IDENTIFICATION OF INTRACELLULAR INTERACTORS OF THE FAT-LIKE CADHERIN CDH-4 IN AXONAL NAVIGATION OF *CAENORHABDITIS ELEGANS* NEURONS

by

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Abstract

The fat-like cadherin CDH-4 in *Caenorhabditis elegans* is a non-classical cadherin with homology to *Drosophila melanogaster* and mouse FAT. Previous work has shown that CDH-4 is important for axon guidance and cell migrations, processes dependent on cell signalling. To further our understanding of CDH-4 signalling, we identified proteins interacting with the intracellular domain of CDH-4 using a yeast two-hybrid screen. We obtained 42 putative interactors of various predicted molecular functions. We began to examine mutants of the candidate genes for defects observed in *cdh-4* mutants with the aim of identifying biologically relevant interactions. None of the 18 candidates tested to date showed significant defects in overall axon guidance, suggesting that these candidates might not act together with *cdh-4*. This study provides the basis to identify components of signalling pathways employed by CDH-4 during axon guidance. This has the potential to uncover novel signalling pathways used by this evolutionarily conserved cadherin.

Keywords: neuronal development, axon guidance, *Caenorhabditis elegans*¸Fat-like cadherin, CDH-4

To Beverley, for all your support and love.

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List of Abbreviations

3-AT	3-aminotriazole
AD	activation domain
ADE	adenine
abLIM	actin-binding LIM protein
BLAST	basic local alignment search tool
BMP	bone morphogenic protein
bp	base pair
C. elegans	*Caenorhabditis elegans*
cAMP	cyclic adenosine monosphosphate
cDNA	complementary DNA
DNA	deoxyribonucleic acid
CFP	cyane fluorescent protein
DB	dna binding domain
DCC	deleted in colorectal cancer
DNC	dorsal nerve cord
E. coli	*Escherichia coli*
ECM	extracellular matrix
Eph	ephrin
EWM	easiest worm medium
fmi	flamingo
GEF	guanine nucleotide exchange factor
GFP	green fluorescent protein
GPCR	G protein coupled receptor
HIS	histidine
IgCAM	Immunoglobulin domain cell adhesion molecule

LEU	leucine
LiAc	lithium acetate
NCAM	neuronal cell adhesion molecule
PCP	planar cell polarity
PCR	polymerase chain reaction
RNA	ribonucleic acid
RNAi	RNA interference
ROBO	roundabout
TGF-β	transforming growth factor β
TRP	tryptophan
unc	uncoordinated
VNC	ventral nerve cord
X-gal	5-bromo-4-chloro-3-indolyl-β-D-galactosidase

1. Introduction

1.1 The nervous system

Found in many metazoans, the nervous system is an incredibly complex and important structure. Composed of specialized cells called neurons, the nervous system is vital for sending and receiving signals throughout the body (Squire, 2013). Signals are transmitted between cells as an electrochemical wave called action potentials. The structure of neurons is designed specifically for this purpose. Neurons are typically polar with multiple branching processes called dendrites at one end and one long process called an axon at the other end. Signals are received at the dendrite and transmitted along the axon to the dendrites of another neuron. A synapse forms between the axon of one nerve cell and the dendrites of another. At the synapse, neurotransmitters are released from the axon and picked up by the dendrites as a means of transmitting the signal between cells (Squire, 2013). In this way, neural circuits are created between multiple neurons to respond to or act on the environment surrounding the organism. The brain, the largest structure of the nervous system, contains a remarkably large number of neuronal cells. The human brain contains an average of 86.1 billion neuronal cells with another 84.6 billion glial support cells (Azevedo et al., 2012). As this is a difficult system to work with, study of nervous system development frequently uses simpler organisms for study. Frequently studied organisms are *Danio rerio*, *Drosophila melanogaster* and *Caenorhabditis elegans* for a variety of reasons. Firstly, it is easier to study defects at the single cell level in these species. Recent studies in zebrafish and *Drosophila* show the advantages of studying axon guidance in model organisms (Chak and Kolodkin, 2013; Kwon et al., 2013). Secondly, genetic screens are faster in these species thanks to shorter generation time. Finally, a wide variety of genetic tools have been developed in these organisms to assist with experiments.

Development of the nervous system begins with specification of neurons from precursors and differentiation into different subtypes (Squire, 2013). These neurons must typically migrate to their final location within the body. Next, axons extend from the developing

neuron and travel along long distances to their final targets. Upon reaching their target, axons synapse with their target cells to establish neural circuits. The process by which axons travel towards their targets is of critical importance for proper formation of neural circuits and is not entirely understood (Park and Shen, 2012).

1.2 The ventral nerve cord of *Caenorhabditis elegans* as a model for axon guidance

The nature of *C. elegans* has made it an interesting choice as a model organism ever since the original work done by Sydney Brenner (Brenner, 1974). As a small (~1mm) and quickly reproducing species, *C. elegans* is an excellent choice to study development. Added to this, the transparency of *C. elegans* and invariant development are important advantages when working with *C. elegans*. Transparency allows for in-vivo visualization of gene expression, protein localization and protein-protein interactions (Chalfie, 1994). Additionally, this transparency allows of in-vivo labelling of particular subsets of cells for analysis. With the invariant development of *C. elegans*, John Sulston mapped the cell lineage from embryo to adult (Sulston and Horwitz, 1977; Sulston et al., 1983). Thanks to this, researchers working with *C. elegans* can identify significant defects at the level of a single cell.

The nervous system of *C. elegans* is a particularly useful system to study development. The nervous system is composed of 302 neurons in the adult hermaphrodite (White et al., 1986). Using electron microscopy, the location and morphology of the entire nervous system has been characterized. The majority of neurons extend axons in the head to form a structure called the nerve ring. Several neurons project their axons posteriorly from the head region. There are fewer neurons in the tail region that extend processes anteriorly towards the head along the ventral midline. Overall, the structure of the nervous system is composed of parallel tracts running either anterior/posterior or dorsal/ventral.

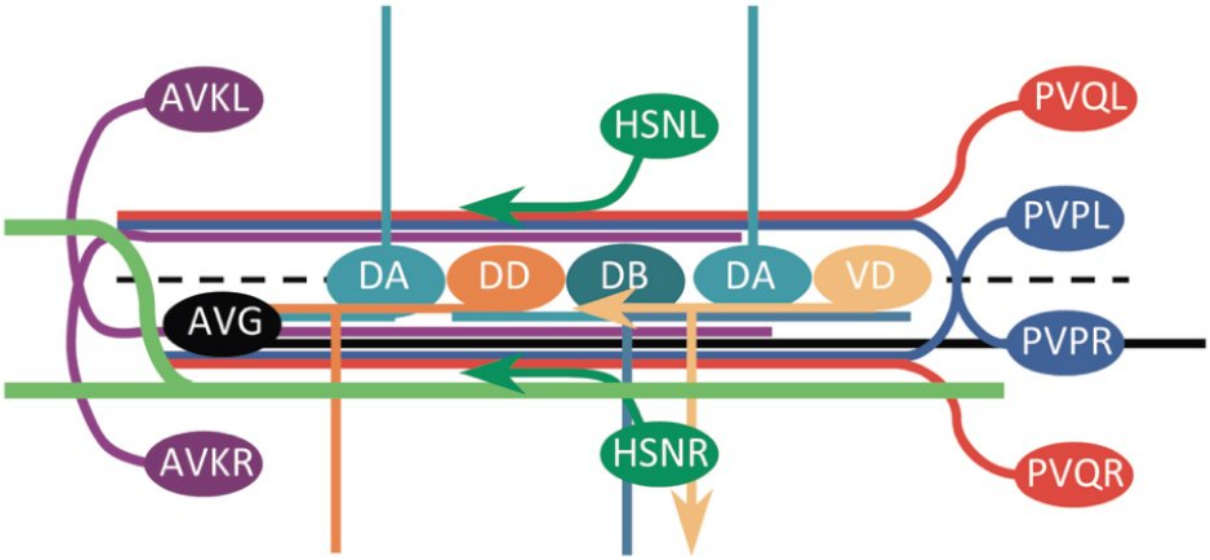


Figure 1. Representation of the *Caenorhabditis elegans* ventral nerve cord. Interneurons exit the nerve ring and enter the VNC in pairs, one on the left and one on the right. Interneurons in the left tract cross into the right tract shortly after entering and travel posteriorly. PVP axons enter the VNC from the posterior, cross the midline and one enters the left tract (PVPR) while the other enters the right (PVPL) where the travel anteriorly. PVQR and PVQL follow PVPL and PVPR respectively. The AVG cell body is located in the VNC and extends an axon along the right tract posteriorly. HSN cell bodies are located on each side of the VNC near the vulva and extend axons anteriorly with one in each tract. AVKL extends its axon posteriorly along the right VNC tract while AVKR extends its axon along the left VNC tract. The DA/DB and DD/VD motorneuron cell bodies are located in the midline and extend their axons along the right VNC tract and send commissures circumferentially towards the dorsal cord. (Steimel, 2010).

There are two large process bundles running in the anterior/posterior direction: the dorsal nerve cord and the ventral nerve cord (VNC) (Figure 1) (White et al., 1976). The dorsal cord runs along the dorsal side of the animal and is composed mainly of motorneuron axons that have traveled from the ventral side. The ventral nerve cord is composed of two tracts that run parallel to each other along the ventral side of the animal. These two tracts contain an asymmetric distribution of axons (White et al, 1976). The right tract contains approximately 50 axons while the left tract contains only 4. The majority of the right tract is composed of interneuron and motorneuron axons. The interneurons axons grow out in pairs, one on the left and one on the right, and extend through the ventral cord from the nerve ring and traveling posteriorly through the animal. Shortly after entering the VNC, interneuron axons cross the

ventral midline and enter the right tract. Motorneuron axons enter the VNC in a different manner. Motorneuron cell bodies are located along the VNC and axons enter the VNC from the location of the cell body. Interneurons connect to motorneurons in the VNC which are core components of the motor circuit allowing for controlled movement of theanimal. There are two interneuron pairs with cell bodies located in the tail region that extend axons anteriorly through the VNC towards the head. These interneuron pairs are PVP and PVQ. A pair of motorneurons, HSN, with cell bodies close to the vulva extend anteriorly. AVK neurons extend posteriorly from the head region towards the tail.

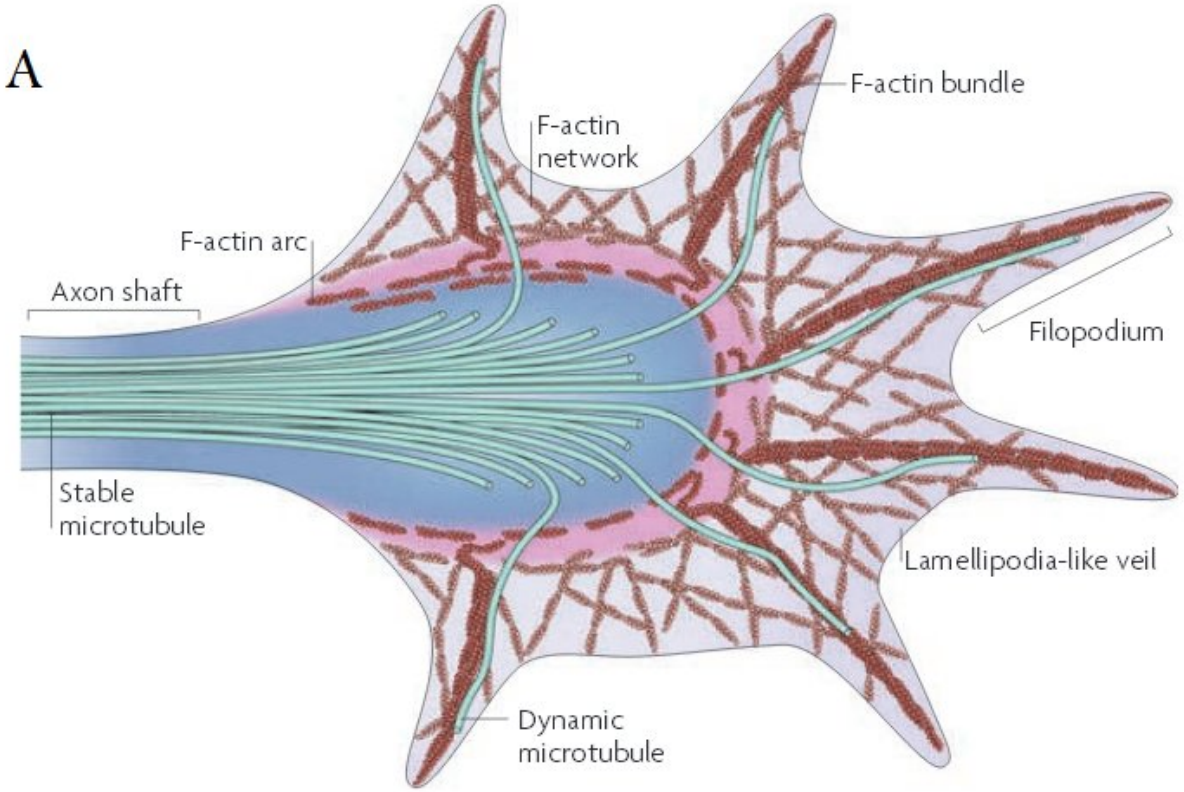
The outgrowth of axons in the ventral nerve cord does not occur simultaneously (Durbin, 1987). Using electron microscopy to reconstruct the VNC in embryos, the order of axon outgrowth was determined. Each tract has one axon that extends first to pioneer the tract. In the right tract, AVG acts as pioneer while other axons are able to follow the path laid down by AVG later. In the left tract, PVPR functions as pioneer and is later followed by PVQL, AVKL and HSNL. Loss of some pioneers has a detrimental effect on the ability of some follower axons to navigate properly in grasshopper sensory neuron migration (Klose and Bentley, 1989).However, when AVG is ablated we find that its loss has a limited impact on the ability of follower navigation, suggesting that followers have the potential to navigate without the pioneer (Hutter, 2003). The requirement of pioneers for axon navigation in *C. elegans* is not entirely understood.

The relatively simple structure of the *C. elegans* VNC makes it an ideal system to study axon guidance decisions at the single cell level. The function of several guidance proteins have been characterized and studied using this system (For a review see Lowery et al, 2009; Myers et al., 2011).

1.3 Axon Guidance

During development, axons must travel long distances in order to reach their targets. Proper guidance of axons is integral to the proper formation of neural circuits. To do so, axons must respond to various cues in their environment. A specialized structure called the growth

cone is located at the tip of the extending axon (Yamada et al., 1970). The growth cone consists of finger like projections called filopodia and an extended sheet like structure called lamellipodia. The structures are composed of an extensive actin microfilament network that is constantly being built up and broken down (Kuczmarski and Rosenbaum, 1978). When this actin network is lost, axons continue to extend but are very disordered (Bentley and Toroian-Raymond, 1986). This led to the understanding cues in the environment can control axon pathfinding by alterations to the actin cytoskeleton of the growth cone (Figure 2). These cues can be in the form of secreted molecules that form a gradient and act as repulsive or attractive signals. Typically, these molecules bind to receptors located at the growth cone. Repulsive cues lead to growth cone collapse while attractive cues help to stabilize the growth cone (Vitriol and Zheng, 2012). Membrane bound proteins can also function in an attractive or repulsive manner in axon guidance. Additionally, differential cell adhesion plays a role in axon guidance as axons will follow paths with the strongest adhesion (Lowery et al., 2009). Typically this means following tracts laid down previously by other axons.


A
F-actin bundle
F-actin
network
F-actin arc
Axon shaft
Filopodium
Stable
microtubule
Lamellipodia-like veil
Dynamic
microtubule

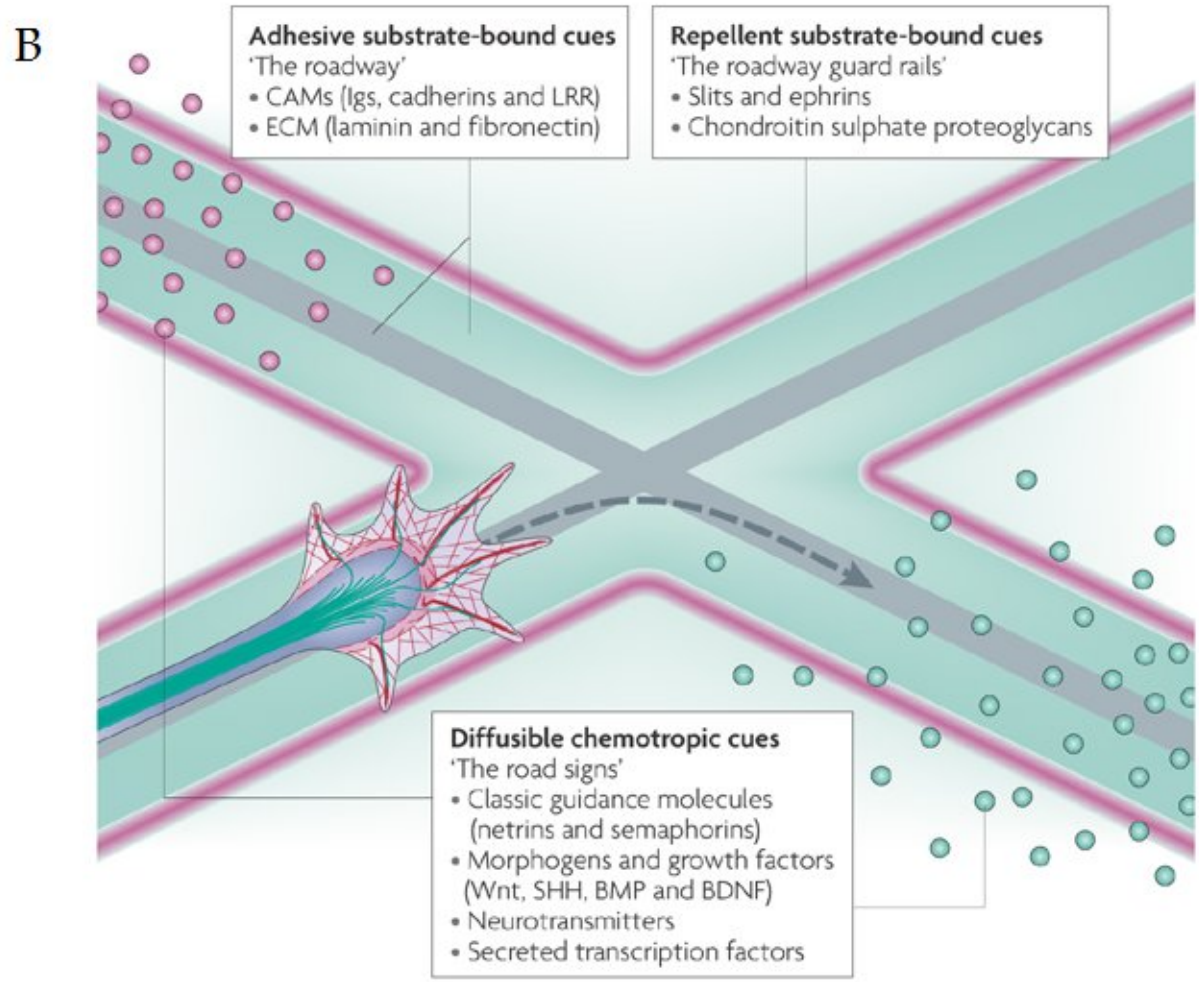


Figure 2. Growth cone structure and interpretation of guidance cues
A) Structure of the growth cone. Bundles of actin form the structure of the filopodia and lamellipodia of the growth cone. Microtubules form the structure of the axon shaft and extend into the filopodia where they remain dynamic at the tips. B) Growth cones respond to diffusible and adhesive cues from their environment in order to make proper guidance decisions towards their target (Lowery et al., 2009).

Several guidance molecules have been identified in a variety of species and seem to be evolutionarily conserved among all animals (Yu and Bargmann, 2001). These molecules can be classified as signalling molecules and receptors, morphogens, and cell adhesion molecules. Addtionally, intracellular signalling molecules play a key role in responding to guidance cues and determining growth (For a review see Lowery et al., 2009; Vitriol and Zheng, 2012). The

integration of all the extracellular signals by intracellular signalling pathways is critical for proper migration of axons.

1.3.1 Cell-signalling and axon guidance

Several classes of signalling molecule and receptors have been identified for their role in axon guidance (Figure 3). These guidance cues are the Ephrins, Netrins, Slit and Semaphorins. Ephrins were originally identified in *Xenopus laevis* as being required in retinotectal mapping of neurons and are membrane bound proteins (Drescher et al., 1995). Ephrins can lead to a repulsive or attractive signal when they interact with their receptor Eph. The Ephrin receptors are also membrane bound and contain a tyrosine kinase domain. An interesting feature of Ephrins is their ability to signal bidirectionally (Holland et al., 1996). In this situation, both Eph and Ephrin expressing cells respond to ligand-receptor interaction. The second class of signalling molecules are UNC-6/netrin identified in *C. elegans* (Hedgecock et al., 1990). In *C. elegans*, UNC-6 is required for dorsal-ventral guidance through interactions with receptors UNC-5 and UNC-40. In vertebrates, netrin is required for chemoattraction in spinal commissural axons, however, netrins have chemorepellant function as well (Tessier-Lavigne et al. 1988, Colamarino and Tessier-Lavigne, 1995). The third class of signalling molecules are Slit. Slit was originally identified in *Drosophila melanogaster* while searching for a midline repulsion cue for commissural axons (Kidd et al., 1999). Slit is a large secreted protein that interacts with its membrane bound receptors roundabout (Robo). In *C. elegans*, the Slit homologue SLT-1 acts as a repulsive cue for axons expressing its receptor SAX-3 (Hao et al., 2001). Another class of guidance molecule are the semaphorins although not all semaphorins are involved in guidance. Semaphorins were originally identified in grasshopper as transmembrane proteins (Kolodkin et al., 1992). However, later secreted semaphorins were identified (Luo et al., 1993). Overall, semaphorins are mainly repulsive signals that function by leading to growth cone collapse. In *C. elegans*, *mab-20* semaphorin homologue shows weak axon guidance defects (Roy et al., 2000).

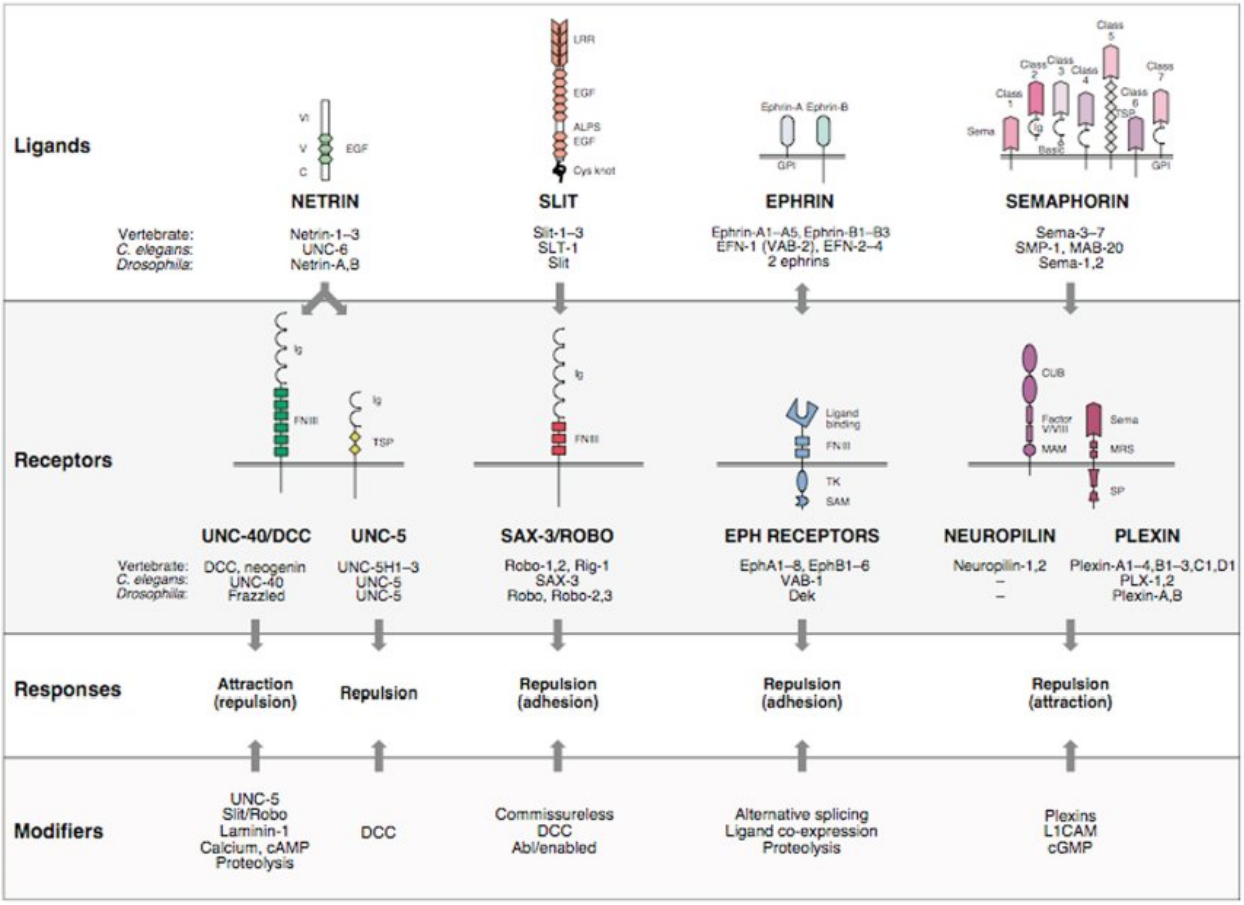


Figure 3. Axon guidance cues.
The top is a representation of the four major axon guidance cue families. Shown below the cues are their receptors in axon guidance. Beneath the receptors the response to the signal is shown. The bottom shows proteins that modify or are involved in the signalling (Yu and Bargmann, 2001).

The role of signalling molecules and receptors in axon guidance has been established in both vertebrate and invertebrate species. The conservation of function across species for most guidance cues suggests these processes developed in an ancestor of both vertebrates and invertebrates.

1.3.2 Morphogens in axon guidance

Several genes previously identified as morphogens and growth factors have been identified to have a role in axon guidance. These include Wnts, Hedgehog and TGFβ. In *Drosophila*, WNT5 functions as a repellent cue through Derailed in commissure axon navigation in the CNS (Yoshikawa et al., 2003). In *C. elegans*, several Wnts and their receptor, Frizzled,

are responsible for proper anterior/posterior axon guidance (Hilliard and Bargmann, 2006; Pan et al., 2006; Zinovyeva et al., 2008). One of five Wnt homologues, EGL-20, has been shown to function as a repellent expressed in the tail of the worm. However, the main function of Wnts appears to be in determining in anterior-posterior polarity of neurons. Hedgehog is another morphogen that has been identified to function in axon guidance (Charron et al., 20to03, Trousse et al., 2001). In vertebrates, Sonic Hedgehog (Shh) has been shown to function as an attractant and repellent in different sets of neurons. In the floor plate of the neural tube, Shh functions as an attractive cue to attract commissures towards the floor plate. In *C. elegans*, several of the Hedgehog pathway components including Hedgehog and Smoothened are missing (Bürglin TR and Kuwabara PE, 2006). To date, no role for hedgehog signaling in *C. elegans* nervous system development has been identified. Finally, members of the TGFβ family of growth factors play important roles in axon guidance. In vertebrates, the roof plate secretes BMP7 that repels axons away and leads them towards the floor plate (Augsburger et al., 1999). In *C. elegans*, a TGFβ homologue UNC-129 is expressed from the dorsal side and required for proper dorsal/ventral guidance of motorneuron axons (Colavita et al., 1998). In this case, UNC-129 functions as a repellent cue.

1.3.3 Intracellular Signalling and Cytoskeletal re-arrangement in axon guidance

Intracellular signalling plays a critical role in response to axon guidance cues in all animals. Guidance signals interpreted by the growth cone are extracellular signals interacting with proteins at the cell surface. Intracellular signalling is required in order to respond to these signals.

Intracellular signalling in axon guidance does not rely solely on proteins as second messenger such as Ca^2+ can also be used. In the guidance of corpus callosal axons, Wnt signalling functions by repulsion through Ca^2+ signalling (Hutchins et al., 2011).

cAMP also plays a role in axon guidance. Protein kinase a and Epac are two proteins that regulate cAMP function in axon guidance (Murray et al., 2009). Early in

development, cAMP allows for attractive signals from netrin while later in development these signals change to repulsion.

Frequent responses to guidance cues are changes to actin and microtubule dynamics. As such, it is not surprising that a large number of cytoskeletal proteins are downstream targets of guidance signalling pathways (Govek et al., 2005; Koh, 2007). These downstream targets include proteins such as Rac, Rho and Ras classes of GTPases.

As previously discussed, cytoskeletal remodelling is important to axon guidance (Figure 4). Therefore, it is no surprise that several actin regulatory proteins have are involved in axon guidance. Actin polymerization is regulated by a wide variety of proteins at both the barbed and pointed ends. Specifically, microinjection of Rac1 and Cdc42 into mammalian cells was able to promote the formation of lamellipodia and filopodia (Kozma et al., 1996). In fact, Rac was found to be involved in Semaphorin signalling (Jin and Strittmatter, 1997). In *C. elegans*, several rac and rho genes: *ced-10, mig-2, rac-2* and *unc-73* show various axon guidance defects (Lundquist et al., 2001). Similarly, other proteins with actin binding activity such as UNC-115 show axon guidance defects as well (Lundquist et al., 1998).

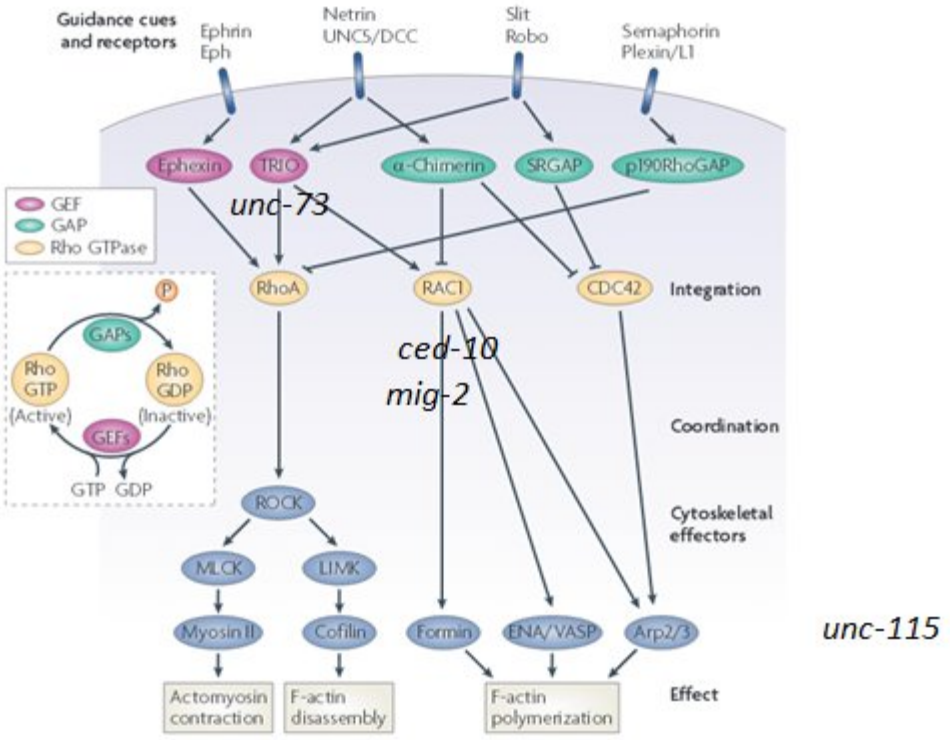


Figure 4. Axon guidance cues and their receptors signal through several intracellular proteins to modify the actin cytoskeleton to alter the trajectory of the axon (Adapted from Lowery et al., 2009).

Additionally, microtubules play an important role in growth cone dynamics and axon guidance. Microtubules are polarized due to their composition of α/β tubulin dimers and contain a minus-end and a plus-end. Microtubules often undergo dynamic instability at their plus-end where the microtubule will undergo rapid depolymerization (Mitchison and Kirschner, 1984). Interestingly, in the axon microtubules are oriented with their plus-ends distally and therefore towards the growth cone (Baas et al., 1991). In this manner, microtubules can be rapidly degraded in response to extracellular cues present near the growth cone. Microtubules are also present in the growth cone although they do not reach the edge of the extending growth cone (Marchisio et al., 1978). More recently, it has been observed that a small number of microtubules extend from the axon into the actin rich regions of the growth cone. Modifications to microtubule dynamics in the growth cone influence growth cone turning (Buck and Zheng,

2002). The involvement of microtubule dynamics in growth cone turning suggests a connection between microtubule dynamics and the actin based structures of the growth cone.

A wide variety of microtubule proteins cause axon guidance defects. For example, there is a connection between netrin signalling and microtubule dynamics. Qu et al. found that netrin promoted interactions between its receptors DCC and TUBB3, a β tubulin (Qu et al., 2013). Netrin signalling promoted microtubule growth in mouse commissural neurons. Proteins regulating microtubule dynamics are also involved in axon guidance. Several plus-end interacting proteins (+TIPs) act downstream in axon guidance pathways. Specifically, Orbit/MAST/CLASP has been shown to act downstream of the tyrosine kinase Abl (Lee et al., 2004). Additionally, MAP-1B, a protein involved in microtubule assembly, is controlled by GSK-3β which is a component of the Wnt pathway. Wnt signalling is capable of increasing microtubule assembly by reducing the activity of GSK-3β (Ciani et al., 2004).

In *C. elegans*, MIG-10/lamellipodin has been found to promote cytoskeletal assembly through both f-actin and microtubules (Quinn et al., 2008). Additionally, CED-10/Rac1 GTPase was responsible for the distribution of MIG-10.

In summary, several intracellular signalling proteins including cytoskeletal organizing proteins are required for proper axon guidance in both vertebrates and invertebrates. Extracellular cues are integrated by these proteins and signals in order to make proper guidance decisions for development of the nervous system.

1.4 Cell adhesion in axon guidance

Cell adhesion molecules can also play an important part in axon guidance and neuronal development. Cell adhesion is required for proper movement of all migrating cells and not just neuronal cells. Addtionally, growth cones make contact and require adhesion for proper guidance. Adhesion can occur in two ways: cell to cell or cell to extracellular matrix (ECM).

1.4.1 The extra-cellular matrix

The growth cone must travel through the ECM in order to reach its target location. The structure and composition of the ECM has been previously shown to positively and negatively affect cell movement (Venstrom and Reichardt, 1993). Components of the ECM are mainly glycoproteins and proteoglycans (Timpl, 1996). These components include collagen, heparin sulfate, and laminin. Components of the ECM function as a permissive substrate allowing for cell movement and guidance (Letourneau et al., 1994). Mutations in these collagens and laminins have significant axon outgrowth and cell migration defects. Specifically, *cle-1* a type XVIII collagen in *C. elegans* has specific axon guidance and cell migration defects (Ackley et al., 2001). *cle-1* mutants have significant HSN cell migration defects and significant DA/DB motorneuron axon guidance defects. The role of collagens in axon guidance is not limited to *C. elegans* or invertebrates. The *stumpy* gene, Collagen XIXa1, in zebrafish is required for proper pathfinding of the primary motorneuron, CaP (Beattie et al., 2000; Hilario et al., 2010). Axons in these mutants proceed to their intermediate targets but fail to extend along their axon specific paths.

Heparan sulfates are another component of the ECM important in cell migrations and axon guidance. A mammalian heparan sulfate homologue, EXT-1, is required for proper midline crossing in major commissural tracts (Inatani et al., 2003). The *C.elegans* heperan sulfate homologue, UNC-52/Perlecan, is also required for proper guidance of pharyngeal motorneurons (Axang et al., 2008). Axons stopped prematurely of their target in *unc-52* mutants.

Nidogen is a glycoprotein conserved in *C. elegans* and vertebrates. There are two closely related mammalian nidogens and a single nidogen in *C. elegans*. Null alleles of *nid-1* in *C. elegans* have defects in positioning of longitudinal nerves including those located in the VNC (Kim and Wadsworth, 2000). Additionally, *nid-1* was required for the decision of one axon, SDQR, to stop moving longitudinally and travel dorsally. In contrast, in rats nidogen plays a role in regulation of schwann cells and their migration. Nidogen induces regenerative axon outgrowth for peripheral nerves (Lee et al., 2009).

Another class of ECM component, Laminins, are also required for proper cell migration and growth of neurons (Forrester and Garriga, 1997; Turney and Bridgman, 2005). Laminins consist of heterotrimers containing α, β, and γ subunits. Vertebrate species contain multiple α, β, and γ chains allowing for over 15 laminin isoforms. However, *C. elegans* contains only two isoforms as there are 2 α, a single β, and a single γ (Pettitt, 2005). These two laminin α chains are EPI-1 and LAM-3. *epi-1* mutants show significant neuronal cell migration defects and are important for formation of the VNC (Forrester and Garriga, 1997). Laminins are also required for proper cell migrations and axon outgrowth in vertebrates (Turney and Bridgman, 2005).

Components of the ECM must interact with the growth cone of the axon in order to have an effect on axon guidance. This is accomplished through interaction with integrins. Integrins are heterodimeric membrane receptors required for anchoring cells to the ECM. Integrins consist of an α and β monomers. Vertebrates contain 18 α and 8 β chains, while *C. elegans* contains only two α and a single β chain. In zebrafish, integrin antagonists caused abnormal branching and truncations to axons when added during outgrowth suggesting a role for integrins in axon guidance (Becker et al., 2003). In *C. elegans* the integrin α subunits, *ina-1* and *pat-2* mutants display defects in cell migrations (Meighan and Schwarzbauer, 2007). Additionally, *ina-1* mutants show defects in neuronal cell migrations and fasciculation of axons (Baum and Garriga, 1997). In the growth cone, integrins tend to localize to filopodia (Myers et al., 2011). Suppression of integrin function through Ras causes significant defects in cell migrations and growth cone collapse (Oinuma et al., 2006). Interestingly, integrins are required for signalling of several axon guidance cues. Guidance of semaphorin 7A requires integrin receptors for proper signalling (Pasterkamp et al., 2003). Similarly, integrin is required for integrating netrin signals for growth cone collapse (Lemons et al., 2013).

Interactions between cells and the ECM are important for proper migration and also in axon guidance. Components of the ECM provide a permissive substrate that allows for adhesion by neurons. In addition to this effect, it seems that the ECM is also important for

binding of some axon guidance cues. Integrins play an important role in both of these processes by binding to components of both the ECM and guidance cues.

1.4.2 IgCAMs

A large class of cell adhesion molecules expressed in the nervous system are the immunoglobulin domain cell adhesion molecules (IgCAMs). IgCAMs contain immunoglobulin domains similar to the variable and constant regions found in antibodies (Harpaz and Chothia, 1994). IgCAMs function as both receptors and cell adhesion molecules in axon guidance. The two netrin receptors, UNC-5 and UNC-40/DCC are both members of the IgCAM family. Additionally, the Slit receptor ROBO is another example of an IgCAM receptor. As discussed above, these proteins play important roles in axon guidance and show the importance of IgCAM receptors in guidance.

IgCAMs also function as cell adhesion molecules in axon guidance. Homophilic binding of these IgCAMs has been shown to be required for proper migration of neural cells. One of the most prominent is neural cell adhesion molecule, NCAM, which was originally identified for its role in cell adhesion in the retina (Hoffman et al., 1982). NCAM has a variety of effects on neuronal development including control of neurite outgrowth (Doherty et al., 1990). Although there is only one vertebrate NCAM gene, there are multiple isoforms due to alternative splicing (Reyes et al., 1991). Additional variability between NCAMs comes from a variety of post-translational modifications that modify the function of a particular NCAM (Conchonaud et al., 2007).

The L1 subfamily of IgCAMs are also involved in guidance of neuronal processes and neuronal migrations (Demyanenko et al., 1999). SAX-7, a *C. elegans* L1 homologue, is required for maintenance of axons in the VNC and positioning of several neuronal cell bodies in the head of the worm (Pocock et al., 2008). This function of SAX-7 appears to be mainly adhesive. A second member of the L1 subfamily, LAD-2, has been shown to function as a co-receptor in axon guidance (Wang et al., 2008).

Another *C. elegans* IgCAM, WRK-1, has been shown to play a role in guidance of axons at the ventral midline (Boulin et al., 2006). Two more IgCAMs, SYG-1 and SYG-2, function together to ensure the proper localization of HSN synapses (Shen et al., 2004). The remaining *C. elegans* IgCAMs have shown limited or no involvement axon guidance to date (Schwarz et al., 2009).

IgCAMs remain important molecules for axon guidance. In vertebrates and invertebrates, IgCAMs function as both receptors and cell-adhesion molecules for neuronal migrations and axon guidance.

1.4.3 Cadherins

Cadherins are a large family of proteins originally identified for calcium dependent adhesion in mouse embryonic cells (Yoshida-Noro et al., 1984). More recently, cadherins were subdivided into major subfamilies based on sequence homology (Nollet et al., 2000). The first major family contains the classical cadherins, atypical/type II cadherins, desmogleins, and desmocollins (Hulpiau and van Roy, 2009). The classical cadherins contain approximately five extracellular cadherin domains, a transmembrane domain, a membrane proximal conserved domain (MPCD) and an intracellular catenin binding site (Stappert and Kemler, 1994). Additionally, these cadherins contain a His-Ala-Val (HAV) sequence for cell adhesion recognition in their extracellular domain (Blaschuk et al., 1990). Members of this family show 68% to 78% sequence similarity with E-cadherin (Nollet et al., 2000). The atypical or type II cadherins show similar domain organizations to those of the classical cadherins but lack the HAV sequence found in classical cadherins. Desmogleins and desmocollins have a similar domain organizations to classical cadherins however both lack the MPCD. Both desmogleins and desmocollins localize to desmosomes where they interact with intermediate filaments. Desmogleins differ from desmocollins in that they contain a proline-rich linker domain, a typical repeat unit domain, and a terminal domain (Koch et al., 1990).

Cadherins lacking a catenin binding site are considered non-classical. The largest subfamily of cadherins are the protocadherins and are placed in this group (Hulpiau and van Roy, 2009). The protocadherins are grouped separately from the seven pass transmembrane cadherins and the FAT cadherins based on cadherin ectodomain similarity (Nollet et al., 2000). These proteins contain 6-34 cadherin domains and do not contain the HAV cell adhesion recognition sequence of the classical cadherins. The protocadherins undergo extensive splicing to produce multiple isoforms (Obata et al., 1998). Expression of the different protocadherins is highly controlled in neuronal cells (Hirayama and Yagi, 2013). It is proposed that controlled expression of protocadherins in different neuronal cells is required for proper connectivity.

One of the cadherins known to function in axon guidance is a classical cadherin N-cadherin. N-cadherin was originally identified by disruption of calcium-dependent adhesion in the brain with the use of N-cadherin specific antibody (Hatta et al., 1985). N-cadherin is expressed in neuronal cells and required for proper guidance of optic nerves in chick embryos (Mastunaga et al., 1988). In zebrafish, the N-cadherin homolog is required for proper patterning of retinal neurites, axon fasciculation and outgrowth (Masai et al., 2003). Mutants of the *Drosophila* N-cadherin homolog also show defects in axon patterning in the central nervous system (Iwai et al., 1997). Among these defects are errors in axon trajectories and fasciculation.

The role of N-cadherin in these species seems to be due to adhesion.

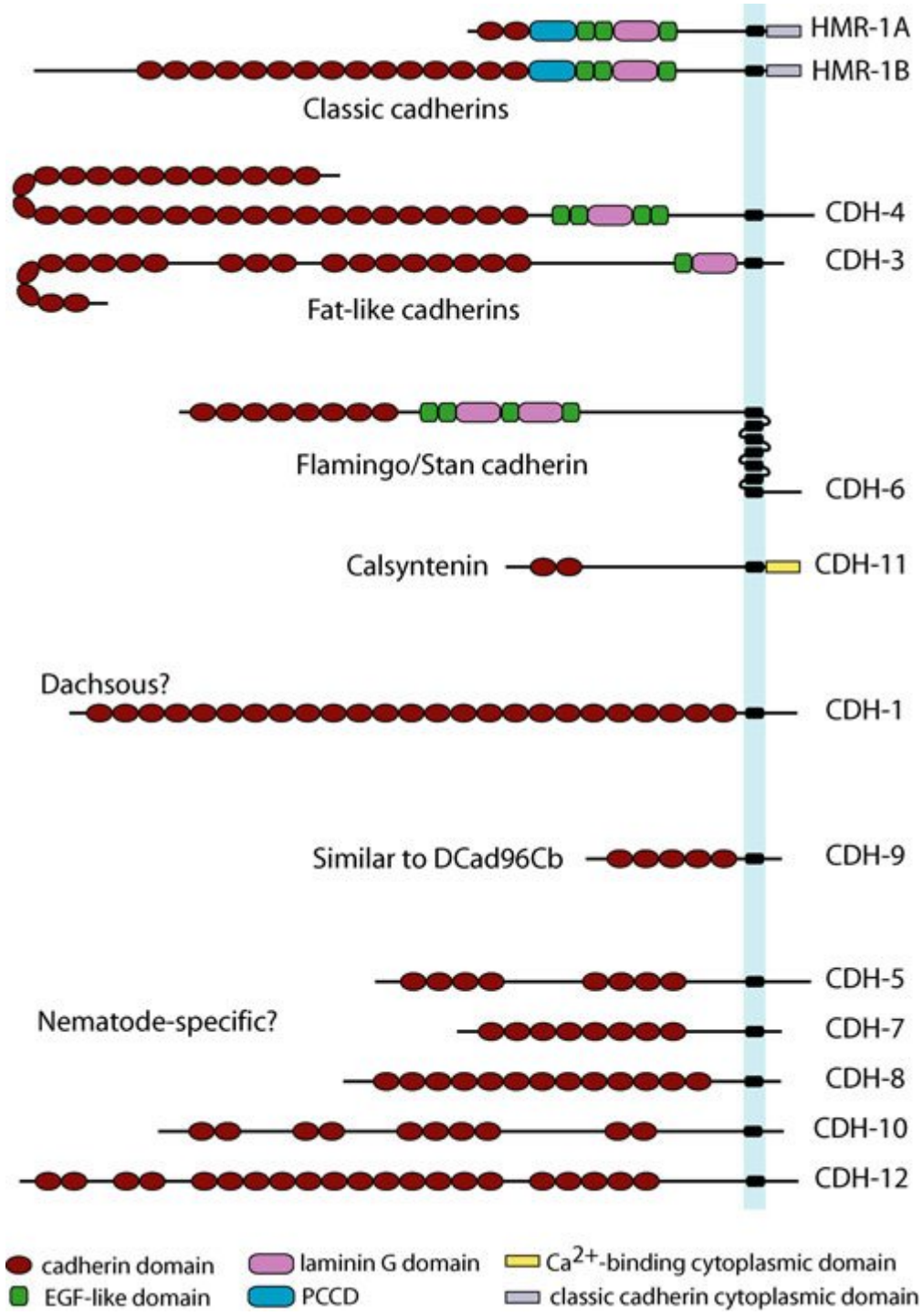


Figure 5. *C. elegans* cadherin proteins.

Domain organization of the *C. elegans* cadherin proteins listed with their homologs. Top to bottom: Classical cadherins: HMR-1a and HMR-1b. Fat-like cadherins: CDH-3 and CDH-4.

Flamingo: CDH-6. Calsyntenin: CDH-11. Dachsous: CDH-1. DCad96Cb:CDH-9. Nematode-specific: CDH-5, CDH-7, CDH-8, CDH-10 and CDH-12. (Pettitt, 2005).

C. elegans contains 12 cadherin genes encoding 13 cadherin proteins (Figure 5) (Hill et al., 2001). Two of these cadherin proteins are classical cadherins HMR-1a and HMR-1b. However, only HMR-1b is expressed in the nervous system. HMR-1b mutants show relatively weak fasciculation and outgrowth defects in neurons (Broadbent and Pettitt, 2002). Another cadherin expressed in the nervous system, *casy-1*, shows the strongest sequence similarity to other species of all *C. elegans* cadherins (Pettitt, 2005). Defects in *casy-1* mutants have been mainly associated with learning in response to salt-chemotaxis (Ikeda et al., 2008; Hoerndli et al., 2009). A third neuronally expressed cadherin in *C. elegans* is the flamingo homolog *fmi-1*. In *Drosophila*, flamingo is involved in proper orientation with the epithelial layer through the planar cell polarity pathway (Chae et al., 1999; Usui et al., 1999). Additionally, flamingo is required for proper axon guidance in the *Drosophila* visual system (Lee et al., 2003; Senti et al., 2003). Flamingo is believed to function as a receptor or co-receptor in these pathways. In *C. elegans*, *fmi-1* show significant defects in guidance of several axons in the ventral nerve cord including HSN and PVP. Additionally, *fmi-1* is required for PVQ axons to follow PVP axons properly (Steimel et al., 2010). *fmi-1* mutants also show defects in synapse formation, size and morphology (Najarro et al., 2012). Despite these defects, *fmi-1* mutants look superficially wildtype.

Cadherins play important roles in axon guidance in vertebrate and invertebrate species. The function of cadherins has been shown to be both as cell adhesion molecules and as receptors for signalling pathways depending on the protein and pathway.

1.5 Fat-like Cadherin: CDH-4

C. elegans contains two fate-like cadherin proteins, CDH-3 and CDH-4 (Hill et al., 2001). *cdh-3* is expressed in a subset of epithelia, vulval and neuronal cells (Pettitt et al., 1996). However, *cdh-3* mutants have only shown defects in morphogenesis and no neuronal defects.

The other fat-like cadherin, CDH-4 has significant neuronal defects when lost (Schmitz et al., 2008). CDH-4 is a fat-like cadherin with homology to *Drosophila* Fat and four Fat cadherins in mammals (Hulpiau and van Roy, 2009). CDH-4 is a 4292 amino acid protein containing 32 cadherin domains, 4 EGF domains, a laminin G domain and a single-pass transmembrane domain (Figure 6). Three mutant alleles have been used in our lab to study *cdh-4* function. *hd40* is a deletion removing the beginning of the gene and is likely a a complete loss-of-function allele although this has not been confirmed by antibody staining due to lack of an antibody. *rh310* and *hd13* are both single base pair mutations that cause a premature stop and form a truncated protein. All three alleles have a similar penetrance of phenotypes. *cdh*-4 mutants show defects in the nervous system, hypodermis and pharynx development (Schmitz et al., 2008). In the nervous system, interneuron axons expressing GFP did not enter the VNC and instead grew laterally (Figure 7). Fasciculation defects, where axons in a bundle are no longer closely packed, were seen in the dorsal cord and the interneurons of the VNC. PVPR and PVQL showed a cross-over phenotype where they would cross into the right tract. In DD/VD motorneurons, axons exited the ventral nerve cord to the left which is not seen in wildtype. *cdh-4* mutants also show non-neuronal defects such as larval lethality, attachment defects of the pharynx and variable morphology defects (Figure 8).

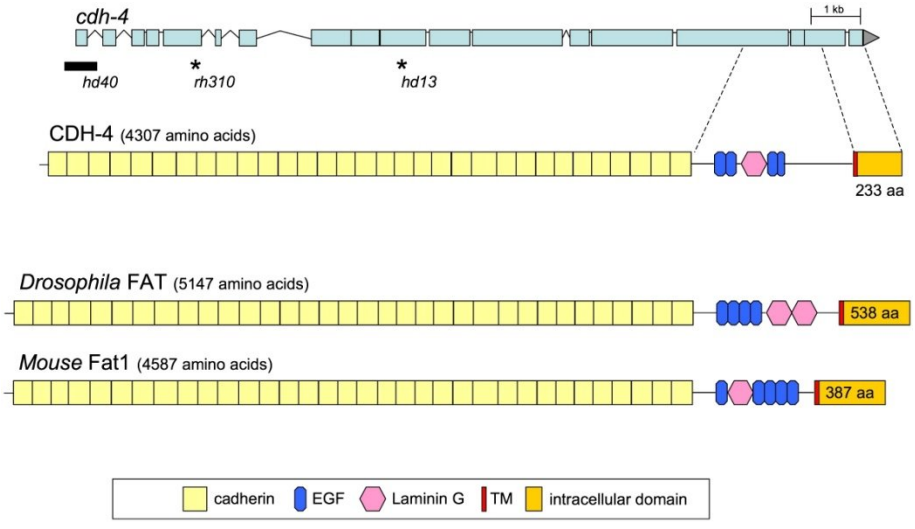


Figure 6. Structure of CDH-4.
The intron-exon structure of *C. elegans cdh-4* is shown with mutants marked. A bar represents the location of *hd40* deletion. Asterisks represent the locations of point mutations *rh310* and *hd13* causing premature stops. Protein domain organization is shown for CDH-4 and its mouse and *Drosophila* homologues, Fat1 and FAT respectively (Schmitz et al., 2008).

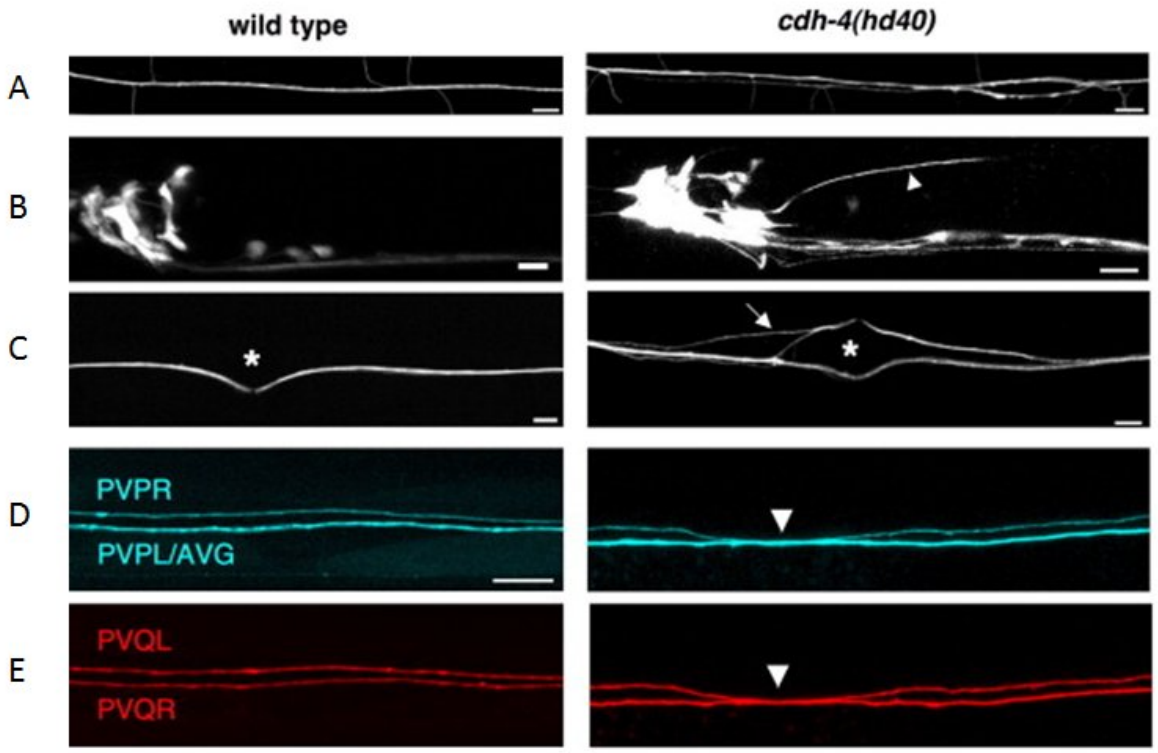
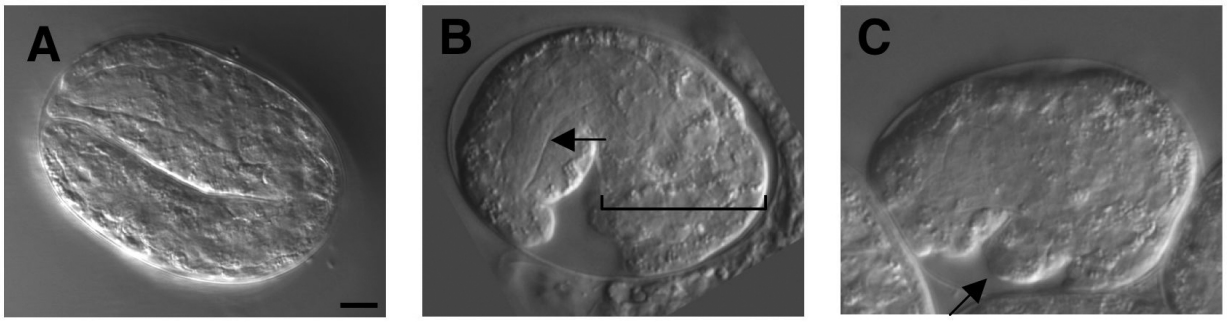
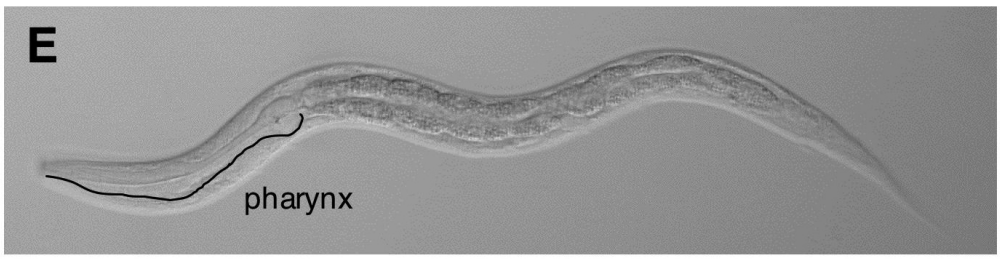


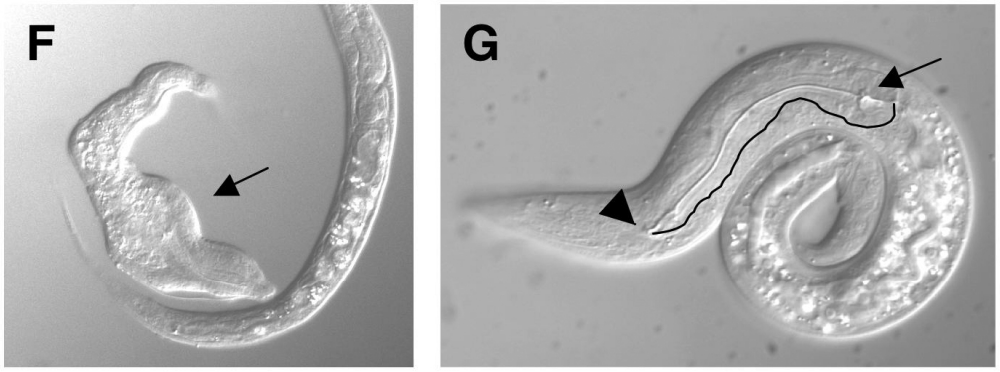
Figure 7. Axonal defects of *cdh-4* mutants. A) The dorsal cord is labeled *F25B3.3::GFP* and forms a tightly bound cord. *cdh-4* mutants show frequent defasciculation, where axons no longer bundle tightly, of this cord B) Interneurons labeled with *glr-1::GFP* enter into the ventral nerve cord and travel posteriorly. *cdh-4* mutant interneurons exit the nerve ring and extend laterally rather than in the VNC (Arrowhead). C) *glr-1::GFP* labeled interneuron axons stay in the right tract in wild type animals. *cdh-4* mutants show frequent cross-over of interneuron axons into the left tract (arrow). The star represents the location of the vulva D) PVPR, PVPL and AVG neurons are labeled with *odr-2::CFP*. Wild type animals show PVPL and AVG axons running along the right ventral cord tract and PVPR along the left tract. *cdh-4* mutants show PVPR axons crossing the ventral midline and entering the right tract (arrowhead). F) PVPR and PVQL neurons are labeled with *sra-6::dsRed*. Wild type animals show PVQR axon extending along the right tract and PVQL axon extending along the left tract. In *cdh-4* mutants, PVQL crosses the ventral midline by following PVPR across in the same location (arrowhead) A) Dorsal view B) Lateral view C-E) Ventral view. Scale bar: 10 μm (Schmitz et al., 2008).



D

	% dead eggs	% surviving adults
wild type	0 ± 0	106 ± 7
cdh-4(hd40)	22 ± 6	22 ± 6
cdh-4(rh310)	11 ± 5	62 ± 8
cdh-4(hd13)	27 ± 4	14 ± 5





H

	% variable abnormal morphology	% pharynx unattached
wild type	0	0
cdh-4(hd40)	12	30
cdh-4(rh310)	15	24
cdh-4(hd13)	22	37

Figure 8. Morphological defects and larval lethality of *cdh-4* mutants.
A-D) Embryonic lethality in *cdh-4* mutants. A) Wild type embryo at the 2-fold stage. B) *cdh-4* mutants fail to elongate properly (bracket). C) *cdh-4* mutants develop protrusions at various locations of the embryo (arrow). Scale bar: 10 μm. D) Table representing the penetrance of

embryonic lethality and percentage of surviving adults. E-H) Larval lethality of *cdh-4* mutants. E) Pharynx of L1 larvae is connected to anterior hypodermis to form a mouth. F) L1 larvae in *cdh-4* mutants show a variable abnormal morphology (arrow). G) *cdh-4* mutants show a pharynx unattached phenotype where the pharynx forms normally (arrow) but does not attach to the anterior hypodermis (arrowhead). H) Table representing the penetrance of larval lethality and pharynx unattached in *cdh-4* mutants.

The CDH-4 homologue in *Drosophila, Fat* causes overgrowth of the imaginal discs when mutated (Bryant et al., 1988). Work with *Fat* has shown that it acts upstream of the Hippo-Warts pathway for control of cell division (Willecke et al., 2006). In this pathway when Hippo is activated, it is able to phosphorylate a non-catalytic protein Mats (Figure 9). Salvador binds to Hippo and Warts, another kinase, to allow activated Hippo to phosphorylate Warts. Phosphorylated Mats also helps with activation of Hippo. Active Warts is able to inhibit the activity of the transcription factor Yorkie and prevent growth. This signalling cassette functions similarly in vertebrates (Zhou et al., 2009). Hippo-warts signalling has not been studied in great detail in *C. elegans*, however it is clear that it has an important role in early development as loss of Warts leads to death in the first larval stage (Cai et al., 2009).

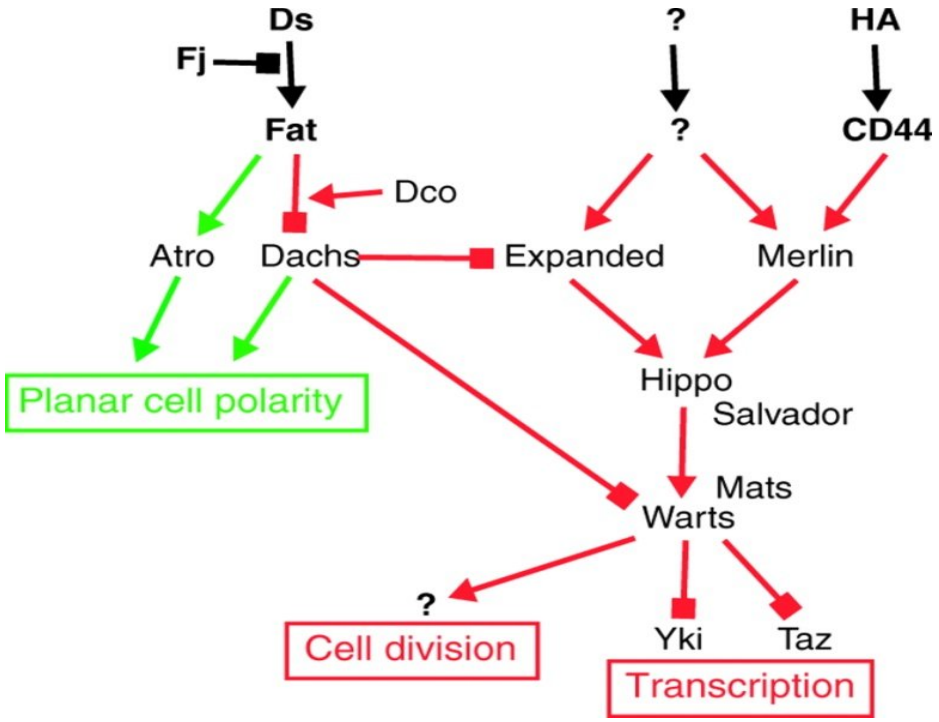


Figure 9. *Drosphila* Fat signals through Dachs and Atro to regulate the planar cell polarity pathway. Fat and Dachs signal to regulate cell division through the Hippo-Warts signalling pathway (Reddy and Irvine, 2008).

Drosophila Fat also signals through the planar cell polarity pathway (PCP) (Yang et al., 2002). The planar cell polarity pathway is required for the orientation of cells within the epithelial layer (Whrii and Tomlinson, 1995). There is a core set of PCP components which are involved in planar cell polarity at all or most locations (Thomas and Strutt, 2012). These components are able to orient cells within the plane of the epithelium by localizing to either proximal or distal locations of the cell. For example in the *drosophila* wing, Frizzled, Dishevelled and Diego localize to the proximal end while Strabismus and Prickled localize to the distal end. Flamingo can be found on both proximal and distal ends. On top of this, other proteins such as Fat interact with the pathway in ways that are not yet entirely known. The impact of Fat on the PCP pathway is partially due to its effect on Yorkie in the Hippo-Warts pathway. In fact, recently a region within the intracellular domain has been identified that is capable of rescuing overgrowth defects when membrane bound (Zhao et al., 2013). Interestingly, this region is not necessary for the PCP function of Fat. Also, research has shown that the extracellular domain of *Drosophila* Fat is not necessary for its function in PCP or cell growth (Matakatsu and Blair, 2006). In *C. elegans*, while many of the PCP pathway components are present, PCP defects have not been identified in corresponding mutants. *C. elegans* Wnts do play a role in anterior posterior guidance decisions in *C. elegans* and do interact with the PCP component *fmi-1*, however, it appears that they do not act through the PCP pathway for these effects (Huarcaya et al., 2013). Additionally, *cdh-4* does not interact genetically with *fmi-1* for this process. Finally, *C. elegans* do not have large epithelia with polarized cells so they may not require a PCP pathway for this purpose.

To examine the possibility that CDH-4 in *C. elegans* was working through one of the pathways identified in *Drosophila* we compared phenotypes between *cdh-4* mutants and

mutants of PCP and Hippo-Warts components. As no strong overlap was found, we concluded that CDH-4 might act through a different, yet to be identified pathway. We therefore decided to try to identify new interaction partners in an unbiased fashion. Yeast two-hybrid screens can be used as an unbiased method of identifying physical interaction partners. Through this method it is possible to identify potential signal transduction components acting through the *cdh-4* pathway. Using the intracellular domain of CDH-4 as bait, we performed a yeast two-hybrid screen.

1.6 Yeast two-hybrid Screen

Yeast two-hybrid screens are a popular method of identifying protein-protein interactions. The approach was developed to take advantage of a few things: the ability to split the GAL4 transcription factor and the use of reporter genes (Fields and Song, 1989). In yeast, the GAL4 transcription factor can be used to activate transcription of genes containing a particular upstream activating sequence. Activation of transcription by GAL4 requires two distinct domains: the DNA binding domain (BD) and the activation domain (AD). It is possible to separate the two domains and attach them to separate proteins. Once these two domains come into close enough contact, they are able to enhance transcription and recruit RNA polymerase. By fusing different proteins to the BD and the AD, researchers are able to test if there is a physical interaction between the proteins (Figure 10). The interaction can then be determined by examining various reporter genes. For example, enzymes required for certain metabolic steps under the GAL4 upstream activating sequence can be used. In this case, positive physical interactions will allow for grow on medium lacking the particular metabolite. This protocol can be scaled up to screen many interactions. For large scale screens, a cDNA library must be prepared and incorporated into plasmid containing either the AD or BD (typically AD). Then yeast containing your protein of interest (bait protein) are transformed with the plasmid library. Positives from this process must later be confirmed.

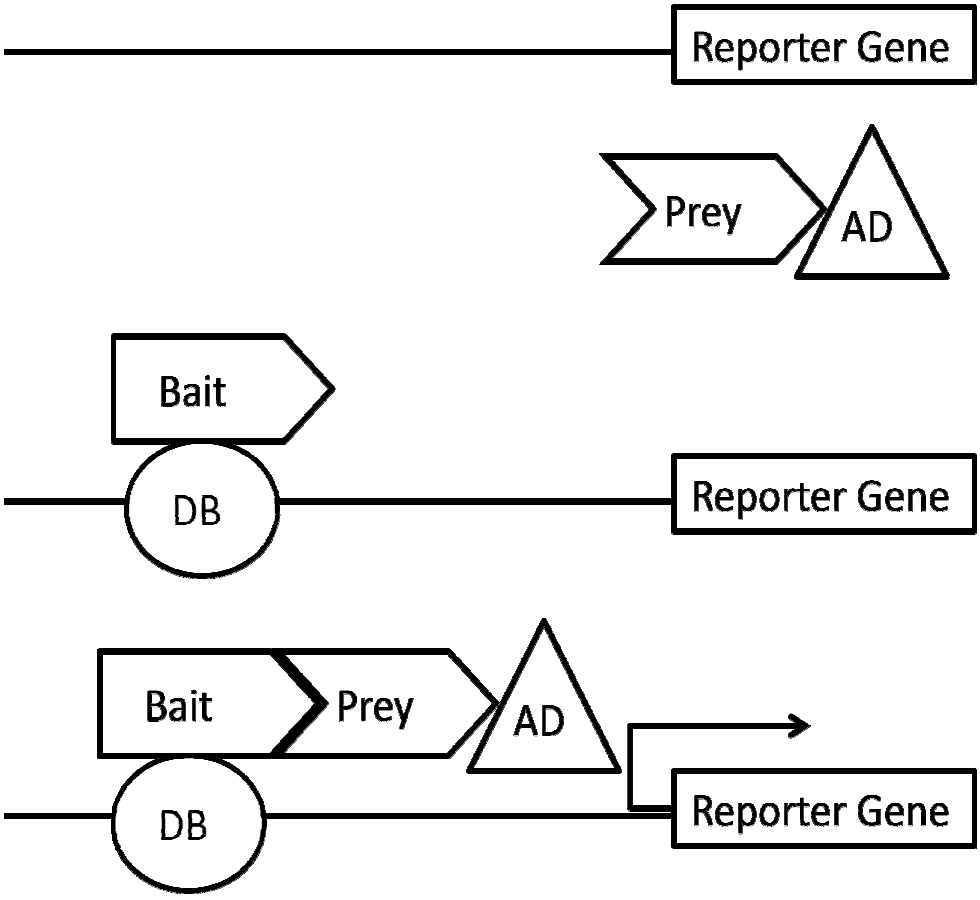


Figure 11. The concept of yeast two-hybrid interactions.

The function of the GAL4 transcription factor can be divided into two function proteins: Activation domain (AD) and DNA binding domain (DB). Expression of reporter genes can be controlled by taking advantage of this division and using fusion proteins. A bait protein is attached to the DB of GAL4. Bait interaction is tested with prey proteins fused to the AD. Interaction between bait and prey allows for reconstitution of the GAL4 transcription factor activity and reporter gene expression.

1.7 Scope of this study

The purpose of this study was to gain insight into signalling pathways used by CDH-4 in axon guidance in *C. elegans*. We initially examined the Hippo-Warts and PCP pathways mutants to identify shared phenotypes. In doing so, we aimed to determine if CDH-4 functions through the same pathways as its *Drosophila* homolog. As hippo-warts and PCP pathway mutants did not show guidance defects characteristic for *cdh-4* mutants, we hypothesized that CDH-4 acts through a different molecular pathway. Consequently we proceeded with an

unbiased approach to identify proteins directly interacting with CDH-4. We used a yeast two-hybrid screen to identify novel biochemical interaction partners of CDH-4 inside the cell. Using the intracellular domain of CDH-4 as bait and screening a *C. elegans* cDNA library, courtesy of Dr. Barstead, as the prey, we identified 42 potential interaction partners and began to test them for an in vivo role in the CDH-4 pathway. Additionally, we tested genetic interactions between *cdh-4* and several intracellular signalling pathways known to play a role in axon guidance.

2. Materials and Methods

2.1 Maintenance and Strains

Strains were maintained following standard conditions (Brenner, 1974). Animals were grown on easiest worm media (EWM) plates with OP50 *Escherichia coli* and grown at 15 ^{o}C and 20^{o}C. EWM plates were prepared by adding 5.9g of EWM (mixed by adding 55g Tris-HCl, 24g Tris-OH, 310g Bacto Peptone, 800mg Cholesterol and 200g NaCl) and 18g agar to a total volume of 1L H2O and poured into plates.

The following integrated fluorescent protein markers were used to examine phenotypes in mutant strains: III: *hdIs26[odr-2::CFP, sra-6::DsRed2]*, *rhIs4[glr-1::GFP, dpy-20(+)].* V: *hdIs29[odr-2::CFP, sra-6::DsRed2], evIs111[F25B3.3::GFP].*

Alleles used for phenotypic description were as follows: I: *unc-73 (hd40), dcp-66 (gk370), tag-210 (gk303), spr-4 (by105)*. II: *dab-1 (gk291), gpb-1 (ok1875)*. III: *gei-1 (tm3626), paa-1 (ok1539)*. IV: *ced-10 (n1993), dcaf-1 (ok1867), mep-1 (q660), T12B3.4 (ok3212)*. V: *gfi-1 (ok2669), tag-153 (ok699), F57A8.1 (gk108), F57F4.4 (ok2599)*. X: *peb-1 (cu9), vang-1 (tm1422), vha-12 (ok821), hpk-1 (pk1393), mig-2 (mu28), gpn-1 (ok377), unc-115 (mn481), aco-1 (jh131), C39D10.7 (ok2758)*.

2.2 DNA Manipulation and Cloning

2.2.1 Agarose gel electrophoresis

Gel electrophoresis was conducted using 1% agarose gels prepared by adding agarose to TBE buffer (89 mM Tris-Borate and 2mM EDTA) containing 0.5 µg/ml ethidium bromide. The sample was heated to dissolve the agarose and cooled to gel state before running.

2.2.2 Plasmid DNA preparation

Plasmid DNA were prepared using the alkaline lysis method (Birnboim and Doly, 1979). Plasmid preparations were prepared using Fermentas kits following the manufacturer's protocols. A larger scale medi-prep was performed to purify cDNA library plasmids using a kit provided by Fermentas.

2.2.3 Cloning

In order to use the CDH-4 intracellular domain as bait in a yeast two-hybrid screen, we cloned the DNA encoding amino acids 4053 to 4292 of *cdh-4* into the bait vector pAS2. pAS2 contains a multiple cloning site with restriction sites for NdeI and SalI. A linear DNA product was produced by amplifying *cdh-4* intracellular domain using PCR. Using ESTs obtained from the Kohara lab as a source of cDNA, we amplified using primers that added a 5' NdeI restriction site and a 3' SalI restriction site. *cdh-4* PCR product and pAS2 were digested for 1 hour at 37 ^{o}C. The two were ligated together overnight at 15 ^{o}C using T4 DNA ligase.

The ligated plasmid was transformed into chemically competent DH5α *E. coli* cells by heat shock. Transformation was confirmed by growth on ampicillin plates and restriction digest.

2.2.4 Transformation of bait vector in PJ694α yeast

Small scale yeast transformations were performed using a lithium acetate and heat shock method. Fresh colonies were picked off a plate and placed in a 1.5 microcentrifuge tube. 10 µl of 10mg/ml salmon testes DNA and 1µg of plasmid DNA were added to the tube. 0.5 ml of PLATE solution (40% PEG, 0.1 M Lithium acetate, 10 mM Tris-HCl and 1 mM EDTA) was added to the tube and vortexed vigourously. Incubate mixture overnight at room temperature. Heat shock the sample at 42 ^{o}C for 15 minutes. Pellet the sample by centrifuging for 10 seconds

at maximum speed. Remove supernatant and resuspend in 200 µl sterile distilled water. Spread on selective plates and grow at 25 °C.

2.3 Yeast-two Hybrid Screen

Yeast strain PJ69-4α (MATα trp1-901 leu2-3,112 ura3-52 his3-200 gal4(deleted) gal80(deleted) LYS2::GAL1-HIS3 GAL2-ADE2 met2::GAL7-lacZ) was maintained on YPD plates (1% Bacto yeast extract, 2% Bacto peptone, 2% dextrose, 2% agar in distilled water) at 30 °C. PJ69-4α was also grown at 30°C in liquid YPD media (1% Bacto yeast extract, 2% Bacto peptone, 2% dextrose in distilled water).

Yeast were also grown in several different drop-out media for selection purposes. Selective dropout media(6.7 g Yeast nitrogren base without amino acids, 20 g agar, 850 ml distilled water, and 100 ml of 10X dropout solution), SD, was used for selection of yeast containing bait or prey plasmids and positive interactions. 10X Dropout solution contained all amino acids except tryptophan, leucine, histidine and adenine and was prepared by adding the following to 1L of distilled water: 200mg arginine-HCl, 300mg isoleucine, 300mg lysine-HCl, 200mg methionine, 500mg phenylalanine, 2000mg threonine, 300mg tyrosine, 200mg uracil and 1500mg valine. The following 10X stocks were prepared to supplement media based on selection choice: 100mg/50 ml L-adenine, 100mg/50 ml L-histidine, 500mg/50 ml L-Leucine and 100mg/50 ml L-Tryptophan. Selection of yeast containing bait was performed by plating yeast cells on plates containing SD supplemented with Leu, His and Ade. Selection of yeast containing prey was performed by plating yeast cells on plates containing SD supplemented with Trp, His and Ade. Medium stringency selection of positive interactions was performed using SD supplemented with Ade. High tringency selection of positive interactions was performed using SD with no supplements.

SD plates were all supplemented with 25 mM 3-aminotriazole (3-AT). HIS3 expression is considered leaky as there are low levels of expression even without positive interactors. 3-AT is

a competitive inhibitor of the HIS3 protein and small concentrations are enough to eliminate the low levels of expression while allowing expression when positive interactions occur.

Large scale transformation of PJ6-4α yeast containing pVH26.01 bait plasmid was performed in the following manner. One yeast colony was picked from an SD-Trp plate less than one week old and incubated overnight at 30^{o}C in 150 ml liquid SD-Trp broth with 250 rpm shaking. Overnight culture was diluted to an OD600 of 0.2-0.3 by adding approximately 850 ml of SD-trp liquid broth. Culture was incubated at 30^{o}C for approximately 3 hours until an OD600 of 0.5. Culture was then separated into 50 ml tubes and centrifuged for 5 minutes at 1000 x g at room temperature. Supernatant was discarded. Cells were resuspended in 500 ml TE buffer (10 mM Tris, 1 mM EDTA). Suspended cells were then centrifuged for 5 minutes at 1000 x g at room temperature. Supernatant was decanted and cells were resuspended in 8 ml of freshly prepared TELiAc (1x TE buffer, 0.1 M Lithium Acetate). 1 mg of purified prey library and 20 mg of salmon testes DNA was added to a 500 ml flask. 8 ml of competent cells prepared above is added to this mix. Next, 60 ml of PEG/LiAc solution (40% PEG, 0.1 mM Lithium Acetate, 1x TE buffer) is added and mixed at high speed. The mixture is incubated 30 mins at 30^{o}C with 200 rpm shaking. 7 ml of DMSO was added and mixed by swirling. Sample is heat shocked at 42^{o}C for 15 mins with occasional swirling. Sample was then chilled on ice for 1-2 minutes and centrifuged at 1000g for 5 minutes at room temperature. Supernatant was discarded and cells were resuspended in 10 ml of YPD liquid broth. This solution was then plated on SD-trp-leu-his plates for medium stringency selection of positive interactions.

Positive interaction candidates were selected for their growth on medium stringency plates. These candidates were replica plated on high stringency plates and tested again for their ability to activate reporter genes. These candidates were also tested using a β–galactosidase activity assay.

Candidates that showed reporter gene activity under the previous three conditions were plated on SD-Leu plates containing cycloheximide. The bait vector backbone contains a gene

cyh2 that confers sensitivity to cycloheximide when the protein is present in yeast cells. Plating candidates on plates containing cycloheximide selected for the loss of bait plasmid. These yeast strains contained candidates lacking bait and were replica plated on SD-leu-his-ade plates to test for ability of prey to auto-activate transcription.

Bait plasmid was re-transformed into candidate strains lacking the bait plasmid that were unable to activate reporter genes. Reporter gene activity was re-tested in these new strains. Candidates that once again activated reporter gene activity were used for PCR to amplify prey DNA and sent for sequencing.

2.4 Polymerase Chain Reaction and DNA Sequencing

Positive candidates identified in the yeast two-hybrid screen were used as template for a colony PCR reaction to amplify prey DNA. 1 mm yeast colonies were transferred to 50 µl of 60U/ml zymolyase in water. The solution was incubated 30 minutes at 37 ^{o}C followed by 95 ^{o}C for 10 minutes. 5 µl of the solution was used as template for a 50 µl PCR reaction. PCR reactions were prepared by mixing the following: 2.5 µl forward primer (10mM), 2.5 µl reverse primer (10mM), 5 µl Taq buffer, 1 µl DNTPs (10 mM), 1.25 units *Taq* polymerase, 2 µl $MgCl_2$ (25mM) and water to total volume of 50 µl. Primers used: The PCR reaction temperatures were:

95 ^{o}C for 30 seconds, followed by 30 cycles of 95 ^{o}C for 30 seconds, 45-68 ^{o}C for 30 seconds, and 68 ^{o}C for 60 seconds. Annealing temperatures were determined by subtracting 5 ^{o}C from the melting temperatures of primers used in the reaction. A final extension of 5 minutes at 68 ^{o}C was performed at the end of the PCR reaction.

PCR products were sent for DNA sequencing through Eurofins Operon with a concentration of approximately 25ng/µl.

3. Results

3.1 Hippo-Warts pathway component mutants do not share neuronal defects of *cdh-4* mutants

Currently, there are no known physical or genetic interactors with *cdh-4* in *C. elegans*. As previously stated, the *cdh-4* homologue in flies Fat is involved in both the Hippo-Warts and the PCP pathway. There is a possibility that *cdh-4* interacts with genes within the Hippo-Warts and PCP pathway. We examined mutants of genes in the Hippo-Warts pathway to see if they were involved in axon guidance. Interneuron and PVP/PVQ axons were labelled with fluorescent proteins by the transgenes *rhIs4* and *hdIs26* respectively which were then crossed into homologues of the Hippo-Warts and PCP pathway components. These genes were then examined for interneuron, PVPR and PVQL crossover defects (Table 1). Crossover defects were scored as axons leaving their proper tract in the VNC and crossing into the opposite tract. Components of the Hippo-Warts pathway did not show significant defects in these areas. Components of the PCP pathway were examined with similar markers by Andreas Steimel (Thesis, 2010). Similarly to the Hippo-Warts pathway, the PCP pathway does not seem to show strong neuronal defects in *C. elegans*. These data together with the disparity in phenotypes between CDH-4 and its mammalian and *Drosophila* homologues and the lack of evolutionary conservation of the intracellular domain of CDH-4 suggest that CDH-4 does not signal through these pathways for axon guidance in *C. elegans*.

Table 1. Comparison of neuronal defects using interneuron, PVP and PVQ markers (hdIs26, rhIs4) between *cdh-4* mutants and components of the Hippo-Warts pathway.

Allele	Homologue	n	Interneuron Cross-over[a] (%)	PVPR[b] (%)	PVQL[c] (%)
wild-type		100	7	5	5
cdh-4 (rh310)	Fat	100	23	66	63
gpn-1 (ok377)	Dally/Dally-like	100	8	6	5
egl-27 (n170)	Atrophin	100	6	8	7
cdh-1 (hd44)	Dachsous	100	8	7	7
wts-1/hT2 (ok753)	Warts	100	10	4	5
T12B3.4	Mats	100	4	6	6

[a]defects scored as interneurons crossing the ventral midline.
[b]defects scored as PVPR axons crossing the ventral midline.
[c]defects scored as PVQL axons crossing the ventral midline

3.2 Use of the CDH-4 intracellular domain as bait in a Yeast two-hybrid screen

Due to the lack of conservation in the intracellular domain of CDH-4 when compared to mouse and *Drosophila*, difference in phenotypes and lack of overlapping phenotypes between Hippo-Warts and PCP genes with *cdh-4*, we hypothesized that CDH-4 acts through a different mechanism in *C. elegans.* A commonly used approach to identify physical interactors is a yeast two-hybrid screen. However, this procedure has typically been limited to soluble proteins and therefore would not work with full length CDH-4, a transmembrane protein. As we are interested in interactions occurring within the cell, we prepared a construct using the intracellular domain of CDH-4 as bait for the procedure.

A plasmid pVH26.01 was produced by cloning a fragment of CDH-4 into pAS1-CYH2. PCR was used to add 5' NdeI and 3' SalI restriction sites to the CDH-4 intracellular domain fragment. These same enzymes were used to incorporate the CDH-4 intracellular fragment into the pAS1-CYH2 vector. The product of this was a fusion protein encoding the CDH-4 intracellular domain and the GAL4 dna binding domain (CDH-4 Intra). This fusion protein could then be used for screening a yeast two-hybrid library.

A cDNA library, courtesy of Dr. Barstead, coding for fusion of *C. elegans* proteins with the GAL4 activation domain was used to identify interaction partners with the CDH-4 Intra. Interaction partners are identified by their ability to activate several reporter genes. Two proteins that physically interact are able to bring the GAL4 activation domain and DNA binding domain into close enough contact to transcribe genes using the GAL4 promoter. The strain used was incapable of synthesizing adenine, histidine, leucine and tryptophan and required media to be supplemented with these amino acids.

The bait vector pAS1-CYH2 contained TRP1, a gene essential for tryptophan synthesis. As such, any yeast cells containing the bait vector were able to grow on plates lacking tryptophan. Similarly, the prey vectors pACTII contained LEU2 a gene essential for leucine biosynthesis and allowed for growth on plates lacking leucine. ADE2, HIS3 and LacZ are all under the GAL4 promoter and expressed upon proper binding between bait and prey fusion proteins.

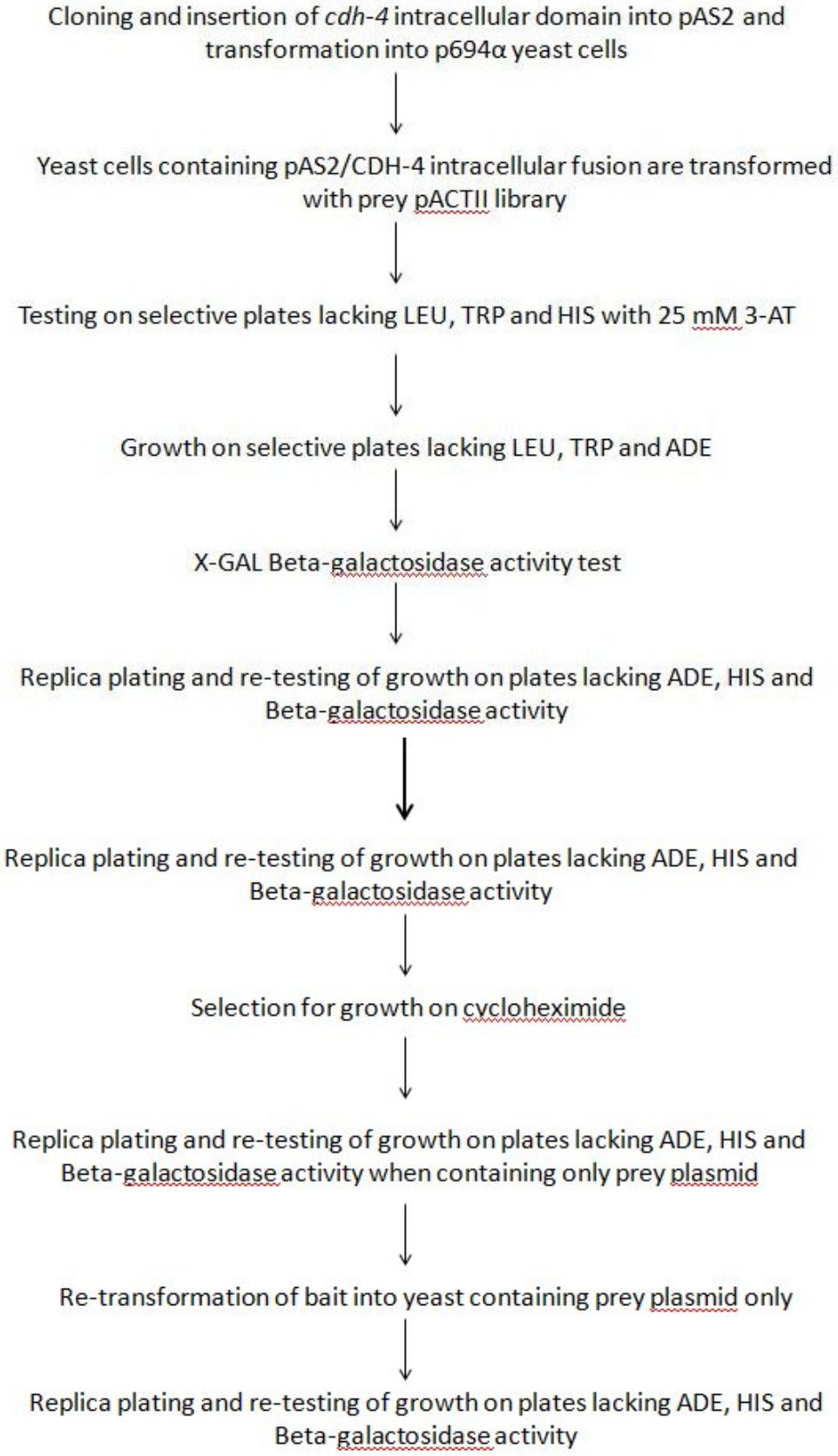

Cloning and insertion of *cdh-4* intracellular domain into pAS2 and transformation into p694α yeast cells
Yeast cells containing pAS2/CDH-4 intracellular fusion are transformed with prey pACTII library
Testing on selective plates lacking LEU, TRP and HIS with 25 mM 3-AT
Growth on selective plates lacking LEU, TRP and ADE
X-GAL Beta-galactosidase activity test
Replica plating and re-testing of growth on plates lacking ADE, HIS and Beta-galactosidase activity
Replica plating and re-testing of growth on plates lacking ADE, HIS and Beta-galactosidase activity
Selection for growth on cycloheximide
Replica plating and re-testing of growth on plates lacking ADE, HIS and Beta-galactosidase activity when containing only prey plasmid
Re-transformation of bait into yeast containing prey plasmid only
Replica plating and re-testing of growth on plates lacking ADE, HIS and Beta-galactosidase activity

Figure 10. Overview of yeast two-hybrid procedure.

Overview of the yeast two-hybrid screening procedure. Candidates were tested for expression of three separate reporter genes (-HIS, -ADE, and beta-galactosidase activity). Candidates were retested and tested for ability to activate expression of reporter genes with only prey plasmid present. Finally, candidates were tested for the ability to activate reporter gene activity after re-transformation of bait plasmid.

3.3 Yeast two-hybrid controls

Several control experiments must be conducted before performing a large scale yeast two-hybrid screen (Table 2). First, new bait plasmids must be tested for their ability to auto-activate reporter genes. This was done by transforming plasmid containing CDH-4 Intracellular into pJ694a yeast and testing reporter gene activity. Yeast containing CDH-4 Intracelluar was unable to grow on –Ade plates but was able to grow weakly on –His plates. This is not uncommon as there are often low levels of HIS3 expression. To compensate for this 25 mM of 3-amino-1,2,4-triazole is added to plates. 3-AT competitively inhibits HIS3 protein reducing false positives due to low expression. Finally, a beta-galactosidase assay can be performed to verify the activation of LacZ. CDH-4 Intra did not lead to beta-galactosidase activity on its own. Additionally. the ability of the prey vector pACTII with CDH-4 intracellular was tested in a similar manner.

Table 2. Control experiments testing reporter gene activity with CDH-4 intracellular domain protein fusion.

Added to pJ694a yeast	Growth on -LEU	Growth on -TRP	Growth on -TRP, -LEU	Growth on -TRP, -LEU, -HIS +25 mM 3-AT	Growth on -TRP, -LEU, -HIS +25 mM 3-AT -ADE	Positive LacZ Assay
none	-	-	-	-	-	-
pAS2	-	+	-	-	-	-
pACTII	+	-	-	-	-	-
GAL4	+	+	+	+	+	+
pAS2 + pACTII	+	+	+	-	-	-
CDH-4 Intracelullar	-	+	-	-	-	-
CDH-4 Intracellular	+	+	+	-	-	-

+ pACTII

Finally, the ability of bait and prey vectors with no inserts must be tested. In the same manner as for bait and prey, yeast cells containing both bait and prey vectors were plated on plates lacking leucine and tryptophan. These were then spread onto plates lacking either adenine or histidine. These showed no growth. Again, ability to activate beta-galactosidase was assayed and it was confirmed that these did not activate it. A positive control using full length GAL4 was used for comparison.

3.4 Removal of false positives

We identified 423 positive interactions initially by the ability to activate expression of all three reporter genes tested. As false positives are a frequent concern in yeast two-hybrid screens, various steps are taken to reduce their presence. The first step is to replica plate the positive interaction to test the ability to activate reporter genes again. 79 positives would not grow after a replica plating (Table 3).

Table 3. Overview of positive interactions identified in yeast two-hybrid screen and removal of false positives.

Description of positive interaction	Number
Positive interaction by all reporter genes	423
No growth when replica plated	79
Growth on selective plates with only prey	68
Growth on selective plates with pAS2 and prey	75
No growth on selective plates after retransformation of bait	125
Candidates identified for sequencing	72
Unique candidates	41

The second step to removing false positives is to test the ability of prey plasmid to activate reporter genes on its own. To do so, the bait plasmid must be removed from the positive yeast strains from the screen. The bait plasmid, pAS2, contains a gene that does not allow the cell to grow on media containing cycloheximide. Plating positive colonies on plates containing cycloheximide and tryptophan but lacking leucine allows for selection of yeast containing on the prey plasmid. Yeast containing only prey plasmid is then re-tested for ability to

activate reporter gene expression by plating on media lacking leucine and histidine. If reporter gene activity is seen, the sample is discarded as the prey has the ability to activate transcription on its own. 68 positive interactions from the screen showed growth when containing only prey plasmid and were discarded. Those positives that showed no growth with prey plasmid only were re-transformed with the empty bait vector pAS2 and once again re-tested. 75 of these positives showed growth and were discarded.

Yeast containing prey that do not auto-activate reporter genes are used for further testing. Bait plasmid can be re-transformed into prey yeast strains and once again tested for ability to activate reporter genes. All three reporter genes activity was tested. 125 candidates that were unable to activate reporter genes were discarded. The remaining candidates that were capable of activating reporter genes were used for colony PCR. Colony PCR was performed to amplify the insert region of the prey plasmid. The colony PCR products were sent for sequencing to identify the prey gene for further analysis. 76 candidates were sequenced using colony PCR and identified 42 unique candidates.

3.5 Molecular Identity of Yeast-two Hybrid Positive Interactors

A total of 42 genes were identified as positive interactors based on the controls and protocol previously stated (Table 4, Figure10). Candidates ranged from a wide variety of predicted molecular functions including kinases, transcription factors and metabolic enzymes. Several candidates were identified multiple times in the screen however there was no clear first choice to follow up on. Candidates were identified by aligning sequence data with the *C. elegans* genome using BLAST (Altschul et al., 1990).

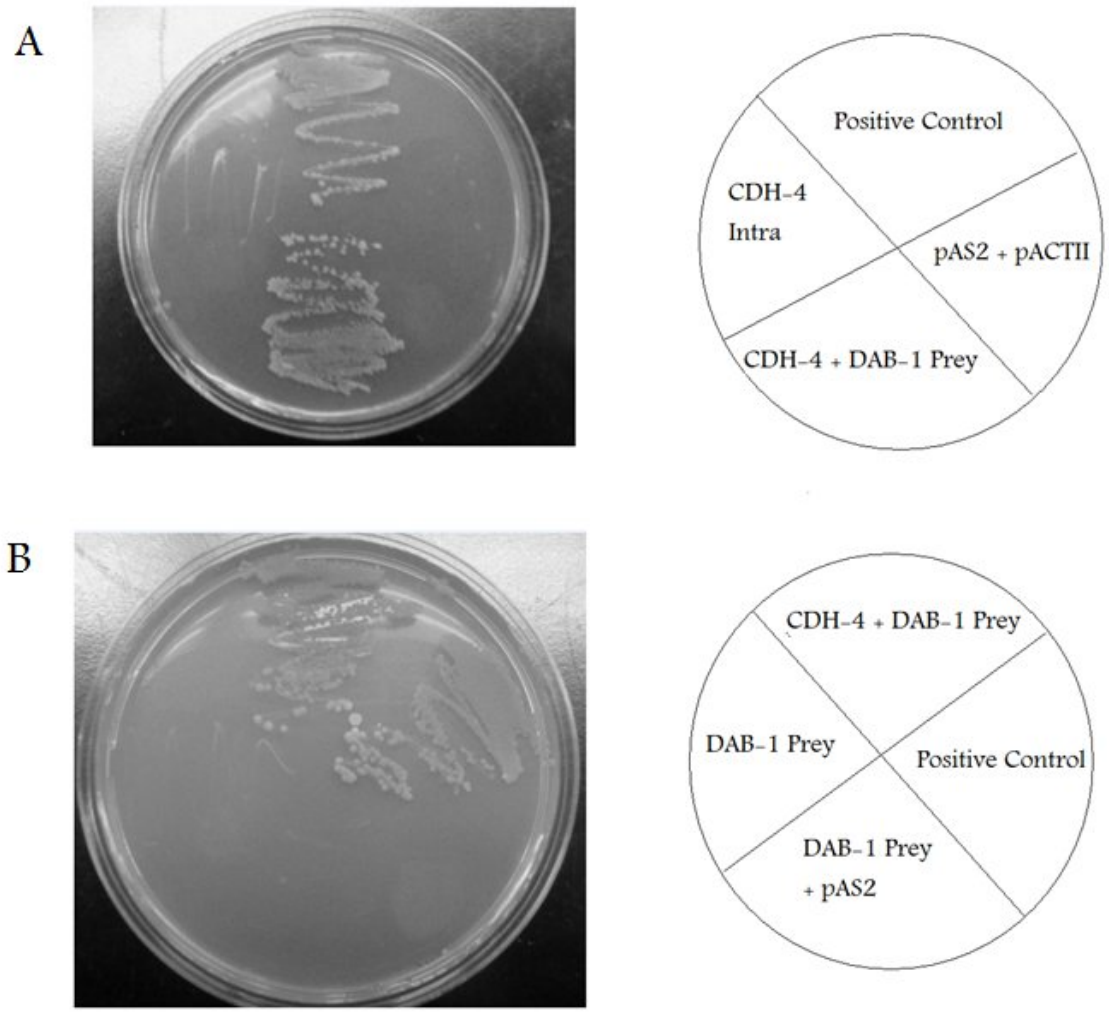


Figure 12. Growth of positive interactions on selective plates.

A) Growth on yeast two-hybrid positive interactions on plates lacking LEU, TRP, HIS and containing 25 mM 3-AT. The positive control GAL4 shows similar growth to the CDH-4 intracellular bait and DAB-1 prey plasmid yeast. No growth visible on bait only and empty bait and prey vectors. B) Growth on yeast two-hybrid positive interactions on plates lacking LEU, TRP, HIS and containing 25 mM 3-AT. DAB-1 prey plasmid alone and with empty bait vector does not show growth on selective plates.

Table 4. Candidates identified as interaction partners with CDH-4 intracellular domain by Yeast two-hybrid screening.

Gene	Location/Function/Domains	Description	Mutant Available (If yes, allele)	Number of times identified
dab-1	intracellular signaling	adaptor protein	gk291	2
gei-1	intracellular signaling	RhoGAP domain	tm3626	1

gpb-1	intracellular signaling	G protein beta subunit	ok1875	3
tag-210	intracellular signaling	predicted GTP binding protein	gk303	2
hpk-1	intracellular signaling	kinase	pk1393	3
paa-1	intracellular signaling	phosphatase 2 A	tm655	2
pph-5	intracellular signaling	protein phosphatase 5	ok3498	2
dcp-66	nuclear protein	nucleosome re-arrangement	gk370	3
dnj-8	nuclear protein	contains dna J domain	No	1
gei-3	nuclear protein	High mobility group transcription factor	gk1233	2
mep-1	nuclear protein	repression of 3' UTR	q660	1
peb-1	nuclear protein	dna binding but involved in proper pharynx development	ok1941	1
spr-4	nuclear protein	suppressor of presenilin	n1299	1
tag-153	nuclear protein	predicted transcriptional regulator	ok699	1
T07C12.1 1	nuclear protein	MADF dna binding domain	gk990	3
C14C11.2	unknown	unknown	pk1358	1
C39D10.7	unknown	multiple chitin binding domains	No	1
D1007.4	unknown	unknown	No	1
F16B4.3	unknown	unknown	No	1
F56A8.3	unknown	unknown	No	1
F57A8.1	unknown	unknown	gk1088	1
F59B1.10	unknown	zinc finger domain usually containing choline kinases	No	7
pqn-32	unknown	prion like domain	No	1
T24B8.7a	unknown	ubiquitin associated domain, ubiquitin hydroxylase	tm1245	1
aagr-2	metabolic	glucosidase reductase	No	1
aco-1	metabolic	acotinase, iron regulation	tm695	1
atp-2	metabolic	ATP synthase component	ok3002	2
B0238.1	metabolic	carboxylesterase	No	2
C48B4.1	metabolic	carboxylesterase	ok2619	2
gln-5	metabolic	glutamine synthetase	No	1

icl-1	metabolic	isocitrate synthase	No	1
ucr-2.3	metabolic	ubiquitinol cytochrome c reductase	ok3073	1
vha-12	metabolic	B subunit of V-ATPase	n2915	1
vit-5	metabolic	lipid transport protein	ok3239	1
emr-1	structural	found in nuclear envelope of cells	ok252	1
dcaf-1	structural	LisH motif usually involved in mt dynamics, Protein/protein interaction	ok1867	1
let-2	secreted	collagen	mn153	8
wrt-1	secreted	warthog	tm1417	1
gfi-1	secreted	21 ET domains, similar to F57F4.4	ok2669	2
F57F4.4	secreted	21 ET domains, similar to gfi-1	ok2599	1
ZK822.4	secreted	signal peptide, DUF	No	2

3.5.1 Intracellular signalling candidates

Eight genes identified were involved in intracellular signalling based on the presence of particular protein domains. Two candidates mapped to the gene *dab-1*. *dab-1* is a 492 amino acid protein that is homologous to the cytoplasmic adaptor protein DISABLED (Le and Simon, 1998; Wormbase, 2013).

Another candidate mapped to the gene *gei-1*. *gei-1* is a protein of 1334 amino acids in length although there are several isoforms. *gei-1* contains a RhoGAP domain and a lipid transfer domain. *gei-1* was found to interact with *gex-2* through a yeast-two hybrid screen. *gex-2* encodes a 1262 amino acid protein that is a ligand for Rac1 and involved in several cell migrations (Soto et al., 2002). Nothing else is currently known of the function or role of *gei-1*.

Three candidates mapped to the gene *gpb-1*. *gpb-1* is a 340 amino acid beta subunit of a heterotrimeric G protein (Wormbase, 2013). *gpb-1* has been shown to be required for proper spindle formation during early cell divisions (Zwaal et al, 1996). Loss of *gpb-1* leads to death in

the first larval stage when provided with maternal *gpb-1*. However, high levels of *gpb-1* expression remain in neurons of adult animals.

tag-210 was identified as another potential candidate by mapping of two positive strains. *tag-210* is an ATPase of 395 amino acids in length (Wormbase, 2013). Currently nothing else is known of the function of *tag-210*.

Three more candidates mapped to *hpk-1*, a dual-specificity protein kinase of 821 amino acids in length. *hpk-1,* Homeodomain interacting Protein Kinase 1, is a homologue of the drosophila protein minibrain (Wormbase, 2013). An RNAi screen in *Drosophila melanogaster* identified minibrain as a regulator of actin-based protrusions in central nervous system derived cell lines (Liu et al., 2009). In *C. elegans*, *hpk-1* mutants show no obvious defects (Chang et al., 2003). However, more recently *hpk-1* has been found to be required in germ-line proliferation and localizes mainly to the nucleus (Berber et al., 2013).

A phosphatase 2A subunit, *paa-1*, was identified by mapping of two candidates. *paa-1* is 590 amino acids in length and an essential protein (Wormbase, 2013). As part of a phosphatase, *paa-1* can be involved in many signalling pathways and processes. However, to date *paa-1* has been identified to be mainly involved in mitotic spindle formation (Song et al., 2011). Interestingly, another phosphatase, *pph-5*, was also identified twice in this screen. However, *pph-5* does not form a complex with *paa-1* and seems to play a different in *C. elegans*. *pph-5* was found to suppress cortical granule exocytosis defects in a *sep-1* hypomorphic background (Richie et al., 2011).

3.5.2 Nuclear localized proteins and transcription factors

A second group of genes, containing nine genes, identified were classified as transcription factors. *peb-1* is one of these transcription factors and was identified by one of the candidate yeast strains. *peb-1* encodes a 427 amino acid protein that is involved in proper pharyngeal development (Thatcher et al., 2001; Wormbase, 2013). *peb-1* is expressed in all cells of the pharynx except for those of the nervous system.

Another transcription factor identified was *dcp-66* and was mapped by three separate candidates. *dcp-66* is a homolog of the NuRD component p66 and involved in nucleosome remodelling and deacetylation. *dcp-66* is required for proper excretory cell differentiation and vulval development (Poulin et al., 2005; Wormbase, 2013).

A single candidate mapped to *mep-1* or Mog interacting, Ectopic P granules-1, a zinc-finger protein of 870 amino acids in length. *mep-1* is required for germline cell differentiation and maturation (Unhavaithaya et al., 2002; Wormbase, 2013). *mep-1* localizes to the nuclei and is expressed highly in the embryo and later in some somatic cells including the dorsal and ventral nerve cords.

The remaining transcription factor candidates have not been studied previously in much detail. *dnj-8* is a protein of 813 amino acids in length containing a dna J binding domain and was identified once in the yeast two-hybrid screen (Wormbase, 2013). *gei-3* was identified twice and is a member of the high mobility group of proteins. There are three isoforms of *gei-3* ranging in size from 291 to 1405 amino acids in length (Wormbase, 2013). *spr-4* or suppressor of presinilin-4 was identified once in the screen (Wormbase, 2013). In *C. elegans*, presenilins are required for proper Notch signalling and their loss causes cell attachment problems and reduced egg laying (Smialowska and Baumeister, 2006). *spr-4* was identified through genetic screens to suppress these defects. *tag-153* was also identified only once in the screen and encodes a protein of 733 amino acids that contains a NOT2 domain involved in transcription. Currently, nothing else is known of *tag-153*. *madf-4* was identified three times in the screen and encodes a transcription factor of 329 amino acids in length containing a MADF domain. Nothing else is currently known of *madf-4* function.

3.5.3 Structural proteins

A structural protein *emr-1* was also identified once in the yeast-two hybrid screen. *emr-1* encodes a protein of 166 amino acids in length that his homologous to the human integral nuclear protein emerin (Wormbase, 2013). *emr-1* localizes to the nuclear membrane and

interacts with lamin (Gruenbaum et al., 2002). Loss of *emr-1* leads to errors in cell division, chromosome segregation and nuclear envelope organization. Identified once in the screen, *dcaf-1*, encodes a relatively large protein of 1701 amino acids in length. *dcaf-1* is predicted to encode a protein containing a Lissencephaly type-1 homology motif (Wormbase, 2013). This motif is predicted to be involved in regulation of microtubule dynamics and protein dimerization (Kim et al., 2004).

3.5.4 Proteins with no predicted function

Another group of genes identified in the screen encode for genes of no predicted function with limited or no structural information based on protein domains. *C14C11.2* encodes a protein with no recognizable domains and a length of 574 amino acids (Wormbase, 2013). *C14C11.2* was identified by mapping on one positive strain from the screen. Also identified once in the screen was *C39D10.7* which encodes 1171 amino acid protein containing multiple chitin binding domains which are predominantly found in proteins with carbohydrate binding activity (Wormbase, 2013). Similarly to the two previous genes, *D1007.4* was also identified only once in the screen. *D1007.4* encodes a protein of 168 amino acids in length and contains no known domains (Wormbase, 2013). *F16B4.3* was identified once through the yeast-two hybrid screen as well. *F16B4.3* encodes a 688 amino acid protein that contains a domain of unknown function. *F56A8.3* was identified once and contains several leucine rich repeats and a transmembrane domain (Wormbase, 2013). *F57A8.1* was identified once and encodes a protein of 269 amino acids in length with no known domains. *F59B1.10* was one of the frequently identified genes from the screen having been identified by seven different strains. *F59B1.10* encodes a protein of 428 amino acids in length containing a choline kinase domain (Wormbase, 2013). No mutants are available for this gene and no other information is known. Identified three times in the screen, *fbxc-41*, encodes a protein of 117 amino acids in length. *fbxc-41* contains a domain of unknown function and otherwise nothing is known of this gene (Wormbase, 2013). One candidate mapped to *pqn-32* which encodes a protein of 592 amino acids in length (Wormbase,

2013). *pqn-32* is predicted to contain a prion domain although nothing else is known of this protein.

3.5.5 Enzymatic proteins

Several genes encoding metabolic proteins were also identified through the yeast-two hybrid screen. *aagr-2* a gene encoding a glycoside hydrolase protein was identified by one candidate. *aagr-2* encodes a protein of 955 amino acids with predicted glucosidase activity (Wormbase, 2013). Another metabolic enzyme identified was *aco-1* and it was also identified only once in the screen. *aco-1* encodes a protein of 887 amino acids in length with aconitase activity (Wormbase, 2013). In *C. elegans*, *aco-1* is required for normal brood size and normal lifespan when under iron stress (Kim et al., 2004). The gene *atp-2* was identified by two candidates in the yeast-two hybrid screen. *atp-2* encodes the beta subunit of an ATP synthase of 538 amino acids in length. *atp-2* is required for viability beyond the L3 larval stage (Tsang et al., 2001; Wormbase, 2013). ATP-2 localizes mainly to the mitochondria, although it is expressed in the cilia of male specific neurons (Hu and Barr, 2005). Also identified twice in the screen was a gene encoding a carboxylesterase, *B0238.1*. The protein is 545 amino acids and there is currently nothing known of this gene other than its predicted function as a carboxylesterase (Wormbase, 2013). *C48B4.1* was identified by two candidates from the screen as well. *C48B4.1* encodes a protein of 659 amino acids in length and contains the middle and N-terminal domains of acetyl-CoA dehydrogenase but lacks the C-terminal domain (Wormbase, 2013). A glutamine synthetase, *gln-5*, was identified once in the yeast-two hybrid screen. *gln-5* encodes a protein of 369 amino acids in length with no known activity other than its function predicted based on structure (Wormbase, 2013). An isocitrate lyase, *icl-1*, was also identified once in the screen. *icl-1* encodes a protein of 968 amino acids in length and is required for embryonic morphogenesis (Murphy et al., 2003; Wormbase, 2013). *T24B8.7* was identified once through mapping of a candidate. *T24B8.7* encodes a protein with multiple isoforms ranging from 1271 to 2938 amino acids in length (Wormbase, 2013). *T24B8.7* is predicted to contain two

domains, a ubiquitin associated domain and a ubiquitin carboxy terminal hydrolase. *vit-5*, a vitellogenin, was identified once in the screen. *vit-5* encodes a protein of 1603 amino acids in length and is predicted to function in lipid transport (Wormbase, 2013). The final metabolic gene identified in the screen was *ucr-2.3* and was only identified once. *ucr-2.3* encodes a ubiquinol-cytochrome C reductase protein of 427 amino acids in length (Wormbase, 2013).

One candidate mapped to the gene *vha-12*. *vha-12* is a 491 amino acid vacuolar proton-translocating ATPase (Wormbase, 2013). The major function of *vha-12* currently identified is the acidification of organelles for neurodegeneration (Syntichaki et al., 2005). *vha-12* has not been identified with a role in cell migrations.

Based on the localization of the above proteins, several would be unlikely to interact with CDH-4 under typical conditions.

3.5.6 Secreted proteins

Five secreted proteins were also identified in the screen. *let-2* was identified 8 times while *wrt-1* was identified once. *let-2* encodes alpha-2 type IV collagen 1759 amino acids in length (Wormbase, 2013). *let-2* is a component of the basement and specifically between muscle and hypodermis (Sibley et al., 1994). *wrt-1* encodes a warthog gene of 484 amino acids in length. Warthog genes are named due to their similarity to hedgehog and expected to function as an intercellular signalling protein (Wormbase, 2013).

Two proteins of very similar structure were identified in the screen. *F57F4.4* and *gfi-1* were identified once and twice respectively. Both genes encode proteins of approximately 2100 amino acids in length composed of a signal peptide and 21 ET domains of unknown function (Wormbase, 2013). *gfi-1* was found to interact with *unc-68* in a yeast-two hybrid screen but this interaction has not been pursued (Sakube and Kagawa, 1999). A final secreted protein was identified, *ZK822.4*, by two candidates from the screen. *ZK822.4* encodes a protein of 453 amino acids in length that contains a signal peptide and a domain of unknown function.

Overall, a large number of proteins with a wide variety of functions were identified. It would be incredibly time consuming to clone and confirm the physical interaction between all candidates. For this reason, a secondary screen was used to focus on interactions related to the neuronal and morphological defects seen in *cdh-4* mutants.

3.6 Identification of phenotypes in yeast-two hybrid candidate mutants

As we obtained a large list of candidates with a variety of predicted molecular functions, we decided to try and identify biologically relevant interactions. As mutants were readily available for many candidates, analysis of mutant phenotypes allowed a quick method of trying to identify biologically relevant interactions. To do so, a pan-neuronal GFP marker *evIs111* was crossed into strains containing mutant alleles of the most promising candidates based on availability of alleles and predicted domain structures. By examining mutants of the candidates for neuronal and morphological defects similar to those found in *cdh-4* mutants, we hoped to reduce the number of candidates necessary for confirmation of physical interaction by co-immunoprecipitation. Co-immunoprecipitation requires cloning both proteins and adding different antibody tags to each. One antibody tag is used to biochemically isolate the protein complex from solution. Next, the second antibody is used to detect the presence of the second protein of interest. If the protein is present you can conclude that the proteins form a complex and likely interact. A pan-neuronal GFP marker allowed for visualization of most neuronal phenotypes present in *cdh-4* mutants. Some candidates could not be tested as mutant alleles are not available for all genes identified in the yeast-two hybrid screen. Thirteen of fourty-two candidates did not have mutant alleles available for testing (Table 5). The stringency of promising candidates selected for examination was relaxed as the most promising candidates showed no overlapping phenotypes with *cdh-4*. Several lethal mutants were also tested. The genes *paa-1*, *mep-1*, *dcaf-1*, *dcp-66*, *peb-1* and *gpb-1* were balanced to allow the strains to propagate. Homozygous mutants were examined, however, there is the presence of maternal proteins in these animals.

Mutants were scored for cross-over defects in the dorsal and ventral nerve cords. Wildtype animals containing only the pan-neuronal marker had 6% of early adult animals with crossovers occurring in the ventral nerve cord (n=100). A crossover was scored when an axon exited either the right or left tract of the VNC and crossed into the opposite tract. The dorsal cord of these same animals showed 3% defasciculation in the dorsal cord (n=100). Defasciculation was scored when one or more axons separated significantly from the main dorsal cord bundle. In comparison, *cdh-4* mutants showed 52% of early adults with ventral cord crossovers and 53% with dorsal cord defasciculation defects (n=100). Fifteen mutants tested were not significantly different from wildtype suggesting that none of these genes are involved in axon guidance (Table 5).

Table 5. Comparison of neuronal phenotypes between *cdh-4* mutants and candidates identified in yeast two-hybrid screen. Axons labeled with a pan-neuronal marker *F25B3.3::GFP*.

Gene	Allele	n	VC Cross-over (%)[a]	Dorsal Cord (%)[b]
n/a	n/a	100	6	3
cdh-4	rh310	100	52	53
hpk-1	pk1393	100	8	4
gei-1	tm3626	100	10	6
tag-210	gk303	100	6	3
aco-1	jh131	100	5	4
dab-1	gk291	100	6	2
F57F4.4	ok2599	100	4	6
gfi-1	ok2669	100	4	5
gpb-1/mIn1	ok1875	100	9	5
dcp-66/hT2	gk370	100	4	4
dcaf-1/nT1	ok1867	100	2	4
mep-1/nT1	q660	100	6	6
vha-12	ok821	100	4	2
spr-4	by105	100	4	4
paa-1/mT1	ok1539	100	6	4
peb-1/dpy-3 unc-2	cu9	100	4	2
C39D10.7	ok2758	100	5	2
tag-153	ok699	100	6	2
F57A8.1	gk1088	100	4	4

[a]defects scored as axons crossing the ventral midline.
[b]defects scored as axons separating from the dorsal cord bundle.

Fifteen mutants were also tested for the most obvious morphological defects present in *cdh-4* mutants. In wildtype animals, we saw no significant abnormal morphology in the L1 larval

stage. In *cdh-4(rh310)* mutants we saw 16% of L1 animals with a variable abnormal morphology. All mutants tested showed 0% penetrance of the variable abnormal morphology in L1 larvae. This phenotype is clearly visible in *cdh-4* mutants suggesting these candidate genes do not function with *cdh-4* in this process (Table 6).

cdh-4 mutants show another visible morphological defect, where the pharynx becomes detached from the mouth region in early larval stages. The pharynx forms normally, but detaches from the mouth as the embryo elongates. Consequently, the hatching larvae are unable to feed and die of starvation. In *cdh-4(rh310)* we saw a penetrance of 28% for the pharynx unattached phenotype in early larval animals (Table 6). As with the other phenotypes tested, the mutants examined in this study showed no significant difference from wildtype for this phenotype. This suggests that perhaps these genes are not involved with *cdh-4* for this function.

Table 6. Comparison of morphological phenotypes between *cdh-4* mutants and candidates identified in yeast two-hybrid screen.

Gene	Allele	n	Abnormal Morphology in L1 (%)[a]	Pharynx Unattached in L1 (%)[b]
n/a	n/a	100	0	0
cdh-4	rh310	100	16	28
hpk-1	pk1393	100	0	0
gei-1	tm3626	100	0	0
tag-210	gk303	100	0	0
aco-1	jh131	100	0	0
dab-1	gk291	100	0	0
F57F4.4	ok2599	100	0	0
gfi-1	ok2669	100	0	0
gpb-1/mIn1	ok1875	100	0	0
dcp-66/hT2	gk370	100	0	0
dcaf-1/nT1	ok1867	100	0	0
mep-1/nT1	q660	100	0	0
vha-12	ok821	100	0	0
spr-4	by105	100	0	0
paa-1/mT1	ok1539	100	0	0
tag-153	ok699	100	0	0

[a] defects scored as significantly different morphology than wildtype
[b] defects scored as pharynx unattached from anterior hypodermis.

3.7 Placing *cdh-4* in a genetic pathway

To complement the yeast-two hybrid screen, we wanted to place *cdh-4* in a genetic pathway with intracellular proteins known to cause axon guidance defects. Axon guidance cues frequently interact with components of cytoskeletal re-arrangement (Lowery et al., 2009). As the phenotypes of *cdh-4* mutants appear to be related to cellular cytoskeleton re-arrangement, we examined mutants from several actin remodelling pathways. *unc-115*, *unc-73*, *mig-2*, and *ced-10* are all components of these various pathways (see Figure 4). *vang-1* was also tested to see if *cdh-4* as it contains significant guidance defects. Double mutants were prepared between the genes listed above and *cdh-4 (rh310)*. Neuronal defects were scored in single mutants and double mutants and compared. Additive penetrance suggests that the genes are acting through separate pathways. We would expect no increase in penetrance if both genes function in the same pathway. Three fluorescent markers were used to examine particular neuronal defects. Using these markers, we examined the penetrance of interneuron crossovers in the ventral nerve cord, extension of interneurons laterally without entering the ventral nerve cord, crossover of PVPR axon from the left tract to the right and crossover of the PVQL axon from the left to the right tract.

Wildtype animals showed a penetrance of 3% for interneurons extending laterally (Table 7). Wildtype animals had a 5% crossover penetrance for interneurons. PVPR and PVQL both had a penetrance of 6% for crossovers. In *cdh-4 (rh310)* mutants, the penetrance of interneurons extending laterally is 20% and a 23% penetrance of interneuron crossovers. PVPR crossovers occurred in 66% of *cdh-4* mutants while PVQL axons had a 63% crossover penetrance.

Table 7. Genetic interactions between *cdh-4* and several axon guidance pathway mutants.
Interneurons labeled with *glr-1::GFP*. PVP labeled with *odr-2::CFP*. PVQ labeled with *sra-6::DsRed*.

Gene	Allele	N	Interneuron lateral (%)[a]	Interneuron crossover (%)[b]	PVPR (%)[c]	PVQL (%)[d]

wild type	n/a	100	3	5	6	6
cdh-4	*rh310*	100	20	23	66	63
unc-115	*mn481*	100	19	15	17	18
cdh-4; unc-115	*rh310; mn481*	100	41	40	80	82
vang-1	*tm1422*	100	5	11	47	47
cdh-4; vang-1	*rh310; tm1422*	100	23	36	93	96
mig-2	*mu28*	100	9	8	64	63
cdh-4; mig-2	*rh310; mu28*	100	27	28	90	93
ced-10	*n1993*	100	9	7	55	57
cdh-4;ced-10	*rh310; n1993*	100	27	28	90	93
unc-73	*rh40*	100	45	22	98	99
cdh-4; unc-73	*rh310; rh40*	100	68	44	97	97

[a] defects scored as interneurons that do not enter the VNC and extend laterally.
[b] defects scored as interneuron axons that cross the ventral midline.
[c] defects scored as PVPR axons that cross the ventral midline.
[d] defects scored as PVQL axons that cross the ventral midline.

unc-115 (mn481) mutants also had defects with interneurons extending laterally with a penetrance of 19%. *unc-115* mutants also had a penetrance of 15% ineterneuron crossover defects. Additionally, PVPR and PVQL crossover defects were 17% and 18% respectively. Interestingly, the *cdh-4;unc-115* double mutants had a penetrance of 41% for interneurons extending laterally (Refer to Figure 5). The double mutants also showed a penetrance of 40% for interneuron crossover defects and therefore the phenotypes is additive. Similarly, the PVPR and PVQL defects in double mutants were additive as well at 80% and 82% respectively. These data suggest that *cdh-4* and *unc-115* act through separate genetic pathways.

vang-1 (tm1422) mutants had a penetrance of 5% of interneurons extending laterally and 11% of interneuron crossovers. PVPR and PVQL both crossed over at a penetrance of 47% in *vang-1* mutants. *cdh-4;vang-1* double mutants had a penetrance of 23% of interneurons extending laterally. 36% of *cdh-4;vang-1* adults had interneuron crossover defects. PVPR crossover defects were 93% in the double mutants while PVQL defects were 92%. The

penetrance is additive in all phenotypes suggesting that these genes act in separate genetic pathways.

mig-2 (mu28) mutants showed a penetrance of 9% of interneurons extending laterally. In these mutants, there was interneuron crossovers in 8% of the adults. The stronger defect in *mig-2* mutants was seen in PVPR and PVQL. 64% of PVPR axons crossed over in *mig-2* mutants. 63% of PVQL axons also crossed over in *mig-2* mutants. *mig-2;cdh-4* double mutants showed a penetrance of 27% defects in interneuron lateral extension. 28% of the adults in these double mutants showed interneuron crossover defects. Defects in PVPR and PVQL of these double mutants were near 100% as they were 95% and 96% respectively. Again, the penetrance seems additive suggesting parallel pathways.

ced-10 (n1993) mutants had slight defects in interneuron lateral extension and crossovers. Interneurons extended laterally in 9% of adults and crossover in 7% of adults. However, as with several other genes there was a significant defect in PVPR and PVQL axons. 55% of adult *ced-10* mutants had crossover defects in PVPR. 57% of adult *ced-10* mutants had crossover defects in PVQL. *ced-10;cdh-4* double mutants showed moderate defects in interneurons extending laterally and crossing over. The penetrance of these defects was 27% and 28% respectively. Defects in PVPR and PVQL in these double mutants were very severe. 90% of adult double mutants had crossover defects in PVPR and 93% of adults had cross over defects in PVQL.

unc-73 (rh40) mutants have strong defects in PVPR and PVQL. Almost all adult animals in *unc-73* mutants have these defects. 98% of *unc-73* adults had defects in PVPR and 99% of *unc-73* adults had defects in PVQL. Additionally, *unc-73* mutants show 45% lateral interneuron defects and 22% interneuron crossover defects. *unc-73;cdh-4* double mutants showed a penetrance of 97% in both of these defects. It is impossible to determine if there is a genetic interaction between these two genes in PVPR or PVQL because the penetrance of *unc-73* defects is so strong. However, we can state that *cdh-4* does not suppress *unc-73* defects.

4. Discussion

4.1 CDH-4 intracellular domain may interact with a variety of proteins

The outcome of the yeast two-hybrid screen suggests CDH-4 interacts physically with a wide variety of proteins. It is important to note that a positive interaction in a yeast two-hybrid screen does not indicate a biologically relevant interaction between both proteins. It is possible that while the proteins have the ability to interact physically, they never come into contact due to spatial or temporal segregation. As such, positive results from a yeast two-hybrid screen have to be confirmed by using independent approaches.

4.1.1 CDH-4 intracellular domain may interact with signalling proteins

DAB-1 is an interesting candidate for interaction with CDH-4 as the interaction occurred within the pleckstrin homology (PH) domain found commonly in proteins involved in intracellular signalling or members of the cytoskeleton. Both clones identified in the screen contained roughly the same sequence within the *dab-1* gene suggesting an interaction within this region. Additionally, DAB-1 homologs can be identified by Blastp searches in many species including *D. melanogaster, M. musculus and H. sapiens* suggesting strong conservation and importance across species. However, most of the sequence homology is centred on the PH domain with some homology among the regions surrounding the PH domain in non-nematode species. The *Drosophila* disabled homolog has been shown to be required for proper growth and guidance of motorneurons and acts upstream of Abl (Song et al., 2010). Disabled-1, the mouse homolog of DAB-1, is required for intracellular signalling of the Reelin signal which controls migration in cortical neurons (Keilani et al., 2012). Despite a role in neuronal migration of vertebrates, in *C. elegans dab-1* has been mainly studied with respect to protein trafficking and the secretory pathway (Holmes et al., 2007; Kamikura and Cooper, 2003). As DAB-1 is believed to function as an adaptor protein in several signalling pathways, it is possible that CDH-4 interacts physically with DAB-1 in this context.

As a protein containing a RhoGAP domain, GEI-1 is another candidate of particular interest. Examination of the domains alone suggests a role for GEI-1 in cytoskeletal re-arrangement which fits with the phenotypes we observe in CDH-4 mutants. Additionally, alignment between the clone identified from the yeast two-hybrid screen overlaps with the RhoGAP and StAR domains of *gei-1*. As nothing is currently known of GEI-1 function in *C. elegans*, examination of its closest homologs in other species provides the most relevant information. By Blastp alignment, GEI-1 contains homology at its N terminus with StAR-related lipid transfer protein 13 or deleted in liver cancer 2 (DLC-2) in vertebrates. DLC-2 activated RhoA and is believed to control cytoskeletal regulation (Nagaraja and Kandpal, 2004). While the homology is limited between the two, it is likely that GEI-1 plays a role in cytoskeletal re-arrangement. As such, physical interaction between GEI-1 and CDH-4 would not be unexpected.

As a potential component of intracellular signalling, *gpb-1* was also a promising candidate. G proteins consist of a trimer of alpha, beta and gamma subunits signal through several intracellular pathways pathways. As a beta subunit of a G protein, *gpb-1* is likely to signal in a similar manner. G proteins typically interact with serpentine receptors in signalling through the alpha subunit. *gpb-1* is one of only two G protein beta subunits in *C. elegans*. Loss of *gpb-1* leads to larval lethality suggesting an important role in development of the worm. Analyzing homology among species using Blastp shows strong conservation between GPB-1 and beta subunits of G proteins in vertebrates. The human G protein beta subunit GNB4 shows 90% sequence similarity with GPB-1. No specific function for GNB4 has been identified, however it is expected to function as a typical G protein beta subunit. Interactions between beta subunits of G proteins and membrane bound proteins are not uncommon. However, frequently these interactions involve transport channels or regulation of the secretory pathway (Wells et al., 2012). A possible interaction between GPB-1 and CDH-4 would not be unexpected. As *gpb-1*

was balanced during our testing, we tested the progeny of heterozygous animals and we cannot conclude that *gpb-1* mutants will show no guidance defects.

tag-210 was another protein with a predicted function in intracellular signalling. *tag-210* belongs to a class of ATPases with similarity to *Obg-like* GTPases. *Obg-like* GTPases are not very well characterized but are members of a highly conserved protein family (Leipe et al., 2002). *tag-210* maintains homology with its human homolog OLA1 along the entire protein length with 67% of amino acids conserved. OLA1 is a P-loop ATPase required for regulation G protein molecular switches. As such, OLA1 and similar ATPases are predicted to regulate a variety of signalling pathways within the cell. Mutants in *Obg-like* GTPases and ATPases show a wide variety of defects in various species despite their strong evolutionary conservation (Czyz and Wegrzyn, 2005). Very little is currently known about these proteins.

The one kinase identified in the screen, *hpk-1*, shares homology with homeodomain interacting protein kinases of other species including *D. melanogaster, M. musculus* and *H. sapiens*. The most closely related human homolog HIPK2 shares sequence similarity mainly within the kinase domain with some similarity in the regions flanking. HIPK2 activates p53 by phosphorylation to promote apoptosis (D'Orazi et al., 2002). HIPK in mammals function in the nucleus to regulate the function of homeodomain transcription factors (Kim et al., 1998). A similar function is seen in *Drosophila* as Hipk, the fly homolog of HIPK, is required to activate Yorkie activity in Hippo pathway tissue growth (Chen and Verheyen, 2012). In flies, Hipk is present in the cytoplasm and nucleus with its entry to the nucleus controlled by post-translational modifications (Huang et al., 2011). In *C. elegans*, *hpk-1* is mainly localized to puncta within the nucleus during embryogenesis and adult animals (Raich et al., 2003). Lower levels of *hpk-1* may be present outside the nucleus, however, it seems the majority of *hpk-1* localizes to the nucleus suggesting CDH-4 and HPK-1 may not interact physically in vivo.

One components of the protein phosphatase 2A complex was identified through the yeast two-hybrid screen. The PP2A complex is a heterotrimeric protein expressed ubiquitously

(Seshacharyulu et al., 2013). PP2A has a wide variety of functions including cell proliferation and signal transduction. In mouse, the PP2A complex has been shown to dephosphorylate Ezrin/Radixin/Moesin and ultimately lead to changes in F-actin (Staser et al., 2013). PP2A are composed of a structural A subunit, a regulatory B subunit and a catalytic C subunit. *paa-1* encodes a protein that functions as the structural component of the PP2A complex in *C. elegans*. PAA-1 is homologous to the human PP2A structural subunit PR65. Researchers found that PR65 was able to interact with the intracellular domain of CXCR2, a chemokine receptor, in cell culture experiments (Fan et al., 2001). It is possible that CDH-4 interacts with the structural subunit of PP2A in *C. elegans*.

Another phosphatase identified through the screen *pph-5* shows some conservation along its entire length with vertebrate serine/threonine protein phosphatase 5. PP5 in humans shows nuclear localization and has been shown to dephosphorylate histone H1 in vitro. Mouse knockout of protein phosphatase 5 showed only minor defects and an increased sensitivity to UV light (Amable et al., 2011). *pph-5* is reported to be localized to the cytoplasm during interphase but localize to the mitotic spindle during cell division (Richie et al., 2011). While PP5 in vertebrates functions mainly in the nucleus, it is possible that PPH-5 has some function in the cytoplasm. For this reason, the physical interaction between PPH-5 and CDH-4 may be biologically relevant.

All of the signalling proteins described above have the potential for biologically relevant interactions with CDH-4. A physical interaction must be confirmed by co-immunoprecipitation before characterizing these interactions further.

4.1.2 CDH-4 intracellular domain may interact with transcription factors

Surprisingly, CDH-4 interacted physically with several transcription factors in the Y2H screen. As all prey were tested for their ability to auto-activate reporter genes, these candidates were not identified by their ability to activate transcription on their own. Additionally, it is possible to screen specifically for transcription factors using yeast one-hybrid or two-hybrid approaches

using transcription factor libraries (Mitsuda et al., 2011). Transcription factors can be identified when using cytoplasmic proteins as bait for a yeast two-hybrid screen. An example of cytoplasmic proteins interacting with transcription factors through a yeast two-hybrid screen would be when a cytoplasmic protein is sequestering a transcription factor outside of the nucleus (for a recent example see: Wu et al., 2013). Additionally, there is the possibility that these transcription factors are typically capable of physically interacting with CDH-4 intracellular domain but never come into contact in a biological context. Having said that, many transcription factors move between the cytosol and the nucleus so an interaction is possible. However, the defects we see in *cdh-4* mutants appear to be directly related to CDH-4 function in adhesion or potentially signalling to reorganize cytoskeleton components. In this sense, we do not expect the *cdh-4* defects to be due to changes in gene transcription through the effect of a transcription factor.

4.1.3 CDH-4 intracellular domain may interact with metabolic proteins

Identification of several metabolic proteins in the yeast two-hybrid screen was somewhat unexpected. The phenotypes we have observed in *cdh-4* mutants do not seem to be associated with the metabolic proteins identified in the screen. Genes such as *ucr-2.3* and *T24B8.7* could interact with CDH-4 in its regulation and not in its function for axon guidance. The genes *aagr-2, gln-5, icl-1, B0238.1, C48B4.1, aco-1,* and *vit-5* seem more likely to be physical interactions that will not occur in vivo. *vit-5* also seems like an unlikely candidate as it's major role is in lipid transport and is mainly expressed in the intestine (McKay et al., 2003). The ATP synthase catalytic subunit, *atp-2*, is yet another unlikely candidate. However, there is the possibility that these are biologically relevant interactions and these genes should be examined as well.

4.1.4 CDH-4 intracellular domain may interact with several proteins of unknown function

It is difficult to decipher the importance of several proteins that contain little or no information regarding them. *D1007.4*, *F16B4.3, F59B1.10* and *ZK822.4* contain no recognizable domains (aside from a signal peptide in *ZK822.4*) and currently have no mutants available.

Testing these candidates must be done by RNAi knockdown of these genes. Additionally, no information regarding expression pattern or putative function is available to evaluate the potential of a biologically relevant interaction with CDH-4. *C14C11.2* and *F57A8.1* on the other hand do have mutants available and are capable of being tested for overlapping phenotypes with *cdh-4* mutants to assess the likelihood of a biologically relevant interaction.

Several other proteins contained a few known domains but it is difficult to assign a putative function. *gfi-1, F57F4.4, pqn-32* and *F56A8.3* each contain domains that do not indicate a potential function. *gfi-1* and *F57F4.4* contain 21 ET domains unique to these two *C. elegans* proteins and contains no known function. *pqn-32* contains a DB domain which is found in several *C. elegans* proteins but again has no known function associated with it. Also, this protein is predicted to contain a "prion"-like domain. Finally, *F56A8.3* contains four leucine-rich repeat domains that are found in a variety of proteins. Leucine rich repeats are frequently involved in protein-protein interactions and may be the cause of physical interaction between F56A8.3 and CDH-4.

4.2 Candidates examined to date do not share overlapping phenotypes with *cdh-4* mutants

Mutant alleles for 18 candidate genes were examined as a secondary screen to reduce the number of candidates for further analysis. The method to screen these candidates was to cross a pan-neuronal GFP marker into the mutant strain and identify any neuronal phenotypes that may overlap with *cdh-4* mutants. Unfortunately, none of the candidates we examined showed an overlapping phenotype with *cdh-4*. However, this does not rule out these genes as candidates entirely for several reasons.

Firstly, these proteins may interact with CDH-4 in processes unrelated to the phenotypes we examined. As such, we would not see neuronal or morphological defects that we tested. A more detailed look at phenotypes, for example Q cell migrations, would be able to

identify overlapping phenotypes between candidates and *cdh-4*. Similarly, *cdh-4* mutants may be responsible for a much larger set of phenotypes than previously described.

Secondly, for three genes (*spr-4, gei-1* and *C39D10.7*) the only available alleles were unlikely to be null. It is possible that these mutations produce a protein that retains sufficient function for proper development of the VNC and larva. In this case, we would not see any defects in the mutants despite the fact that the gene plays a role in these processes.

Thirdly, it is very difficult to rule out the lethal genes we examined in this study. In some cases, the animals die during larval development making it more difficult to determine if defects have or will occur. Additionally, a major concern stems from the fact that there will be a maternal contribution in all balanced lines. In this case, it is possible that the maternal protein is sufficient for normal development. For this reason, a better method for characterizing these mutants would be to use tissue specific RNA interference (RNAi). Briefly, RNA complementary to the messenger RNA for a protein of interest can be expressed in specific tissues. In doing so, double stranded RNA is degraded leading to loss of messenger RNA. The loss of messenger RNA significantly reduces the amount of translated protein produced. This would allow us to look at the effects in neuron specifically and hopefully avoid the issues with lethality.

Finally, genetic redundancy remains a concern by masking defects of candidate mutants. Using Treefam to identify closely related family members, we identified closely related proteins for 19 of the candidates identified (Table 8) (Li et al., 2006). The possibility of redundancy remains a strong for these genes. Mutations in one member of the family may cause no detectable phenotype because of partially redundant function with other family members. Recently this was observed in two closely related axon guidance receptors in *C. elegans* (Unsoeld et al., 2013). *ddr-1* mutants showed no guidance defects on their own, but significantly enhanced the defects of *ddr-2* mutants. This suggests that *ddr-1* plays a role in axon guidance, but acts in part redundantly with *ddr-2*. Similarly, some significant interactions

with CDH-4 may not be visible under our current approach analyzing single mutants. To address this problem, double mutants of family members for candidates would be required.

Table 8. Candidates with closely related paralogs in *C. elegans* identified using TreeFam.

Gene	Family members
gpb-1	*gpb-2*
hpk-1	*mbk-2, T04C10.1, C36B7.1, C36B7.2*
spr-4	*C28G1.4*
tag-153	*ntl-2*
T07C11.12	*H20J04.3*
F16B4.3	*Y48G1BM.6, Y54G2A.21, AC8.10, AC8.3, F25G6.1*
pqn-32	29 genes including *nhr-246*
aagr-2	*aagr-1*
B0238.1	12 genes
C48B4.1	8 genes including *acox-1*
gln-5	*gln-3, gln-6, C45B2.5, K03H1.1, F26D10.10*
ucr-2.3	*ucr-2.1, ucr-2.2*
vha-12	*Y110A7A.12*
vit-5	*vit-2, vit-6, F59D8.2, F59D8.1, C04F6.1, K09F5.2*
let-2	*gon-2, F54D1.5, C05C12.3*
wrt-1	*C29F3.2, ZK1037.10, ZK678.5, B0344.2, F52E4.6*
gfi-1	*F57F4.4*
F57F4.4	*gfi-1*
ZK822.4	18 genes

4.3 *cdh-4* acts in none of the known axon guidance pathways we tested

Independently from the yeast two-hybrid screen, we were interested in placing *cdh*-4 in a genetic pathway. In doing so, we aimed to gain additional candidates for protein-protein interactions and insight into the function of CDH-4. In order to place *cdh-4* in a genetic pathway, we studied several double mutants with *cdh-4* and various genes involved in axon guidance and cytoskeletal remodelling. The cytoskeletal remodelling genes tested were a Rac1 homolog *ced-10*, a Rac/Rho *unc-73*, Rho GTPase *mig-2*, and abLIM *unc-115*. *vang-1* was also tested as it is a member of the *Wnt* pathway with weak guidance defects.

The intracellular signalling genes were chosen as candidates as all have significant guidance defects in the VNC. Specifically, each gene has significant defects in PVP and PVQ axons. Additionally, genes were examined for an ability to enhance interneuron defects present in *cdh-4* mutants.

Double mutants with *cdh-4* were examined for defects in lateral extension of interneuron axons from the nerve ring, interneuron crossover defects, PVPR and PVQL crossover defects. *cdh-4* single mutants have highly penetrant defects in PVPR and PVQL making it difficult to assess changes in penetrance (in particular increased penetrance) in double mutants. All double mutants examined increased the penetrance of these defects close to 100%.

Examination of lateral interneuron extension and interneuron crossover defects provides more information with respect genetic interactions with *cdh-4*. All of the genes tested aside from *cdh-4* showed no defects in lateral interneuron extension. Examination of the double mutants in all but *vang-1* showed an additive effect in the penetrance of lateral interneuron defects. *vang-1*;*cdh-4*double mutants showed no significant differences in lateral interneuron extension. This is expected as *vang-1* mutants show no significant defects in lateral interneuron extension. In the case of the other genes tested, the additive phenotype suggests that *cdh-4* functions in a parallel pathway to these genes.

Mild interneuron crossover defects were present in all genes tested. Examination of the double mutants again showed an additive effect of penetrance. This suggests that similarly to lateral interneuron extension, for interneuron navigation in the VNC *cdh-4* acts in a separate genetic pathway than the genes examined.

One interesting possibility for genetic interaction that remains is *unc-34*/enabled. Enabled is a component of the VASP cytoskeletal re-arrangement pathway and a strong candidate for being a component in the CDH-4 pathway. Additionally, there are several other phenotypes present in *cdh-4* mutants that remain to be tested for genetic interactions.

4.4 Conclusions and outlook

We found that neither mutants in components of the Hippo-Warts pathway nor those of the PCP pathway share phenotypes with *cdh*-4 mutants suggesting *cdh-4* in *C. elegans* uses a different signalling pathway. We examined known signalling pathway components involved in axon guidance and tested them for genetic interactions with *cdh-4*. We discovered that *cdh-4* does not act in the same genetic pathway as *unc-73, mig-2, unc-115, vang-1,* and *ced-10*. Using a yeast two-hybrid screen we were able to identify 42 potential physical interaction partners with the intracellular domain of CDH-4. We tested 18 of these candidates for overlapping phenotypes with *cdh-4* mutants but were not able to identify any.

A closer study of all candidates is required to determine which of them interact with CDH-4 in a biologically relevant context. For some candidates, mutations will have to be isolated first to determine a potential role in CDH-4 signalling. Aternatively, tissue specific RNAi can be used for targeted knock-down of particular candidates (especially those causing lethality) specifically in neurons to examine their effects on axon guidance. Systematic analysis of candidates and their closely related family members might be required to detect biological interactions masked by redundancy.

Finally, examining *cdh-4* mutants for additional phenotypes would be useful in identifying relevant interactions. Using a wide variety of fluorescent markers it is possible to examine migration and axon guidance of additional neurons as well as other aspects of neuronal development such as synapse formation. Closer examination of non-neuronal tissues might also reveal additional phenotypes, where candidates from the yeast two-hybrid screen might interact with CDH-4.

In summary, this study provides a basis for identification of intracellular interactions of CDH-4 in *C. elegans*. We have identified a list of candidate genes, some of which are likely involved in CDH-4 signalling in axon guidance.

References

Ackley, B. D., Crew, J. R., Elamaa, H., Pihlajaniemi, T., Kuo, C. J., & Kramer, J. M. (2001). The NC1/endostatin domain of caenorhabditis elegans type XVIII collagen affects cell migration and axon guidance. *The Journal of Cell Biology, 152*(6), 1219-1232.

Altschul, S. F., Gish, W., Miller, W., Myers, E. W., & Lipman, D. J. (1990). Basic local alignment search tool. *Journal of Molecular Biology, 215*(3), 403-410. doi:10.1016/S0022-2836(05)80360-2

Amable, L., Grankvist, N., Largen, J. W., Ortsater, H., Sjoholm, A., & Honkanen, R. E. (2011). Disruption of serine/threonine protein phosphatase 5 (PP5:PPP5c) in mice reveals a novel role for PP5 in the regulation of ultraviolet light-induced phosphorylation of serine/threonine protein kinase Chk1 (CHEK1). *The Journal of Biological Chemistry, 286*(47), 40413-40422. doi:10.1074/jbc.M111.244053; 10.1074/jbc.M111.244053

Augsburger, A., Schuchardt, A., Hoskins, S., Dodd, J., & Butler, S. (1999). BMPs as mediators of roof plate repulsion of commissural neurons. *Neuron, 24*(1), 127-141.

Avruch, J., Zhou, D., Fitamant, J., Bardeesy, N., Mou, F., & Barrufet, L. R. (2012). Protein kinases of the hippo pathway: Regulation and substrates. *Seminars in Cell & Developmental Biology, 23*(7), 770-784. doi:10.1016/j.semcdb.2012.07.002; 10.1016/j.semcdb.2012.07.002

Axang, C., Rauthan, M., Hall, D. H., & Pilon, M. (2008). Developmental genetics of the C. elegans pharyngeal neurons NSML and NSMR. *BMC Developmental Biology, 8*, 38-213X-8-38. doi:10.1186/1471-213X-8-38; 10.1186/1471-213X-8-38

Azevedo, F. A., Carvalho, L. R., Grinberg, L. T., Farfel, J. M., Ferretti, R. E., Leite, R. E., et al. (2009). Equal numbers of neuronal and nonneuronal cells make the human brain an isometrically scaled-up primate brain. *The Journal of Comparative Neurology, 513*(5), 532-541. doi:10.1002/cne.21974; 10.1002/cne.21974

Baas, P. W., Slaughter, T., Brown, A., & Black, M. M. (1991). Microtubule dynamics in axons and dendrites. *Journal of Neuroscience Research, 30*(1), 134-153. doi:10.1002/jnr.490300115

Baum, P. D., & Garriga, G. (1997). Neuronal migrations and axon fasciculation are disrupted in ina-1 integrin mutants. *Neuron, 19*(1), 51-62.

Beattie, C. E., Melancon, E., & Eisen, J. S. (2000). Mutations in the stumpy gene reveal intermediate targets for zebrafish motor axons. *Development (Cambridge, England), 127*(12), 2653-2662.

Becker, T., McLane, M. A., & Becker, C. G. (2003). Integrin antagonists affect growth and pathfinding of ventral motor nerves in the trunk of embryonic zebrafish. *Molecular and Cellular Neurosciences, 23*(1), 54-68.

Bentley, D., & Toroian-Raymond, A. (1986). Disoriented pathfinding by pioneer neurone growth cones deprived of filopodia by cytochalasin treatment. *Nature, 323*(6090), 712-715. doi:10.1038/323712a0

Berber, S., Llamosas, E., Thaivalappil, P., Boag, P. R., Crossley, M., & Nicholas, H. R. (2013). Homeodomain interacting protein kinase (HPK-1) is required in the soma for robust germline proliferation in C. elegans. *Developmental Dynamics : An Official Publication of the American Association of Anatomists, 242*(11), 1250-1261. doi:10.1002/dvdy.24023; 10.1002/dvdy.24023

Birnboim, H. C., & Doly, J. (1979). A rapid alkaline extraction procedure for screening recombinant plasmid DNA. *Nucleic Acids Research, 7*(6), 1513-1523.

Blaschuk, O. W., Sullivan, R., David, S., & Pouliot, Y. (1990). Identification of a cadherin cell adhesion recognition sequence. *Developmental Biology, 139*(1), 227-229.

Boulin, T., Pocock, R., & Hobert, O. (2006). A novel eph receptor-interacting IgSF protein provides C. elegans motoneurons with midline guidepost function. *Current Biology : CB, 16*(19), 1871-1883. doi:10.1016/j.cub.2006.08.056

Brenner, S. (1974). The genetics of caenorhabditis elegans. *Genetics, 77*(1), 71-94.

Broadbent, I. D., & Pettitt, J. (2002). The C. elegans hmr-1 gene can encode a neuronal classic cadherin involved in the regulation of axon fasciculation. *Current Biology : CB, 12*(1), 59-63.

Bryant, P. J., Huettner, B., Held, L. I.,Jr, Ryerse, J., & Szidonya, J. (1988). Mutations at the fat locus interfere with cell proliferation control and epithelial morphogenesis in drosophila. *Developmental Biology, 129*(2), 541-554.

Buck, K. B., & Zheng, J. Q. (2002). Growth cone turning induced by direct local modification of microtubule dynamics. *The Journal of Neuroscience : The Official Journal of the Society for Neuroscience, 22*(21), 9358-9367.

Burglin, T. R., & Kuwabara, P. E. (2006). Homologs of the hh signalling network in C. elegans. *WormBook : The Online Review of C.Elegans Biology,* , 1-14. doi:10.1895/wormbook.1.76.1

Cai, Q., Wang, W., Gao, Y., Yang, Y., Zhu, Z., & Fan, Q. (2009). Ce-wts-1 plays important roles in caenorhabditis elegans development. *FEBS Letters, 583*(19), 3158-3164. doi:10.1016/j.febslet.2009.09.002; 10.1016/j.febslet.2009.09.002

Chae, J., Kim, M. J., Goo, J. H., Collier, S., Gubb, D., Charlton, J., et al. (1999). The drosophila tissue polarity gene starry night encodes a member of the protocadherin family. *Development (Cambridge, England), 126*(23), 5421-5429.

Chak, K., & Kolodkin, A. L. (2014). Function of the drosophila receptor guanylyl cyclase Gyc76C in PlexA-mediated motor axon guidance. *Development (Cambridge, England), 141*(1), 136-147. doi:10.1242/dev.095968; 10.1242/dev.095968

Chalfie, M., Tu, Y., Euskirchen, G., Ward, W. W., & Prasher, D. C. (1994). Green fluorescent protein as a marker for gene expression. *Science (New York, N.Y.), 263*(5148), 802-805.

Chang, S., Johnston, R. J.,Jr, & Hobert, O. (2003). A transcriptional regulatory cascade that controls left/right asymmetry in chemosensory neurons of C. elegans. *Genes & Development, 17*(17), 2123-2137. doi:10.1101/gad.1117903

Charron, F., Stein, E., Jeong, J., McMahon, A. P., & Tessier-Lavigne, M. (2003). The morphogen sonic hedgehog is an axonal chemoattractant that collaborates with netrin-1 in midline axon guidance. *Cell, 113*(1), 11-23.

Chen, J., & Verheyen, E. M. (2012). Homeodomain-interacting protein kinase regulates yorkie activity to promote tissue growth. *Current Biology : CB, 22*(17), 1582-1586. doi:10.1016/j.cub.2012.06.074; 10.1016/j.cub.2012.06.074

Chen, M. X., McPartlin, A. E., Brown, L., Chen, Y. H., Barker, H. M., & Cohen, P. T. (1994). A novel human protein serine/threonine phosphatase, which possesses four tetratricopeptide repeat motifs and localizes to the nucleus. *The EMBO Journal, 13*(18), 4278-4290.

Ciani, L., Krylova, O., Smalley, M. J., Dale, T. C., & Salinas, P. C. (2004). A divergent canonical WNT-signaling pathway regulates microtubule dynamics: Dishevelled signals locally to stabilize microtubules. *The Journal of Cell Biology, 164*(2), 243-253. doi:10.1083/jcb.200309096

Colamarino, S. A., & Tessier-Lavigne, M. (1995). The axonal chemoattractant netrin-1 is also a chemorepellent for trochlear motor axons. *Cell, 81*(4), 621-629.

Colavita, A., Krishna, S., Zheng, H., Padgett, R. W., & Culotti, J. G. (1998). Pioneer axon guidance by UNC-129, a C. elegans TGF-beta. *Science (New York, N.Y.), 281*(5377), 706-709.

Conchonaud, F., Nicolas, S., Amoureux, M. C., Menager, C., Marguet, D., Lenne, P. F., et al. (2007). Polysialylation increases lateral diffusion of neural cell adhesion molecule in the cell membrane. *The Journal of Biological Chemistry, 282*(36), 26266-26274. doi:10.1074/jbc.M608590200

Czyz, A., & Wegrzyn, G. (2005). The obg subfamily of bacterial GTP-binding proteins: Essential proteins of largely unknown functions that are evolutionarily conserved from bacteria to humans. *Acta Biochimica Polonica, 52*(1), 35-43. doi:055201035

Demyanenko, G. P., Tsai, A. Y., & Maness, P. F. (1999). Abnormalities in neuronal process extension, hippocampal development, and the ventricular system of L1 knockout mice. *The Journal of Neuroscience : The Official Journal of the Society for Neuroscience, 19*(12), 4907-4920.

Doherty, P., Cohen, J., & Walsh, F. S. (1990). Neurite outgrowth in response to transfected N-CAM changes during development and is modulated by polysialic acid. *Neuron, 5*(2), 209-219.

D'Orazi, G., Cecchinelli, B., Bruno, T., Manni, I., Higashimoto, Y., Saito, S., et al. (2002). Homeodomain-interacting protein kinase-2 phosphorylates p53 at ser 46 and mediates apoptosis. *Nature Cell Biology, 4*(1), 11-19. doi:10.1038/ncb714

Drescher, U., Kremoser, C., Handwerker, C., Loschinger, J., Noda, M., & Bonhoeffer, F. (1995). In vitro guidance of retinal ganglion cell axons by RAGS, a 25 kDa tectal protein related to ligands for eph receptor tyrosine kinases. *Cell, 82*(3), 359-370.

Durbin, R.M. (1987). Studies on the development and organisation of the nervous system of Caenorhabditis elegans, PhD thesis, pp. 150: Cambridge, UK.

Fan, G. H., Yang, W., Sai, J., & Richmond, A. (2001). Phosphorylation-independent association of CXCR2 with the protein phosphatase 2A core enzyme. *The Journal of Biological Chemistry, 276*(20), 16960-16968. doi:10.1074/jbc.M009292200

Fields, S., & Song, O. (1989). A novel genetic system to detect protein-protein interactions. *Nature, 340*(6230), 245-246. doi:10.1038/340245a0

Fields, S. (1993). The two-hybrid system to detect protein-protein interactions. *Methods, 5*(2), 116-124. Retrieved from http://www.sciencedirect.com/science/article/pii/S1046202383710169

Fokkens, L., Botelho, S. M., Boekhorst, J., & Snel, B. (2010). Enrichment of homologs in insignificant BLAST hits by co-complex network alignment. *BMC Bioinformatics, 11*, 86-2105-11-86. doi:10.1186/1471-2105-11-86; 10.1186/1471-2105-11-86

Forrester, W. C., & Garriga, G. (1997). Genes necessary for C. elegans cell and growth cone migrations. *Development (Cambridge, England), 124*(9), 1831-1843.

Govek, E. E., Newey, S. E., & Van Aelst, L. (2005). The role of the rho GTPases in neuronal development. *Genes & Development, 19*(1), 1-49. doi:10.1101/gad.1256405

Gruenbaum, Y., Lee, K. K., Liu, J., Cohen, M., & Wilson, K. L. (2002). The expression, lamin-dependent localization and RNAi depletion phenotype for emerin in C. elegans. *Journal of Cell Science, 115*(Pt 5), 923-929.

Hao, J. C., Yu, T. W., Fujisawa, K., Culotti, J. G., Gengyo-Ando, K., Mitani, S., et al. (2001). C. elegans slit acts in midline, dorsal-ventral, and anterior-posterior guidance via the SAX-3/Robo receptor. *Neuron, 32*(1), 25-38.

Harpaz, Y., & Chothia, C. (1994). Many of the immunoglobulin superfamily domains in cell adhesion molecules and surface receptors belong to a new structural set which is close to that containing variable domains. *Journal of Molecular Biology, 238*(4), 528-539. doi:10.1006/jmbi.1994.1312

Hatta, K., Okada, T. S., & Takeichi, M. (1985). A monoclonal antibody disrupting calcium-dependent cell-cell adhesion of brain tissues: Possible role of its target antigen in animal pattern formation. *Proceedings of the National Academy of Sciences of the United States of America, 82*(9), 2789-2793.

Hedgecock, E. M., Culotti, J. G., & Hall, D. H. (1990). The unc-5, unc-6, and unc-40 genes guide circumferential migrations of pioneer axons and mesodermal cells on the epidermis in C. elegans. *Neuron, 4*(1), 61-85.

Hilario, J. D., Wang, C., & Beattie, C. E. (2010). Collagen XIXa1 is crucial for motor axon navigation at intermediate targets. *Development (Cambridge, England), 137*(24), 4261-4269. doi:10.1242/dev.051730; 10.1242/dev.051730

Hill, E., Broadbent, I. D., Chothia, C., & Pettitt, J. (2001). Cadherin superfamily proteins in caenorhabditis elegans and drosophila melanogaster. *Journal of Molecular Biology, 305*(5), 1011-1024. doi:10.1006/jmbi.2000.4361

Hilliard, M. A., & Bargmann, C. I. (2006). Wnt signals and frizzled activity orient anterior-posterior axon outgrowth in C. elegans. *Developmental Cell, 10*(3), 379-390. doi:10.1016/j.devcel.2006.01.013

Hirayama, T., & Yagi, T. (2013). Clustered protocadherins and neuronal diversity. *Progress in Molecular Biology and Translational Science, 116*, 145-167. doi:10.1016/B978-0-12-394311-8.00007-8; 10.1016/B978-0-12-394311-8.00007-8

Hoerndli, F. J., Walser, M., Frohli Hoier, E., de Quervain, D., Papassotiropoulos, A., & Hajnal, A. (2009). A conserved function of C. elegans CASY-1 calsyntenin in associative learning. *PloS One, 4*(3), e4880. doi:10.1371/journal.pone.0004880; 10.1371/journal.pone.0004880

Hoffman, S., Sorkin, B. C., White, P. C., Brackenbury, R., Mailhammer, R., Rutishauser, U., et al. (1982). Chemical characterization of a neural cell adhesion molecule purified from embryonic brain membranes. *The Journal of Biological Chemistry, 257*(13), 7720-7729.

Holland, S. J., Gale, N. W., Mbamalu, G., Yancopoulos, G. D., Henkemeyer, M., & Pawson, T. (1996). Bidirectional signalling through the EPH-family receptor nuk and its transmembrane ligands. *Nature, 383*(6602), 722-725. doi:10.1038/383722a0

Holmes, A., Flett, A., Coudreuse, D., Korswagen, H. C., & Pettitt, J. (2007). C. elegans disabled is required for cell-type specific endocytosis and is essential in animals lacking the AP-3 adaptor complex. *Journal of Cell Science, 120*(Pt 15), 2741-2751. doi:10.1242/jcs.03474

Hu, J., & Barr, M. M. (2005). ATP-2 interacts with the PLAT domain of LOV-1 and is involved in caenorhabditis elegans polycystin signaling. *Molecular Biology of the Cell, 16*(2), 458-469. doi:10.1091/mbc.E04-09-0851

Huang, H., Du, G., Chen, H., Liang, X., Li, C., Zhu, N., et al. (2011). Drosophila Smt3 negatively regulates JNK signaling through sequestering hipk in the nucleus. *Development (Cambridge, England), 138*(12), 2477-2485. doi:10.1242/dev.061770; 10.1242/dev.061770

Huarcaya Najarro, E., & Ackley, B. D. (2013). C. elegans fmi-1/flamingo and wnt pathway components interact genetically to control the anteroposterior neurite growth of the VD GABAergic neurons. *Developmental Biology, 377*(1), 224-235. doi:10.1016/j.ydbio.2013.01.014; 10.1016/j.ydbio.2013.01.014

Hulpiau, P., & van Roy, F. (2009). Molecular evolution of the cadherin superfamily. *The International Journal of Biochemistry & Cell Biology, 41*(2), 349-369. doi:10.1016/j.biocel.2008.09.027; 10.1016/j.biocel.2008.09.027

Hutchins, B. I., Li, L., & Kalil, K. (2011). Wnt/calcium signaling mediates axon growth and guidance in the developing corpus callosum. *Developmental Neurobiology, 71*(4), 269-283. doi:10.1002/dneu.20846; 10.1002/dneu.20846

Hutter, H. (2003). Extracellular cues and pioneers act together to guide axons in the ventral cord of C. elegans. *Development (Cambridge, England), 130*(22), 5307-5318. doi:10.1242/dev.00727

Ikeda, D. D., Duan, Y., Matsuki, M., Kunitomo, H., Hutter, H., Hedgecock, E. M., et al. (2008). CASY-1, an ortholog of calsyntenins/alcadeins, is essential for learning in caenorhabditis elegans. *Proceedings of the National Academy of Sciences of the United States of America, 105*(13), 5260-5265. doi:10.1073/pnas.0711894105; 10.1073/pnas.0711894105

Inatani, M., Irie, F., Plump, A. S., Tessier-Lavigne, M., & Yamaguchi, Y. (2003). Mammalian brain morphogenesis and midline axon guidance require heparan sulfate. *Science (New York, N.Y.), 302*(5647), 1044-1046. doi:10.1126/science.1090497

Iozzo, R. V. (1998). Matrix proteoglycans: From molecular design to cellular function. *Annual Review of Biochemistry, 67*, 609-652. doi:10.1146/annurev.biochem.67.1.609

Iwai, Y., Usui, T., Hirano, S., Steward, R., Takeichi, M., & Uemura, T. (1997). Axon patterning requires DN-cadherin, a novel neuronal adhesion receptor, in the drosophila embryonic CNS. *Neuron, 19*(1), 77-89.

Jin, Z., & Strittmatter, S. M. (1997). Rac1 mediates collapsin-1-induced growth cone collapse. *The Journal of Neuroscience : The Official Journal of the Society for Neuroscience, 17*(16), 6256-6263.

Keilani, S., Healey, D., & Sugaya, K. (2012). Reelin regulates differentiation of neural stem cells by activation of notch signaling through disabled-1 tyrosine phosphorylation. *Canadian Journal of Physiology and Pharmacology, 90*(3), 361-369. doi:10.1139/y2012-001; 10.1139/y2012-001

Kidd, T., Bland, K. S., & Goodman, C. S. (1999). Slit is the midline repellent for the robo receptor in drosophila. *Cell, 96*(6), 785-794.

Kim, M. H., Cooper, D. R., Oleksy, A., Devedjiev, Y., Derewenda, U., Reiner, O., et al. (2004). The structure of the N-terminal domain of the product of the lissencephaly gene Lis1 and its functional implications. *Structure (London, England : 1993), 12*(6), 987-998. doi:10.1016/j.str.2004.03.024

Kim, S., & Wadsworth, W. G. (2000). Positioning of longitudinal nerves in C. elegans by nidogen. *Science (New York, N.Y.), 288*(5463), 150-154.

Kim, Y. H., Choi, C. Y., Lee, S. J., Conti, M. A., & Kim, Y. (1998). Homeodomain-interacting protein kinases, a novel family of co-repressors for homeodomain transcription factors. *The Journal of Biological Chemistry, 273*(40), 25875-25879.

Kim, Y. I., Cho, J. H., Yoo, O. J., & Ahnn, J. (2004). Transcriptional regulation and life-span modulation of cytosolic aconitase and ferritin genes in C.elegans. *Journal of Molecular Biology, 342*(2), 421-433. doi:10.1016/j.jmb.2004.07.036

Klose, M., & Bentley, D. (1989). Transient pioneer neurons are essential for formation of an embryonic peripheral nerve. *Science (New York, N.Y.), 245*(4921), 982-984.

Koch, P. J., Walsh, M. J., Schmelz, M., Goldschmidt, M. D., Zimbelmann, R., & Franke, W. W. (1990). Identification of desmoglein, a constitutive desmosomal glycoprotein, as a member of the cadherin family of cell adhesion molecules. *European Journal of Cell Biology, 53*(1), 1-12.

Koh, C. G. (2006). Rho GTPases and their regulators in neuronal functions and development. *Neuro-Signals, 15*(5), 228-237. doi:10.1159/000101527

Kolodkin, A. L., Matthes, D. J., O'Connor, T. P., Patel, N. H., Admon, A., Bentley, D., et al. (1992). Fasciclin IV: Sequence, expression, and function during growth cone guidance in the grasshopper embryo. *Neuron, 9*(5), 831-845.

Kozma, R., Ahmed, S., Best, A., & Lim, L. (1996). The GTPase-activating protein n-chimaerin cooperates with Rac1 and Cdc42Hs to induce the formation of lamellipodia and filopodia. *Molecular and Cellular Biology, 16*(9), 5069-5080.

Kuczmarski, E. R., & Rosenbaum, J. L. (1979). Studies on the organization and localization of actin and myosin in neurons. *The Journal of Cell Biology, 80*(2), 356-371.

Kwon, H. B., Fukuhara, S., Asakawa, K., Ando, K., Kashiwada, T., Kawakami, K., et al. (2013). The parallel growth of motoneuron axons with the dorsal aorta depends on Vegfc/Vegfr3 signaling in zebrafish. *Development (Cambridge, England), 140*(19), 4081-4090. doi:10.1242/dev.091702; 10.1242/dev.091702

Le, N., & Simon, M. A. (1998). Disabled is a putative adaptor protein that functions during signaling by the sevenless receptor tyrosine kinase. *Molecular and Cellular Biology, 18*(8), 4844-4854.

Lee, H., Engel, U., Rusch, J., Scherrer, S., Sheard, K., & Van Vactor, D. (2004). The microtubule plus end tracking protein Orbit/MAST/CLASP acts downstream of the tyrosine kinase abl in mediating axon guidance. *Neuron, 42*(6), 913-926. doi:10.1016/j.neuron.2004.05.020

Lee, H. K., Seo, I. A., Suh, D. J., & Park, H. T. (2009). Nidogen plays a role in the regenerative axon growth of adult sensory neurons through schwann cells. *Journal of Korean Medical Science, 24*(4), 654-659. doi:10.3346/jkms.2009.24.4.654; 10.3346/jkms.2009.24.4.654

Lee, R. C., Clandinin, T. R., Lee, C. H., Chen, P. L., Meinertzhagen, I. A., & Zipursky, S. L. (2003). The protocadherin flamingo is required for axon target selection in the drosophila visual system. *Nature Neuroscience, 6*(6), 557-563. doi:10.1038/nn1063

Leipe, D. D., Wolf, Y. I., Koonin, E. V., & Aravind, L. (2002). Classification and evolution of P-loop GTPases and related ATPases. *Journal of Molecular Biology, 317*(1), 41-72. doi:10.1006/jmbi.2001.5378

Lemons, M. L., Abanto, M. L., Dambrouskas, N., Clements, C. C., Deloughery, Z., Garozzo, J., et al. (2013). Integrins and cAMP mediate netrin-induced growth cone collapse. *Brain Research, 1537*, 46-58. doi:10.1016/j.brainres.2013.08.045; 10.1016/j.brainres.2013.08.045

Letourneau, P. C., Condic, M. L., & Snow, D. M. (1994). Interactions of developing neurons with the extracellular matrix. *The Journal of Neuroscience : The Official Journal of the Society for Neuroscience, 14*(3 Pt 1), 915-928.

Li, H., Coghlan, A., Ruan, J., Coin, L. J., Heriche, J. K., Osmotherly, L., et al. (2006). TreeFam: A curated database of phylogenetic trees of animal gene families. *Nucleic Acids Research, 34*(Database issue), D572-80. doi:10.1093/nar/gkj118

Liu, T., Sims, D., & Baum, B. (2009). Parallel RNAi screens across different cell lines identify generic and cell type-specific regulators of actin organization and cell morphology. *Genome Biology, 10*(3), R26-2009-10-3-r26. Epub 2009 Mar 5. doi:10.1186/gb-2009-10-3-r26; 10.1186/gb-2009-10-3-r26

Lowery, L. A., & Van Vactor, D. (2009). The trip of the tip: Understanding the growth cone machinery. *Nature Reviews.Molecular Cell Biology, 10*(5), 332-343. doi:10.1038/nrm2679; 10.1038/nrm2679

Lundquist, E. A., Herman, R. K., Shaw, J. E., & Bargmann, C. I. (1998). UNC-115, a conserved protein with predicted LIM and actin-binding domains, mediates axon guidance in C. elegans. *Neuron, 21*(2), 385-392.

Lundquist, E. A., Reddien, P. W., Hartwieg, E., Horvitz, H. R., & Bargmann, C. I. (2001). Three C. elegans rac proteins and several alternative rac regulators control axon guidance, cell migration and apoptotic cell phagocytosis. *Development (Cambridge, England), 128*(22), 4475-4488.

Luo, Y., Raible, D., & Raper, J. A. (1993). Collapsin: A protein in brain that induces the collapse and paralysis of neuronal growth cones. *Cell, 75*(2), 217-227.

Marchisio, P. C., Osborn, M., & Weber, K. (1978). The intracellular organization of actin and tubulin in cultured C-1300 mouse neuroblastoma cells (clone NB41A3). *Journal of Neurocytology, 7*(5), 571-582.

Masai, I., Lele, Z., Yamaguchi, M., Komori, A., Nakata, A., Nishiwaki, Y., et al. (2003). N-cadherin mediates retinal lamination, maintenance of forebrain compartments and patterning of retinal neurites. *Development (Cambridge, England), 130*(11), 2479-2494.

Matakatsu, H., & Blair, S. S. (2006). Separating the adhesive and signaling functions of the fat and dachsous protocadherins. *Development (Cambridge, England), 133*(12), 2315-2324. doi:10.1242/dev.02401

Matsunaga, M., Hatta, K., Nagafuchi, A., & Takeichi, M. (1988). Guidance of optic nerve fibres by N-cadherin adhesion molecules. *Nature, 334*(6177), 62-64. doi:10.1038/334062a0

McKay, S. J., Johnsen, R., Khattra, J., Asano, J., Baillie, D. L., Chan, S., et al. (2003). Gene expression profiling of cells, tissues, and developmental stages of the nematode C. elegans. *Cold Spring Harbor Symposia on Quantitative Biology, 68*, 159-169.

Meighan, C. M., & Schwarzbauer, J. E. (2007). Control of C. elegans hermaphrodite gonad size and shape by vab-3/Pax6-mediated regulation of integrin receptors. *Genes & Development, 21*(13), 1615-1620. doi:10.1101/gad.1534807

Mitchison, T., & Kirschner, M. (1984). Dynamic instability of microtubule growth. *Nature, 312*(5991), 237-242.

Mitsuda, N., Ikeda, M., Takada, S., Takiguchi, Y., Kondou, Y., Yoshizumi, T., et al. (2010). Efficient yeast one-/two-hybrid screening using a library composed only of transcription factors in arabidopsis thaliana. *Plant & Cell Physiology, 51*(12), 2145-2151. doi:10.1093/pcp/pcq161; 10.1093/pcp/pcq161

Murphy, C. T., McCarroll, S. A., Bargmann, C. I., Fraser, A., Kamath, R. S., Ahringer, J., et al. (2003). Genes that act downstream of DAF-16 to influence the lifespan of caenorhabditis elegans. *Nature, 424*(6946), 277-283. doi:10.1038/nature01789

Murray, A. J., Tucker, S. J., & Shewan, D. A. (2009). cAMP-dependent axon guidance is distinctly regulated by epac and protein kinase A. *The Journal of Neuroscience : The Official Journal of the Society for Neuroscience, 29*(49), 15434-15444. doi:10.1523/JNEUROSCI.3071-09.2009; 10.1523/JNEUROSCI.3071-09.2009

Myers, J. P., Santiago-Medina, M., & Gomez, T. M. (2011). Regulation of axonal outgrowth and pathfinding by integrin-ECM interactions. *Developmental Neurobiology, 71*(11), 901-923. doi:10.1002/dneu.20931; 10.1002/dneu.20931

Nagaraja, G. M., & Kandpal, R. P. (2004). Chromosome 13q12 encoded rho GTPase activating protein suppresses growth of breast carcinoma cells, and yeast two-hybrid screen shows its interaction with several proteins. *Biochemical and Biophysical Research Communications, 313*(3), 654-665.

Najarro, E. H., Wong, L., Zhen, M., Carpio, E. P., Goncharov, A., Garriga, G., et al. (2012). Caenorhabditis elegans flamingo cadherin fmi-1 regulates GABAergic neuronal development. *The Journal of Neuroscience : The Official Journal of the Society for Neuroscience, 32*(12), 4196-4211. doi:10.1523/JNEUROSCI.3094-11.2012; 10.1523/JNEUROSCI.3094-11.2012

Nollet, F., Kools, P., & van Roy, F. (2000). Phylogenetic analysis of the cadherin superfamily allows identification of six major subfamilies besides several solitary members. *Journal of Molecular Biology, 299*(3), 551-572. doi:10.1006/jmbi.2000.3777

Obata, S., Sago, H., Mori, N., Davidson, M., St John, T., & Suzuki, S. T. (1998). A common protocadherin tail: Multiple protocadherins share the same sequence in their cytoplasmic domains and are expressed in different regions of brain. *Cell Adhesion and Communication, 6*(4), 323-333.

Oinuma, I., Katoh, H., & Negishi, M. (2006). Semaphorin 4D/Plexin-B1-mediated R-ras GAP activity inhibits cell migration by regulating beta(1) integrin activity. *The Journal of Cell Biology, 173*(4), 601-613. doi:10.1083/jcb.200508204

Pan, C. L., Howell, J. E., Clark, S. G., Hilliard, M., Cordes, S., Bargmann, C. I., et al. (2006). Multiple wnts and frizzled receptors regulate anteriorly directed cell and growth cone migrations in caenorhabditis elegans. *Developmental Cell, 10*(3), 367-377. doi:10.1016/j.devcel.2006.02.010

Park, M., & Shen, K. (2012). WNTs in synapse formation and neuronal circuitry. *The EMBO Journal, 31*(12), 2697-2704. doi:10.1038/emboj.2012.145; 10.1038/emboj.2012.145

Pasterkamp, R. J., Peschon, J. J., Spriggs, M. K., & Kolodkin, A. L. (2003). Semaphorin 7A promotes axon outgrowth through integrins and MAPKs. *Nature, 424*(6947), 398-405. doi:10.1038/nature01790

Pettitt, J. (2005). The cadherin superfamily. *WormBook : The Online Review of C.Elegans Biology,* , 1-9. doi:10.1895/wormbook.1.50.1

Pettitt, J., Wood, W. B., & Plasterk, R. H. (1996). Cdh-3, a gene encoding a member of the cadherin superfamily, functions in epithelial cell morphogenesis in caenorhabditis elegans. *Development (Cambridge, England), 122*(12), 4149-4157.

Pocock, R., Benard, C. Y., Shapiro, L., & Hobert, O. (2008). Functional dissection of the C. elegans cell adhesion molecule SAX-7, a homologue of human L1. *Molecular and Cellular Neurosciences, 37*(1), 56-68. doi:10.1016/j.mcn.2007.08.014

Poulin, G., Dong, Y., Fraser, A. G., Hopper, N. A., & Ahringer, J. (2005). Chromatin regulation and sumoylation in the inhibition of ras-induced vulval development in caenorhabditis elegans. *The EMBO Journal, 24*(14), 2613-2623. doi:10.1038/sj.emboj.7600726

Qu, C., Dwyer, T., Shao, Q., Yang, T., Huang, H., & Liu, G. (2013). Direct binding of TUBB3 with DCC couples netrin-1 signaling to intracellular microtubule dynamics in axon outgrowth and guidance. *Journal of Cell Science, 126*(Pt 14), 3070-3081. doi:10.1242/jcs.122184; 10.1242/jcs.122184

Quinn, C. C., Pfeil, D. S., & Wadsworth, W. G. (2008). CED-10/Rac1 mediates axon guidance by regulating the asymmetric distribution of MIG-10/lamellipodin. *Current Biology : CB, 18*(11), 808-813. doi:10.1016/j.cub.2008.04.050; 10.1016/j.cub.2008.04.050

Raich, W. B., Moorman, C., Lacefield, C. O., Lehrer, J., Bartsch, D., Plasterk, R. H., et al. (2003). Characterization of caenorhabditis elegans homologs of the down syndrome candidate gene DYRK1A. *Genetics, 163*(2), 571-580.

Reddy, B. V., & Irvine, K. D. (2008). The fat and warts signaling pathways: New insights into their regulation, mechanism and conservation. *Development (Cambridge, England), 135*(17), 2827-2838. doi:10.1242/dev.020974; 10.1242/dev.020974

Reyes, A. A., Small, S. J., & Akeson, R. (1991). At least 27 alternatively spliced forms of the neural cell adhesion molecule mRNA are expressed during rat heart development. *Molecular and Cellular Biology, 11*(3), 1654-1661.

Richie, C. T., Bembenek, J. N., Chestnut, B., Furuta, T., Schumacher, J. M., Wallenfang, M., et al. (2011). Protein phosphatase 5 is a negative regulator of separase function during cortical granule exocytosis in C. elegans. *Journal of Cell Science, 124*(Pt 17), 2903-2913. doi:10.1242/jcs.073379; 10.1242/jcs.073379

Richie, C. T., Bembenek, J. N., Chestnut, B., Furuta, T., Schumacher, J. M., Wallenfang, M., et al. (2011). Protein phosphatase 5 is a negative regulator of separase function during cortical granule exocytosis in C. elegans. *Journal of Cell Science, 124*(Pt 17), 2903-2913. doi:10.1242/jcs.073379; 10.1242/jcs.073379

Roy, P. J., Zheng, H., Warren, C. E., & Culotti, J. G. (2000). Mab-20 encodes semaphorin-2a and is required to prevent ectopic cell contacts during epidermal morphogenesis in caenorhabditis elegans. *Development (Cambridge, England), 127*(4), 755-767.

Sakube, Y., & Kagawa, H. (1999). Identification and characterization of proteins associated with the ryanodine receptor in caenorhabditis elegans [Abstract]. *International Worm Meeting,*

Schmitz, C., Wacker, I., & Hutter, H. (2008). The fat-like cadherin CDH-4 controls axon fasciculation, cell migration and hypodermis and pharynx development in caenorhabditis elegans. *Developmental Biology, 316*(2), 249-259. doi:10.1016/j.ydbio.2008.01.024; 10.1016/j.ydbio.2008.01.024

Senti, K. A., Usui, T., Boucke, K., Greber, U., Uemura, T., & Dickson, B. J. (2003). Flamingo regulates R8 axon-axon and axon-target interactions in the drosophila visual system. *Current Biology : CB, 13*(10), 828-832.

Seshacharyulu, P., Pandey, P., Datta, K., & Batra, S. K. (2013). Phosphatase: PP2A structural importance, regulation and its aberrant expression in cancer. *Cancer Letters, 335*(1), 9-18. doi:10.1016/j.canlet.2013.02.036; 10.1016/j.canlet.2013.02.036

Shen, K., Fetter, R. D., & Bargmann, C. I. (2004). Synaptic specificity is generated by the synaptic guidepost protein SYG-2 and its receptor, SYG-1. *Cell, 116*(6), 869-881.

Sibley, M. H., Graham, P. L., von Mende, N., & Kramer, J. M. (1994). Mutations in the alpha 2(IV) basement membrane collagen gene of caenorhabditis elegans produce phenotypes of differing severities. *The EMBO Journal, 13*(14), 3278-3285.

Smialowska, A., & Baumeister, R. (2006). Presenilin function in caenorhabditis elegans. *Neuro-Degenerative Diseases, 3*(4-5), 227-232. doi:10.1159/000095260

Song, J. K., Kannan, R., Merdes, G., Singh, J., Mlodzik, M., & Giniger, E. (2010). Disabled is a bona fide component of the abl signaling network. *Development (Cambridge, England), 137*(21), 3719-3727. doi:10.1242/dev.050948; 10.1242/dev.050948

Song, M. H., Liu, Y., Anderson, D. E., Jahng, W. J., & O'Connell, K. F. (2011). Protein phosphatase 2A-SUR-6/B55 regulates centriole duplication in C. elegans by controlling the levels of centriole assembly factors. *Developmental Cell, 20*(4), 563-571. doi:10.1016/j.devcel.2011.03.007; 10.1016/j.devcel.2011.03.007

Soto, M. C., Qadota, H., Kasuya, K., Inoue, M., Tsuboi, D., Mello, C. C., et al. (2002). The GEX-2 and GEX-3 proteins are required for tissue morphogenesis and cell migrations in C. elegans. *Genes & Development, 16*(5), 620-632. doi:10.1101/gad.955702

Squire, L. R. (2012). Fundamental neuroscience. Amsterdam ; Boston: Academic Press/Elsevier.

Stappert, J., & Kemler, R. (1994). A short core region of E-cadherin is essential for catenin binding and is highly phosphorylated. *Cell Adhesion and Communication, 2*(4), 319-327.

Staser, K., Shew, M. A., Michels, E. G., Mwanthi, M. M., Yang, F. C., Clapp, D. W., et al. (2013). A Pak1-PP2A-ERM signaling axis mediates F-actin rearrangement and degranulation in mast cells. *Experimental Hematology, 41*(1), 56-66.e2. doi:10.1016/j.exphem.2012.10.001; 10.1016/j.exphem.2012.10.001

Steimel, A., Wong, L., Najarro, E. H., Ackley, B. D., Garriga, G., & Hutter, H. (2010). The flamingo ortholog FMI-1 controls pioneer-dependent navigation of follower axons in C. elegans. *Development (Cambridge, England), 137*(21), 3663-3673. doi:10.1242/dev.054320; 10.1242/dev.054320

Sulston, J. E., & Horvitz, H. R. (1977). Post-embryonic cell lineages of the nematode, caenorhabditis elegans. *Developmental Biology, 56*(1), 110-156.

Sulston, J. E., Schierenberg, E., White, J. G., & Thomson, J. N. (1983). The embryonic cell lineage of the nematode caenorhabditis elegans. *Developmental Biology, 100*(1), 64-119.

Syntichaki, P., Samara, C., & Tavernarakis, N. (2005). The vacuolar H+ -ATPase mediates intracellular acidification required for neurodegeneration in C. elegans. *Current Biology : CB, 15*(13), 1249-1254. doi:10.1016/j.cub.2005.05.057

Tessier-Lavigne, M., Placzek, M., Lumsden, A. G., Dodd, J., & Jessell, T. M. (1988). Chemotropic guidance of developing axons in the mammalian central nervous system. *Nature, 336*(6201), 775-778. doi:10.1038/336775a0

Thatcher, J. D., Fernandez, A. P., Beaster-Jones, L., Haun, C., & Okkema, P. G. (2001). The caenorhabditis elegans peb-1 gene encodes a novel DNA-binding protein involved in morphogenesis of the pharynx, vulva, and hindgut. *Developmental Biology, 229*(2), 480-493. doi:10.1006/dbio.2000.9978

Thomas, C., & Strutt, D. (2012). The roles of the cadherins fat and dachsous in planar polarity specification in drosophila. *Developmental Dynamics : An Official Publication of the American Association of Anatomists, 241*(1), 27-39. doi:10.1002/dvdy.22736; 10.1002/dvdy.22736

Timpl, R. (1996). Macromolecular organization of basement membranes. *Current Opinion in Cell Biology, 8*(5), 618-624.

Trousse, F., Marti, E., Gruss, P., Torres, M., & Bovolenta, P. (2001). Control of retinal ganglion cell axon growth: A new role for sonic hedgehog. *Development (Cambridge, England), 128*(20), 3927-3936.

Tsang, W. Y., Sayles, L. C., Grad, L. I., Pilgrim, D. B., & Lemire, B. D. (2001). Mitochondrial respiratory chain deficiency in caenorhabditis elegans results in developmental arrest and increased life span. *The Journal of Biological Chemistry, 276*(34), 32240-32246. doi:10.1074/jbc.M103999200

Turney, S. G., & Bridgman, P. C. (2005). Laminin stimulates and guides axonal outgrowth via growth cone myosin II activity. *Nature Neuroscience, 8*(6), 717-719. doi:10.1038/nn1466

Unhavaithaya, Y., Shin, T. H., Miliaras, N., Lee, J., Oyama, T., & Mello, C. C. (2002). MEP-1 and a homolog of the NURD complex component mi-2 act together to maintain germline-soma distinctions in C. elegans. *Cell, 111*(7), 991-1002.

Unsoeld, T., Park, J. O., & Hutter, H. (2013). Discoidin domain receptors guide axons along longitudinal tracts in C. elegans. *Developmental Biology, 374*(1), 142-152. doi:10.1016/j.ydbio.2012.11.001; 10.1016/j.ydbio.2012.11.001

Usui, T., Shima, Y., Shimada, Y., Hirano, S., Burgess, R. W., Schwarz, T. L., et al. (1999). Flamingo, a seven-pass transmembrane cadherin, regulates planar cell polarity under the control of frizzled. *Cell, 98*(5), 585-595.

Venstrom, K. A., & Reichardt, L. F. (1993). Extracellular matrix. 2: Role of extracellular matrix molecules and their receptors in the nervous system. *FASEB Journal : Official Publication of the Federation of American Societies for Experimental Biology, 7*(11), 996-1003.

Vitriol, E. A., & Zheng, J. Q. (2012). Growth cone travel in space and time: The cellular ensemble of cytoskeleton, adhesion, and membrane. *Neuron, 73*(6), 1068-1081. doi:10.1016/j.neuron.2012.03.005; 10.1016/j.neuron.2012.03.005

Wang, X., Zhang, W., Cheever, T., Schwarz, V., Opperman, K., Hutter, H., et al. (2008). The C. elegans L1CAM homologue LAD-2 functions as a coreceptor in MAB-20/Sema2 mediated axon guidance. *The Journal of Cell Biology, 180*(1), 233-246. doi:10.1083/jcb.200704178; 10.1083/jcb.200704178

Wehrli, M., & Tomlinson, A. (1995). Epithelial planar polarity in the developing drosophila eye. *Development (Cambridge, England), 121*(8), 2451-2459.

Wells, C. A., Zurawski, Z., Betke, K. M., Yim, Y. Y., Hyde, K., Rodriguez, S., et al. (2012). Gbetagamma inhibits exocytosis via interaction with critical residues on soluble N-ethylmaleimide-sensitive factor attachment protein-25. *Molecular Pharmacology, 82*(6), 1136-1149. doi:10.1124/mol.112.080507; 10.1124/mol.112.080507

White, J. G., Southgate, E., Thomson, J. N., & Brenner, S. (1976). The structure of the ventral nerve cord of caenorhabditis elegans. *Philosophical Transactions of the Royal Society of London.Series B, Biological Sciences, 275*(938), 327-348.

White, J. G., Southgate, E., Thomson, J. N., & Brenner, S. (1986). The structure of the nervous system of the nematode caenorhabditis elegans. *Philosophical Transactions of the Royal Society of London.Series B, Biological Sciences, 314*(1165), 1-340.

Willecke, M., Hamaratoglu, F., Kango-Singh, M., Udan, R., Chen, C. L., Tao, C., et al. (2006). The fat cadherin acts through the hippo tumor-suppressor pathway to regulate tissue size. *Current Biology : CB, 16*(21), 2090-2100. doi:10.1016/j.cub.2006.09.005

Wu, Y., Liao, S., Wang, X., Wang, S., Wang, M., & Han, C. (2013). HSF2BP represses BNC1 transcriptional activity by sequestering BNC1 to the cytoplasm. *FEBS Letters, 587*(14), 2099-2104. doi:10.1016/j.febslet.2013.04.049; 10.1016/j.febslet.2013.04.049

Yamada, K. M., Spooner, B. S., & Wessells, N. K. (1970). Axon growth: Roles of microfilaments and microtubules. *Proceedings of the National Academy of Sciences of the United States of America, 66*(4), 1206-1212.

Yang, C. H., Axelrod, J. D., & Simon, M. A. (2002). Regulation of frizzled by fat-like cadherins during planar polarity signaling in the drosophila compound eye. *Cell, 108*(5), 675-688.

Yoshida-Noro, C., Suzuki, N., & Takeichi, M. (1984). Molecular nature of the calcium-dependent cell-cell adhesion system in mouse teratocarcinoma and embryonic cells studied with a monoclonal antibody. *Developmental Biology, 101*(1), 19-27.

Yoshikawa, S., McKinnon, R. D., Kokel, M., & Thomas, J. B. (2003). Wnt-mediated axon guidance via the drosophila derailed receptor. *Nature, 422*(6932), 583-588. doi:10.1038/nature01522

Yu, T. W., & Bargmann, C. I. (2001). Dynamic regulation of axon guidance. *Nature Neuroscience, 4 Suppl*, 1169-1176. doi:10.1038/nn748

Zhao, X., Yang, C. H., & Simon, M. A. (2013). The drosophila cadherin fat regulates tissue size and planar cell polarity through different domains. *PloS One, 8*(5), e62998. doi:10.1371/journal.pone.0062998; 10.1371/journal.pone.0062998

Zhou, D., Conrad, C., Xia, F., Park, J. S., Payer, B., Yin, Y., et al. (2009). Mst1 and Mst2 maintain hepatocyte quiescence and suppress hepatocellular carcinoma development through inactivation of the Yap1 oncogene. *Cancer Cell, 16*(5), 425-438. doi:10.1016/j.ccr.2009.09.026; 10.1016/j.ccr.2009.09.026

Zinovyeva, A. Y., Yamamoto, Y., Sawa, H., & Forrester, W. C. (2008). Complex network of wnt signaling regulates neuronal migrations during caenorhabditis elegans development. *Genetics, 179*(3), 1357-1371. doi:10.1534/genetics.108.090290; 10.1534/genetics.108.090290

Zwaal, R. R., Ahringer, J., van Luenen, H. G., Rushforth, A., Anderson, P., & Plasterk, R. H. (1996). G proteins are required for spatial orientation of early cell cleavages in C. elegans embryos. *Cell, 86*(4), 619-629.