RNAi suppressor screen to identify novel genetic interactors with the sydn-1/pfs-2 neurodevelopmental pathway of Caenorhabditis elegans and construction of plasmid vectors for yeast-two-hybrid and in vivo analyses

Mitchell Lee
Western Washington University

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RNAi suppressor screen to identify novel genetic interactors with the sydn-1/pfs-2 neurodevelopmental pathway of Caenorhabditis elegans and construction of plasmid vectors for yeast-two-hybrid and in vivo analyses

By

Mitchell Lee

Accepted in Partial Completion
Of the Requirements for the Degree
Master of Science

Kathleen L. Kitto, Dean of the Graduate School

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MASTER’S THESIS

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Mitchell Lee
October 7, 2012
RNAi suppressor screen to identify novel genetic interactors with the sydn-1/pfs-2 neurodevelopmental pathway of Caenorhabditis elegans and construction of plasmid vectors for yeast-two-hybrid and in vivo analyses

A Thesis
Presented to
The Faculty of
Western Washington University

In Partial Fulfillment
Of the Requirements for the Degree
Master of Science

By
Mitchell Lee
October 2012
PROPER DEVELOPMENT OF NEURONAL CIRCUITS ARE CRUCIAL FOR NERVOUS SYSTEM FUNCTIONING.

A novel pathway regulating axon and synapse development in Caenorhabditis elegans through nuclear 3’-end polyadenylation of nascent mRNA has recently been uncovered (Van Epps et al., 2010). In this pathway, the protein product of the gene synaptic defective enhancer (sydn-1) negatively regulates polyadenylation factor subunit homolog (PFS-2), an evolutionarily conserved scaffolding protein in a multi-protein complex involved in mRNA 3’-end processing. Although 3’-end processing of mRNA has a regulatory role in many cellular processes, regulation of synapse and axon development via this cellular mechanism has not been characterized.

An RNAi screen was performed using C. elegans to identify genetic suppressors in this regulatory pathway. This screen was conducted in a sensitized genetic background that allowed for targeted gene silencing and visual screening for suppression of a neuronal phenotype resulting from SYDN-1 mediated misregulation of PFS-2. We identified seven novel genetic interactors. One of these genes, ELAV-type RNA binding protein family (etr-1), encodes a highly conserved protein that is involved in 3’-end alternative splicing during muscle development. The human homolog of ETR-1, CUGBP, elav-like family member (CELF1), is implicated in the development of the genetic disease myotonic dystrophy.

Vectors were produced to further study the function of these proteins in the context of the SYDN-1/PFS-2 pathway. One vector contains etr-1 cDNA fused to a gene encoding GFP, and the other contains CELF1 cDNA fused to the yeast-two-hybrid GAL4 DNA-binding domain, to be used for in vivo localization and yeast-two-hybrid assays, respectively.
ACKNOWLEDGMENTS

I am deeply indebted to many individuals for support of my research and the composition of this thesis. Foremost, I would like to thank my thesis advisor, Lynn Pillitteri. Through numerous conversations with, and critical feedback from, Lynn, I have become a better writer, thinker, and researcher. My thesis committee has been a constant source of support and has kept me on track during this process. For that I would like to thank Joann Otto, Jackie Rose, and again, Lynn Pillitteri. The detailed microscopy training I’ve received while at Western (and some fascinating conversations on the philosophy of science) would not have been possible without the engagement and guidance of David Leaf.

I am grateful to my research mentor Heather Van Epps for allowing me into her lab, teaching me numerous different molecular and genetic techniques, and introducing me to the much-adored nematode, *Caenorhabditis elegans*. I thank Genie Ankoudinova and Erika Bjorklund for help performing the RNAi suppressor screen. Ben Allen performed previous yeast-two-hybrid analyses using ETR-1 and PFS-2.

Lastly, my family and friends (particularly Matthew Loeffelholz and fellow Biology grad students) have been a vital support structure for this venture into the unknown that has been higher education. Most importantly in this regard, I would like to thank Cambee Jines for her care and support.
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<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>3' UTR</td>
<td>3' -Untranslated region</td>
</tr>
<tr>
<td>ASE</td>
<td>Amphid sensilla neuron</td>
</tr>
<tr>
<td>ASEL</td>
<td>Amphid sensilla neuron left</td>
</tr>
<tr>
<td>ASER</td>
<td>Amphid sensilla neuron right</td>
</tr>
<tr>
<td>BDM</td>
<td>2,3-butanedione monoxime</td>
</tr>
<tr>
<td>cAMP</td>
<td>cyclic adenosine monophosphate</td>
</tr>
<tr>
<td>cGMP</td>
<td>cyclic guanine monophosphate</td>
</tr>
<tr>
<td>CPE</td>
<td>cytoplasmic polyadenylation element</td>
</tr>
<tr>
<td>CPEB</td>
<td>cytoplasmic polyadenylation element binding protein</td>
</tr>
<tr>
<td>CPF</td>
<td>Cleavage and polyadenylation factor</td>
</tr>
<tr>
<td>CPSF</td>
<td>Cleavage and polyadenylation specificity factor</td>
</tr>
<tr>
<td>CstF</td>
<td>Cleavage stimulation factor</td>
</tr>
<tr>
<td>DBD</td>
<td>DNA binding domain</td>
</tr>
<tr>
<td>DIC</td>
<td>Differential interference contrast microscopy</td>
</tr>
<tr>
<td>dsRNA</td>
<td>double-stranded RNA</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>EMS</td>
<td>ethyl methanesulfonate</td>
</tr>
<tr>
<td>EtBr</td>
<td>ethidium bromide</td>
</tr>
<tr>
<td>GABA</td>
<td>γ-aminobutyric acid</td>
</tr>
<tr>
<td>gDNA</td>
<td>genomic DNA</td>
</tr>
<tr>
<td>GFP</td>
<td>Green fluorescent protein</td>
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<tr>
<td>GTP</td>
<td>Guanosine triphosphate</td>
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<tr>
<td>IPTG</td>
<td>Isopropyl β-D-1-thiogalactopyranoside</td>
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<tr>
<td>LB</td>
<td>lysogeny broth</td>
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<td>LB_AMP</td>
<td>lysogeny broth + ampicillin</td>
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<tr>
<td>LB_SPEC</td>
<td>lysogeny broth + spectinomycin</td>
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<tr>
<td>MCS</td>
<td>Multiple cloning site</td>
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<tr>
<td>miRNA</td>
<td>micro RNA</td>
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<tr>
<td>mRNA</td>
<td>messenger RNA</td>
</tr>
<tr>
<td>NGM</td>
<td>Nematode growth media</td>
</tr>
<tr>
<td>PABP</td>
<td>Polyadenylation binding protein</td>
</tr>
<tr>
<td>PAP</td>
<td>Polyadenylation polymerase</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase chain reaction</td>
</tr>
<tr>
<td>poly(A)</td>
<td>Polyadenosine monophosphate</td>
</tr>
<tr>
<td>RBP</td>
<td>RNA-binding protein</td>
</tr>
<tr>
<td>RdRP</td>
<td>RNA-dependent RNA polymerase</td>
</tr>
<tr>
<td>Acronym</td>
<td>Definition</td>
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<tr>
<td>RISC</td>
<td>RNA-induced silencing complex</td>
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<tr>
<td>RNA-Seq</td>
<td>RNA sequencing</td>
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<tr>
<td>RNAi</td>
<td>RNA interference</td>
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<tr>
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<td>Endoribonuclease</td>
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<td>RNP</td>
<td>Ribonucleoprotein</td>
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<td>RNA-recognition motif</td>
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<tr>
<td>RT-PCR</td>
<td>Reverse-transcriptase PCR</td>
</tr>
<tr>
<td>siRNA</td>
<td>short interfering RNA</td>
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<tr>
<td>SOC</td>
<td>Super optimal broth with catabolite repression</td>
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<tr>
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<td>single-stranded RNA</td>
</tr>
<tr>
<td>TAE</td>
<td>Tris-acetate-EDTA</td>
</tr>
<tr>
<td>TD-PCR</td>
<td>Touchdown PCR</td>
</tr>
<tr>
<td>Znf</td>
<td>Zinc-finger</td>
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LITERATURE REVIEW

1.1 Axon and synapse formation

Nervous system function is established through the production of circuits composed of neurons. Neurons are a specialized cell-type that transmit electrochemical signals via long projections called axons and form connections with other cells at sites known as synapses (Sanes et al., 2005; Sanes et al., 2011). Axon and synapse development are the two major processes that shape neuronal circuitry and architecture. Axonal growth is directed by structures at their tips known as growth cones. Growth cones contain actin cytoskeletal elements (lamellipodia and filopodia) at their distal ends and interact with proximal microtubule elements that form and stabilize the nascent axon (Schaefer et al., 2008; Smith, 1988; Tanaka et al., 1995).

The growth cone perceives extracellular guidance cues which are ultimately processed as either positive or negative chemotactic signals. Secreted proteins, such as netrins, slits, semaphorins, and ephrins, as well as small molecules like nitric oxide elicit chemotactic signaling by triggering intracellular Rho GTPases and other signal transduction pathways (Fukura et al., 2000; Gordon-Weeks, 2004; Lowery and Van Vactor, 2009; O'Donnell et al., 2009). Zinc-finger (Znf) and LIM homeodomain transcription factors and local concentrations of second messengers, such as cAMP, cGMP, and Ca\(^{2+}\) ions, in the growth cone are responsible for axon outgrowth and pathfinding (Herrera et al., 2003; Nicol et al., 2011; Pak et al., 2004; Polleux et al., 2007; Shelly et al., 2010; Yoshida et al., 2009). Growth cone activity is dynamic; filopodial actin filaments collapse in certain directions while new actin filaments are synthesized in other directions, allowing the growth cone to
scan the extracellular milieu for chemical signals (Sanes et al., 2011). Directionality and movement of the growth cone is dependent on peripheral actin filaments and microtubules (Baas et al., 2006; Rajnicek et al., 2006; Tanaka and Kirschner, 1995). Synaptogenesis (the formation of new synapses) is initiated once a specific set of signals is received by the growth cone.

Synapses are sites of intercellular communication (Sanes et al., 2011). Synapses can be formed between two different components of nervous tissue, such as between an axon and a dendrite, or between nervous and muscle tissue, as is the case with motor neurons which communicate with muscle fibers. During synaptogenesis, specific changes occur in the growth cone and the target cell which facilitate the transmission of chemical and electrical signals. For instance, synaptic vesicles are shuttled from the axon and form the presynaptic active zone, whereas neurotransmitter receptors, scaffolding proteins, and cell adhesion molecules form the postsynaptic density at the target site (Gerrow and El-Husseini, 2006; Kennedy, 2000; Ziff, 1997).

1.2 Regulation of axon and synapse formation

1.2.1 Regulation of axon and synapse formation via cytoplasmic mRNA regulation and processing. mRNA regulation is an important means of producing fine-tuned responsiveness in developing nervous tissue. The growth cone must quickly respond to external and internal cues to properly guide axon growth and determine proper targets for synaptogenesis. Growth cone responses are mediated, in part, through local mRNA regulation and transport via microRNAs (miRNAs), RNA-binding proteins (RBPs), and cytoplasmic polyadenylation. miRNAs are 17-25 base pair RNA fragments that can bind the
miRNA-mediated gene regulation is broadly conserved throughout eukaryotes (Molnar et al., 2007; Pasquinelli et al., 2000). In *C. elegans*, over 100 miRNAs have been identified (Ruby et al., 2006). Numerous miRNA-regulating pathways are involved in early cell-fate specification. Larval development in *C. elegans* occurs in stages, L1-L4, which are separated by molts of a collagenous cuticle (Riddle et al., 1997). Progression through these stages is directed by heterochronic genes that establish cell-fates (Lee et al., 1993; Olsen and Ambros, 1999; Wightman et al., 1993). An important positive regulator of the L1 to L2 juvenile stage transition is the transcription regulator *abnormal cell lineage* (*lin-14*) (Lee et al., 1993; Wightman et al., 1993). LIN-14 is highly expressed at the L1 stage, but expressed at a much lower level at the L2 stage (Hong et al., 2000). Overexpression of LIN-14 results in cells that maintain an L1 developmental program and do not progress to L2. LIN-14
translation is negatively regulated by the miRNA, lin-4, which binds to the 3'UTR of lin-14 transcripts and inhibits translation initiation (Olsen and Ambros, 1999).

Nervous tissue-specific examples of miRNA regulation include the C. elegans miRNA (mir-124) (Clark et al., 2010). The conserved functions of mir-124 have not been elucidated, but multiple lines of evidence suggest that this miRNA functions as a neuronal cell-fate specification factor. mir-124 downregulates non-neuronal mRNAs in neural stem cells and is thought to upregulate neuronal differentiation mRNAs, (reviewed in Saba and Schratt, 2010). Recently, the Xenopus laevis microRNA mir-124 has been found to play a role in axonal migration by altering growth cone sensitivity to the secreted chemosensory molecule semaphorin (Baudet et al., 2012).

In C. elegans nervous tissue, miRNAs are used in establishing left/right asymmetry between the amphid sensilla (ASE) class of sensory neurons (Chang et al., 2004; Chang et al., 2003; Johnston and Hobert, 2003). There are two ASE neurons, ASE left (ASEL) and ASE right (ASER), and these are the main sensors responsible for perceiving K⁺, Na⁺, Cl⁻, and water soluble small molecules (Bargmann and Horvitz, 1991; Hall and Altun, 2008; Pierce-Shimomura et al., 2001). These neurons asymmetrically express membrane-bound guanylate cyclases that are involved in chemosensory function (Yu et al., 1997). Asymmetry is established through two miRNAs, laterally symmetric (lsy-6) and mir-273 which are expressed in an asymmetric manner between ASE neurons. In ASER, mir-273 is expressed and binds to the 3'UTR of the Znf transcription factor, dorsal intercalation and elongation defect (die-1), resulting in its repression and production of the guanylate cyclase (GCY-5). In ASEL, mir-273 is not expressed and DIE-1 accumulates (Chang et al., 2004). DIE-1
expression results in the production of LSY-6 in these neurons and, ultimately, the synthesis of GCY-7, an asymmetrically-expressed guanylate cyclase. Through the differential expression of these two miRNAs, specific cellular identities are established for these neurons.

RNA-binding proteins (RBPs) are a class of proteins whose function is to form complexes with RNA molecules. A subset of RBPs function by binding to mRNAs in the cytoplasm, resulting in varied types of posttranscriptional regulation (Agnes and Perron, 2004). RBP binding to an mRNA often represses or promotes translation while preventing degradation of the transcript. Additionally, certain RBPs localize mRNAs to discrete subcellular areas (Jung et al., 2012; Wang et al., 2010). When an RBP dissociates from the mRNA, this transcript can then be translated in situ. This mechanism facilitates fine-tuned production of protein in precisely localized cellular regions, regulating synaptic protein expression and stability (Casadio et al., 1999; Kiebler and DesGroseillers, 2000; Martin et al., 1997; Schuman, 1999; St. Johnston, 1995). In axon development, RBPs facilitate translation of neuronal transcripts which function in neurite outgrowth and growth cone navigation (Antic et al., 1999; Aranda-Abreu et al., 1999; Kuwako et al., 2010; Lin and Holt, 2007; Lin and Holt, 2008; Welshhans and Bassell, 2011). A different set of RBPs exert posttranscriptional control through alternative splicing of pre-mRNA. This processing step is performed in the nucleus by the spliceosome, which is composed of ribonucleoproteins (RNPs) and secondary factors that determine pre-mRNA binding and splice-site usage (Black, 2003; Chen and Manley, 2009; Wahl et al., 2009).
Once a transcript has left the nucleus, changes in mRNA 3’ polyadenylation state in the cytoplasm up- or downregulate mRNA translation. The 3’ polyadenosine monophosphate (poly(A)) tail of mature mRNA slowly degrades while in the cytoplasm. Over time, the poly(A) tail becomes so short that translational initiation is hampered due to inefficient binding of poly(A)-binding protein (PABP) to the poly(A) tail (Mendez and Richter, 2001; Villalba et al., 2011). Expansion of the poly(A) tail by cytoplasmic cleavage and polyadenylation specificity factor (CPSF), poly(A) polymerase (PAP), and other factors promotes translation of the transcript. Conversely, deadenylating factors in the nucleus and cytoplasm degrade the poly(A) tail, thereby hampering translation (Zhang et al., 2010). Regulation via cytoplasmic polyadenylation involves binding to cis-elements, nucleotide sequences that are part of the mRNA transcript, known as cytoplasmic polyadenylation elements (CPEs). CPEs are bound by CPE-binding (CPEB) proteins, which contain a set of conserved RNA recognition motifs (RRMs) (Hafer et al., 2011). One well-characterized mechanism of CPEB function involves phosphorylation of CPEB which drives polyadenylation of a bound transcript. Phosphorylated CPEB and translation initiation factors bind to elements in the mRNA 3’-UTR that facilitate protein synthesis (Richter, 2007; Villalba et al., 2011). CPEB proteins are involved in *Xenopus laevis* retinal axon outgrowth, asymmetrical neural cell division in *Drosophila melanogaster*, and synaptic formation and stability in *Drosophila melanogaster* (Hafer et al., 2011; Lin et al., 2009; Mastushita-Sakai et al., 2010; Wu et al., 1998).

1.2.2 Regulation of axon and synapse formation via nuclear RNA processing. mRNA transcription requires multiple processing steps. At the 5’-end of the pre-mRNA transcript, a
cap composed of a 7-methyl guanosine residue is attached. This “capping” of nascent pre-mRNA transcripts is thought to function primarily as a protection from 5’-3’ exonuclease-mediated degradation (Furuichi et al., 1977; Shimotohno et al., 1977). 5’-end capping also functions in export of mRNA transcripts from the nucleus as well as other aspects of RNA processing (Cooke and Alwine, 1996; Izaurralde et al., 1994; Jarmolowski et al., 1994).

For mRNA to be properly translated into protein, introns must be removed. This is because the polymerase involved in mRNA transcription, RNA polymerase II, cannot distinguish between introns and exons while transcribing DNA into pre-mRNA. The removal of introns is known as splicing and it is performed by a multi-protein complex known as the spliceosome (Wahl et al., 2009). In addition to splicing out introns, exons can also be spliced out of a pre-mRNA transcript to form different isoforms of a protein. This process is known as alternative splicing and is a major regulatory mechanism driving animal development (Elliott and Grellscheld, 2006; Grabowski and Black, 2001).

The final nuclear processing step that must be completed for a pre-mRNA transcript to become functional mRNA is cleavage at its 3’-end and polyadenylation. Polyadenylation is the process of adding between 100-250 adenosine monophosphate residues to the 3’-end of the nascent pre-mRNA transcript before it is transported out of the nucleus (Colgan and Manley, 1997; Proudfoot, 2004; Zhao et al., 1999). Cleavage and nuclear polyadenylation play roles in export from the nucleus, translation efficiency, and stability of the mRNA transcript itself (Jacobson and Peltz, 1996; Lewis et al., 1995; Sachs et al., 1997; Wickens et al., 1997). The nuclear cleavage and polyadenylation machinery is a set of large, multi-protein complexes. The function of these complexes is conserved across taxa, but the
individual protein components and the total number of proteins involved in these complexes vary (MinvielleSebastia et al., 1997; Preker et al., 1997; Shi et al., 2009). Dysfunctions in cleavage and nuclear polyadenylation are associated with numerous diseases such as muscular dystrophy, cancer, thrombophilia, and thalassemias (Danckwardt et al., 2008; Mayr and Bartel, 2009).

Nuclear polyadenylation is regulated by both cis- and trans-elements. There are three major cis-elements directing polyadenylation, a poly(A) signal composed of a hexamer, most commonly the sequence AAUAAA, the cleavage and poly(A) addition site, which is 11-23 bps downstream of the poly(A) signal, and a GU- or U-rich region, found 10-30 bps from the cleavage and poly(A) addition site (Edwalds-Gilbert et al., 1997; Gil and Proudfoot, 1984; Hart et al., 1985; Liu et al., 2001; McDevitt et al., 1986; Sadofsky and Alwine, 1984; Zhao et al., 1999). Poly(A) site strength has a profound effect on the type of transcripts produced by influencing protein isoform synthesis and the overall ability of the transcriptional machinery to produce functional transcripts (Black, 2003; Denome and Cole, 1988; Edwalds-Gilbert et al., 1993; Edwalds-Gilbert et al., 1997; Tian et al., 2005). This is often due to the binding of trans-elements, proteins that functionally interact with pre-mRNA during the cleavage and polyadenylation reaction (Tian et al., 2005).

Recently, it was found that synapse and axon formation in C. elegans is regulated, in part, through genes associated with nuclear 3’ polyadenylation (Van Epps et al., 2010). Synaptic defective enhancer (sydn-1) was identified through an ethyl methanesulfonate (EMS) mutagenesis screen as an enhancer of locomotion defects in a regulator of presynaptic morphology (rpm-1) null mutant C. elegans strain. sydn-1 single mutants
produce a phenotype with narrowing axonal diameter, axonal protrusions that extend off of
commissural axons (dorsoventrally-migrating axons that travel from one nerve cord to
another), and a large reduction of presynaptic sites known as puncta (Van Epps et al., 2010).

SYDN-1 is a worm-specific polyproline protein. SYDN-1 contains no recognized
domains as determined by NCBI and SMART bioinformatic resources (Bradshaw et al.,
1998; Letunic et al., 2012; Schultz et al., 1998). SYDN-1 mode of action is thought to reside
in multiple polyproline repeat motifs that are found throughout the C-terminal region. There
are five regions with six or more prolines, and 35.6% of the 233 C-terminal amino acids are
prolines. Polyproline binding is an important regulatory feature of proteins such as the actin-
binding protein profilin and tryptophan/tryptophan (WW) domain-containing proteins
(Ingham et al., 2005; Ostrander et al., 1999).

A second EMS mutagenesis screen in a sydn-1; synapse defective (syd-2) double
mutant background strain determined that a partial loss-of-function mutation in
polyadenylation factor subunit homolog (pfs-2) suppressed the neuronal phenotype caused by
sydn-1 loss-of-function (Van Epps et al., 2010). PFS-2 is a conserved protein component of
the machinery that performs nuclear cleavage and polyadenylation (known in yeast as
cleavage and polyadenylation factor (CPF) and in mammals as cleavage and polyadenylation
specificity factor (CPSF)) (Ohnacker et al., 2000; Shi et al., 2009; Simpson et al., 2003; Vo et
al., 2001; Zhao et al., 1999). pfs-2 encodes a protein containing seven WD40-repeat domains
(a domain consisting of tryptophan/aspartate amino acid repeats every 40 amino acid
residues) which often serve as scaffolds for multi-protein interactions (Neer et al., 1994;
Ohnacker et al., 2000; Smith et al., 1999). The pfs-2 mutation identified in the EMS screen
altered one of these conserved WD40 repeat sequences (Van Epps et al., 2010). PFS-2 has been shown to physically interact with 32 different proteins through various \textit{in vitro} biochemical assays (Stark et al., 2006). The total number of proteins that interact with PFS-2 in \textit{C. elegans} and what roles they may have in the SYDN-1/PFS-2 pathway are not known.

1.3 Two models for SYDN-1/PFS-2 function

Models describing SYDN-1 negative regulation of PFS-2 can be generated from published reports (Van Epps et al., 2010). SYDN-1 may directly bind to PFS-2 and cause degradation of PFS-2 (Figure 1a). In support of this, SYDN-1 partially colocalizes with PFS-2 and a SYDN-1 loss-of-function mutation results in accumulation of PFS-2 protein in the nucleus. Misregulation of pre-mRNA processing in this model results from excessive polyadenylation due to overexpression of PFS-2 and stabilization of the cleavage and polyadenylation machinery. Excessively polyadenylated mRNAs are likely to be more stable in the cytoplasm and may perhaps be overly translated, thereby altering the biochemical environment of the cell.

A second model of SYDN-1/PFS-2 function suggests that SYDN-1 binds with PFS-2, or some other cleavage and polyadenylation subunit resulting in a change in cleavage and polyadenylation (Figure 1b-d.). A disruption in cleavage and poly(A) processing should result in an overall decrease in mature mRNA production. SYDN-1 could act to recruit or block an unknown cleavage and polyadenylation trans-element (Figure 1c). If SYDN-1/PFS-2 interaction leads to a change in cis-element binding efficiency, this could favor transcriptional termination at one poly(A) site over another, leading to a change in translated
protein isoforms (Figure 1d). Either of these models could explain the sydn-1 phenotypes. These models, however, do not address how SYDN-1 alters the nuclear abundance of PFS-2.
Figure 1. Two models of PFS-2/SYDN-1 interaction. A) In the most parsimonious model, SYDN-1 binds to PFS-2 causing the degradation of PFS-2 and large-scale cellular downregulation of cleavage and polyadenylation activity. B-D) In other models, SYDN-1 is an auxiliary cleavage and polyadenylation component that could act by recruiting or sequestering other factors (protein X) or binding to PFS-2 directly. SYDN-1, in these models, causes a change from one isoform being produced (B) to a different protein isoform being produced when present in the nucleus (C-D). SYDN-1 may also result in no protein being produced when it is expressed.
A broad set of transcript targets would be potentially affected if PFS-2 was absent or misregulated. Because this pathway is poorly understood, it is likely that further proteins are involved in this regulatory pathway. To discover additional components that regulate synapse and axon formation through the SYDN-1/PFS-2 polyadenylation pathway, as well as targets of PFS-2 misregulation, an RNAi suppressor screen was performed in a \textit{sydn-1;syd-2} mutant background. One of these genes, \textit{etr-1}, encodes a highly conserved RBP. Homologs of \textit{etr-1} and \textit{pfs-2} physically interact with each other in \textit{Arabidopsis thaliana} to regulate floral timing (Simpson et al., 2003). Construction of additional genetic tools to characterize ETR-1 function in \textit{C. elegans} and determine the conservation of PFS-2 binding with ETR-1 homologs was done for future use.
INTRODUCTION

1. The use of Caenorhabditis elegans in biological research

The soil nematode Caenorhabditis elegans (or simply “the worm”, as it is lovingly referred to by many) is an ideal organism for studying numerous questions currently being asked in 21st century biology. C. elegans is incredibly conducive to laboratory conditions, having a small size (1-1.5 mm), quick development (≈3 days from embryo to embryo-laying adult at 20°C), and the ability to subsist solely on the bacteria Escherichia coli (E. coli) as a food source (Brenner, 1974). C. elegans cultures are able to withstand being frozen in liquid nitrogen or stored in -80°C incubation, due to the existence of a spore-like resting phase known as dauer (Wood, 1988). C. elegans has two sexes: the predominant hermaphrodite, and the less common male. Genetically, C. elegans are diploid and contain six chromosomes (five autosomes, and one sex chromosome) (Sulston and Brenner, 1974). Individuals that are homozygous at a given loci can be quickly created and maintained through hermaphrodite selfing and genetic variation can be introduced through sexual reproduction using males. C elegans are transparent and transgenic organisms can be created with relative ease, therefore, they are well-suited for fluorescent labeling and visualization, as well as analyses using differential interference contrast (DIC) microscopy.

One of the most notable features of C. elegans is its nearly invariant development (Brenner, 1974; Riddle et al., 1997). The hermaphrodite worm contains 959 somatic cells (Wood, 1988). Cell lineage mapping has traced each of these 959 cells back to the embryo (Sulston et al., 1983; Wood, 1988). The 302 neurons in the hermaphrodite have also been mapped via serial electron micrographs to create a complete neuronal wiring diagram (White...
et al., 1986). This level of understanding has allowed researchers to define incredibly
detailed and nuanced neuronal phenotypes.

2. RNA interference

2.1 Mechanism

In eukaryotic cells, two types of double-stranded RNAs (dsRNAs) are found:
exogenous dsRNAs which are introduced into the cell often via viral infection, or
endogenous dsRNAs which are produced either as a precursor in posttranscriptional gene
silencing processes or through the movement of transposons (Bartel, 2009; Sijen and
Plasterk, 2003; Tabara et al., 1999; Watanabe et al., 2006; Wilkins et al., 2005). RNA
interference (RNAi) is a gene-silencing process by which dsRNA is enzymatically degraded
into short-interfering RNAs (siRNAs) that are used as templates for the enzymatic
degradation of complimentary mRNA transcripts (Fire et al., 1998; Martinez et al., 2002).
RNAi can be broken into two phases: the initiation phase, where siRNAs are produced, and
the effector phase, where posttranscriptional gene-silencing occurs through the binding of
these siRNAs to cognate mRNA transcripts.

The initiation phase of RNAi involves the production of siRNAs from dsRNA
precursors. This is performed by the bidentate endoribonuclease Dicer, which is a member
of the RNase III nuclease family (Bernstein et al., 2001). The subset of RNase III proteins to
which Dicer belongs contains 1-2 dsRNA binding domains, two catalytic domains, and a
PAZ domain (named for the proteins Piwi, Argonaute, and Zwille/pinhead) which has been
suggested to function in the aggregation of protein complexes and binding of the cleaved
dsRNA product (Cerutti et al., 2000; Ma et al., 2004). Dicer forms a homodimeric structure
and cleaves dsRNA into multiple duplex 21-25 siRNAs. siRNAs have a unique two-nucleotide overhang on their 3’ ends that is anchored by PAZ domain-containing proteins (Ma et al., 2004). The structure of Dicer determines the size of the siRNAs that are produced via the distance between the PAZ domain and the RNase III domains (MacRae et al., 2006). Numerous different protein cofactors and dsRNA-binding proteins (dsRBPs) are also involved in this process (Chendrimada et al., 2005; Forstemann et al., 2005; Haase et al., 2005; Hiraguri et al., 2005; Parker et al., 2006; Parrish and Fire, 2001). In addition to the production of siRNAs, Dicer is also responsible for the production of microRNAs (miRNAs) which cause post-transcriptional gene silencing in a similar manner to siRNAs, except that miRNAs can bind the 3’ UTR of the genes they target and do not perfectly base-pair (Grishok et al., 2001; Hutvagner et al., 2001).

Coordination between the initiation and effector phases is still being experimentally determined, but unique protein cofactors are required, (reviewed in Gaynor et al., 2010). A protein cofactor complexes with the PAZ domain of Dicer and facilitates the interaction of Dicer’s PAZ domain and the PAZ domain from a component of the RNA-induced silencing complex (RISC) (Elbashir et al., 2001a; Liu et al., 2003; MacRae et al., 2006). The siRNA held by the Dicer complex is transferred to the inactive RISC. For RISC to be activated, the passenger RNA strand must be degraded so that the guide strand can be used to complement cognate mRNAs. The best-supported theory concerning this degradation is that the passenger RNA strand is the first target of the RISC complex and additional protein factors are involved in removing oligonucleotide fragments from the passenger strand (Liu et al., 2009; Matranga et al., 2005; Preall and Sontheimer, 2005; Rand et al., 2005).
In the effector phase of RNAi, the guide strand loaded into RISC is used to target complementary mRNAs for degradation (Martinez et al., 2002). The main enzymatic component of RISC complexes are members of the eukaryotic-conserved Argonaute family of proteins (Hammond et al., 2001; Liu et al., 2004; Rivas et al., 2005). Members of this protein family contain conserved PAZ and PIWI domains (Hock and Meister, 2008). Those Argonaute members that are involved in RNAi use the PAZ domain for binding of siRNAs generated by dicer activity and the PIWI domain for “slicer” activity, or cleavage of mRNAs (Lingel et al., 2003; Ma et al., 2004; Parker et al., 2004; Song et al., 2003; Song et al., 2004; Yan et al., 2003; Yuan et al., 2005). Once a complimentary mRNA has base-paired with the guide strand, the mRNA is cleaved via a hydrolysis reaction 10 nucleotides from the 5’ end of the guide strand (Elbashir et al., 2001b; Martinez and Tuschl, 2004). Although RISC enzymatic activity has been established in vitro using only an Argonaute protein and a bound guide strand, other protein components are required for efficient recycling of RISC through the degradation of cleavage products (Martinez and Tuschl, 2004; Orban and Izaurralde, 2005; Rivas et al., 2005).

2.2 Function and Use of RNAi in C. elegans

RNAi confers a robust, non-autonomous response in C. elegans, even at very low concentrations (Fire et al., 1998). It is thought that dsRNA is polymerized de novo using single-stranded RNA (ssRNA) from the original dsRNA as a primer and the target mRNA as template (Mango, 2001). This generates dsRNA that can then be processed by the Dicer complex. This process is known as transitive RNAi and has been well-documented in C. elegans (Alder et al., 2003; Sijen et al., 2001). Transitive RNAi requires an RNA-dependent
RNA polymerase (RdRP) to catalyze the synthesis of dsRNA. These types of RdRPs have been found to function in posttranscriptional gene silencing in numerous plants, *Neurospora crassa*, and *C. elegans*, but not in mammals or *Drosophila melanogaster* (Birchler, 2009; Cogoni and Macino, 1999; Roignant et al., 2003; Smardon et al., 2000).

The first RNAi experiments in *C. elegans* were performed using dsRNA that was purified and injected into somatic and gonad tissues (Fire et al., 1998). Although this method conferred robust reduction in transcript accumulation, purification of RNA and injection via micropipette are laborious processes. Shortly thereafter, it was discovered that dsRNA can trigger RNAi in worms via soaking the animals in dsRNA solutions or feeding them *E. coli* that express dsRNA transcribed from vectors using the bacteriophage T7 polymerase (Tabara et al., 1998; Timmons and Fire, 1998). The process of triggering RNAi through uptake of dsRNA from outside the organism is known as environmental RNAi. In *C. elegans*, dsRNA is initially imported into epithelial cells of the intestinal lumen, then through other somatic cells via two transmembrane channels, systemic RNA interference defective (SID-1 and SID-2) (Winston et al., 2002; Winston et al., 2007). Many other components of the non-autonomous RNAi pathway, such as vesicle trafficking proteins, have been identified (reviewed in Whangbo and Hunter, 2008). Optimization of feeding-based dsRNA delivery has facilitated widespread use of *C. elegans* in high-throughput reverse genetic screening (Kamath et al., 2001). In neuronal contexts, RNAi is inefficient without the use of sensitizing genetic mutations that facilitate expression of dsRNA in this tissue type (Kamath et al., 2003; Kennedy et al., 2004; Simmer et al., 2002; Tavernarakis et al., 2000; Timmons et al., 2001). Using strains containing background mutations in these genes, screens were
performed that identified genes associated with GABAergic motor neurons (neurons which innervate muscle cells) and synapses in *C. elegans* (Figure 2) (Sieburth et al., 2005; Vashlishan et al., 2008). GABAergic motor neurons produce the neurotransmitter γ-aminobutyric acid (GABA). There are 26 GABAergic neurons in *C. elegans* and most are inhibitory (Riddle et al., 1997).
Figure 2. Fluorescence micrograph and diagram of *C. elegans* γ-aminobutyric acid (GABA)-expressing nervous tissue. A) Fluorescence micrograph, scale bar = 0.1 mm, B) diagram of *Caenorhabditis elegans* GABA-expression neuron, and C) enlarged view of posterior-most D-type motor neurons (Schuske et al., 2004).
The Ahringer RNAi clone library contains short sequence fragments for ~86% of *C. elegans* genes which have been cloned into the feeding vector L4440 (Fraser et al., 2000; Kamath and Ahringer, 2003). Flanking the multiple cloning site (MCS) of L4440 are two T7 RNA polymerase sites, which allow amplification of complimentary ssRNA from the plasmid. The complimentary ssRNAs are thermodynamically driven to base pair and form dsRNA, which can be processed by Dicer.

The purpose of this study was to determine novel genetic interactors in the *sydn-1/pfs-2* pathway. An RNAi screen was performed to identify suppressors of severe uncoordinated (*unc*) behavioral and axonal branching phenotypes produced in a *sydn-1; syd-2* genetic background. Over 700 gene clones from chromosomes II and III of the Ahringer clone library were tested and 10 novel genetic interactors were identified. Three-dimensional reconstructions of GFP-labeled GABAergic motor neurons of suppressor animals were created using DeltaVision® microscopy. One of these genes, *ELAV-type RNA binding protein family* (*etr-1*), was selected for further analysis. I have constructed an *etr-1::GFP*-containing vector that will be used for *in vivo* colocalization analysis and a yeast-two-hybrid DNA-binding domain (DBD) vector containing the human homolog of *etr-1*, *CUGBP, elav-like family member* (*CELF1*), to be used to analyze the conservation of protein: protein interactions between ETR-1 homologs and PFS-2. A better understanding of ETR-1 localization can provide insights into its function in pre-mRNA processing. By understanding the conservation of protein: protein interactions, the evolutionary and biochemical importance of the functional interaction between ETR-1, PFS-2, and their homologs can be ascertained.
METHODS

RNAi suppressor screen

Protocols for the RNAi screen were adapted from Kamath and Ahringer (2003). To perform the RNAi suppressor screen, overnight cultures of *E. coli* containing the gene clone in the vector L4440 (Figure 3) from the Ahringer RNAi library were prepared in lysogeny broth (LB) with 75 µg/ml ampicillin (LB<sub>AMP</sub>). A separate plasmid, phv-24, containing *pfs-2* gDNA was used to amplify *pfs-2* dsRNA (Van Epps et al., 2010). Bacteria were incubated in a 37°C shaking incubator at ≈120 RPM for 12-16 hours. After incubation, 45 µl aliquots of individual clones were dispensed onto a top and bottom well of a six-well Greiner Cellstar® tissue culture plate (BioExpress, Kaysville, UT USA). Plates were filled with nematode growth medium (NGM) agar plus 50 µg/ml ampicillin and 1mM IPTG. This was repeated for each gene clone to be screened. After the cultures were alliquoted, the six-well plates were placed in a room temperature cabinet for 48 hours. 1:50 and 1:100 dilutions of dsRNA-producing *E. coli* were performed for *etr-1* and *pfs-2* by taking a volume from an overnight culture of *E. coli* and adding it to LB<sub>AMP</sub>. After incubating at room temperature for 48 hours, embryos were harvested from gravid *C. elegans* adults and placed on the top wells of the six-well culture plates. In order to prepare embryos, multiple plates containing gravid CZ8828 (see description under Strains) animals were washed from NGM plates with M9 to release animals into solution. This solution was then centrifuged using a clinical centrifuge (Damon/IEC Division, Needham Heights, MA USA) for 1 minute at ≈1000x g to pellet the worms. The supernatant was poured off and the worms were resuspended in a 0.25 M NaOH, 20% (v/v) bleach solution. This solution was placed on a rocking nutator for 10
minutes then centrifuged at \( \approx 1250 \times g \) for 1 minute to pellet embryos. The supernatant was poured off and the embryos were resuspended in 5 ml nanopure \( H_2O \). The resuspension was centrifuged at \( \approx 1250 \times g \) for 1 minute and the \( H_2O \) was removed. Embryos were resuspended in 5 ml of fresh nanopure \( H_2O \). Using a sterile Pasteur pipet, one drop of this embryo solution was used to seed the top well of the six-well culture plates. The culture dishes containing plated embryos were incubated at 15°C for six days. This was a two-generation RNAi screen, so five gravid adults from each clone were transferred from the top well to the bottom well of the culture plates and returned to the 15°C incubator for another six days until the F2 generation developed into L4-early adults. These animals were used in the behavioral and axonal assays (Figure 4).

**Behavioral assay**

To assess behavioral differences between worms treated with an empty vector control (L4440) and RNAi vectors, a multi-step behavioral assay was developed. First, animals were observed under an Olympus SZ61 dissecting scope (Olympus America, Center Valley, PA USA) for five minutes to assess independent movement. Subsequently, 3-5 animals were chosen at random and gently tapped behind the head with a 0.5 mm Platinum wire pick. These animals were monitored for 1-2 minutes to observe differences in nictation and locomotory movement compared to the empty vector control animals. 3-5 animals were simultaneously chosen to be tapped on their posterior ends and similarly observed. Subsequently, the culture plate was tapped 3-5 times against a table and all animals on the bottom wells (F2 generation) were monitored for 1-2 minutes. All observations were compiled for each treatment type and each was given a score of 0, +, or -. Those that were
given a ‘0’ were deemed behaviorally identical to L4440. Animals given a ‘-‘ or ‘+’ were counted as enhancers or suppressers of the behavioral phenotype, respectively.

**Axonal assay**

Two different methods were used to place animals on pads for the axonal screen. For the first, a thin 5% agar pad was made out of molten 5% agar on a standard microscope slide. This slide was flanked on both sides with microscope slides containing a single piece of tape on their faces. A second microscope slide was placed perpendicular on top of the slide with the molten agar and pressed down so that each end of the microscope slide rested on the taped slides flanking the agar slide. A 2 µl droplet of 30 mg/ml 2,3-butanedione monoxime (BDM) in M9 was added on agar to anesthetize animals. 6-10 animals were placed in the droplet on the slide using a 0.5 mm platinum wire pick. Care was taken to minimize the amount of *E. coli* transferred with the animals.

For the second method (based on Evans, 2006), a drop of 2% agar was placed on a 24 x 50 mm coverslip and a second coverslip was used to flatten the agar drop into a thin sheet. The top coverslip was removed and the agar sheet was allowed to dry overnight at room temperature. One µl of mineral oil or a drop of Halocarbon oil 700 (Sigma-Aldrich, St. Louis, MO USA) was placed on the agar sheet and 6-10 animals were placed in the oil and pressed onto the agar sheet with a 0.5 mm wire pick to affix the animals to the dried agar.

Axonal branching was quantified as the number of branched GABAergic commissural axons identified over total GABAergic commissural axons observed per animal using green fluorescent protein (GFP). The significance between empty vector control animals and potential suppressors was tested using a one-tail t-test with unequal variance.
using Excel 2007 (Microsoft®, Redmond, WA USA). Multiple comparisons were controlled for with a Bonferroni-adjusted significance level. Commissural axons of the GABAergic D-type motor neurons were visualized in animals given a ‘+’ rating using an Olympus BX60 compound microscope with a Mercury-100W illuminator (Chiu Technical corporation, Kings Park, NY USA) and an Olympus UPlanFL N 60x/1.25 oil immersion objective (Olympus America, Center Valley, PA USA).

Strains

Two different strains were used in the screen: CZ8828 (sydn-1(ju541); syd-2(ju37); eri-1(mg366); P_{unc-25}GFP (juIs76)) and CZ4778 (sydn-1(ju541); P_{unc-25}GFP (juIs76)). CZ8828 contains a null mutation for our gene of interest, sydn-1, and also a null mutation in syd-2 which enhances the axonal branching phenotype, a neuronal RNAi-sensitizing mutation, enhanced RNAi (eri-1(mg366)), and a promoter-GFP fusion, P_{unc-25}GFP, which expresses GFP throughout the GABAergic nervous tissue. All gene clones found to confer behavioral and axonal suppression in CZ8828 were rescreened using CZ4778 (sydn-1(ju541); P_{unc-25}GFP (juIs76)). Axonal branching was tested in this strain to show that phenotypic suppression is caused by loss-of-function in sydn-1, isolated from any confounding effects due to the syd-2 mutant background. Commissural axon protrusions, in addition to axon branching, that occurred on commissural axons were included in the quantification of total commissural axon branching for strain CZ4778.

DNA sequencing

The L4440 plasmid was isolated from all positive suppressors, as well as the empty vector control and phv-24, the pfs-2-expressing feeding vector used by Van Epps et al., using
PureYield™ Plasmid Miniprep System (Promega, Madison, WI USA). Sequencing was performed using Nevada Genomics Center (RENO, NV, USA: http://www.ag.unr.edu/genomics/). The M13F primer was used for sequencing (Table 1).

3D reconstructions of GABAergic neurons and DIC imaging

Three-dimensional reconstructions of commissural axons were created for each suppressor using an Olympus IX70 inverted compound microscope (Olympus America, Center Valley, PA USA) with the DeltaVision® computer core (Applied Precision, Issaquah, WA USA) and softWoRx® 4.1.2 imaging software (Applied Precision, Issaquah, WA USA). Worms were placed on 5% agarose pads, anesthetized, and visualized with an Olympus UPlanApo 40x/0.85 objective (Olympus America, Center Valley, PA USA) and a FITC filter set. Z stack images were collected at 0.3-1 µm intervals for a total of 25-60 images for each animal. Differential interference contrast (DIC) images were taken with the same objective. All images collected were 512x512 pixels in dimension. The exposure time was determined using the Find function or by manually setting to 0.1 second for GFP imaging and 0.01 second for DIC imaging. The collect panels option was selected and the start and stop points were set such that they created opposite corners of a rectangle containing the entire animal. Overlap between panels was determined by taking the border rolloff in volumetric pixels (voxels) from a previously acquired and deconvolved 400x total magnification 512x512 image and multiplying by two. For all images, this overlap was 32 pixels. After acquisition, all files were then deconvolved for 10 cycles using Enhanced ratio method, medium noise filtering, and automatic correction. Images were then cropped based on the initial dimensions of the images subtracted by the overlap. Panels were stitched based on a nearest
neighbor interpolation method. Pixel size was adjusted to 1.15, overlap was averaged, and panel flattening was applied. Images were optimized using Imaris® x64 imaging software (Bitplane Inc., South Windsor, CT USA).
Figure 3. Plasmid map of RNAi feeding vector L4440 from Ahringer clone library (Fire, 2012). All RNAi inserts from this library are at the EcoRV site in the multiple clone site (MCS) (EcoRV site not shown above). Restriction map taken from Addgene plasmid repository, http://www.addgene.org/1654/.
Figure 4. Experimental design for RNAi suppressor screen. *E. coli* strain HT115(DE3) from the Ahringer clone library containing the L4440 vector, either empty or with a *C. elegans* gene cloned within, were plated onto six-well NGM + AMP + IPTG media. Each clone was plated onto a top and bottom well. Above inset are individual *E. coli* clones from clone library. Green and red inserts indicate genes screened. Yellow dots indicate *E. coli* colonies growing on agar. Left inset is a 40X magnification of embryos plated on *E. coli*. Black lines indicate adult *C. elegans* on wells. After two days growth at room temperature (22-25°C), embryos isolated from *C. elegans* were plated onto top wells and allowed to develop for six days at 15°C. Five L4/early adult animals from each treatment were transferred to bottom wells and allowed to produce progeny. After six days, L4 animals were behaviorally screened for increased movement relative to the empty vector control. ‘X’ marks points where screening is halted for a given gene clone. Animals that show increased movement in response to RNAi were further screened for reduction of axonal branching relative to the empty vector control. Whole animal 3D reconstructions of the GABAergic neural circuit were made for each suppressor identified.
Isolation of etr-1 and CELF1 cDNA

etr-1 and CELF1 cDNAs were isolated from sequence-verified plasmids using hot-start PCR. Primers etr-1_F1 and etr-1_R4 was used (see Table 1) to generate an etr-1 cDNA fragment which lacks the terminal stop codon (etr-1-taa). CELF1_F1 and CELF1_R1 were used to amplify full-length cDNA for CELF1. Products were amplified using Phusion® high-fidelity DNA polymerase (Thermo Fisher Scientific, Lafayette, CO USA) and a touchdown polymerase chain reaction (TD-PCR) thermocycler program. TD-PCR starts with a high annealing temperature and gradually brings the temperature down over a period of several cycles to allow for greater specificity in primer annealing. Amplification of etr-1-taa was performed as follows: initial denaturing step at 95°C for 2 minutes, and 30 cycles of 95°C for 30 seconds, 59°C for 30 seconds and 72°C for 1 minute for elongation. The annealing temperature was dropped by 0.5°C per cycle for 15 cycles and maintained at 56°C for the remaining 15 cycles. A final elongation at 72°C for 10 minutes was performed. CELF1 amplification followed the same protocol except the initial annealing temperature was set to 60°C and reduced to 55°C after 15 cycles. The PCR products were run on a 2% agarose tris base, acetic acid, EDTA (TAE) gel containing 250 ng/ml ethidium bromide (EtBr) at 125V and visualized using UV radiation. Bands of ≈1.5kbs (corresponding in size to etr-1-taa and CELF1) were excised and purified using the UltraClean™ DNA Purification Kit (MO BIO Laboratories, Carlsbad, CA USA). PCR product concentration was determined using a NanoDrop™ 1000 (Thermo Fisher Scientific, Wilmington, DE USA). Absorbance at 260 nm was used to determine nucleic acid concentration and the ratio
of absorbance at 260 nm and 280 nm was used to determine protein contamination for each sample.

*Adding 3’ adenosine overhangs*

To facilitate cloning into the Gateway® entry vector pCR8®, 3’ adenine overhangs were added to the PCR product. 284 ng of gel-purified *etr-1-taa* cDNA or 486 ng of *CELF1* cDNA was added to 1 µl 10X *Taq* PCR buffer with 1.5 mM MgCl₂, 1 µl 2.5 mM dNTPs and 0.5 U of *Taq* polymerase in a PCR tube and incubated at 72°C for 15 minutes. Products were stored at -20°C until use.

*TOPO® reaction and transformation into chemically competent E. coli*

For the TOPO® (Invitrogen, Carlsbad, CA USA) reaction, 1 µl of gel purified cDNA (either *etr-1-taa* or *CELF1*) with added 3’ A overhangs, 1 µl of salt solution, 3 µl of nanopure H₂O and 1 µl of the linearized entry vector pCR8 were added to a PCR tube and incubated at room temperature (20-22°C) for 15 minutes. 2 µl of this reaction was transformed into 50 µl One Shot® TOP10 *E. coli* (Invitrogen, Carlsbad, CA USA). Cells were incubated on ice for 30 minutes, placed at 42°C for 30 seconds, then returned to ice for 2 minutes. 250 µl of super optimal broth with catabolite repression (SOC) medium was added and the cells were incubated in a shaking incubator at 37°C and 150 rpm for one hour. Cells were plated onto room temperature LB plates containing 100 mg/ml spectinomycin (LB_SPEC) and incubated overnight at 37°C. A subset of colonies that grew on the LB_SPEC plates were removed and grown in 3-5 mls 100 mg/ml LB_SPEC liquid broth overnight in a shaking incubator at 37°C and 150 rpm.
**Plasmid isolation**

PureYield™ Plasmid Miniprep System (Promega, Madison, WI USA) was used for all plasmid isolations using the manufacturer’s high-volume culture protocol. Briefly, 1-1.5 mls of culture was placed in a 1.5 ml microcentrifuge tube and centrifuged for 30 seconds at 17000x g. The supernatant was then removed and resuspended in another 1-1.5 ml of culture and centrifuged again under the same conditions. Culture volumes of up to 4 ml were processed in this way for plasmid preparations.

**Confirmation of insert DNA and test of orientation**

Hot-start PCR was used to verify incorporation and orientation of *etr-1-taa* and *CELF1* cDNA into pCR8®. For *etr-1-taa*, M13F (-21) and *etr-1_R5* primers were used (Table 1). Thermocycler conditions were 94°C for 2 minutes, 94°C for 30 seconds, 53°C for 30 seconds, and 72°C for 30 seconds, for 25 cycles. Products were run on a 1% agarose TAE gel. For *CELF1*, GW1 and *CELF1_R1* primers were used. TD-PCR was performed at 94°C for 30 seconds, 59°C for 30 seconds, and 72°C for 1 minute. The annealing temperature was reduced by 0.5°C per cycle for 15 cycles and 56°C for the remaining 15 cycles. A final elongation at 72°C for 5 minutes was then performed for all PCR reactions. Products were run on a 1% agarose TAE gel. Once verified, *etr-1-taa* in pCR8 was named pML2 and *CELF1* in pCR8 was named pML3.

**LR clonase reaction**

An LR clonase reaction (Invitrogen, Carlsbad, CA USA) was performed to transfer *etr-1-taa* and *CELF1* from entry vectors to destination vectors containing a gene encoding GFP and the yeast-two-hybrid DNA-binding domain, respectively. For pML2, 145 ng was
added to 187 ng of the destination vector pCZGY51, 5 µl of pH 8.0 TE buffer, and 2 µl of Gateway® LR clonase II enzyme mix (Invitrogen, Carlsbad, CA USA). The reaction was incubated at 25°C for two hours. Subsequently, 1 µl of 20 mg/ml Proteinase K (New England BioLabs Inc., Ipswich, MA USA) was added and incubated at 37°C for 10 minutes. The same protocol was followed for pML3 cloning, except 146 ng of pML3 and 120 ng of the destination vector pCZGY52 was used.

One µl of each of the clonase reactions were separately transformed into One Shot® TOP10 E. coli (Invitrogen, Carlsbad, CA USA). Resistant E. coli were selected by growing cells in LB_AMP. Individual colonies were collected and grown in LB_AMP for ~16 hours at 37°C at 200 rpm. Plasmids were purified as described above and the insert was verified by PCR. Once verified, etr-1-taa in pCZGY51 was named pML5 and CELF1 in pCZGY52 was named pML4.

DNA sequencing

Sequencing of pML4 and pML5 were performed by Nevada Genomics Center (RENO, NV, USA http://www.ag.unr.edu/genomics/). For pML4, CELF1_F1-F3 and CELF1_R1, R3-R4 was used. For pML5, etr-1_F1-F4 and etr-1_R4-R5 were used (Table 1).

Creation of glycerol stocks

Glycerol stocks of pML2-pML5 for -80°C storage were created by transforming each plasmid of interest separately into TOP10 E. coli then taking 900 µl of overnight culture and adding it to 300 µl of sterile 50% glycerol in a cryotube. The tubes were sealed, inverted 2-3 times to mix the solution and stored at -80°C.
Table 1. Primers used for sequencing of RNAi clone library constructs and amplification and analysis of *etr-1-taa* and *CELF1* cDNA sequences.

<table>
<thead>
<tr>
<th>Name</th>
<th>Sequence (5’→3’)</th>
<th>Starting location</th>
<th>+/- strand</th>
</tr>
</thead>
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<td>-</td>
<td>+</td>
</tr>
<tr>
<td>GW1</td>
<td>GTTGCAACAAATTGATGAGCAGAAATGC</td>
<td>-</td>
<td>+</td>
</tr>
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RESULTS

sydn-1; syd-2 RNAi suppressor screen

To identify additional genes involved with the sydn-1/pfs-2 neurodevelopmental pathway, dsRNA-producing clones from the Ahringer clone library were tested for their ability to suppress the severe unc behavioral phenotype produced by the sydn-1;syd-2 double mutant. 710 clone library wells from chromosome II and one well from chromosome III of the Ahringer clone library were tested in this RNAi suppressor screen (Table 2). 84% (597) of wells tested produced animals that were screened in the behavioral assay. The remaining 114 wells (16%) were not screened due to E. coli growth failure, starvation of C. elegans, or contamination of plates. A total of 51 wells (7.2%) failed to grow E.coli after overnight culture. Thirty-three wells were not screened behaviorally because the animals had starved by the time of the behavioral assay. This could be due to delayed screening, or some type of phenotypic suppression resulting in greater brood sizes or increased movement. Perception of low food source by C. elegans results in broad changes in gene expression (Kang and Avery, 2009). Since it is unknown how this change in gene expression may affect behavior in the sydn-1; syd-2 double mutant, these animals are not suitable for behavioral screening. The remaining thirty-three wells were contaminated by fungus or other secondary bacterial growth before behavioral screening could be conducted and were not screened due to potential confounding affects produced by the interaction of the worm with the contaminating organism (Table 2).

Blind screening was performed on plates 3, 4, 7, 8, and 9 of chromosome II of the Ahringer clone library. Out of this blind screen, many F-box domain-containing proteins
were found to confer behavioral suppression. In light of this result, an additional screen of wormbase.org-annotated F-box domain-containing proteins on chromosome II was performed. In total, 21 F-box domain-containing proteins were behaviorally screened, which represents 3.8% (21/557) of F-box domain-containing proteins in *C. elegans* (Wormbase release ws227 http://www.wormbase.org/). The large number of F-box proteins in *C. elegans* is in stark contrast to *Homo sapiens*, *Danio rerio*, and *Mus musculus*, which contain between 73-85 F-box encoding genes (Ensembl release 64 http://uswest.ensembl.org/index.html).

*etr-1* was not identified in the blind screen, but was tested based on results from Simpson et al. which determined that *Arabidopsis thaliana* homologs of ETR-1 and PFS-2 physically interact (Simpson et al., 2003). *etr-1* conferred both behavioral and axonal suppression. The *etr-1* paralog *excretory canal abnormal* (*exc-7*) was also tested. *exc-7* is expressed in a subset of GABAergic motor neurons (VA, VB, and AS) (Fujita et al., 1999). *exc-7* knockdown resulted in behavioral suppression the *sydn-1/syd-2* phenotype, but not the branching axonal phenotype. The mRNA *cleavage and polyadenylation factor 1a subunit* (*clpf-1*) was also screened. As with *exc-7*, *clpf-1* knockdown suppressed the behavioral phenotype, but not the axonal phenotype (Table 3).
Table 2. Clones tested in RNAi suppressor screen separated by location in the Ahringer RNAi clone library.

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‘Plate’ is used to denote the 384-well clone library plate from which clones were tested. ‘Tested’ refers to any well from an Ahringer RNAi clone library plate for which screening was attempted. ‘Screened’ refers to those clones that were screened behaviorally (and axonally, when appropriate). Not all tested wells were screened. There were three primary reasons for this: no E. coli was cultured from a well, animals were starved by the time of the behavioral screen, or the RNAi plate was contaminated with mold or some other secondary bacteria at some point.
Primary screen: analysis of behavioral assay

This screen used *C. elegans* strain CZ8828 (sydn-1(ju541); syd-2(ju37); eri-1(mg366); P\_unc-25GFP (juIs76)). eri-1 encodes an RNase that degrades the two-nucleotide 3’ overhangs of siRNAs, preventing proper loading of siRNA into RISC and thereby negatively regulating RNAi (Kennedy et al., 2004). The null mutation, eri-1(mg366) in this strain facilitates robust RNAi in nervous tissue, where RNAi efficiency is typically low (Kamath et al., 2003; Kennedy et al., 2004; Tavernarakis et al., 2000; Timmons et al., 2001). The promoter-GFP fusion, P\_unc-25GFP drives GFP expression in GABAergic motor neurons. This strain contains two null mutations affecting neuronal function, sydn-1(ju541) and syd-2(ju37). syd-2 is involved in presynaptic protein localization and acts synergistically with sydn-1 to produce excessive branching of commissural axons (Van Epps et al., 2010; Zhen and Jin, 1999).

Of the 597 clones screened in the behavioral assay, 92.9% (555) caused animals to exhibit either a more severe phenotype (either an increase in uncoordinated (unc) behavior, sterility, or lethality) or a visually undetectable difference in unc phenotype. The remaining 7.0% (42) demonstrated some degree of suppression of the unc phenotype compared to control animals grown on *E. coli* containing the empty L4440 vector (Table 3, Figure 5). Interestingly, animals grown on *E. coli* expressing pfs-2 dsRNA (phv-24) did not show suppression of the unc behavioral phenotype. This is the result of PFS-2 being necessary for normal development. Based on previous RNAi screens, reduction in pfs-2 expression in a wildtype background results in locomotion defects, larval arrest, embryonic lethality and sterility (Rual et al., 2004; wormbase.org version 230 http://www.wormbase.org/). In this
study, animals grown on *etr-1* dsRNA-expressing *E. coli* survived and showed reduction in *unc* behavior, in addition to an observed reduction in brood size. Reduction in *etr-1* expression in other screens is known to cause sterility, embryonic lethality, and paralysis (Ceron et al., 2007; Kamath et al., 2003b; Rual et al., 2004). Those animals that survived in our screen showed suppression of *unc* behavior. Based on these results, 1:50 and 1:100 dilutions of *E. coli* containing phv-24 and *etr-1::L4440* were used for behavioral assays. Animals grown on these dilutions showed increases in brood size and suppression of *unc* behavior.
Figure 5. Results of behavioral assay from RNAi suppressor screen. 597 gene clones from chromosomes II and III of the Ahringer feeding library were assayed for severe uncoordinated (unc) behavior using *C. elegans* strain CZ8828 (*sydn-1*(*ju541*); *syd-2*(*ju37*); *eri-1*(*mg366*); *P* _unc-25GFP_ (*juIs76*_). ‘0’ refers to no change compared to control vector, ‘enhancer’ describes unc behavior worse than control vector, and ‘suppressor’ describes unc behavior reduced compared to vector control.
Table 3. Results of behavioral assay from RNAi suppressor screen using *C. elegans* strain CZ8828 (*sydn-1(ju541); syd-2(ju37); eri-1(mg366); P\text{unc-25}GFP (juIs76)). Wells screened from chromosomes II and III of the Ahringer clone library for suppression of severe uncoordinated movement divided into three categories: ‘0’ refers to no change compared to control vector, ‘enhancer’ describes unc behavior worse than control vector, and ‘suppressor’ describes unc behavior reduced compared to vector control.

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II3E01 II3L11 II3L17 II3D23 II7L07 II4L13 II4P02
II3E02 II3L12 II3B18 II3F22 II7F07 II4K12 II4P03
II3D02 II3E07 II3A18 II3P22 II7I07 II4D03 II4J04
II3F06 II3E16 II3G19 II3E23 II7H07 II4D01 II4J05
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</table>
Secondary screen: analysis of axonal assay

All 42 genes that suppressed the *unc* phenotype of the *sydn-1/syd-2* double mutant were screened for suppression of axonal branching relative to animals grown on the empty vector control, L4440, by analyzing GFP-labeled GABAergic motor neurons. Ten genes conferred a reduction in GABAergic commissural axon branching compared to L4440 (Table 4, Figure 6, 7, 8). This represents 1.7% of genes screened in the behavioral assay (10/597). The greatest suppression was conferred by reduction of *pfs-2* expression (Figure 6, 7, 8). Animals with this gene knocked-down showed a 84.5% average reduction in axonal branching (n=51) compared with L4440 animals (n=93). This result is expected, as phenotypes generated by SYDN-1 misregulation are thought to be directly mediated by expression of PFS-2. The next strongest suppressor, *ZC239.6*, produced a 77.0% reduction in commissural axon branching (n=20), relative to L4440 animals. The remaining eight suppressors reduced commissural axon branching between 39.8% and 71.0% relative to L4440 animals.

The dilutions of *pfs-2* and *etr-1* that were performed, in addition to allowing for better resolution in behavioral screening, function as an important control for responsiveness of the axonal branching phenotype relative to dsRNA concentration. With lower concentrations of dsRNA produced by *E. coli*, the degree of suppression of commissural axon branching should also be reduced. Undiluted *pfs-2* culture confers robust suppression of axonal branching in *C. elegans* and is significantly different (p < 0.001) from both 1:50 (n=20) and 1:100 (n=20) dilutions, as determined by a Bonferroni-corrected one-tail t-test with unequal variance (Figure 6). *pfs-2* 1:50 and 1:100 dilutions are not significantly different from each
other, but there is a trend between greater dilution and an increase in axonal branching. Similarly, undiluted etr-1 culture is significantly different from the 1:100 dilution (n=21, p <0.01) and an overall trend of less axonal branching suppression with more dilute E. coli is evident.

To make dilutions, cultures of dsRNA-producing E. coli are typically diluted with overnight culture of the common feeding E. coli strain OP50 which does not express dsRNA (Kamath et al., 2001; Van Epps et al., 2010). For this study, cultures were diluted with the same LB<sub>AMP</sub> liquid media from which the dsRNA producing E. coli were grown leaving less E. coli in the diluted samples. Using this method may result in animals that exhaust their food source sooner and starve before screening can be completed. Starvation response is associated with changes in gene expression and an increase in what is known as the “bag of worms” phenotype, in which embryos are not expelled from a gravid hermaphrodite and hatch within the animal, eventually bursting out and killing the parent (Riddle et al., 1997). An increase in this phenotype would be expected if these animals were exhausting their food supply (Fay, 2006). Although the number of animals exhibiting the bag of worms phenotype per well was not quantified, it did not appear that there was a greater incidence of this phenotype in the dilutions, compared to any other treatment.
Table 4. Ahringer RNAi clone library positions and gene names for clones found to confer behavioral and axonal suppression in *C. elegans* strain CZ8828 (*sydn-1(ju541); syd-2(ju37); eri-1(mg366); *P*~*unc-25*~*GFP* (*juIs76*))

<table>
<thead>
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<th>Ahringer clone library position</th>
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<tr>
<td>II1G15</td>
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</tr>
<tr>
<td>II7C12</td>
<td><em>ceh-57</em></td>
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<tr>
<td>II1H01</td>
<td><em>fbxc-33</em></td>
</tr>
<tr>
<td>II3M18</td>
<td><em>fbxc-53</em></td>
</tr>
<tr>
<td>II3P18</td>
<td><em>F10G7.10</em></td>
</tr>
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<td>II3B06</td>
<td><em>F31D5.6</em></td>
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<tr>
<td>II7O07</td>
<td><em>srb-6</em></td>
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<tr>
<td>II3C17</td>
<td><em>ZC239.6</em></td>
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</table>
Figure 6. Suppression of sydn-1;syd-2 axonal branching phenotype via RNAi in C. elegans strain CZ8828 (sydn-1(ju541); syd-2(ju37); eri-1(mg366); Punc-25GFP (juIs76)). Suppression determined by quantifying number of commissural axons displaying excessive branching over the total number of commissural axons visualized per animal. Bars represent average +/- standard error. All treatments are significantly different from the L4440 empty vector control (p < 0.001) using Bonferonni-corrected one-tail t-test with unequal variance. For the empty vector control, 93 animals were screened. For each experimental treatment, n ≥ 20 except for R06F6.8, for which n = 19.
Figure 7. Imaging of RNAi-produced axonal suppression in strain CZ8828 (sydn-1(ju541); syd-2(ju37); eri-1(mg366); P\textunderscore unc-25\textunderscore GFP (juls76)) using DeltaVision® fluorescence microscopy. For each animal, a DIC image and an image of GFP-labeled GABAergic motor neurons showing varying degrees of commissural axon branching was taken. Strains juls76 and CZ4778 were imaged to document wild-type and sydn-1 single mutant GABAergic motor neuron structure, respectively.
pfs-2 1:50
pfs-2 1:100
etr-1 1:50
ceh-57
fbxc-53
Figure 8. Posterior-most GABAergic commissural motor neurons showing RNAi-produced axonal branching suppression in strain CZ8828 (sydn-1(ju541); syd-2(ju37); eri-1(mg366); \(P_{unc-25}GFP\) (juIs76)) using DeltaVision® fluorescence microscopy. Wild-type strain juIs76 (\(P_{unc-25}GFP\)), strain CZ4778 sydn-1(ju541); \(P_{unc-25}GFP\) (juIs76)), L4440 empty vector control and suppressors from RNAi screen imaged. Scale bar = 50 µm.
The axonal branching phenotype in strain CZ8828 (sydn-1(ju541); syd-2(ju37); eri-1(mg366); P\textsubscript{unc-25}GFP (juIs76)) is produced by the loss of both sydn-1 and syd-2; therefore, it is possible for suppression of the phenotype to result from changes involving syd-2 expression, instead of sydn-1. In order to better understand the source of suppression, a second RNAi axonal screen was performed using the identified suppressors and the strain CZ4778 (sydn-1(ju541); P\textsubscript{unc-25}GFP (juIs76)). Although some suppressors demonstrated a significant reduction in axonal branching compared to the empty vector control, the etr-1 and pfs-2 dilution controls did not show a graded response between dsRNA concentration and phenotypic suppression (Figure 9). As pfs-2 knockdown treatment does not affect the axonal phenotype and suppression of axonal branching in this strain is not dependent on dsRNA concentration, it is unlikely that the response assayed is due to RNAi-induced gene silencing.

There are a few reasons that this may be the case.

First, the axonal phenotype in the sydn-1 single mutant strain is more nuanced than the double mutant strain. Instead of excessive branching of commissural axons, small protrusions from the nerve cords and commissural axons were more commonly observed (Figure 6,7). Those protrusions that occurred on commissural axons were included in the quantification of total commissural axon branching. Additionally, given the small size of the protrusions in the CZ4778 background (5-10 µm), it was possible to overlook branch points during axonal screening, which would introduce experimentally-derived variability into the axonal assay. Finally, strain CZ4778 is not sensitized for neuronal RNAi. eri-1 in the genetic background of CZ8828 plays an important role in facilitating neuronal RNAi. In fact, this gene was initially discovered in a mutagenesis screen designed to uncover C.
*C. elegans* mutants with susceptibility to neuronal RNAi (Kennedy et al., 2004). The lack of positive results from a screen in strain CZ4778 may be the result of the lack of sensitizing mutations in neuronal RNAi.
Figure 9. Suppression of sydn-1 axonal phenotype via RNAi in C. elegans strain CZ4778 (sydn-1(ju541); Punc-25GFP (juls76)). Suppression determined by quantifying the number of commissural axons displaying excessive branching or ectopic axonal protrusions over the total number of commissural axons visualized per animal. Bars represent average +/- standard error. Asterisk indicates treatments with p< 0.001 compared to L4440 empty vector control (vector) using Bonferroni-corrected one-tail t-test with unequal variance. For the empty vector control, 86 animals were screened. For each experimental treatment, n ≥ 10 except for F10G7.10, for which n = 7.
Analysis of DNA sequencing

L4440 plasmids carrying RNAi insertions were sequenced to confirm gene identities (Figure 10). All sequences generated Phred (Q20) scores greater than 29, which is an indication of highly reliable (99-99.9%) sequencing results (Ewing and Green, 1998; Ewing et al., 1998).

When compared to NCBI publicly available data, some sequenced clones produced ambiguous results. *BTB and MATH domain containing (bath-8), E-box C (fbxc-33)*, and *ZC239.6* inserts contain sequence similarity with other genes (Table 5). The Ahringer clone library was constructed using the GenePairs primer sets (Kamath and Ahringer, 2003). These primers were originally designed to amplify 1-1.5kbs sequences for microarray construction. Given the large size of the fragments produced, these primers produce highly specific DNA sequences. For RNAi screening, however, these 1-1.5kbs gene clones are diced into short 21-25 bps fragments. If these fragments share sequence similarity to other *C. elegans* genes (as is the case with paralogs), then off-target effects are likely to result. For this reason, further analysis of these genes was not performed.
Figure 10. DNA sequences of genetic suppressors identified in RNAi suppressor screen using *C. elegans* strain CZ8828 (*sydh-1(ju541); syd-2(ju37); eri-1(mg366); Punc-25 GFP (juIs76)). All gene clones except *pfs-2* were isolated from the Ahringer clone library (Kamath and Ahringer, 2003). EcoRV site used for cloning is highlighted in yellow in L4440 empty vector. Modified EcoRV site “GATT” at beginning of each sequence indicates the end of L4440 and the beginning of gene insert. Megablast alignment was used to verify gene identity (NCBI BLAST http://blast.ncbi.nlm.nih.gov/). All clone e-value < 10^{-90}.
**ceb-57**

GATTGAGATTTTCATCCACTGGGAGTCATTTGACTCTCTCAATACGGGAAACGGCAAGAGAAAATGGCTCCAT
TAAGGGCGACGCTAAGATTTTTCATTTTGATTTCTGCCGGGAAAGAATTTTTCTTTCTCTTCTCATTTTAC
CACCAAGATCCAACTTCATCTTCATTTTGTGACAATTGCAGAAGGGCCTGGCCTTCGGGAAACGCAGCTG
TTTTCGATTGCTGAAACTTCCGGGACTAACCTGGGACTTCTGGGAAAGGACGCTTCTTCATTTATG
ATTATGAAAAGAAATTCCGATGAAATTGCAGAGGCTGAGCATACAAGAAATTTGAATGATCCGTGAACTT
CCCAATTATTCATCCAAAAACAGTTGATTGACAGATGATCAAGTTAGAGAAGGTTTGAGGAAATATATTGTG
GGATGTTTTAAAAATTTTTATGTACATATATTTTTTAGAATACTTCAATCTTAAAGAATGTATCCTATGAGAA
AAACGTTA

**fbxc-33**

GATTCGAAAACAAGAGCCTACGGCCTCGTGTGCTGCTGCTGCTGCAACGGGAAACCTGCAATTCGAAGCTGCTG
CTACAAATAGAAGCTTACCCAACTGCTTTTATAGTCTGAAACTGGCAGAGCCCTGCTTCTCCCAAAAT
AAATGGAAAGAAATTCCGATGAAATTGCAGAGGCTGAGCATACAAGAAATTTGAATGATCCGTGAACTT
CCCAATTATTCATCCAAAAACAGTTGATTGACAGATGATCAAGTTAGAGAAGGTTTGAGGAAATATATTGTG
GGATGTTTTAAAAATTTTTATGTACATATATTTTTTAGAATACTTCAATCTTAAAGAATGTATCCTATGAGAA
AAACGTTA

**fbxc-53**

GATTCGAAAACAAGAGCCTACGGCCTCGTGTGCTGCTGCTGCTGCAACGGGAAACCTGCAATTCGAAGCTGCTG
CTACAAATAGAAGCTTACCCAACTGCTTTTATAGTCTGAAACTGGCAGAGCCCTGCTTCTCCCAAAAT
AAATGGAAAGAAATTCCGATGAAATTGCAGAGGCTGAGCATACAAGAAATTTGAATGATCCGTGAACTT
CCCAATTATTCATCCAAAAACAGTTGATTGACAGATGATCAAGTTAGAGAAGGTTTGAGGAAATATATTGTG
GGATGTTTTAAAAATTTTTATGTACATATATTTTTTAGAATACTTCAATCTTAAAGAATGTATCCTATGAGAA
AAACGTTA

**F10G7.10**

GATTGGTTCCATCCGTGGTTGATTGACCTCTCGGATCAGAAACGGCACAGAGGAAATTCTTCTCTTCTTCT
TTGTTGAGCCGAGGAGGGTGGTTTGTATTCTGCTGGAACGCTGAAACTGGCAGAGCCCTGCTTCTCCCAAAAT
AAATGGAAAGAAATTCCGATGAAATTGCAGAGGCTGAGCATACAAGAAATTTGAATGATCCGTGAACTT
CCCAATTATTCATCCAAAAACAGTTGATTGACAGATGATCAAGTTAGAGAAGGTTTGAGGAAATATATTGTG
GGATGTTTTAAAAATTTTTATGTACATATATTTTTTAGAATACTTCAATCTTAAAGAATGTATCCTATGAGAA
AAACGTTA

69
Table 5. Genes that share homology with suppressors identified in this screen. Gene clone names and names of homologous sequences are given.

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<td>fhxc-31</td>
</tr>
<tr>
<td>ZC239.6</td>
<td>F22E5.13</td>
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</tbody>
</table>

Gene homology was considered sufficient if it spanned ≥20bps.
Identification of novel genetic interactors with the sydn-1/pfs-2 pathway

Seven genetic interactors were identified from the Ahringer clone library (Table 6). *etr-1* is conserved among metazoans and encodes an RNA-binding protein. In *C. elegans*, ETR-1 is necessary for survival and plays a role in body wall muscle development. Expression analysis using a 1 kb upstream promoter region fused to a gene encoding GFP displayed fluorescence in body wall muscles only (Milne, 1999). When the splice variant ETR-1a is expressed using a body wall muscle promoter, this protein localizes to the nucleus only (Meissner et al., 2011). There are six other splice variants of ETR-1.

Orthologs of *etr-1* in *Danio rerio*, *Xenopus laevis*, and *Homo sapiens* encode proteins involved in alternative splicing of pre-mRNA transcripts (Ladd et al., 2001; Suzuki et al., 2002). The human homolog of ETR-1, CELF1, is associated with myotonic dystrophy, a genetic disease characterized by severe muscle wasting (Ho et al., 2005; Ranum and Day, 2004; Wang et al., 2007). Interaction between homologs of ETR-1 and PFS-2 in *Arabidopsis thaliana* has been shown (Simpson et al., 2003). In this model system, the ETR-1 homolog, FCA, interacts with FY, the PFS-2 homolog to regulate floral timing.

*C. elegans* homeobox (*ceh-57*) is a *C. elegans*-specific homeobox domain-containing protein. Homeobox domains are often associated with transcription factors. Expression data using a GFP-labeled CEH-57 determined that it is localized throughout the nervous system and expressed throughout development, but strongest expression is visualized during the L1-L3 juvenile stages (Hunt-Newbury et al., 2007). No specific function has been assigned to this protein.
fbxc-53 encodes an uncharacterized, Caenorhabdid-specific protein containing an F-box domain and a domain of unknown function, duf3557. F-box proteins are important scaffolding proteins involved in ubiquitination (Bai et al., 1996; Kipreos and Pagano, 2000). F-box proteins facilitate the interaction between ubiquitin ligase enzymes that interacts with the F-box domain, and a target protein that interacts with other domains on the F-box protein. Together with other protein cofactors, F-box proteins and the ubiquitin ligase constitute the E3 ubiquitin ligase complex. The F-box protein LIN-23 is a negative regulator of axonal outgrowth in GABAergic neurons (Mehta et al., 2004). Animals containing a mutation in \textit{lin-23} show axonal outgrowth defects similar to those seen in the \textit{sydn-1;syd-2} double mutant. In contrast to \textit{lin-23}, \textit{fbxc-53} suppresses axonal misregulation in GABAergic neurons. Our study, combined with these results, suggest multiple potential roles for ubiquitination in proper axonal development.

\textit{F31D5.6} encodes a 58 amino acid long, Caenorhabdid-specific protein initially thought to be part of a larger F-box containing gene, but it was determined via EST and blastX data that this was not likely to be the case (Wormbase.org version 231 http://www.wormbase.org/db/get?name=WBGene0017949;class=Gene). No \textit{F31D5.6} paralogs exist in \textit{C. elegans}, but it shows limited similarity to sections of a TetR family transcriptional regulator found in \textit{Neurospora} (ascomycete), \textit{Burkholderia} (proteobacteria), and \textit{Sordaria macrospora} (ascomycete) (NCBI http://blast.ncbi.nlm.nih.gov/Blast.cgi). Beyond this weak sequence similarity, no other data have been published about this protein’s function.
*F10G7.10* encodes a metazoan-conserved E3 ubiquitin ligase that is similar to E3 ubiquitin ligases involved in the n-end rule ubiquitination pathway. This pathway recognizes destabilizing N-terminal amino acid residues (n-degrons) and targets proteins containing these n-degrons for polyubiquitination and subsequent proteasome-mediated degradation (Bartel et al., 1990; Gonda et al., 1989; Varshavsky, 1997). *F10G7.10* encodes two different types of Znf domains: an N-recognin Znf and a RING-type Znf. In the n-end rule pathway, N-recognin type Znf binds to the n-degron and the RING Znf promotes the synthesis of the polyubiquitin chain onto an internal lysine residue (Freemont, 2000; Joazeiro et al., 1999). Although *F10G7.10* and its homologs share similar domain architecture to n-end rule ubiquitin ligases, evidence suggests that these proteins do not recognize n-degron-containing proteins (Tasaki et al., 2007). Interestingly, this same study found that the mouse homolog, *Ubr3*, is heavily expressed in sensory neurons using a β-galactosidase reporter assay (Tasaki et al., 2007). Recently, the human UBR3 protein has been shown to bind and promote ubiquitin-mediated degradation of the DNA repair enzyme APE1 by targeting multiple n-terminal lysine residues for polyubiquitination (Meisenberg et al., 2012). It is not known what, if any, other types of substrates are targeted by this type of ubiquitin ligase.

*Serpentine receptor class Beta (srb-6)* encodes a Caenorhabditis-specific seven-transmembrane receptor protein. Generally, SRB receptor proteins are found in chemosensory neurons where they respond to a variety of environmental cues (Hall and Altun, 2008). The particular function or SRB-6 has not been studied.

*R06F6.8* encodes the *C. elegans* homolog of RIC1, a yeast factor required for the transcription of ribosomal proteins and rRNAs (Mizuta et al., 1997). The function of
R06F6.8 has not been characterized in *C. elegans*. R06F6.8 interacts with factors involved in γ-tubulin binding, wnt signaling, and a large ribosomal subunit, as determined by high-throughput yeast-two-hybrid analysis (Li et al., 2004). A deletion in this gene results in lethality or sterility in wildtype animals (Wormbase.org version 230 http://www.wormbase.org/).
Table 6. Gene names and descriptions of DNA sequence-verified gene clones found to confer suppression of neuronal phenotype in strain CZ8828 (sydn-1 (ju541), \(P_{unc-25}\)GFP (juls76), eri-1 (mg366), syd-2 (ju37)) through behavioral and axonal assays in RNAi suppressor screen of selected regions of Chromosome II in *C. elegans*.

<table>
<thead>
<tr>
<th>Gene Name</th>
<th>Description</th>
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<tr>
<td>ceh-57</td>
<td>Homeobox</td>
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<tr>
<td>etr-1</td>
<td>RNA-binding protein CUGBP1/BRUNO</td>
</tr>
<tr>
<td>fbxc-53</td>
<td>F-box</td>
</tr>
<tr>
<td>(F10G7.10)</td>
<td>E3 ubiquitin ligase, Zn binding</td>
</tr>
<tr>
<td>(F31D5.6)</td>
<td>Unknown, potential transcription factor</td>
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<tr>
<td>(R06F6.8)</td>
<td>Ribosome control 1 homolog</td>
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<tr>
<td>srb-6</td>
<td>seven transmembrane receptor protein</td>
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<tr>
<td>Total Positives</td>
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</table>
Production of etr-1 and CELF1 vectors for future use

To investigate the role of ETR-1 in SYDN-1/PFS-2 function and characterize the binding affinity of CELF1, the human homolog of ETR-1, expression vectors containing the coding regions of these genes were produced. PCR confirmed that cDNAs of etr-1 and CELF1 were successfully cloned into pML2-pML5 (Table 7). PCR-based orientation tests were performed for pML2 and pML3 using a forward primer upstream of the insert in the vector backbone and a reverse primer within the insert (Figure 11). Sequencing of pML2 and pML3 confirmed that both etr-1-taa and CELF1 were amplified and cloned into the pCR8® entry vector without generating mutations (Figure 12). For pML2, all sequence reads generated Q20 scores greater than 40. pML3 generated sequence reads with Q20 scores greater than 30.
Figure 11. 1% agarose gel images showing amplification and correct orientation for cloned genes. A) Amplification and orientation tests for *etr-1-taa*-containing vectors. From left to right, HiLo® DNA marker, *etr-1-taa* amplification from pML2, forward orientation test of pML2 using M13F and *etr-1*_R5, reverse orientation test using M13F and *etr-1*_F3, and *etr-1-taa* amplification from pML5. B) Amplification and orientation tests for *CELF1*-containing vectors. From left to right, HiLo® DNA marker, *CELF1* amplification from pML3, forward orientation test of pML3 using GW1 and *CELF1*_R1, reverse orientation test using GW1 and *CELF1*_F1, and *CELF1* amplification from pML4.
Figure 12. DNA sequencing results for cloning of *etr-1-taa* and *CELF1* in entry vector pCR8®. A) Annotated *etr-1* isoform e. B) Annotated *CELF1* variant 3. Yellow highlighted sequences represent forward primers and green highlighted sequences represent reverse primers.
Table 7. Plasmids constructed with vector backbone and cDNA insert.

<table>
<thead>
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<td>pML2</td>
<td>pCR8\textsuperscript{R}::etr-1-taa</td>
</tr>
<tr>
<td>pML3</td>
<td>pCR8\textsuperscript{R}::CELF1</td>
</tr>
<tr>
<td>pML4</td>
<td>pCZGY52::CELF1</td>
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<tr>
<td>pML5</td>
<td>PCZGY51::etr-1-taa</td>
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</table>
DISCUSSION

Potential roles for novel genes implicated in the sydn-1/pfs-2 pathway

Transcription factors are well-known regulators of neurodevelopmental genes. In the sydn-1/pfs-2 pathway, three potential transcription factors were identified, ceh-57, F31D5.6, and R06F6.8, which suppress the neuronal defects generated by PFS-2 misregulation. If sydn-1 loss-of-function results in broad upregulation of pre-mRNA processing as described by the direct interaction model of SYDN-1/PFS-2 function (Figure 1), then reducing expression of specific transcription factors may result in fewer pre-mRNA transcripts being produced. This may result in a net balance of mature specific mRNA transcripts. R06F6.8 is required for transcription of ribosomal proteins and reduced expression of this transcription factor would result in fewer active ribosomes and could ameliorate PFS-2 misregulation by reducing proteins produced from these overabundant transcripts. The second model of SYDN-1/PFS-2 interaction suggests that PFS-2 misregulation results in differential isoform production. This model is also compatible with phenotypic suppression resulting from knockdown of transcription factors. This would ultimately depend on what genes are regulated by these transcription factors and whether further deleterious effects are likely to be produced by broad knockdown of these genes. Further experiments will be necessary to elucidate which model is correct.

Two genes, fbxc-53 and F10G7.10, were identified as ubiquitination-related proteins. Interestingly, all of our ambiguous genes contain either F-box domains, or other domains associated with ubiquitination, so a larger role for ubiquitination in the sydn-1/pfs-2 pathway is likely to result from further analysis. Two models for the role of ubiquitination in the
sydn-1/pfs-2 pathway involve ubiquitination factors as being either downstream targets of PFS-2 misregulation, or as regulatory components within the pathway itself. If a ubiquitination factor is either over-produced, or an aberrant isoform is generated, genetic knockdown would suppress this misregulation. If the direct interaction model of SYDN-1/PFS-2 interaction is correct, ubiquitination factors as downstream targets may provide an explanation for why sydn-1 single mutants produce minimal effects in non-neuronal tissues, given broad-scale overexpression of mRNAs. Overexpression of ubiquitination factors may act as a buffer in certain tissues by degrading proteins that are overexpressed due to an increase in mature mRNA production. In motor neurons, we do not see this type of buffering and genetic knockdown of ubiquitination factors promotes phenotypic suppression.

In the differential isoforms model, ubiquitination may regulate a negative controller of some factor that binds PFS-2 and promotes the use of certain cis-elements in the cleavage and polyadenylation reaction. Genetically knocking down the ubiquitination factor would result in an increased accumulation of this negative regulator and a decrease in the PFS-2-binding factor. This would result in the decrease of a particular isoform (Figure 13).

etr-1 is a good candidate for a regulatory component within the sydn-1/pfs-2 pathway. This gene encodes an RBP whose homologs are associated with alternative splicing. Binding of ETR-1 to PFS-2 may result in the formation of particular isoforms of a protein, as is the case with ETR-1 and PFS-2 homologs in Arabidopsis thaliana. If ETR-1 binding is affected by SYDN-1, this would be strong support for the differential isoforms model of SYDN-1/PFS-2 interaction. One caveat concerning ETR-1 is that it has not been found in nervous tissue. Two separate experiments have localized ETR-1 to muscle tissue only (Meissner et
al., 2011; Milne, 1999). There are, however, seven different isoforms of the ETR-1 protein and only one has been tested (a promoter::GFP fusion was used for the other).
Figure 13. Model of ubiquitination-mediated protein degradation resulting in suppression of PFS-2 misregulation, under the differential isoforms model of SYDN-1/PFS-2 interaction. X represents some factor which, when bound to PFS-2, results in the production of certain protein isoforms. Y is a proposed negative regulator of X. Reduction in the abundance of X in a sydn-1 loss-of-function mutant will reduce expression of X-dependent isoforms. If sydn-1 null mutant phenotypes are produced by a buildup X-dependent isoforms, degradation of X could restore wildtype levels of X-dependent isoforms.
ETR-1 variants may be tissue-specific and previous experiments have not facilitated ETR-1 neuronal expression. Driving \textit{etr-1::GFP} expression using a panneuronal promoter and examining neuronal localization relative to \textit{pfs-2} expression would provide insight into ETR-1 localization and interaction with PFS-2. \textit{In vitro} protein: protein interaction studies using the yeast-two-hybrid system have shown weak interaction between ETR-1 and PFS-2 (Allen, 2011). Examination of the binding capabilities of the human homolog CELF1 with PFS-2, and with the human homolog of PFS-2, WDR33, could establish an evolutionarily-conserved protein: protein interaction that is found within \textit{Arabidopsis thaliana}, \textit{Caenorhabditis elegans}, and \textit{Homo sapiens}.

\textit{Further experiments to test SYDN-1/PFS-2 interaction models}

If the direct interaction model is correct, in a \textit{sydn-1} mutant background, overall nuclear cleavage and polyadenylation will be increased. A higher rate of RNA processing will likely lead to an increase in mature mRNA transcripts; either maintained within the nucleus or exported into the cytoplasm. This could be tested via traditional microarray or RNA sequencing (RNA-Seq) analysis. RNA-seq is a burgeoning technique used for transcriptome analysis and allows for transcriptome resolution down to the allelic and isoform level (reviewed in Wang et al., 2009). Fractionated mRNA or cDNA created via reverse-transcriptase PCR (RT-PCR) is flanked with adaptors specific to a high throughput DNA sequencing platform and deep-sequenced (Wang et al., 2009). To understand PFS-2 and SYDN-1 activity using this technology, mRNA or cDNA preparations from developmentally synchronized animals, both wildtype and \textit{sydn-1} mutants, could be sequenced and transcriptome variation at the isoform level could be assessed. Change in
overall transcript level, or a change in specific isoforms between these two populations would be indicative of SYDN-1 influence on pre-mRNA processing.

Biochemical analysis will be an important experimental technique in elucidating the SYDN-1/PFS-2 pathway. Biochemical pulldown assays could be performed with SYDN-1 and PFS-2 as independent baits. These assays will allow a better understanding of the *C. elegans* cleavage and polyadenylation machinery and its regulation. This type of analysis has been attempted, but results were not definitive (Allen, 2011). In addition to these experimental strategies, bioinformatic analyses could be done to evaluate models that rely on differential protein isoform synthesis. In order for SYDN-1 binding to cause the accumulation of certain protein isoforms, different cis-elements (such as poly(A) hexamer sequences) within a given gDNA sequence must be selected for or against. If the above model is correct, then targets of PFS-2 mediated misregulation should contain different types of poly(A) signals. These signals could be identified in *C. elegans* gDNA by using basic searches of existing genome databases, such as the *C. elegans* resource wormbase.org.

An additional RNAi screen using our identified suppressors and the muv strain *lin-15AB(n765)* could help elucidate where in the SYDN-1/PFS-2 pathway our suppressors may reside (Cui et al., 2008). This strain has a unique mutation in an operon containing *lin-15A* and *lin-15B*, two genes involved in specifying vulval cell fates (Clark et al., 1994; Huang et al., 1994). The mutation is a knocked-in poly(A) site from the gene *H18N23.2* which is inserted into the *lin-15B* transcript. The *lin-15A* transcript is downstream of *lin-15B*. As a result of this mutation, both transcript levels are greatly reduced. These factors encode dual vulva cell fate repressors, so when both transcripts are greatly reduced, a multivulval
phenotype is observed. Factors involved in the cleavage and polyadenylation specificity factor (CPSF) and the cleavage stimulation factor (CstF) have been shown to abrogate the multivulval phenotype when knocked down (Cui et al., 2008; Van Epps et al., 2010). Using the suppressors we identified in our screen, we can test whether any of these factors are components of the CPSF or CstF, or if these suppressors are targets of regulation by the SYDN-1/PFS-2 polyadenylation pathway, by examining whether there is a change in the multivulval phenotype when a particular suppressor is knocked down.
BIBLIOGRAPHY


