fsn-1 mutants

Although fsn-1 and rpm-1 animals have defects in their nervous system, they exhibit almost no obvious morphological and behavioral difference from wild type animals except for a slightly shorter body length (Liao et al., 2004). sad-1 encodes a serine/theronine kinase which is involved in presynaptic vesicle clustering and termination of axon outgrowth, however it does not act in the same pathway as fsn-1 (Crump et al., 2001). Like fsn-1 or rpm-1, sad-1 mutants have almost normal locomotion except for a somewhat uncoordinated backing (Crump et al., 2001). In contrast to either fsn-1 or sad-1 single mutants, the fsn-1;sad-1 double mutant animals are severely defective in locomotion and shorter (Figure 6). In the Zhen lab, we use this synthetic phenotype to isolate mutations that can suppress the behavioural phenotype by suppressing either fsn-1 or sad-1. The advantage of using this phenotype is mainly practical. It is much quicker and less technically challenging to screen animals for a suppression of paralyzed motion than to examine their synaptic morphology on a large scale. We took a forward genetics approach and used EMS to mutagenize the fsn-1;sad-1 animals and then screened their progeny for suppressors. We isolated F2 progeny that had an improved locomotion. We then re-isolated the suppressor in a fsn-1 single mutant background through backcrossing, and examine their synaptic phenotype using juIs1 to identify fsn-1-specific suppressors.
**Figure 6. Gross phenotype of *fsn-1* and related mutants.** *hp246* suppresses *fsn-1* and *rpm-1* enhanced phenotype. Animal genotypes are listed to the left and their corresponding phenotypes on the right. The images are taken using the 40 times magnification of a light microscope and display the gross phenotype of the animals.
3.3 *hp246* suppresses *fsn-1* and *rpm-1* phenotypes

*hp246* was isolated in the behavioural screen for suppressors of *fsn-1; sad-1* defects as outlined above (preformed by Dr. Edward Liao, Maja Salihbegovic and Dr. Wesley Hung). The *hp246* single mutant animals look normal in synaptic morphology, they are however loopy and move with exaggerated body bends (Figure 5, Figure 6). *hp246* is potentially a novel suppressor of *fsn-1* and *rpm-1* and if so its suppressing effect on *fsn-1* and *rpm-1* would then be uncharacterized, therefore I characterized the suppression of *hp246* on the *fsn-1* and *rpm-1* phenotypes.

I have analyzed the synaptic morphology defect of *fsn-1;hp246* to determine whether *hp246* suppresses *fsn-1* or *sad-1*. The *juIs1* phenotype of *fsn-1;hp246* appears nearly wild type, rescuing the over- and under-grown synaptic defects exhibited by *fsn-1* mutants (Figure 5). In wild type animals, there are on average 130 +/-2.1 juIs1 puncta and *fsn-1* have 101 +/- 3.5. *fsn-1;hp246* animals have on average a punctum number of 122 +/- 2.3, whereas the *hp246* on its own exhibits no difference in synapse number (124 +/- 3.2) from wild type (p<0.001)(Figure 5).

I used Punctaanalyzer to further characterize the level of suppression of *hp246* on the *fsn-1* synaptic phenotype. *hp246* also rescues the *fsn-1* distribution of distance between the puncta. *fsn-1* animals have a broader range of inter-punctum distances (Figure 5c). Wild-type animals have a tighter distribution of these inter-punctum distances, which suggests that these synapses are distributed evenly. *hp246;fsn-1* animals exhibit the same sharp peak, indicating a restoration of synapse distribution (Figure 5c).

3.4 *hp246* does not function genetically upstream of the Insulin/IGF signaling pathway

The Insulin/IGF signaling pathway negatively regulates the FOXO family transcription factor DAF-16 by preventing its translocation into the nucleus where it functions both as an activator and repressor of gene transcription (Lin et al., 2001). In *fsn-1;hp246* double mutants the over
and under-developed synaptic regions along the dorsal cord of *fsn-1* animals are greatly improved to a more even spacing and size of synapses like wild type animals (Figure 5). Mutations in members of the Insulin/IGF signaling pathway can also suppress *fsn-1* synaptic defects (Hung et al., in preparation). I therefore hypothesized that *hp246* could be a member of this pathway. I examined whether *hp246* functions through the Insulin/IGF signaling pathway by examining the *daf-16;fsn-1;hp246* triple mutant animals. If *hp246* functions in the Insulin/IGF signaling pathway genetically upstream of DAF-16 then its suppression of *fsn-1* should be dependent on *daf-16*. *daf-16;fsn-1;hp246* animals synaptic puncta look almost wild type, with even spacing and size (Figure 7). Given that the suppression of the *fsn-1* synaptic defects is not dependent on DAF-16, *hp246* does not function upstream of *daf-16*. 
Figure 7. *hp246* suppresses *fsn-1* synaptic morphology defect independently of *daf-16*. A) A visualization of the GABAergic NMJ morphology by the synaptic vesicle marker *juls1* in wild-type, *fsn-1(hp1)*, *hp246* and *fsn-1;hp246(hp1)*, *daf-16;fsn-1* and *daf-14;fsn-1;hp246* animals. Scale bar, 5 μm. B) Quantification of the total number of *juls1* puncta in the DD motorneurons: n =15. P<0.001 compared with wild type (One way ANOVA). Error bars show standard deviation.
3.5 \textit{hp246} is a mutation that disrupts the kinase domain of PMK-3

In order to find the gene where \textit{hp246} is located I used single nucleotide polymorphism (SNP) mapping (Wicks et al., 2001). As genomic markers I used identified single nucleotide polymorphisms (SNPs) between the \textit{C. elegans} strain CB4856 (Hawaiian) and N2 Bristol strain that altered restriction enzyme sites (‘snip-SNPs’), and that were easily detectable as RFLPs. I utilized a set of 48 primers flanking SNPs that are evenly spaced across the \textit{C. elegans} genome (Davis et al., 2005). \textit{fsn-1} as well as \textit{fsn-1} suppressors were isolated in the N2 derived background, and animals were crossed with the Hawaiian strain. I re-isolated the \textit{fsn-1;sad-1} paralyzed animals in the F2 progeny, and then suppressors in the F3. Around the loci of the suppressor, \textit{fsn-1} and \textit{sad-1} the SNPs will have the N2 polymorphisms, however the rest of the genome will have a heterozygous Hawaiian/N2 profile. Therefore searching for the N2 enriched genomic regions allowed me to locate these suppressor genes.

In total, I re-isolated 135 \textit{fsn-1;sad-1;hp246} suppressor lines from the N2/CB4856 hybrid lines. I performed snip-SNP mapping by collecting the DNA into 13 pools, each representing 10 or 11 SNP lines. Analysis of the snip-SNP mapping revealed \textit{hp246} to be on Chr. IV between the genetic positions +1.40 and +7.46 (Figure 8). The region contains \textit{pmk-3}, a component of the MAP kinase signaling pathway, and a known suppressor of \textit{rpm-1} (Figure 9) (Nakata et al., 2005).

I sequenced \textit{pmk-3} in the \textit{hp246} animals, and identified a missense mutation within the kinase domain of \textit{pmk-3}. The \textit{hp246} mutation converts a highly conserved aspartic acid to an asparagine mutation (D312N) directly in the kinase domain (Figure 10). How this mutation leads to the loss of the protein function is unknown. It is possible that the protein carrying this mutation cannot fold properly. Alternatively, the mutated residue is predicted to be within 9 Å from the catalytic site and may therefore lead to a disruption of the catalytic activity.
Figure 8. Genome wide SNP mapping of hp246. Each row represents the genetic DNA composition of one DNA pool with 10 mapping lines all carrying hp246 based on suppression of fsn-1,sad-1 behavioural defects. Pools were genotyped at the mapping location listed at the top of each column. The six chromosomes are separated and each was genotype at six different snip-SNP sites except for chromosome V, which was genotyped at 5 sites. Grey indicates a heterzygous genotype, green an Bristol/N2 genotype, blue a Hawaiian/CB4856 genotype and white an unclear result. An enrichment of N2 SNP profile indicates the location of hp246. N2 enrichment was found at IV:1 indicating the locus of hp246. N2 enrichment also found at rpm-1 V:1.6 and syd-2 X:2.23.
Figure 9. Mapping results of suppressors. A drawing of the chromosomal positions that each mutant allele is mapped to. The red bars indicate the region where the mutation mapped to and the known suppressor in that region is marked. Six to eight SNP markers were used on each chromosome and regions are either spanning one or two SNP marker regions. *hp246, hp180, hp192 and hp195 are known suppressors of *fsn-1*. All other alleles were isolated as suppressors of *daf-16;fsn-1;sad-1*. Drawing is not to scale.
Figure 10. Protein alignment of PMK-3 and DLK-1 displaying loss of function mutations. ClustalW multiple sequence alignment of a portion of the kinase domain of the C. elegans genes A) pmk-3 and B) dlk-1. Blast searches were performed, orthologues were chosen from closely related species as well as more divergent species. A) The highlighted aspartic acid residue is mutated to an asparagine in hp246 animals. B) The region containing hp195 mutation is highlighted. The conserved glycine is mutated to a glutamic acid in hp195. The hp192 and hp180 mutations are indicted and both cause a premature stop codon. The blue shading indicates percent identity between the aligned sequences, dark being highly identical and light being less identical. Kinase region predicted using SMART sequence analysis, alignment created using Jalview. Drawing is not to scale.
3.6 *hp180*, *hp192* and *hp195* are all missense alleles of *dlk-1*

Using the same method, I characterized additional suppressors that had been isolated from the *fsn-1;sad-1* suppressor screen that lead to the identification of *hp246*. Given that *hp246* is not a novel suppressor of *fsn-1* I began work with uncharacterized alleles. The three alleles *hp180*, *hp192* and *hp195* are suppressors of the *fsn-1* synaptic morphology defect (isolated and confirmed by Dr. Ed Liao and Dr. Wesley Hung). By snip-SNP mapping, I concluded that all mapped to the right side of Chromosome I; *hp180* and *hp192* mapped from +5.06 to +13.96 and *hp195* from -1.04 to +13.96 (Figure 11). We focused on a candidate gene in this shared region called *dlk-1*, another member of the MAP kinase pathway, and a known suppressor of *rpm-1* and *fsn-1* (Figure 9). We confirmed all three alleles failed to complement *dlk-1*(*hp251*) based on suppression of the *fsn-1;sad-1* paralysed phenotype.

I have identified mutations in *dlk-1* in *hp180*, *hp192* and *hp195* by sequencing. DLK-1 is 928 amino acids in length, and harbours a kinase domain at amino acids 135-377. In *hp180* there is a missense mutation at amino acid Lys469 resulting in a premature termination codon. Similarly in *hp192* there is a premature termination codon at amino acid Trp287 inside the kinase domain. In *hp195*, there is a missense mutation in the kinase domain (G188E), which is a highly conserved residue across many species (Figure 10).
Figure 11. SNP mapping of suppressing mutations. Each row represents one DNA pool of mapping lines. The allele designation is listed at the beginning of each row, the ‘S’ indicates the mapping lines carry the suppressor, the ‘U’ indicates the lines do not carry the suppressor and therefore are uncoordinated. Pools were genotyped at the mapping location listed at the top of each column. The six chromosomes were separated and each was genotyped at eight different SNP sites. Grey indicates a heterozygous genotype, green an N2 genotype, blue a CB4856 genotype and white an unclear result. The loci where the suppressing pool genotype is Bristol/N2 and the uncoordinated pool genotype is Hawaiian/CB4856 are denoted by the red box. They represent regions where mutations are located. All lines carry fsn-1; sad-1 in the background and genotype as N2/Bristol at the loci fsn-1(III:-2.33) and sad-1(X:12.66).
3.7 \textit{daf-16;fsn-1;sad-1} suppressor screen

In order to generate additional and potentially novel alleles of \textit{fsn-1} suppressors we conducted a new \textit{fsn-1} suppressor screen. As outlined in the introduction, the Insulin/IGF signaling pathway is also regulated by \textit{fsn-1}. The active Insulin/IGF signaling pathway negatively regulates \textit{daf-16} thus mutants of this pathway suppress \textit{fsn-1} defects by activating \textit{daf-16}. To enrich for suppressors that function genetically in parallel, or downstream of DAF-16, the screen was performed using \textit{daf-16;fsn-1;sad-1} animals (performed by Dr. Mei Zhen and Tetyana Pekar). The \textit{fsn-1;sad-1} double mutants are severely defective in locomotion and development. They move awkwardly and seldom and have shortened bodies (Figure 6). The \textit{daf-16;fsn-1;sad-1} triple mutant has the same phenotypes but are more severe and fully penetrant in both phenotypes. In this screen 69 mutants that suppress the \textit{daf-1;fsn-1;sad-1} phenotype to varying degrees were isolated.

The MAP kinase pathway mutants \textit{dlk-1}, \textit{mkk-4} and \textit{pmk-3} are suppressors of \textit{rpm-1} and are found in \textit{fsn-1} suppressor screens as seen in the case of \textit{pmk-3}(hp246) and \textit{dlk-1}(hp180, hp192, hp195). To first determine whether any of the 69 mutants isolated in the \textit{daf-16;fsn-1;sad-1} screen were MAP kinase members I used non-complementation tests with the loss of function mutants \textit{pmk-3}(hp182), \textit{dlk-1}(hp195) and \textit{mkk-4}(ok1545) (Figure 12). The mutants fell into 3 major categories; dominant suppressors, MAP kinase pathway suppressors, and suppressors that are likely to be non-MAP kinase pathway components. The dominant suppressors, when crossed with \textit{fsn-1} males always produced male progeny that were not paralyzed and failed to complement in all of the non-complementation tests. The MAP kinase pathway suppressors failed to complement one of the three \textit{mapk} mutants. The non-MAP kinase pathway alleles complemented all of the tested MAP kinase mutants. I further tested the gene \textit{mak-2}, which is downstream of the MAP kinase pathway for non-MAP kinase pathway suppressors (Yan et al., 2009).

Summarized in Table 1 are the results of the non-complementation test. 64\% of the mutants are candidate MAP kinase pathway members, 28\% are dominant and 9\% are recessive suppressors that do not fall into genes of the MAPK signaling pathway or MAK-2 (complete results Table 2). The genetic identities of dominant alleles are unknown, as well as those recessive suppressors not in the MAP kinase pathway.
Figure 12. Non-complementation crosses and *fsn-1* cross carried out in screening *daf-16;fsn-1;sad-1* alleles.

A) A schematic of the complementation crosses that were carried out on the mutants isolated as suppressors of *daf-16;fsn-1;sad-1*. In this case the known gene being tested is *dlk-1*, the same strategy was used for *pmk-3*, *mkk-4* and *mak-2*. *hp606* represents an unknown allele being tested. B) *fsn-1* cross to determine if unknown allele is on the X chromosome.

A

\[
\text{dlk-1;fsn-1lll;sad-1X} \quad \text{x} \quad \text{hp606;fsn-1lll;sad-1X} \\
\]

\[
\begin{align*}
\text{dlk-1; fsn-1; sad-1} & \quad \text{hp606; sad-1} \\
\text{hp606; fsn-1; sad-1} & \quad \text{dlk-1; fsn-1; sad-1} \\
\end{align*}
\]

If animals move like wild type, If animals are nearly paralyzed
then *fsn-1;sad-1* phenotype is suppressed, then *fsn-1;sad-1* phenotype is not suppressed,
hp606 fails to complement *dlk-1*, hp606 complements *dlk-1*,
and *hp606* is an allele of *dlk-1* and *hp606* is not an allele of *dlk-1*

If *hp606* complements all tested genes then it is dominant

B

\[
\text{fsn-1lll} \quad \text{x} \quad \text{hp606;fsn-1lll;sad-1X} \\
\]

\[
\begin{align*}
\text{hp606; fsn-1; sad-1} & \quad \text{or} \quad \text{fsn-1; hp606 sad-1} \\
\text{hp606;/fsn-1} & \quad \text{or} \quad \text{fsn-1; } \text{hp606 sad-1} \\
\end{align*}
\]

If males are nearly paralyzed If males move like wild type,
then *fsn-1;sad-1* phenotype is not suppressed then *fsn-1;sad-1* phenotype is suppressed
*hp606* is not on the X chromosome *hp606* is on the X chromosome
Table 1. A summary of the tentative identities of the alleles from the \textit{daf-16};\textit{fsn-III};\textit{sad-IX} screen:

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### Table 2

A list of the results from the non-complementation tests against MAP kinase mutants for new alleles suppressing daf-16; fsn-1; sad-1.

<table>
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Table 2. **A list of the results from the non-complementation tests against MAP kinase mutants for new alleles suppressing daf-16;fsn-1;sad-1.** Each allele was subjected to non-complementation tests with *dlk-1, mkk-4* and *pmk-3* as well as a cross with *fsn-1* males as displayed in Figure 12. Alleles that complemented all genes were also subjected to non-complementation tests with *mak-2*. The conclusions listed were determined after all non-complementation tests were completed.

<table>
<thead>
<tr>
<th>Allele</th>
<th>Gene</th>
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<th>Location</th>
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<td>F</td>
</tr>
<tr>
<td>hp601</td>
<td>dlk-1</td>
<td>U</td>
<td>F</td>
</tr>
</tbody>
</table>

Legend:  
(U) uncoordinated - on X chromosome or dominant  
(S) suppressed – not on X chromosome  
(F) failed to complement – same gene as tested  
(C) complemented – not the gene tested  
(N/D) not determined

### 3.7.1 Investigating the location of the dominant suppressors

The genetic position of the recessive alleles that complement all the MAP kinase mutants, and the dominant alleles that fail to complement all the MAP kinase mutants, remain unknown. I have been able to rule out the possibility that the recessive alleles are in the MAP kinase pathway making them attractive for further investigation. The dominant suppressor alleles could not be determined definitively by non-complementation tests so I performed rough snip-SNP mapping on 8 of the 19 dominant alleles to identify their genomic region. Locating the genomic region of the dominant alleles will help to determine whether they map to a region where a known suppressor is located and thus are likely that suppressor or whether they map to a region where no known suppressor is located and thus the allele may represent a novel suppressor of *fsn-1*.

Based on my mapping data, I concluded that the alleles *hp576, hp595* and *hp611* map to right arm of chromosome I. *hp576* maps to a large region, nearly the entire right arm of Chr. I (+5.06 - +26.21). *hp595* and *hp611* both mapped to the region +5.06 to +13.96 (Figure 11). A known suppressor of *fsn-1* on Chr. I is the MAPKKK *dlk-1* (+13.61) (Figure 9).
The alleles hp615, hp616 and hp617 all map to the center of Chr. X. hp615 and hp616 map to the region from -4.04 to +8.23, and hp617 maps between -8.13 to +2.36 (Figure 11). The MAPKK mkk-4 is a known suppressor and located on X (+1.73) (Figure 9).

The alleles hp618 and hp626 both map to the left end of Chr. IV to a region from -29.30 to -16.07 (Figure 11). The downstream component of the MAPK pathway mak-2 locates at -23.23 (Figure 9). mak-2 mutants are known suppressors of rpm-1 (Yan et al., 2009). Sequencing analyses of the respective MAPK genes would be necessary to determine whether these dominant suppressors fall into these, or other unknown genes.
4 Discussion

In order to deepen our understanding of presynaptic development, we have sought to identify direct or indirect targets of FSN-1, a F-box protein known to regulate synaptogenesis (Liao et al., 2004). We identified *pmk-3(hp246), dlk-1(hp180), dlk-1(hp192)* and *dlk-1(hp190)*, members of the MAP kinase signaling pathway, and known suppressors of *rpm-1* (Nakata et al., 2005) to also suppress *fsn-1*. We are interested in finding additional targets and signaling pathways regulated by FSN-1, and, as outlined below we hypothesize that many remain to be identified. We continued our efforts to find these new genes using the *daf-16;fsn-1;sad-1* suppressor screen. This project has concluded with identifying the alleles from the screen that are MAP kinase genes, with the remaining alleles as putative novel suppressors of *fsn-1*.

4.1 The *daf-16;fsn-1;sad-1* suppressor screen

The preliminary results of the *daf-16 I;fsn-1 III;sad-1 X* suppressor screen are promising. Of the 69 mutants isolated 6 (8.7%) complemented all the known *fsn-1* suppressors in the MAP kinase pathway *dlk-1, pmk-3, makk-4* or *mak-2*, and could represent novel *fsn-1* suppressor genes. The known Insulin/IGF signaling pathway suppressors that depend on *daf-16* can also be ruled out because the *daf-16* mutation is included the background (Hung et al., in preparation). Importantly in the complementation crosses the mutants segregate loss of function alleles of both *fsn-1* and *sad-1*, suggesting that these mutations are not intragenic suppressors (Figure 12).

*daf-16;fsn-1;sad-1* triple mutant animals exhibit movement defects that are more severe and penetrant than *fsn-1;sad-1* animals alone. There is a possibility of isolating weak suppressors that revert the triple animals back to a *fsn-1;sad-1* phenotype. Such a suppressing allele could be downstream of *daf-16* and possibly part of a retrograde signal between the Insulin/IGF signaling pathway and the presynaptic terminal, including even the MAP kinase signaling pathway (Figure 13). Of the 6 potentially novel alleles, 4 are weak in their suppression of the movement defects of *daf-16;fsn-1;sad-1* (*hp636, hp587, hp605 and hp631*). This is in contrast to the case of MAPK mutants, all are very strong suppressors of *fsn-1;sad-1* and *daf-16;fsn-1;sad-1* movement defects. They should be examined further as candidates for functioning downstream of *daf-16*. 
Figure 13. A schematic of signaling pathways that interact with FSN-1: FSN-1/RPM-1 act in a SCF complex to negatively regulate DLK-1. FSN-1 genetically interacts with DAF-2 possibly independent of RPM-1. We hypothesize that the FOXO transcription factor DAF-16 is regulating genes that serve as a retrograde signal to the presynaptic terminal.
4.2 Dominant suppressors of daf-16;fsn-1;sad-1 may be dominant negative alleles

Nineteen dominant suppressors were isolated in the daf-16;fsn-1;sad-1 screen. The heterozygous mutant alleles exhibit the same effect as homozygous recessive mutations on suppression of the fsn-1;sad-1 paralyzed movement. The fsn-1 phenotype is in part attributed to an increase in the MAPK signalling pathway activity (Nakata et al., 2005). Recessive alleles of MAP kinase genes that suppress fsn-1 are loss-of-function alleles that perturb the MAP kinase pathway. Based on the rough mapping data, many of the dominant alleles fall into regions where genes in the MAP kinase pathway reside. If they represent dominant mutant alleles of MAP kinase genes, then they too must be somehow perturbing the pathway’s activity.

There are a few different situations in which a dominant allele can behave that should be considered in the case of the MAP kinase pathway alleles. Haploinsufficiency of known pathway components can be ruled out as there are known recessive alleles and importantly the deletion alleles are recessive suppressors. It is also unlikely that these are hypermorphs as over expression of these p38 MAPK proteins leads to a fsn-1 like phenotype (Nakata et al., 2005). This suggests that an abundance of p38 MAPK activity does not explain fsn-1 suppression. It is possible that these dominant alleles are dominant negative alleles. They could be competing with the wild type copies but have lost part of their function therefore reducing the amount of active kinases being targeted to levels low enough to sufficiently deactivate the pathway. The mutated residues could cause the protein to have lost its kinase activity or be interfering with the phosphorylation site similar to let-60 dominant alleles that lose their GDP binding ability but out-compete the wild-type proteins as targets to cause vulva developmental defects in C. elegans (Han and Sternberg, 1991).

4.3 SCF<sup>FSN-1</sup> negatively regulates the MAP kinase pathway

The results of all our mapping, screening and non-complementation tests suggest the dominating target of the SCF<sup>FSN-1</sup> is the MAP kinase pathway. Not surprisingly this pathway is also the most
well defined pathway regulated by SCF^{FSN-1} (Nakata et al., 2005; Po et al.). The result of the daf-16:fsn-1;sad-1 suppressor screen in this study revealed 64% of the mutants isolated to be in the MAP kinase pathway, and 28% to be dominant that remain to be further characterized. The defects exhibited by fsn-1 and rpm-1 mutants are attributed to a decrease in the E3 ligase activity, and thus increased activity of its targets, the MAP kinase pathway and specifically DLK-1 (Nakata et al., 2005).

### 4.4 MAP Kinases associated proteins may suppress fsn-1

In C. elegans synaptic development, one of the p38 MAPK signalling pathways includes the MAPKKK DLK-1, the MAPKK MKK-4, and the MAPK PMK-3 (Nakata 2005). The MAPKAPK, MAK-2 is downstream of PMK-3 and stabilizes the mRNA encoding CEBP-1, a bZip protein related to CCAAT/enhancer binding proteins (Yan et al., 2009). As discussed earlier, there are 5 fsn-1 suppressing alleles isolated which data suggests are not in the p38 MAPK signalling cascade based on the above known members. However it is possible that other proteins could be associated with this pathway in synapse development; it is therefore prudent to consider what is known about the proteins involved in the p38 MAPK pathway and their interactors. I hypothesize that p38 MAPK signalling pathway associated proteins could also suppress fsn-1 defects but may have not been discovered yet. This may be because the genes are small and therefore mutations are rare, or because the null allele is lethal but a partial loss-of-function can suppress fsn-1.

In C. elegans, in addition to presynaptic development, the p38 MAPK signaling pathway has been shown to be involved in axon termination and neurite regeneration (Grill et al., 2007; Hammarlund et al., 2009). All these processes are related to microtubule instability and interestingly microtubule defects in touch receptor neurons cause a reduction in overall protein levels. This reduction requires the p38 MAPK pathway (DLK-1, MKK-4, PMK-3) and the downstream transcription factor CEBP-1(Bounoutas et al., 2011). It is unclear how the MAPKs are interacting with microtubules, but there may be a feedback system in place as DLK in mice has been shown to reduce microtubule stability through both JNK (Eto et al., 2010) and p38 MAPKs (Lewcock et al., 2007). It is possible that other microtubule proteins like MEC-7 (β-tubulin) and MEC-12 (α-tubulin) or microtubule associated proteins are interacting with MAPKs
and could also act as suppressors of *fsn-1*. The cytoskeleton provides a large negatively charged surface on which many signaling molecules localize including MAPKs, PI3Ks, cAMP-dependent kinases, phospholipases, GTPases and transcription factors (Janmey, 1998). Any microtubule associated proteins could potentially interact with this p38 MAPK signaling pathway and similarly act as suppressors of *fsn-1* defects.

## 4.5 RPM-1 and FSN-1 interact with additional signaling pathways

The MAP kinase pathway mutants can suppress the *fsn-1* and *rpm-1* enhanced behavioural defects, as well as their synaptic morphology defects, likely due to their importance in synaptic development (Nakata et al., 2005). However, lines of evidence persist in *C. elegans*, *Drosophila*, zebrafish and mice supporting the notion that the MAP kinase pathway is not the only target of SCF*FSN-1*. There are no gross changes in DLK levels in *Phr1* and *Fbxo45* knockout mice (Bloom et al., 2007; Saiga et al., 2009). Moreover, in the double mutant mouse *Phr1;dlk* the CNS defects of *Phr1* mice are not suppressed (Bloom et al., 2007). Although there is rescue, the *rpm-1* and *highwire* mutant synaptic defects are not fully suppressed by *dlk-1* and *wallenda* loss of function mutations (Wu et al., 2007; Zhen et al., 2000). RPM-1 has also been shown to interact with the a guanine nucleotide exchange factor GLO-4 in a parallel fashion to FSN-1 (Grill et al., 2007). The zebrafish *rpm-1* orthologue *esrom* has defects in axon outgrowth and targeting, but these defects are not associated with aberrant p38 signaling (Hendricks and Jesuthasan, 2009). *fsn-1* mutants have a role in apoptosis displaying increased levels of the single *C. elegans* ancestral p53 family member CEP-1 and a hypersensitivity phenotype to ENU-induced apoptosis that is suppressed in *cep-1* mutants (Gao et al., 2008). Additionally the mammalian transcription factor and p53 family member p73 is ubiquitinated by SCF*FBXO-45* both in vivo and in vitro (Peschiaroli et al., 2009).

It is clear that there are additional pathways, possibly stage specific or cell-type specific that PHR proteins are regulating, as SCF*FSN-1* or independent of FSN-1. Indeed, FSN-1 may also act independently of RPM-1 in *C. elegans* at the synapse. Candidate suppressors screens have revealed both *scd-2* and *daf-2* as suppressors of *fsn-1* synaptic morphology defects, but
interestingly these suppressors do not suppress \textit{rpm-1} defects nor do they suppress the enhanced \textit{fsn-1; sad-1} behavioural defects (Hung et al., in preparation; Liao et al., 2004).

4.6 Insulin/IGF and MAP kinase signaling pathways may interact during synapse development

The Insulin/IGF signaling pathway is an important signaling pathway to be considered in synaptic development (Figure 13). The formation of \textit{en passent} NMJs of \textit{C. elegans} nervous system rely on body wall muscles to extend processes termed muscle arms towards the VD and DD motor axons (Dixon and Roy, 2005; White et al., 1986). \textit{daf-2} mutant animals exhibit a supernumery muscle arm phenotype that is dependent on the transcription factor DAF-16 (Dixon et al., 2008). As mentioned above, \textit{daf-2} mutants also suppress the \textit{fsn-1} synaptic morphology defects; however, they have no obvious synaptic morphology defect of their own (Hung et al., in preparation). Our lab has preliminary data that the Insulin/IGF signaling pathway likely functions genetically upstream of the MAP kinase pathway and thus part of a retrograde signal from the postsynaptic to presynaptic terminal at NMJs (Hung et al., in preparation). It is possible that the Insulin/IGF signaling pathway is one of multiple pathways that feed into and activates the MAP kinase signaling pathway (Figure 13).
5 Future Direction

5.1 Future research with unknown \textit{daf-16;fsn-1;sad-1} suppressors

The \textit{daf-16;fsn-1;sad-1} screen revealed six unknown recessive mutants and 19 unknown dominant mutants that require further investigation. Eight of the dominant alleles have been rough mapped (Figure 9) to regions where some known suppressors are located (Nakata et al., 2005). These alleles should be further examined by sequencing and possibly with transgenic rescue. Of the unknown recessive suppressors \textit{hp606} should be tested as \textit{cebp-1}, the CCAAT/enhancer binding protein, as it is a suppressor of \textit{rpm-1} that also locates on the X chromosome, and is not \textit{mkk-4} (Yan et al., 2009). The MAP kinase pathway acts to stabilize the mRNA encoding CEBP-1 and it is the most downstream component known of the MAP kinase pathway (Yan et al., 2009). However, this should be further confirmed by sequencing and transgenic rescue. The remaining 11 dominant alleles and five recessive alleles should also be rough mapped to determine whether they are known suppressors or warrant further mapping and investigation as novel suppressors. After rough mapping, if any of the alleles are likely to be novel then it would be prudent to determine whether they suppress \textit{fsn-1;sad-1} without \textit{daf-16} in the background, then determine whether they suppress \textit{fsn-1} or \textit{sad-1} defects.

5.2 Identify novel \textit{fsn-1} suppressors through carrying out alternative screens

The \textit{fsn-1;sad-1} behavioural phenotype is attractive for genetic suppressor screens because the phenotype is easily recognized with low power dissecting microscopes. The nearly paralyzed movement of the animals is obvious and suppressors can be easily identified based on their improved movement. The vast majority of the mutant animals isolated using this phenotype are the MAP kinase mutants. This brings up the question of whether or not there are more suppressors of \textit{fsn-1} beyond the MAP kinase genes. Both \textit{daf-2} and \textit{scd-2} robustly suppress \textit{fsn-1} synaptic morphology defects but are not a strong suppressor of paralysis exhibited by \textit{fsn-1;sad-1}. Therefore it may be possible to isolate additional novel suppressors using alternative
and more sensitive screening strategies. The suppressor screens could be adapted by using addition phenotypes of \textit{fsn-1} to screen for suppressors.

Screening for genetic modifiers of the \textit{juIs1} synaptic morphology defect of \textit{fsn-1} animals is one possible alternative. This phenotype is fully penetrant and easily recognizable, but it requires screening under a high-power compound microscope. It is therefore technically more laborious, making the screen a lengthier process. The same holds true of screening with other neuronal phenotypes, such as the mild defect in extensions of the ALM and PLM mechanosensory touch neurons in \textit{fsn-1} animals (Grill et al., 2007). The ALM neuron cell body is located slightly anterior to the vulva and its axon extends to the head (Chalfie and Thomson, 1979). In \textit{fsn-1} animals, the ALM axon fails to terminate; it can turn and continue to extend in the posterior direction instead (Grill et al., 2007). The PLM cell body is located in the tail region of the animal, and extends its axon anteriorly. The axon does not pass the ALM cell body and it has a synaptic branch extending ventrally roughly half way along the axon shaft (Chalfie and Thomson, 1979). In \textit{fsn-1} mutants the PLM axon can extend past the ALM cell body, sometimes hooking dorsally and failing to extend the synaptic branch (Grill et al., 2007). These phenotypes have a penetrance below 20\% but can be enhanced to nearly 100\% with a second mutation to the guanine nucleotide exchange factor \textit{glo-4} (Grill et al., 2007). It is therefore possible to identify suppressors of \textit{fsn-1: glo-4} mutants screening for reversion of PLM defects.
6 Conclusion

The identification of the neural specific E3 ubiquitin ligase complex SCF^{FSN-1} has provided a stepping stone to discovering addition factors involved in synaptic development (Liao et al., 2004; Schaefer et al., 2000; Zhen et al., 2000). Suppressor screens like ours have revealed the MAP kinase genes as the major target of SCF^{FSN-1} and indeed levels of DLK-1 are increased in the absence of SCF^{FSN-1} (Nakata et al., 2005). Additionally, the suppressors scd-2 and daf-2 have also been found – although less is known about how they are affecting synaptic development (Hung et al., in preparation; Liao et al., 2004). scd-2 codes for a receptor tyrosine kinase homologous to the proto-oncogene anaplastic lymphoma kinase (ALK), which when mutated can cause non-Hodgkin’s lymphoma (Morris et al., 1997). The insulin receptor DAF-2 regulates the activity of the global transcription factor DAF-16 which likely controls a retrograde signaling factor that affects synaptic development (Figure 13). Presumably, factors interacting with either scd-2 or daf-2 could be found in fsn-1 suppressor screens. It is possible that one of the unknown alleles isolated in our screen codes for such a factor and could therefore help us understand how the fsn-1 suppressors are affecting synaptic development.
References


Morris, S.W., Naeve, C., Mathew, P., James, P.L., Kirstein, M.N., Cui, X., and Witte, D.P. (1997). ALK, the chromosome 2 gene locus altered by the t(2;5) in non-Hodgkin's lymphoma, encodes a novel neural receptor tyrosine kinase that is highly related to leukocyte tyrosine kinase (LTK). Oncogene 14, 2175-2188.


Po, M.D., Hwang, C., and Zhen, M. PHRs: bridging axon guidance, outgrowth and synapse development. Curr Opin Neurobiol 20, 100-107.


Appendix

1 Abbreviations

Ach – Acetylcholine
AGE – Age alteration
AKT – AKT kinase family
ALK – Anaplastic Lymphoma Kinase
ATP – Adenosine triphosphate
AZ – Active Zone
CAM – Cellular Adhesion Molecule
CASK – CamK/SH3/guanylate kinase domain protein
CAZ – Cytomatrix at the Active Zone
CEBP – CCAAT/enhancer binding protein
CEP – C. elegans p53-like protein
CGC – Caenorhabditis Genomics Centre
CNS – Central Nervous System
DAF – Abnormal Dauer Formation
DLK – DAP (Death Associated Protein kinase) Like Kinase
DFsn – Drosophil fsn-1 orthologue
ECM – Extracellular Matrix
ERC – ELKS-Rab6-interacting protein CAST
EMS – Ethane Methyl Sulfonate
ENU - N-ethyl-N-nitrosourea
FSN – F-box Synaptic Protein
GABA – γ-amino-butyric-acid
GAD – Glutamic Acid Decarboxylase
GFP – Green Fluorescent Protein
GLO – Gut granule LOss
Hiw – Highwire
IGF – Insulin Growth Factor
KLC – Kinesin light chain
LAR – leukocyte common antigen related protein
LIN – Abnormal cell lineage
Liprin – LAR interacting protein
LRR – Leucine-rich repeat
LTP – Long Term Potentiation
MAGUK – Membrane associated guanylate kinases
MAK – MAP Kinase Activated protein Kinase
MAPK(KK) – Mitogen activated protein kinase (kinase kinase)
MKK – Mitogen Activated Protein Kinase Kinase
NCRR – National Centre for Research Resources
NGM – Nematode Growth Medium

NID – Nidogen (basement membrane protein)

NMJ – Neuromuscular junction

PAM – Protein Associated with Myc

PAR – Prostate apoptosis response

PDK – Phosphoinositide Dependent Kinase class protein kinase

PHR – Pam, Highwire, RPM-1

PMK – p38 Map Kinase family

PSD – Postsynaptic density

PTP – protein tyrosine phosphatase

Rbx – RING box protein

RFLP – Restriction Fragment Length Polymorphism

RIM – Rab3 Interacting Molecule

RING – Really Interesting New Gene

RPM – Regulator of Presynaptic Morphology

RPTP – Receptor Protein Tyrosine Phosphotase

SAD – Synapses of the Amphids Defective

SCD – Suppressor of Constitutive Dauer

SCF – Skp1 Cullin F-box

SKR – Skp1 related (ubiquitin ligase complex component)
SNAP – Synaptosome associated protein

SNARE–Soluble N-ethylmaleimide-sensitive fusion (NSF) attachment protein receptor

SNB – Synaptobrevin

SNP – Single Nucleotide Polymorphism

SPRY – Sp1a and Ryanodine receptor

SYD – Synapse Defective

Ub - Ubiquitin

UNC – Uncordinated

UPS – Ubiquitin Proteasome System

2 Strains used in this study

Bristol/N2

Hawaiian/CB4856

CZ2333  juIs1

ZM15  fsn-1(hp1) III ;juIs1 IV

ZM235  juIs1 IV ;rpm-1(ju44)

ZM1220  pmk-3 (hp246) IV

ZM234  fsn-1(hp1) III;juIs1 IV;sad-1(ky289) X

CZ1338  juIs1 IV;rpm-1(ju44) V;syd-2(ok217) X

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