A Role for the *Caenorhabditis elegans* Adenylate Forming Domain protein Disco-Interacting Protein 2 (DIP-2) in the Maintenance of Neuron Morphology

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I declare that the research presented here is my own original work unless otherwise specified. None of this material has been previously used for the purpose of obtaining any other degree.

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Abstract

Post-developmentally, neurons are very long lived and, while shown to be capable of plasticity-promoting remodeling of dendritic structures, are thought to maintain relatively stable morphologies over long animal lifespans. The molecular mechanisms that maintain neuronal morphology remain poorly understood. Members of the highly conserved DIP2 protein family contain an N-terminal DNA methyltransferase-associated protein 1 (DMAP1) binding domain and two adenylate-forming (AFD) domains. Using two loss-of-function alleles, we show that DIP-2 acts to maintain neuronal morphology in *C. elegans*. *dip-2* mutants display a progressive increase in ectopic neurite sprouting and branching post-development. DIP-2 acts in a DMAP1 binding-domain-independent manner to maintain neuronal morphology post-developmentally and DIP-2 overexpression can suppress the normal increase in neuronal sprouting defects observed in aging worms. Endogenous DIP-2 is widely expressed in neuronal and epithelial tissue but acts cell-autonomously in neurons. Finally, *dip-2* neuronal defects are modified by loss of insulin/IGF-1 signalling (IIS) and DIP-2 acts in parallel with the SAX-2/Furry-SAX-1/NDR kinase pathway to regulate neuronal morphology.

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List of Abbreviations and Gene Names

AFD	Adenylate forming domain
ALM	Anterior lateral microtubule
ANOVA	Analysis of variance
AVM	Anterior ventral microtubule
cDNA	Complimentary deoxyribonucleic acid
DIP2	Disco-interacting protein 2
DMAP1	DNA methyltransferase associated protein-1
DVB	Dorsal-rectal ganglion neuron
ECM	Extracellular matrix
EMS	Ethylmethanesulfonate
FSTL1	Follistatin like protein 1
FUDR	5’ fluorodeoxyuridine
GFP	Green florescent protein
HSN	Hermaphrodite specific neuron
HSP	Heat shock protein
IIS	Insulin-like/IGF signalling
nde	Neurite outgrowth defective
NGM	Nematode growth media
PCR	Polymerase chain reaction
PLM	Posterior lateral microtubule
PVM	Posterior ventral microtubule
RFP	Red florescent protein
SNV	Single nucleotide variant
VNC	Ventral nerve cord
wt	Wild type

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1. Introduction

Neuronal morphology

Neurons possess morphologies that reflect their individual roles. These morphologies are shaped by factors that include: soma shape, axon and dendritic branching patterns, and the orientation that branches take in relation to their neighboring cells. The mechanisms that control neuronal morphology are diverse and include key molecules like the collapsin response mediator protein (CRMP), GSK3β, Rho kinases and the transcription factors Cux1 and 2 (Jan and Jan 2010; Polleux and Snider, 2010; Arikkarth, 2012; Bilimoria and Bonni, 2013). Neuron morphologies contribute to the establishment of neuronal connections and information processing in the nervous system (reviewed in Spruston, 2008; Jan and Jan, 2010). The mammalian cortex and hippocampus for example are comprised of multiple layers of neurons with unique morphologies reflective of their specific firing patterns and targets (Kasper et al., 1994). These patterns are often determined by dendritic arborization patterns within a particular neuronal population or layer of the brain (Vetter et al., 2001; Mainen and Sejnowski, 1996). Additionally, the length and layout of dendritic branches is important for the organisation of synaptic inputs (De Jong et al., 2012). Perturbations in the establishment and maintenance of neuronal morphology, therefore, impact cognitive function and behaviour in cases of aging (Allard et al., 2011), trauma (Yap et al., 2017) and disease (McFadden and Minshew, 2013).

The maintenance of vertebrate neuronal morphology

Neurons show relatively little turnover post development, often surviving over the entire animal lifespan. During this period, they undergo various challenges including chemical, proteotoxic and physical stress that can have adverse effects on neuronal connections and neuronal function (Bénard and Hobert, 2009; Kagias et al., 2012). Events during adulthood such as physical growth and injuries can additionally add to the stresses that an organism faces. Despite plasticity-promoting remodeling of dendritic and synaptic structures (Bono et al., 2017) and the intrinsic capacity to initiate regrowth of axons (He and Jin, 2016); the general consensus was previously that neurons largely maintain relatively stable morphologies over the adult lifespan. Recent studies have called this theory into question. Furthermore, a number of other features within neuronal cells require active maintenance; for example cells require survival factors like neurotrophins to prevent autophagy throughout their lifespans, and additionally require proper Golgi body trafficking to maintain neuron polarity and neurite identity (Xu et al., 2000; LaMonte et al., 2002).

These lines of evidence reinforce data that has suggested that neuronal morphology requires active maintenance. For example, in mice with deletions of Abelson kinases, dendrites start off normally and progressively loose branches from about 3 weeks of age (Moresco et al., 2005). The mechanisms that facilitate this maintenance are diverse in their source; including extrinsic factors like Wnt5A (Chen et al., 2017) and BDNF (Gorski et al., 2003; Matsunaga et al., 2004), or intrinsic factors like δ-catenin (Matter et al., 2009). The diversity is also reflected in the roles that the intrinsic factors play within neurons. Loss of the adherens junction complex member δ-

catenin (Matter et al., 2009), or the hemophilic cell adhesion molecule DSCAM result in morphology changes in the cerebral cortex or retinal ganglion and bipolar cells, respectively (Fernandes et al., 2016; Simmons et al., 2017). Alternatively, the intrinsic capacity of neurons to propagate action potentials in their circuitry appears to be involved in maintenance; as dendritic architecture and branching patterns within the rat adult cortex become altered post-developmentally in cases of deafferentation (Tailby et al., 2005).

Maintenance mechanisms appear to co-opt pathways that are important for the development of neurites. During development, neurite growth is tightly regulated through the regulation of the cytoskeleton by a combination of extracellular, intrinsic and timing specific mechanisms (reviewed in Schaefer et al., 2008; Polleux and Snider, 2010). For example, activity dependant mechanisms are employed during early development of the vertebrate nervous system (Goodman and Shatz, 1993). In a number of places, including neuromuscular junctions and the visual cortex, early development is initiated without the need for neuronal activity; but requires proper firing patterns for the maturation of architecture. In part, this maturation impacts the outgrowth of neurites through the modulation of the cytoskeleton (Wagh et al., 2015). This relationship between development and maintenance is possibly employed by the cell to address the need for repair mechanisms, which require temporal activation of neurite growth. Several studies have shown that proteins with inhibitory roles for neurite development can persist throughout adulthood and can be themselves regulated in cases of nervous system trauma (Franquinho et al., 2017). This suggests

that other proteins involved in regulating neurite growth during development may remain to be studied in this novel context.

C. elegans: an important model organism for neurobiology

C. elegans is a well-established model organism for studies in developmental neurobiology (Tissenbaum, 2015; Brenner, 1974). This is due to a variety of factors, including a 3 day life cycle, small size and large brood sizes, all of which combine for relatively low culture costs. *C. elegans* are also transparent, facilitating the analysis of developmental processes using fluorescence microscopy.

Additionally, *C. elegans* is particularly suited to studies involving the nervous system. The worm contains 302 neurons, about one third of its 959 somatic adult cells (Chen et al., 2013). There is also a completely mapped and annotated connectome including the location of all chemical and electrical synapses (Azulay, Itskovits and Zaslaver, 2016; White et al., 1986). Many studies have also shown that development and function of the nervous system involves many conserved development pathways including those mediating polarity, axon guidance, synaptic development, neuronal transmission and information processing (for a review of developmental mechanism in *C. elegans*, see Bentley and Barker, 2016; Chisholm et al., 2016). These advantages have made *C. elegans* an important model for neurodegenerative diseases such as Alzheimer's Disease, Parkinson's Disease, Amyotrophic Lateral Sclerosis (ALS) and Huntington's Disease, and for neurodevelopmental diseases such as Autism (Schmeisser and Parker, 2018; Alexander, Marfil and Li, 2014).

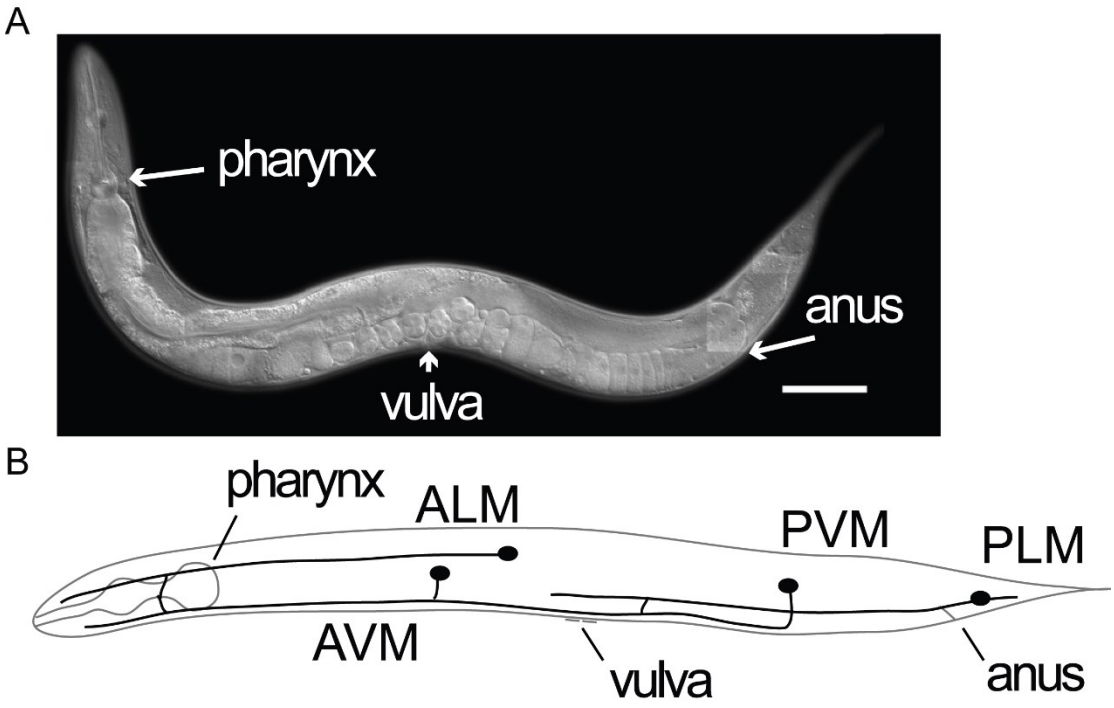

Figure 1: Anatomy of C. elegans and the (mechanosensory) touch receptor neurons. (A) Nomarski (DIC) image of a C. elegans adult with anatomical features indicated (Picture taken by the Chin-Sang lab) (B) Schematic of the touch neurons in the adult worm nervous system.

The *C. elegans* touch (mechanosensory) neurons

Given their simple morphologies, the (mechanosensory) touch neurons are extensively used as a model for neuronal morphology studies in *C. elegans* (Figure 1 B). These are a set of 6 mechanosensory neurons which includes pairs of anterior lateral microtubule (ALM) and posterior lateral microtubule (PLM) neurons, the anterior ventral microtubule (AVM) and the posterior ventral microtubule (PVM) neurons. The left and right ALMs are located at lateral positions, just anterior to the vulva, and project axons anteriorly to connect to the nerve ring in the head (Syntichaki and Tavernarakis, 2004). The left and right PLMs, located in the tail, project axons anteriorly and also extend a single branch to the ventral nerve cord (VNC). AVM and PVM are located at lateral body positions in the anterior and posterior body and project axons ventrally into the ventral nerve cord (VNC) and then anteriorly to the nerve ring. The ALMs and PLMs differentiate and migrate to their final positions during embryogenesis. In contrast, AVM and PVM are derived from neuroblast cell divisions in the 1st larval stage during their migration to their final positions (Syntichaki and Tavernarakis, 2004; Chalfie and Sulton, 1981).

Maintenance of neuronal morphology in *C. elegans*

Studies in the nematode *Caenorhabditis elegans* have also demonstrated that mechanisms exist to maintain neuronal morphology. Loss of neuronal maintenance proteins results in either ectopic neurite growth (Knobel et al., 2001) or spontaneous loss of neurite arbours (Neumann and Hillard, 2014). Often these phenotypes, like those

observed in vertebrates, get progressively worse over time, or can be rescued transiently during adulthood (Neumann and Hillard, 2014; Knobel et al., 2001).

Adhesive proteins act to maintain neuronal position and morphology

Axon tracts and neuronal cell bodies must adhere to neighbouring tissue to avoid being displaced by daily activity-induced mechanical stresses (Bénard et al., 2006). Cell adhesion proteins have been shown to be maintain neuronal soma and axon position in *C. elegans* (reviewed in Bénard and Hobert, 2009). These proteins include the L1CAM homologue SAX-7 and the secreted immunoglobulin superfamily proteins DIG-1, ZIG-4, ZIG-5 and ZIG-8 (Aurelio et al., 2002; Wang et al., 2005; Bénard et al., 2006). In part, some of these proteins appear to counteract the physical stress that organisms are subjected to throughout their lives. *sax-7* and *dig-1* mutants, which are paralysed, tend to show less neuronal soma and axon position defects than their non-paralysed wild type counterparts (Sasakura et al., 2005; Bénard et al., 2006). While the mechanism behind this maintenance is yet unknown, cell adhesion appears to play an important role. For example DIG-1 is part of the basement membrane and is thought to interact with neuron specific factors on the cell surface of neurons to maintain axon and soma positions in the VNC (Bénard et al., 2006). Likewise ZIG-5 and ZIG-8 appear to regulate the adhesive L1CAM homolog SAX-7 in properly adhering neurons to their neighboring cells in the head and VNC (Bénard et al., 2012).

Cell adhesion proteins have also been shown to be important for establishing and/or maintaining neuronal morphology in *C. elegans*. A genetic screen for touch

neuron morphology defects identified several mutants that displayed increased axon branching and ectopic neurite formation (Pan et al., 2011). One of these genes (*mec-5*) encodes a collagen, while another (*mec-2*) produces a membrane stomatin-like protein. In these mutants, neuronal morphology defects were shown to result from loss of neuron attachment to the underlying epidermis rather than other possibilities such as compromised neuronal activity (Pan et al., 2011). Similar neuron attachment defects were observed in other mutants that disrupt cell adhesion such as *him-4/*hemicentin and *fbl-1/*fibulin*,* which both encode ECM proteins that are not required for proper touch neuron function.

Maintenance of neuronal morphology is dependent on neuronal activity

The evoked activity of neurons has also been shown to be critical for the maintenance of neuronal morphology in *C. elegans*. Cyclic nucleotide gated channels, such as the hyperpolarization-activated cyclic nucleotide-gated channels HCN1 and HCN2 in mammals, are important for signal transduction (Gamo et al., 2015). Similarly, mutations in cyclic nucleotide-gated channels cause an ectopic neurite phenotype in a subset of sensory neurons in worms (Coburn et al., 1998, Peckol et al., 1999). The α and β subunits of a cyclic nucleotide gated channel in *C. elegans* are encoded by the genes *tax-2* and *tax-4*, and are required for chemosensory behavior. Continuous expression of TAX-2 and TAX-4 are required post-developmentally to maintain sensory neuron morphology. The *C. elegans mec* screen described above also identified *mec-4* and *mec-10*, which encode mechanosensory Na channels (Pan et al., 2011). *mec-4* and *mec-10* mutants display ectopic axon branching and neurites, similar to defects in *mec* mutants

that disrupt adhesion proteins. Similar defects are also found in mutants of the hyperpolarizing potassium channel SLO-1 or in worms where SLO-1 is overexpressed, both of which are predicted to compromise sensory evoked neuronal activity.

Regulation of microtubule dynamics and neuronal maintenance

Regulation of the cytoskeleton is important in all aspects of nervous system development, including neurite outgrowth (reviewed in Polleux and Snider, 2010; Sainath and Gallo, 2015; Schelski and Bradke, 2017). Following development, regulation of cytoskeletal dynamics continues to be important for maintaining neuronal morphology. Mutations in microtubule binding proteins like the microtubule associated proteins (MAPs) PTRN-1and PTL-1 result in defects similar to those of *mec* and ion channel genes (Chew, 2014; Chuang et al., 2014). PTRN-1 encodes a minus-end binding protein, which binds to microtubules and prevents their disassembly throughout the neuron including at presynaptic sites (Chuang et al., 2014; Richardson et al., 2014). Likewise PTL-1 is responsible for increasing the stability of microtubules, likely in the same manner that its homolog Tau which strengthens the interface between tubulin heterodimers (Chew, 2014; Kadavath et al., 2015)

The effects of aging on mechanosensory neuron morphology in *C. elegans*

In *C. elegans*, a number of neuron subtypes such as the touch neurons, exhibit a progressive increase in structural defects during the normal course of aging that is similar to those caused by loss of neuronal maintenance factors. These include ectopic outgrowths from axons and cell bodies, as well as irregular soma shape and beading or

focal enlargements along axon processes (Pan et al., 2011; Tank et al., 2011; Toth et al., 2012). This neuronal aging process can be genetically dissociated from normal organismal lifespan and be regulated by specific genetic programs such as the insulin-like/IGF signaling (IIS) pathway as well as enhanced by loss of nerve-substratum cell adhesion and neuronal activity (Pan et al., 2011). Active mechanisms therefore exist to protect neuronal morphology from the normal physiological effects of aging.

The observation that neurons display an age-dependent increase in structural defects has led to investigations into whether changes in worm lifespan can modulate these structural changes. The conserved insulin-like/IGF signaling (IIS) pathway has been implicated in lifespan regulation in animals (Lapierre and Hansen, 2012). In *C. elegans*, the IIS pathway involves insulin receptor/DAF-2 activation of a PI3K/AGE-1-AKT kinase cascade to mediate FOXO/DAF-16 retention in the cytoplasm (Gao et al., 2011; Lapierre and Hansen, 2012; Kim and Web, 2016). A number of insulin-like agonists or antagonists bind to the DAF-2/IGF-1 insulin receptor. This cascade takes place largely at the membrane; where PI3K generated PIP_3 leads to PDK-1 activation, which in turn activates AKT1/2 and SGK-1 activation (Gao et al., 2011; Lapierre and Hansen, 2012). AKT, as well as SGK-1 phosphorylates DAF-16/FOXO to maintain its presence in the cytoplasm (Figure 2 A). Inhibition of this cascade allows DAF-16/FOXO to translocate and transcribe several targets that produce a longer lifespan, increase autophagy to address cytotoxic protein aggregation, restrict microtubule stability and inhibit neurite outgrowth during development (Christensen et al., 2011; Lapierre and Hansen, 2012; Nechipurenko and Broihier, 2012).

A

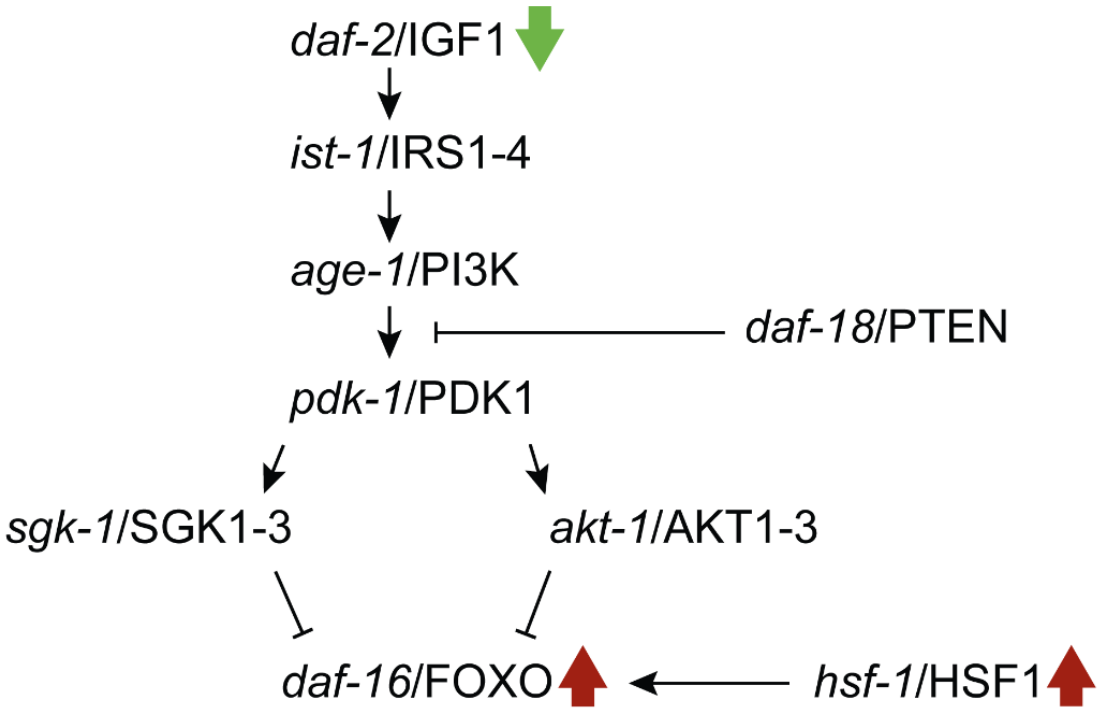

Figure 2: Mutants of the Insulin/IGF-like Signaling (IIS) pathway cause ectopic neurite and branching defects. In both C elegans and mammals, binding of an insulin-like agonist to the daf-2/IGF1 receptor initiates the canonical insulin/IGF-like signaling (IIS) pathway that retains daf-16/FOXO in the cytoplasm and prevents transcription of target genes. The heat shock factor hsf-1/HSF1 can enhance daf-16/FOXO activity. Loss of daf-16 or hsf-1 results in increased defects in aged C. elegans to a minor or significant degree, respectively. Conversely, loss of daf-2 is known to decrease the prevalence of the branching and ectopic neurites.

Several studies have shown that IIS mutants show alteration in age-dependant morphology defects (Pan et al., 2011; Tank et al., 2011; Toth et al., 2012). Mutations in the insulin receptor IGF-1/*daf-2* and PI3K/*age-1* are sufficient to significantly expand the lifespan of C. elegans (Dorman et al., 1995). Additionally, while wild type worms develop additional branching and long ectopic neurites in the touch neurons later in adulthood, mutations in *daf-2* reduce these defects. This suppression is facilitated by the IIS pathway as the neuroprotective effects of *daf-2* are lost in *daf-16/FOXO* mutants (Tank et al., 2011). Likewise, loss of the heat shock transcription factor HSF-*1*, which functions as a co-regulator of DAF-16 activity can enhance morphology defects (Pan et al., 2011; Toth et al., 2012). The suppression of neuronal morphology defects in *daf-2* mutants is also disassociated from its role in lifespan extension as neuron specific expression of *daf-2* is sufficient to supress neurite branching (Tank et al., 2011). Furthermore, mutants that extend lifespan independently of the IIS pathway are insufficient to supress neuronal morphology defects in the same way as *daf-2* (Tank et al., 2011; Pan et al., 2011).

Disco Interacting Protein 2 (DIP-2)

Members of the Disco Interacting Protein 2 (DIP2) family are newly identified regulators of neuronal morphology (Z. Ding and A. Colavita, unpublished, Nitta et al., 2017). In *C. elegans*, mutations in *dip-2* (originally named *neurite outgrowth defective (nde)-5*) were identified in a genetic screen for neuronal morphology defects (Carr et al., 2016). In *nde-5* mutants, the normally bipolar VC4 and VC5 neurons display additional

neurite-like outgrowth from their cell bodies. Zhao Hua Ding (a former graduate student in the Colavita lab) found that *nde-5* encoded the worm homologue of DIP2 and that *dip-2/nde-5* mutants displayed neuronal morphology defects in many neurons including the touch neurons. Further work in the lab identified two additional loss of function alleles (Figure 3)

The DIP2 family, which includes three human genes (DIP2A, DIP2B and DIP2C), is highly conserved across animal phyla and consist of proteins containing a DNA methyltransferase-associated protein 1 (DMAP1) binding domain and two class I superfamily adenylate forming domains (AFD) (Mukhopadhyay et al., 2002) (Figure 3 A). DMAP1 binding domains are predicted to interact with the transcriptional co-repressor DNA methyltransferase associated protein 1 (DMAP1) and its binding partner DNA methyltransferase 1 (DNMT1) (Rountree et al., 2000; Lee et al., 2010). AFD domains, most commonly found in most acyl-CoA synthetases, modify acyl-adenylates during fatty acid biosynthesis (Schmelz and Naismith, 2009).

DIP2: roles in the nervous system

DIP2 was initially identified in *Drosophila* based on protein binding to *disconnected* (*disco*), a transcription factor implicated in axon guidance (Mukhopadhyay et al., 2002). DIP2 family members are broadly expressed in neural tissue (Mukhopadhyay et al., 2002; Zhang et al., 2015) but DIP2 functions in the nervous system are just beginning to be understood. DIP2 was recently shown to regulate axon bifurcation and guidance in mushroom body (MB) neurons (Nitta et al.,

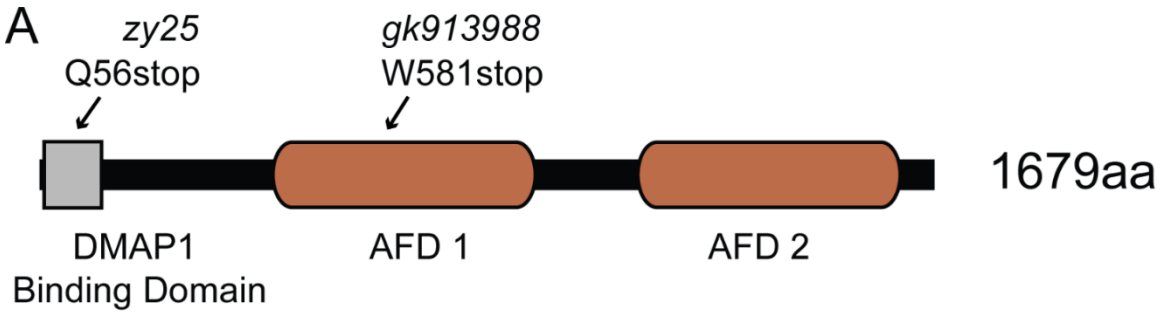

Figure 3: Schematic of the full length DIP-2 protein showing domain structure and location of nonsense mutations. DIP-2 contains methyltransferase associated protein 1 (DMAP1) binding domain and two adenylate forming domains (AFDs).

2017; Nitta and Sugie, 2017). A subset of MB axons bifurcate at a stereotypical location to form the two lobes of the MB (Nitta et al., 2017). In *dip2* mutants, MB neurons display multiple axon branches at this location. This role was shown to be cell autonomous and independent of the DMAP1 binding domain. Nitta et al. (2017) further showed that the AFD domains are required for DIP2 to suppress axon bifurcation. AFD are commonly found in acyl-CoA synthetases (Schmelz and Naismith, 2009). Nitta et al. (2017) showed that loss of dip2 in *Drosophila* results in decreased acyl CoA levels consistent with a role as an acyl-CoA synthetase. Furthermore, loss of function mutations in *acetyl-CoA synthase* (*AcCoAS*) and the *long chain fatty acid-CoA ligase bubblegum-like* (*bgm-l*) show similar MB neuron defects (Nitta et al., 2017) as do mutations in the phospholipase D homolog *gliakit* (Nitta and Sugie, 2017) further suggesting that DIP2 function involves fatty acid synthesis and/or metabolism. Taken together, findings in flies and worms indicate that DIP2 family proteins play evolutionarily conserved roles in regulating neuronal morphology by blocking ectopic protrusions from axons and cell bodies.

Consistent with nervous system expression and defects in worm and fly models, DIP2 genes have been associated with various neurological disorders in humans. Copy number variations in DIP2A has been linked to increased risk for autism spectrum disorder (Egger et al., 2013). Studies have found that increased methylation in DIP2A was linked with significant risk for Alzheimer's disease (De Jager et al., 2014), while deficiencies in DIP2B were found to affect fragile site FRA12A; possibly through methylation (Winnepenninckx et al., 2007). Finally, recent genomic studies in China

identified a mutation in DIP2A in populations with developmental dyslexia (Kong et al., 2016).

DIP2: roles in non-neuronal tissue

In addition to roles in nervous system development, DIP2 has been shown to play diverse biological roles. DIP2A has been identified as a cell surface receptor for the secreted glycoprotein follistatin-like 1 (FSTL1) and is involved in mediating its effects on cell growth and apoptosis through a protein kinase B (AKT) dependent pathway (Ouchi et al., 2010). Additionally, decreased DIP2A levels correlate with increased c-FOS expression (Tanaka et al., 2010; Murakami et al., 2012; Liang et al., 2014). The ability of DIP2A to decrease c-FOS mirrors that of FSTL1. Both ultimately act to supress the translation of proapoptotic proteins, matrix mellanoproteinases and prostaglandins; which are important in inflammatory tissue destruction. While the function of FSTL1-DIP2A mediated apoptosis regulation is not well understood, some recent findings suggest DIP2 may play a role in oncogenic suppression. For example, DIP2A has been shown to be upregulated in Hepatocellular carcinoma tissue and was found in a screen for mutations in Du145 docetaxel resistant prostate cancer cells (Jin et al., 2017; Ma et al., 2016). Furthermore, a recent study has implicated FSTL1 activity in the suppression of AsPC-1 pancreatic cancer growth, which could be likely mediated through DIP2A (Viloria et al., 2016).

Previous work has described a second role for DIP2 in regards to its regulation of transcription. The presence of a DMAP1 binding domain indicates that the protein

possibly acts to regulate transcription. MicroRNA 133b has been shown to downregulate DIP2B in expanding KIT K5 cell populations through changes in methylation (Hayashi et al., 2017). This also matches work in RKO cells, which show hyper methylation with the loss of DIP2C (Larsson et al., 2017). Despite this, little is known about how DIP2 might regulate transcription. Previous studies have largely focused on the membrane localization and FSTL1 related role of the protein; though the presence of a DMAP1 binding domain suggests that DIP2 might possess nuclear localization either in a temporal or tissue specific manner.

Does DIP-2 act to maintain neuron morphology?

Findings in flies and worms have indicated that DIP2 is a novel regulator of neuronal morphology. We were interested in further characterizing the role of DIP-2. Specifically, we were interested in 1) determining if DIP-2 is involved in maintaining neuronal morphology, 2) using CRISPR/Cas9-mediated gene editing to knock-in GFP into the *dip-2* locus to assess endogenous expression and localization pattern, 3) determining if DIP-2 acts cell autonomously, 4) asking if DMAP1 binding and AFD domains are required for neuronal morphology and 5) identifying additional genes or genetic pathways that act with DIP-2 to regulate neuronal through both a forward and reverse genetic approach. Taken together, these objectives will help to clarify a novel protein involved in neuronal maintenance and potential provide novel opportunities to study nervous system diseases in their relation to maintenance.

2. Materials and Methods

C. elegans Strains and Genetics

C. elegans strains were cultured using standard methods (Brenner, 1974). The Bristol N2 strain was used as wild type along with the following alleles and transgenes, which were either constructed with common genetic methods or obtained from the Caenorhabditis Genetics Center: LGI: *dip-2(zy25, zy26, ok885, gk913988, zy70[GFP::DIP-2])*, *zdIs5[mec-4p::GFP]*, *daf-16(mu86)*. LGII: *age-1 (hx546), eat-2(ad453).* LGIII: *daf-2(e1370), sax-2(ot10).* LGIV: *daf-18(ok480), fem-3(hc17), isp-1(qm150), zyIs47[DIP-2::GFP + myo-2p::mCherry]*, *zdIs4[mec-4p::GFP]*, *zdIs13[tph-1p::GFP]*. LGX: *sax-1(ky491). zyIs3[flp-10p::GFP + odr-1p::mCherry]* (linkage unknown). Extrachromosomal transgenes: *zyEx44[dip2p::gfp + myo-2p::mCherry]*, *zyEx53[rgef-1p::DIP-2::SL2::tagRFP + myo-2p::mCherry]*, *zyEx55[dpy-7p::DIP-2::SL2::tagRFP + myo-2p::mCherry]*, *zyEx57[mec-4p::DIP-2::GFP + myo-2p::mCherry]*, *zyEx65[dip-2p::DIP-2::SL2::tagRFP + myo-2::mCherry]*, *zyEx67[dip-2p::DIP-2 DMAP1Δ::SL2::tagRFP + myo-2::mCherry]*, *zyEx69-70[dip-2p::DIP-2 AFD1Δ::SL2::tagRFP + myo-2::mCherry]*, *zyEx72-73[dip-2p::DIP-2 AFD2Δ::SL2::tagRFP + myo-2::mCherry]*, *zyEx75-77[col-19p::DIP-2::GFP + myo-2::mCherry]*, *zyEx78[dip-2p::DIP-2::GFP + myo-2::mCherry]*, *zyEx79[dip-2p::DIP-2 DMAP1Δ::GFP + myo-2::mCherry]*, *zyEx81[dip-2p::DIP-2 AFD1Δ::GFP + myo-2::mCherry]*, *zyEx82[dip-2p::DIP-2 AFD2Δ::GFP + myo-2::mCherry]*, *zyEx83-84[hsp-16.2p::DIP-2::SL2::tagRFP + myo-2p::mCherry]*.

Nematode Growth Media

Worms were grown on nematode growth media (NGM) agarose plates seeded with *Escherichia coli* OP50 as a food source (Brenner, 1974). NGM plates are a basic nutrient media solidified into a 60mm petri plate. To make the media; 3g NaCl, 17g bacterial grade agarose and 2.5g bacterial grade peptone are added to 975mL of deionized water and autoclaved. Media was cooled to 60°C. Cholesterol (1ml of 5mg/mL in 95% ethanol), CaCl2 (1ml of 1M), MgSO4 (1ml of 1M) and KPO4 (25ml of 1M) were added before pouring. Plates were allowed to solidify for two days, after which *E. coli* OP50 was added and allowed to grow for two more days.

Molecular Biology and Transgenic Strains

A *dip-2* cDNA and a *dip-2p::DIP-2::GFP* translational reporter were previously generated (Z. Ding and A. Colavita, unpublished). Briefly, a 5,046 bp cDNA corresponding to the DIP-2b transcript (F28B3.4b, Wormbase) was isolated from mixed stage total RNA by RT-PCR using SL1 trans-spliced lead (5'ggtttaattacccaagtttgag) and *dip-2* C-terminal end (5'ttacatgtgatacgctacgtatat) primers, and cloned into pBluescript. 2.458 kb of regulatory sequence upstream the *dip-2* start codon was PCR amplified and inserted into the pPD95.77 vector (A gift from Andy Fire, Stanford University). This construct was modified using Gibson assembly to insert the *dip-2* cDNA (excluding stop codon) upstream and in-frame with GFP to generate the *dip-2p::DIP-2::GFP* translational reporter.

Expression constructs containing *dip-2* (2458bp), neuronal *rgef-1* (3443bp) (Altun-Gultekin et al., 2001) and epidermal *dpy-7* (434bp) (Myers and Greenwald, 2007)

promoters were made using three fragment Gibson assembly in which PCR-amplified promoters and the 5046bp *dip-2* cDNA (including the stop codon) were ligated upstream of *SL2::tagRFP* from a linearized *pSM-SL2::tagRFP* vector (a gift from Chiou-Fen Chuang, University of Illinois at Chicago). Overlap PCR extension was used to fuse the touch neuron *mec-4* (1020bp) (Duggan et al., 1998) promoter to DIP-2::GFP. DIP-2 domains were identified using HMMER (Finn et al., 2011) and domain deletion constructs were made using Gibson assembly in which either the *dip-2p::dip-2(cDNA)::SL2tagRFP* or *dip-2p::DIP2::GFP* plasmid was the backbone and overlap PCR was used to delete *dip-2* cDNA nucleotides 7-345, 1279-2736 and 3256-4641 to generate the DMAP1 (amino acids 3-115), AFD1 (amino acids 428-912) and AFD2 (amino acids 1086-1547) in-frame deletion constructs respectively. Gibson assembly was used to insert a *dip-2(cDNA)::SL2tagRFP* PCR fragment into the EcoRV site of pPD49.78 to generate *hsp-16.2p::DIP-2::SL2::tagRFP*. Extrachromosomal transgenes were generated by injecting each construct at 10 ng/µl with 5 ng/µl of pCFJ90 (*myo-2p::mCherry*) co-injection marker and 115 ng/ul pBluescript using standard germline transformation protocols (Mello and Fire, 1995). Microinjection and plasmid construction were done by Nathaniel Noblett and Tony Roenspies.

CRISPR/Cas9-mediated 'knock-in' of GFP into *dip-2*

The CRISPR/Cas9-based approach described in Dickinson et al. 2015 was used to insert an in-frame GFP cassette at the N-terminus of endogenous *dip-2* to generate *dip-2(zy70[gfp::dip-2])*. The Feng Zhang lab CRISPR design tool (http://crispr.mit.edu) was used to identify the *dip-2* N terminal target site (5'N_{20}-**NGG**-3')

GTTGCAGATAATGAATGATC**CGG** where **NGG** is the PAM site. Overlap fusion PCR was used to generate the *cas-9-dip-2-sgRNA* plasmid containing *C. elegans* germline promoter driven Cas9 and *dip-2-**sgRNA*** (5'GTTGCAGATAATGAATGATC-**GTTTTAGAGCTAGAAATAGCAAGT**) cassettes. The *gfp::dip-2* homology-directed repair (HDR) plasmid was made using three fragment Gibson assembly in which *dip-2* homology arms containing 508bp upstream and 800bp downstream of the *dip-2* ATG start codon were PCR amplified from N2 genomic DNA and fused such that they flanked the GFP in a linearized GFP cassette-containing plasmid described in Dickinson et al. 2015. The *cas-9-dip-2-sgRNA* plasmid (50ng/µl), *gfp::dip-2* HDR plasmid (10ng/µl) and Pmyo-2::mCherry (pCFJ90) (2.5ng/µl) co-injection marker were injected into N2 worms and knock-in animals identified as described (Dickinson et al. 2015).

Fluorescence microscopy and quantification of neuronal morphology defects

Fluorescence imaging was performed using a Zeiss AxioImager 2 microscope, MRm AxioCam camera and Axiovision Rel. 4.8 software. Worms were paralysed using 200 µM of the acetylcholine receptor agonist levamisole and mounted on 2% agarose pads. Levamisole treatment leads to muscle contraction and paralysis; allowing for the imaging of neurons in vivo while the worm remains alive.

For each population of worms, the number of touch neuron cell bodies that possessed at least one ectopic neurite were scored. These were then converted to percentages and compared to those in other strains. Ectopic neurites and axon branches (in PVM) were considered significant in L4 and adult stages if the neurite length was >10 µm and ectopic neurites with secondary branching were recorded as severe. In L1 stage

worms, any ectopic ALM cell body protrusion >0 µm was scored as an ectopic neurite (Figure 4).Each populations of worms was grown at 20°C or 25°C (as indicated in figure legends).

For the analysis of age-dependence on neuronal morphology phenotypes, worm populations were synchronized by bleaching and then grown on NGM plates supplemented with 16 µM of the antimetabolite 5' fluorodeoxyuridine (FUdR). In *C. elegans*, FUDR is used to chemically induce sterility during aging or lifespan studies in order to avoid a "bag-of-worms" phenotype in which eggs hatch inside the worm (Apfeld and Kenyon, 1999).

Heat shock induced gene expression

Heat shock experiments were performed according to published protocols (Link et al., 1999; Zevian and Yanowitz, 2014). Newly hatched L1 (synchronized as a 0-3 hours post hatching cohort) worms from *dip-2 (gk913988)* or *dip-2 (gk913988)*; *Ex[hsp-16.2p::DIP-2]* grown at 20°C were transferred to 25°C and the percentage of ALM ectopic neurites in each population was scored 48 hours later (day 1 adult). Embryo heat shock: Synchronized embryos were transferred to 35°C for 15 minutes, heat shocked for 2 hours at 35°C and then transferred to 25°C for 46 hours. L1 heat shock: Newly hatched L1 were transferred to 35°C for 15 minutes, heat shocked for 2 hours at 35°C and then transferred to 25°C for 46 hours. The additional 15 minutes at 35°C was used to allow the agar to equilibrate to 35°C from 25°C as previously shown (Zevian and Yanowitz, 2014).

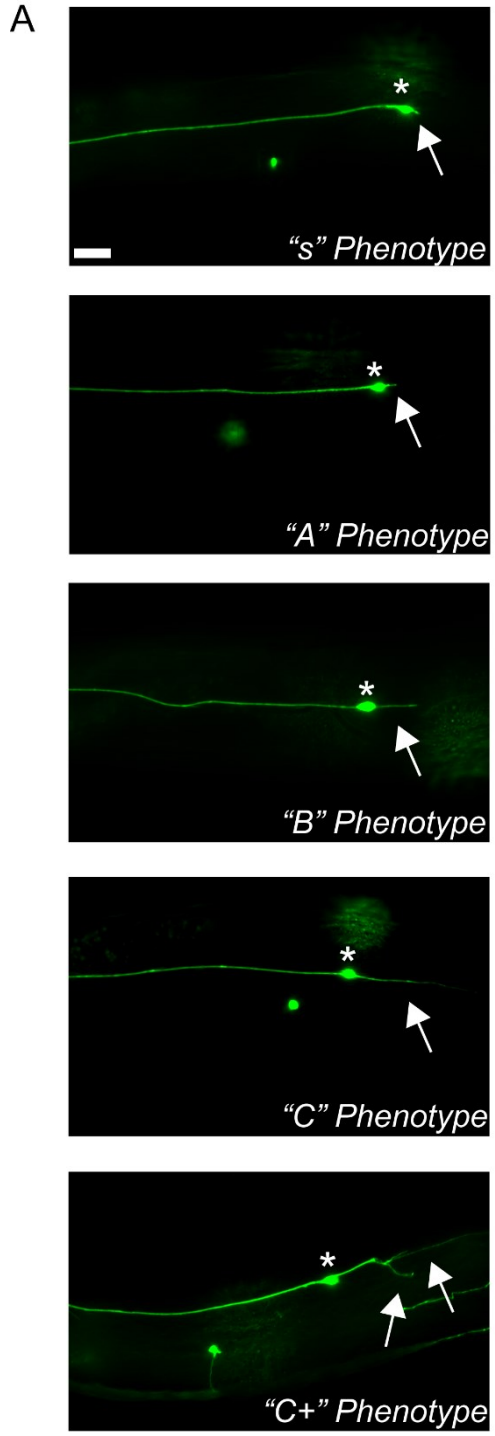

Figure 4: Scoring scheme for the unbranched, unipolar touch neurons. (A) Neurites were scored based on their relative length to the cell body. Neurites were scored "s" if they had a neurite of <5um in length, "A" had a neurite of 5um in length, "B" had a neurite of about 10um in length, "C" had a neurite of 15um in length or greater. (E) Neurites scored "C+" constituted a "severe" category, possessing a neurite of 15um in length or greater that had additional branches. "A" and "s" neurites, were discounted in when scoring ALM neurons in stages older than L1. White arrows indicate ectopic neurites, white asterisk indicates ALM neuron cell body. Scale bar = 20um.

Genetic screen for ectopic neurite defects in touch neurons

ALMs were screened for morphology defects, including ALM ectopic neurites, in 3 day old *fem-3 (hc17ts); zdIs5[mec-4p::GFP]* adults grown at 25°C. *fem-3(hc17ts)* is a temperature sensitive mutant that causes sterility at restrictive temperatures and is therefore useful for preventing 'bag-of-worm' phenotypes in older worms. The *zdIs5* reporter was used to visualize the mechanosensory neurons. *fem-3(hc17ts); zdIs5* worms were mutagenized with the alkylating agent ethylmethanesulfonate (EMS) as described (Brenner, 1974). EMS mutagenesis was performed by Tony Roenspies and Zhegian (Alex) Chen assisted with preforming the genetic screen. The F1 progeny of these worms were transferred to new NGM plates (3 F1 worms/plate) and allowed to lay eggs at 20°C. Each individual F2 generation plate at this plate consisting of L3 and L4 stage worms were then split, with half allowed to grow at 20°C and half at the restrictive temperature of 25°C. Worms on the 25°C plates were assessed for neuronal morphology defects approximately 3 to 4 days later when most worms were at least 3 day old adults. Screening was performed as follows: Worms were immobilized with levamisole (200 µM) and mounted under a coverslip on slides (2% agarose pad) and examined by epifluorescence on an AxioImager 2 microscope to identify plates containing *dip-2*-like neuronal morphology defects. Individual worms were then recovered from the corresponding plate maintained at 20°C, allowed to grow and propagate for several days and then rescreened using epifluorescence on a Zeiss SteREO Discovery V8 stereoscope. New *dip-2*-neuronal morphology like mutants were outcrossed at least 3 times and genomic DNA prepared for whole genome sequencing

(WGS) as described (Flibotte, et al., 2010). WGS was performed at the Centre for Applied Genomics (The Hospital for Sick Children, Toronto, ON). Single-nucleotide variants (SNVs) were identified with assistance from Stephane Flibotte (Department of Zoology, University of British Columbia) as described (Flibotte, et al., 2010).

Statistical Analysis

Statistics concerning the percentage of ectopic neurites in a given population of worms was performed using Graph Pad Prism 6 and Microsoft Excel. One-way ANOVA with Tukey's test for multiple comparisons or independent t-test were performed as appropriate. To compare the "severe" ALM defects a two sample proportion test was used with an ANOVA to assess possible significance.

3. Results

***dip-2* mutants display neuronal morphology defects**

The adenylate forming domain protein DIP-2 has been shown to regulate neuronal morphology in a small subset of *C. elegans* neurons (Z. Ding and A. Colavita, unpublished). To determine the extent of neuronal defects in *dip-2* mutants, we examined some of the original subset of neurons as well as additional neurons for morphological defects in young adult animals. We examined the six (mechanosensory) touch neurons and the DVB and HSN motor neurons for characterization as these neurons have simple, predominately unipolar morphologies and can be visualized individually with cell-specific GFP reporter transgenes. We examined morphology defects in *zy25* and *gk913988*, two punitive *dip-2* null alleles. The *zy25* and *gk913988*

alleles introduce nonsense alleles that are predicted to result in premature protein terminations at Q56 and W581 respectively.

The six touch neurons (ALML/R, AVM, PVM and PLML/R) mediate responses to gentle touch stimuli. ALM and PLM extend a single anteriorly-directed axon while AVM and PVM extend ventrally-directed axons which then extend anteriorly along the ventral nerve cord (VNC) (Syntichaki and Tavernarakis, 2004). We found that touch neuorns displayed highly penetrant morphology defects in *dip-2* mutants. These included the formation of ectopic neurites from cell bodies and ectopic axon branching (Figure 5 C).

For example, 69.2% ($p<0.001$) and 73.1% ($p<0.001$) of ALM neurons in *gk913988* and *zy25* mutants respectively show ectopic cell body neurites compared to 10% in *wt* neurons. Ectopic cell body neurites were also observed in PVM and occasionally in PLM and AVM neurons (Figure 5 E). Both the ALM and PVM ectopic neurite defects were significantly rescued by expressing a DIP2::GFP (*zyIs47)* transgene (Figure 5 D and E). PVM, which sends a single axon ventrally towards the ventral nerve cord (VNC) and then anteriorly along the VNC, also showed a strong axon branching phenotype (Figure 5 F). In *dip-2* mutants, a large proportion of PVM axons (85.6% in *dip-2(gk913988)* and 89.1% in *dip-2(zy25)*) bifurcated at the VNC and extended both anteriorly and a short distance posteriorly along the nerve cord (thick arrow in Figure 5 C bottom-right panel) compared to *wt* (1.3%).

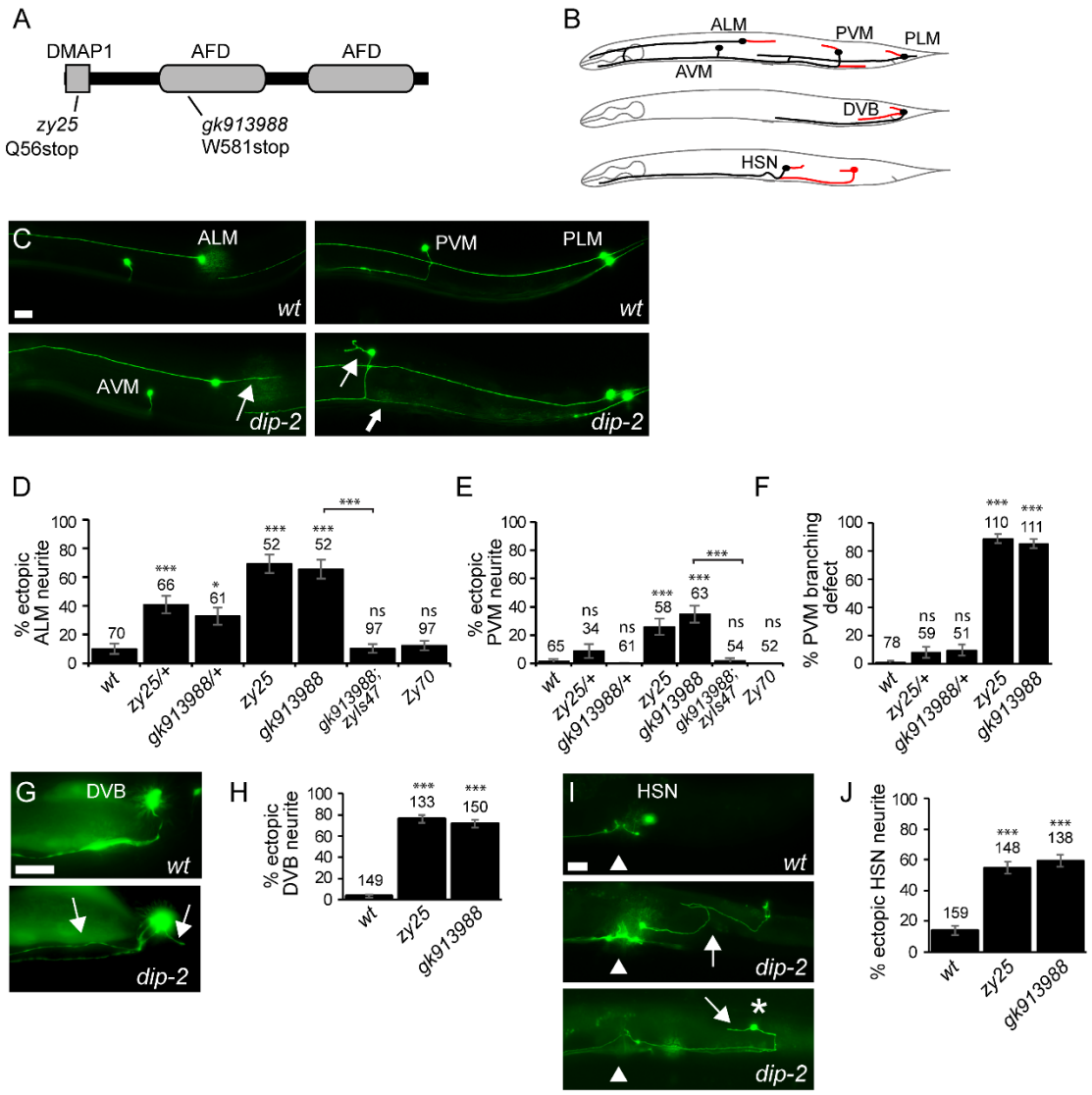

*Figure 5: DIP2 suppresses ectopic neurite growth and branching in multiple neuron types. (A) Schematic of DIP2 protein showing mutants used in the study. (B) Schematic of the touch neuron, DVB and HSN neurons, with common defects in red. (C) Wild type and dip-2 (gk913988) anterior and posterior touch neuron. Arrows indicate ectopic neurites. (D-F) Quantification of the percentage of ectopic neurites in ALM (D) and PVM (E) at 1 day of adulthood. (F) Quantification of bifurcated PVM neurons. (G) Wild type and dip-2 (gk913988) DVB neurons. (H) Quantification of DVB ectopic neurites at 1 day after adulthood. (I) Wild type and mutant dip-2(gk913988) HSN neurons. Vulva is indicated by the white arrow head, arrows indicate ectopic neurites, and asterisk indicates neuron with migration and ectopic neurite phenotype. (J) Quantification of HSN ectopic neurites at 1 day after adulthood. The number of worms in each population is indicated above the appropriate bar. Error bars indicate SEs of. Significance compared to wild type using a one-way ANOVA with Tukey's test for multiple comparisons. *p<0.05, **p<0.01, ***p<0.001. Scale bar = 20um.*

HSN and DVB neurons also displayed ectopic neurites from cell bodies in *dip-2* mutants. The DVB are a pair of motor neurons in the tail that regulate the enteric muscles responsible for defecation (McIntire et al., 1993). The HSN are a pair of bilaterally located motor neurons that innervate vulval muscles to regulate egg laying (Sulston et al., 1983; Waggoner et al., 1998). Both HSN and DVB neurons extend a single axon ventrally into the VNC that extend anteriorly to the nerve ring (Figure 5 G and I). We found that 71.3% of DVB neurons in *dip-2(gk913988)* mutants displayed ectopic cell body neurites and axon branching defects (Figure 5 H). Ectopic axon branches were especially pronounced at the point where the process turned anterior, and would occasionally loop or form multiple branches off of the same neurite (Figure 5 G bottom panel). Similarly, 59.4% ($p<0.001$) of HSN neurons in *dip-2(gk913988)* displayed ectopic neurites (Figure 5 J).

In addition to morphology defects, HSN neurons also displayed a migration phenotype in *dip-2* mutants (Z. Ding and A. Colavita; unpublished). The bilaterally-located HSN neurons are born in the posterior of the embryo and migrate anteriorly during embryogenesis to their final positions adjacent to the vulva (Sulston et al., 1983). In *dip-2* mutants, HSN neurons display an under-migration phenotype (Figure 5 I bottom panel), resulting in posteriorly displaced positions in young adult animals. Indeed, *dip-2* adults often displayed a bag-of-worms phenotype that is likely the result of morphology and migration-related changes in the HSN and VC egg-laying circuitry (Waggoner et al., 1998).

***dip-2* mutants display an age-dependent increase in neuronal morphology defects**

Following embryogenesis *C. elegans* proceed through 4 larval stages (L1-L4) before reaching adulthood (Alexander, Marfil and Li, 2014; Brenner, 1974). While examining the touch neurons in *dip-2* mutants we noticed that morphology defects appeared to be both more frequent and more severe in older animals compared to younger animals (Figure 6 A). We therefore examined touch neuron morphology at different developmental stages ranging from newly hatched L1 to day 5 old adults. ALM and PLM neurons are born embryonically and have undergone primary axon outgrowth and guidance by the time of hatching. AVM and PVM arise post-embryonically and attain their final positions and morphologies by the 2nd larval stage (Chalfie and Sulston, 1981). We found that in *dip-2(gk913988)* mutants the percentage of ALM neurons displaying ectopic neurites increased from approximately 14% in L1 to 71% in day 1 old adults and 98% in day 5 old adults (Fig. 6 B). This contrasts with normal age-dependent morphology changes in *wt* touch neurons (Pan et al., 2011; Tank et al., 2011; Toth et al., 2012) which in ALMs manifested as approximately 5.3% displaying ectopic neurites in day 1 old adults to 35.6% in day 5 old adults. Overexpression of wild type *dip-2* from the *zyIs47* transgene blocked this increase such that only 12.0% of ALMs displayed ectopic neurites in day 5 old adults (Fig. 6 B). Age-dependent increases in morphology defects were also seen in PLM, AVM and PVM neurons (Figure 6 C and D). Strikingly, ectopic ALM neurites in *dip-2* mutants undergo secondary branching or develop arbor-like morphologies (Figure 6 A bottom-middle panel) with increasing age (Figure 6 B). These findings are consistent with *dip-2* acting to establish and/or maintain neuronal morphology.

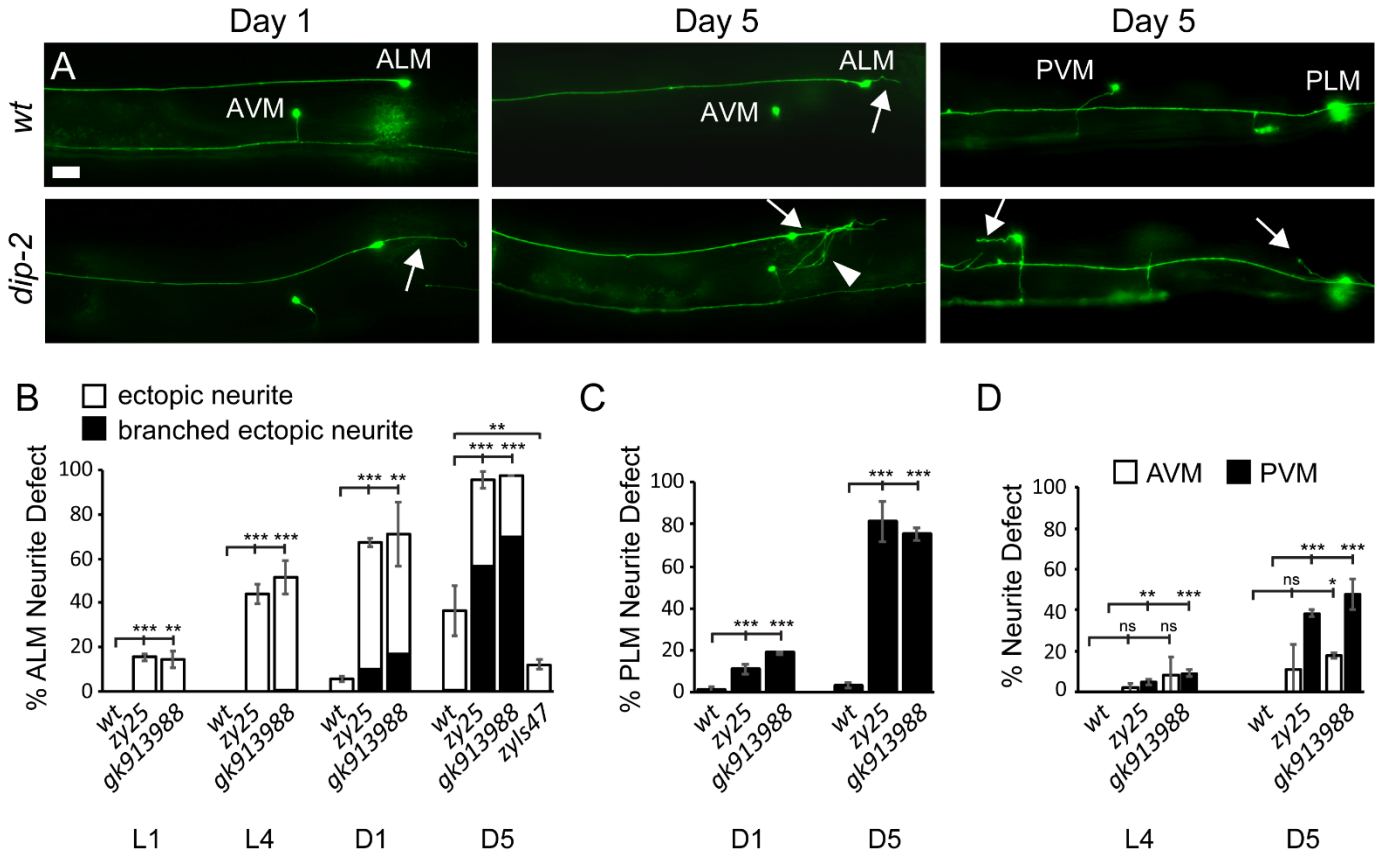

*Figure 6: DIP2 is required to maintain touch neuron morphology during aging. (A) Typical defects observed in a wild type and dip-2(gk913988) backgrounds. White arrows depicting ectopic growth, arrow head indicated branched ectopic neurites. (B-D) Quantification of ALM (B), PLM (C), AVM and PVM (D) ectopic neurites in wild type and dip-2 mutant strains. Age of the animals is indicated for stages of development or days of adulthood. Error bars indicate SD for n=3 trials. Significance compared to wild type using a one-way ANOVA with Tukey's test for multiple comparisons. *p<0.05, **p<0.01, ***p<0.001. Scale bar = 20um.*

DIP-2 acts post-developmentally to maintain neuronal morphology

ALM neurons arise during embryogenesis and have undergone primary axon outgrowth prior to hatching (Chalfie and Sulston, 1981). If DIP2 acts to maintain neuronal morphology, then expression of DIP2 at post-developmental stages should be sufficient to rescue morphology defects in *dip-2* mutants. We therefore asked if restoration of DIP-2 transgene expression in *dip-2(0)* larvae after the establishment of normal ALM morphology in the embryo mitigated the ectopic neurite phenotype in adult animals. Temporal control of gene expression was achieved using the *hsp-16.2* heat shock (HS)-inducible promoter to drive *dip-2* transgene expression (Link et al., 1999). HS-induced DIP-2 expression in *dip-2* mutants was conducted at the L1 stage and visualization in day 1 old adults (Figure 7 A). We found that this resulted in a modest but significant reduction in the proportion of ALM neurons with ectopic neurites compared to non-heat shocked *dip-2* mutants. For example, heat shock at the L1 stage decreased ALM morphology defects to either 48.1% ($p<0.001$) (Figure 7 B) compared to 74.7% in non-heat shocked worms. These findings indicate, at least to some extent, that DIP-2 is required post-developmentally in ALM neurons to maintain neuronal morphology.

DIP-2 is localised to the cytosol of neurons and the membrane of epidermal cells

The fly and vertebrate Dip2 proteins are broadly expressed in the brain and other regions of the central nervous system (Zhang et al., 2015; Mukhopadhyay et al., 2002) and localized to the plasma membrane (Nitta et al., 2017; Ouchi et al., 2010). *dip-2* also shows widespread neuronal expression in *C. elegans* as demonstrated using

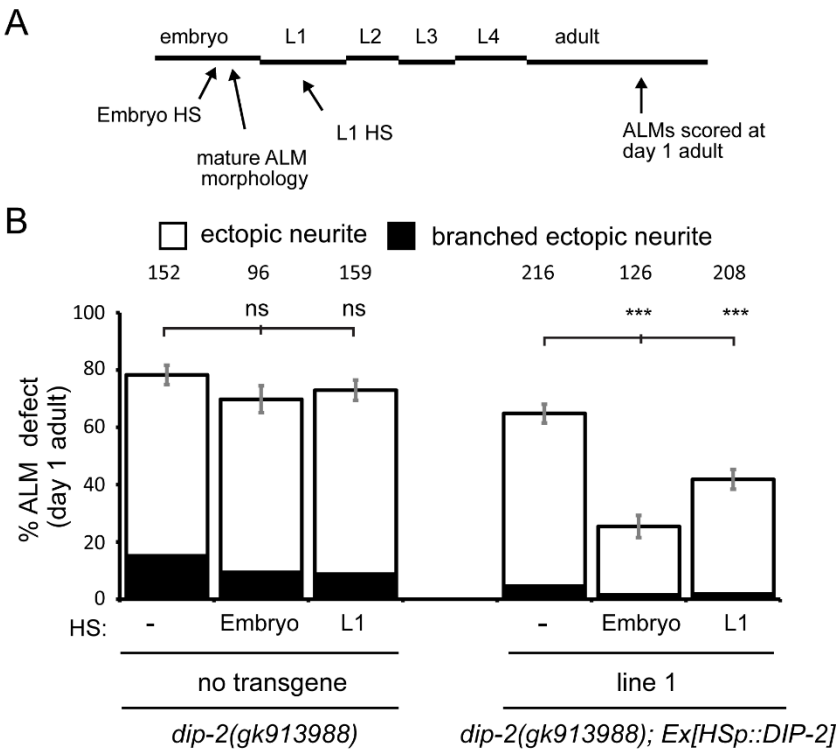

*Figure 7: Transient expression of DIP2 is sufficient to partially suppress ectopic neurite levels. (A) Experimental outline indicating significant points of interest, including the timing of ALM morphology development, the timing of embryonic and L1 heat shocks and when the percentage of ectopic neurites in each worm population was scored. (B) Quantification of ALM morphology and ectopic neurite formation in dip-2(gk913988) and two independent dip-2(gk913988); Ex[hsp-16.2p::DIP2::RFP] lines that have either been subject to non-heat shocked conditions, an embryonic or a L1 stage heat shock. The number of worms in each population is indicated above the appropriate bar. Error bars indicate SEs of proportion for the indicated number of individuals. Each heat shock data set was compared to the non-heat shocked control using a one-way ANOVA with Tukey's test for multiple comparisons. **p<0.01, ***p<0.001.*

transcriptional *(zyEx44[dip2p::GFP])* and translational *(zyIs47[dip-2p::DIP2::GFP])* reporter transgenes (Z. Ding and A. Colavita, unpublished).

Protein expression or localization patterns obtained using transgenically-expressed GFP fusion proteins are sometimes incomplete owing to the omission of important genomic regulatory elements or misleading due to overexpression artifacts. Therefore, to validate the *dip-2* expression patterns obtained using transgenic lines, we used a CRISPR/Cas9-mediated approach (Dickinson et al., 2015) to insert an N-terminal in-frame GFP into the native *dip-2* locus to generate *dip-2(zy70[GFP::DIP-2])*. We confirmed that GFP did not disrupt DIP-2 function as *dip-2(zy70)* did not display neuronal morphology defects (Figure 5 D).

We found that the expression pattern from *dip-2(zy70[GFP::DIP-2])* resembled the previously characterized transgene expression pattern in the nervous system except for more widespread expression in epithelial cells. In larvae to adult animals, *dip-2* was expressed in most head, body and tail neurons including motor neurons in the VNC (Figure 8 A), HSN, PVD (Figure 8 C) and the mechanosensory touch neurons (Figure B and C). *dip-2* was also prominently expressed in epithelial cells such as embryonic epidermal cells (Figure 8 E), larval seam cells (Figure 8 f) and those that comprise the spermatheca and vulval organs (Figure 4 D). In neuronal cells, endogenous GFP::DIP-2 was localized to the cytoplasm of axons and cell somas and appeared to be mostly excluded from nuclei (see Figure 8 C for a typical example).

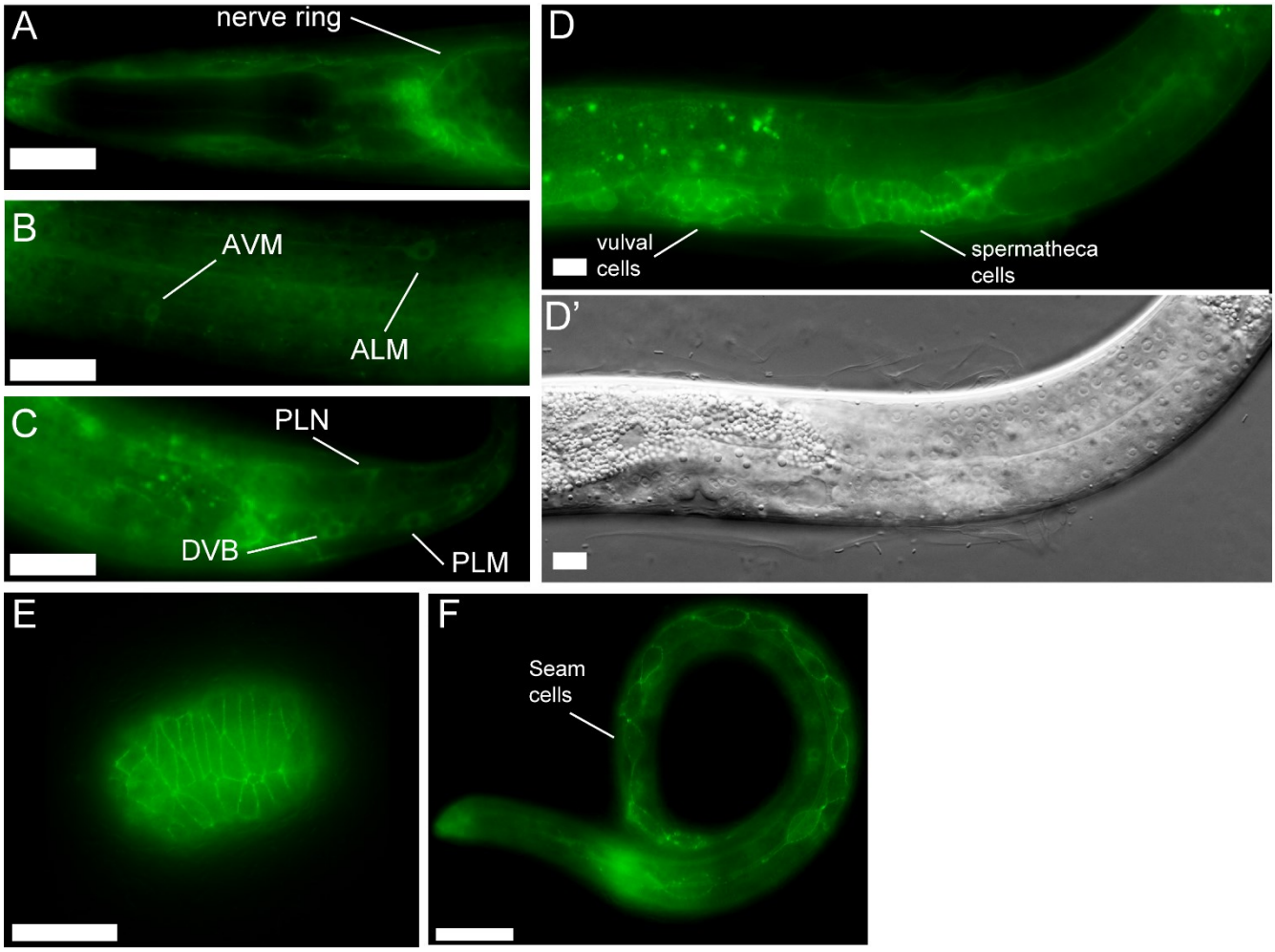

Figure 8: DIP2 localization pattern. Expression of the protein using an endogenous DIP2::GFP (zy70) background. (A-C) Dip2 is localised to the cytoplasm of neurons in the (A) Head, (B) Anterior Touch Neurons and (C) Tail Neurons. (D-F)Epithelial expression of DIP2 is localized to the cell surface. (D) Bean stage embryo. (E) Epithelial seam cells in L4 stage worm. (F and F') Vulval and spermathecal cells of L4 stage worms. Scale bar = 20um.

In contrast, it localized primarily to the membrane of epithelial cells (Figure 8 D, E and F). These distinct localization patterns may be an indication that DIP-2 functions via divergent molecular mechanisms in neuronal versus non-neuronal cells.

DIP-2 acts cell-autonomously to regulate neuronal morphology

Widespread expression of *dip-2* in neurons suggested an intrinsic role in regulating neuronal morphology. We therefore asked if cell-autonomous expression of DIP2 was sufficient to regulate touch neuron morphology. We generated transgenic lines in which DIP2 was expressed from either pan-neuronal (*Prgef-1*), mechanosensory touch neuron (*Pmec-4*), or pan-epithelial (*Pdpy-7*) promoters and assessed them for their ability to rescue ALM and PVM morphology defects in *dip-2(gk913988)* mutants (Figure 9). We found that *dip-2* expression pan-neuronally ($p<0.001$) or specifically in touch neurons ($p<0.001$) but not epidermal cells rescued ALM ectopic neurites and PVM axon bifurcation defects. These results indicate that *dip-2* acts in a cell-autonomous manner to inhibit ectopic neurite formation and axon bifurcation.

DIP-2-mediated regulation of neuronal morphology requires AFD but not the DMAP1-binding domain

By sequence prediction, DIP-2 proteins contain a DMAP1 binding domain and two AFD domains. However, little is known about how each domain contributes to DIP2 function. DMAP1 is a transcriptional co-repressor and a binding partner of DNA methyltransferase 1 (DNMT1) suggesting a role in the epigenetic regulation of gene expression (Rountree et al., 2000; Lee et al., 2010).

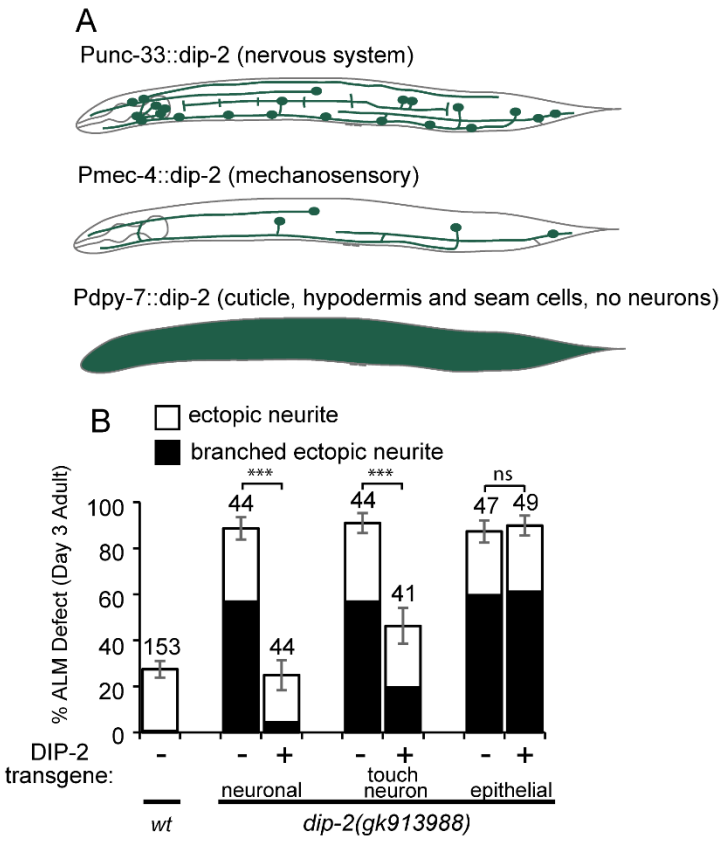

*Figure 9: DIP2 functions cell autonomously in inhibiting neurite growth in the ALM. (A) Expression pattern of the promoters used to rescue dip-2(gk913988) in the experiment is shown in green. (B) Extrachromosomal cell specific rescue of ALM ectopic sprouting defect. The number of worms in each population is indicated above the appropriate bar. Error bars indicate SEs of proportion for the indicated number of animals. Significance compared between siblings using a two-tailed t-test. ***p<0.001.*

AFD domains, most commonly found in acyl-CoA synthetases in higher eukaryotes, activate fatty acids as acyl-adenylates during fatty acid synthesis (Schmelz and Naismith; 2009). We therefore generated domain-specific DIP-2 deletions to test their requirements in regulating neuronal morphology (Fig. 10 A). We found that the DMAP1-binding domain was dispensable for DIP-2 function as expression of a DMAP1 binding domain-deleted transgene was able to completely rescue neuronal morphology defects in *dip-2* mutants (Fig. 10 B and C). In contrast, deletion of either AFD domain completely abolished the ability of DIP-2 to rescue morphology defects.

The cytoplasmic subcellular localization of DIP-2::GFP DMAP1-binding and AFD deleted proteins did not appear to qualitatively differ from full length (FL) protein in late-stage neurons (Fig. 10 D). However, AFD1 and AFD2-deleted DIP-2::GFP protein did show a partial or complete loss of plasma membrane localization respectively in embryonic epidermal cells compared to DMAP1-binding deleted or FL protein (Fig. 10 E). These findings suggest that a DMAP1-mediated epigenetic pathway is not involved in regulating neuronal morphology while AFD domains play an essential role.

Mutations in the insulin/IGF pathway suppress *dip-2* neuronal morphology defects

One of the most prominent pathways involved in longevity and metabolism is the Insulin-like Signalling (IIS) pathway which involves *daf-2*/IGF-1 activation of an *age-1*/PI3K-mediated signalling cascade that serves to inhibit the transcription of *daf-16*/FOXO targets (Gao et al., 2011; Lapierre and Hansen, 2012; Kim and Web, 2016).

Previous work has shown that this pathway is involved in neuron specific

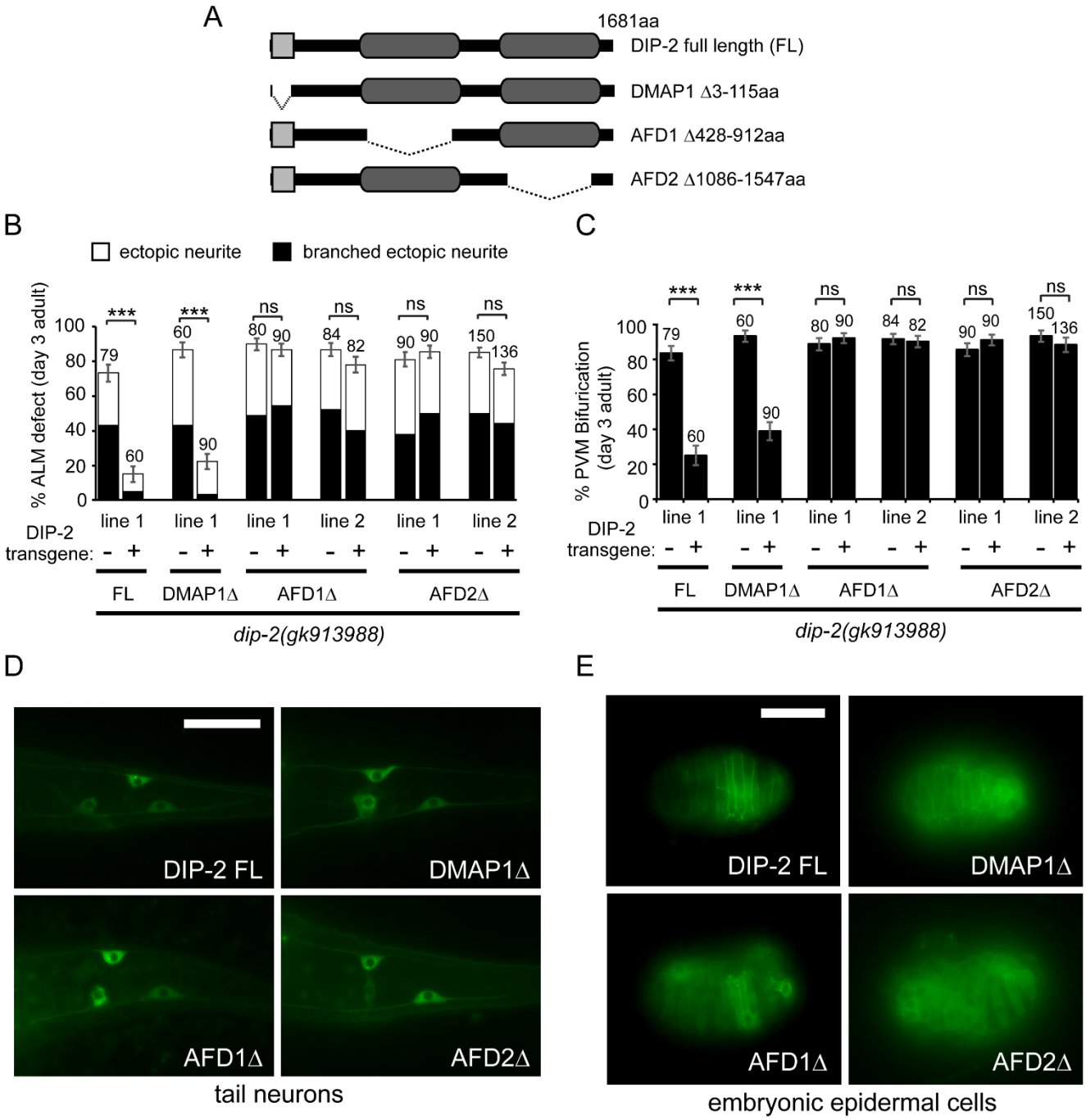

*Figure 10: The DMAP binding domain is dispensable for suppression of ectopic growth and does not affect localization of the protein in vivo. (A) Schematic of the DIP2 extrachromosomal constructs used for rescue experiments showing deleted domains. (B - C) Extrachromosomal rescue of dip-2(gk913988) ALM ectopic neurite (B) and PVM bifurcation (C) defects. (D) Expression of DIP2 in L4 tail neurons and in epidermal cells during embryogenesis. The number of worms in each population is indicated above the appropriate bar. Error bars indicate SEs of proportion for the indicated number of animals. Significance compared between siblings using a two-tailed t-test. ***p<0.001. Scale bar = 20um.*

suppression of age-dependent defects, including ectopic neurite formation and branching (Tank et al., 2011; Pan et al., 2011; Toth et al., 2012). As we found that *dip-2* neuronal morphology defects increase with age, we next asked whether this increase could be mitigated by mutations in the IIS pathway. We found that at day 5, ALM neurons in both *age-1(hx546)*; *dip-2(gk913988)* and *daf-2(e1370)*; *dip-2(gk913988)* appeared to show slightly less ectopic neurite defects compared to *dip-2* single mutants (Figure 11 A and B). However, we found that these double mutants displayed a significant decrease in the proportion of the highly branched category of ectopic ALM neurites. For example, the proportion of branched ectopic ALM neurites decreased from 57.3% to 18.1% ($p<0.001$) in age-*1(hx546)*; *dip-2(gk913988)* animals. Similarly, branched neurites decreased from 63.5% to 8.2% ($p<0.001$) in *daf-2(e1370)*; *dip-2(gk913988)* animals. To verify that this suppression was the result of loss of IIS signaling, we asked if suppression was dependent on the downstream transcription factor DAF-16; as its subcellular localization is under the control of DAF-2 and AGE-1. We found that the proportion of branched ectopic ALM neurons in *age-1(hx546)*; *dip-2(gk913988)*; *daf-16(mu86)* triple mutants were not significantly different from the proportion in *dip-2(gk913988)* single mutants (Figure 11 A). *daf-2(e1370)*; *dip-2(gk913988)*; *daf-16(mu86)* triple mutant displayed a similar restoration of branched ectopic neurites compared to the *daf-2(e1370)*; *dip-2(gk913988)* double, although this was a partial restoration as they remained significantly different from *dip-2(gk913988)* single mutants ($p<0.01$) (Figure 11 B). These results suggest that the IIS pathway may be involved in promoting secondary ectopic neurite branching in *dip-2* mutants. Given that

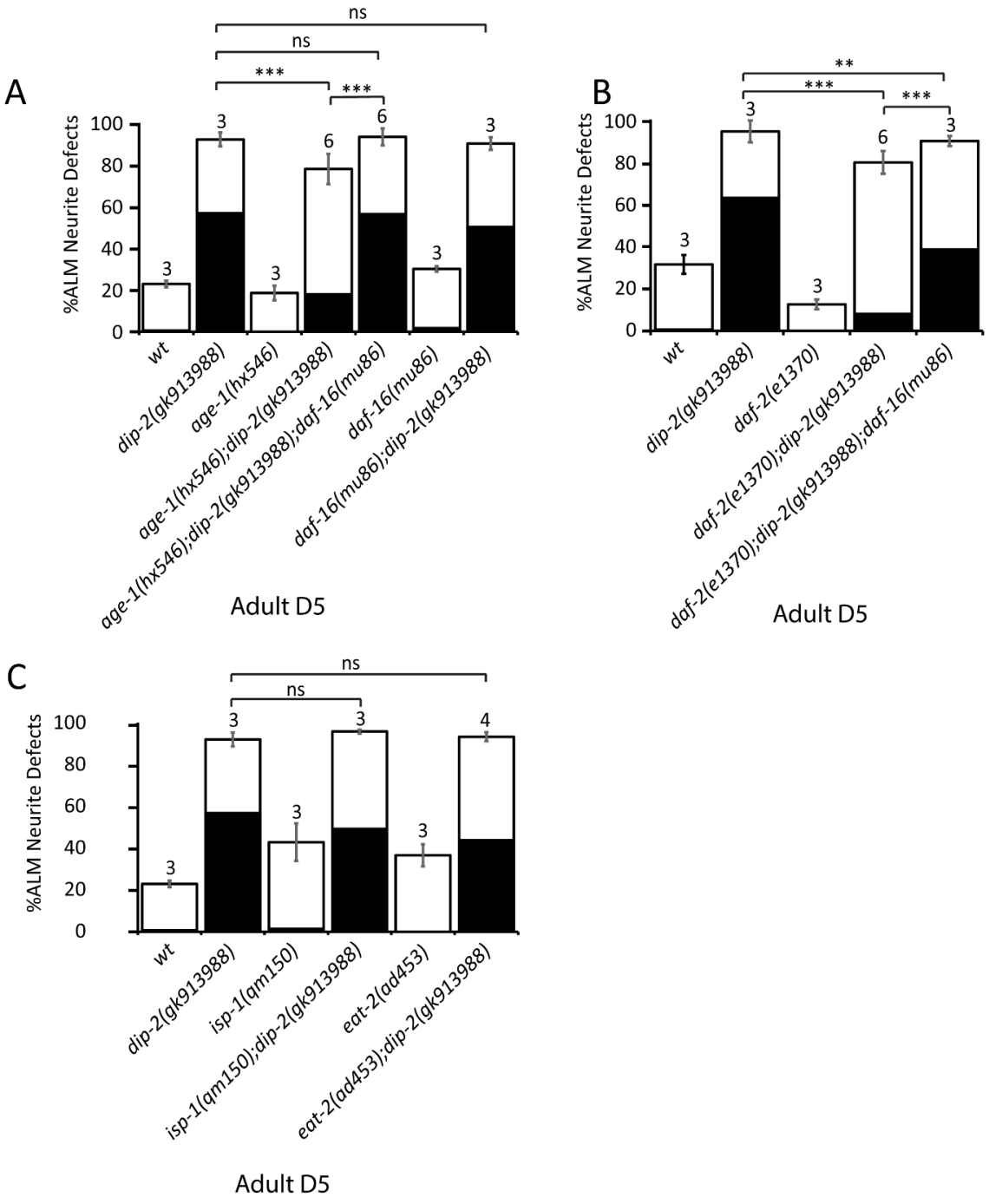

*Figure 11: Insulin Like Signaling (ILS) Pathway mutants are able to suppress the severe ectopic neurite defect of dip-2 mutants. (A) ALM ectopic sprouting at in strains grown at 25°C on day 5 of adulthood. (B) ALM ectopic sprouting at in temperature sensitive strains on day 5 of adulthood (C) ILS Independent aging mutants grown at 25°C on day 5 of adulthood. Error bars indicate SDs for n=3 trials. Significance of between the severe ectopic neurite defects (black bars) were compared using a one-way ANOVA with Tukey's test for multiple comparisons. **p<0.01, ***p<0.001.*

loss of IIS signaling results in lifespan extension, it was possible that the suppression of ectopic neurite branching in *daf-2; dip-2* and *age-1; dip-2* mutants may simply have been a secondary consequence of increased lifespan. If true, mutations in other genes that result in increased lifespan should also suppress *dip-2* ectopic neurite branching. *eat-2* encodes an ion channel that regulates pharyngeal muscle function (Raizen et al., 1995). Loss of *eat-2* results in a dietary restriction-mediated increase in lifespan (Lakowski and Hekimi, 1998). *isp-1* encodes a mitochondrial complex III protein, mutations in which result in increased lifespan (Feng et al., 2001). We found that ectopic neurite branching in *eat-2; dip-2* and *isp-1; dip-2* double mutants were not significantly different from *dip-2* single mutants, suggesting that lifespan extension by itself is not sufficient to supress ectopic neurite branching (Figure 11 C).

A genetic screen for new components of a DIP-2 or parallel pathway that regulate neuronal morphology

In the mechanosensory ALM neurons, *dip-2* mutants display an age-dependent increase in ectopic neurites and branching (Figure 12 A). Our work has identified DIP-2 as a new protein that maintains stereotypical neuronal morphology. In order to identify new factors that act in a common or parallel pathway with *dip-2* we carried out a forward genetic screen for mutants that displayed *dip-2*-like neuronal morphology defects (*nde*) mutants.

ALMs were screened for morphology defects in 3 day old *fem-3(hc17ts); zdIs5[mec-4p::GFP]* adults (see rationale for *fem-3* in Materials and Methods and screen

outline in Figure 12 A). A mutagenesis screen of approximately 7200 genomes identified 15 mutants that displayed strong ectopic neurite defects in the ALM neurons (Figure 12 B and C). Whole genome sequencing (WGS) was used to identify the relevant genes. WGS revealed multiple single nucleotide variants (SNVs) in each mutant. We therefore focused on SNVs that disrupted the same gene in multiple mutants hypothesizing that these are likely to represent bona fide regulators of neuronal morphology. As expected, we identified new *dip-2* mutants (2 alleles). We also identified multiple mutations that disrupted the HEAT/Armadillo domain containing protein SAX-2 (3 alleles), the microtubule minus-end binding protein PTRN-1 (3 alleles) and the β tubulin homolog MEC-7 (2 alleles), all of which have been previously shown to inhibit ectopic neurite formation in ALM (Zallen et al., 2000; Kirszenblat et al., 2013; Chuang et al., 2014). Of the 5 mutants that contained unique SNVs we took a candidate approach and examined existing mutations for ALM morphology defects. In this manner, we identified the tubulin-associated protein COEL-1 as a regulator of ALM morphology. A list of the 5 genes we identified in our genetic screen for ALM morphology defects is provided in Table 1.

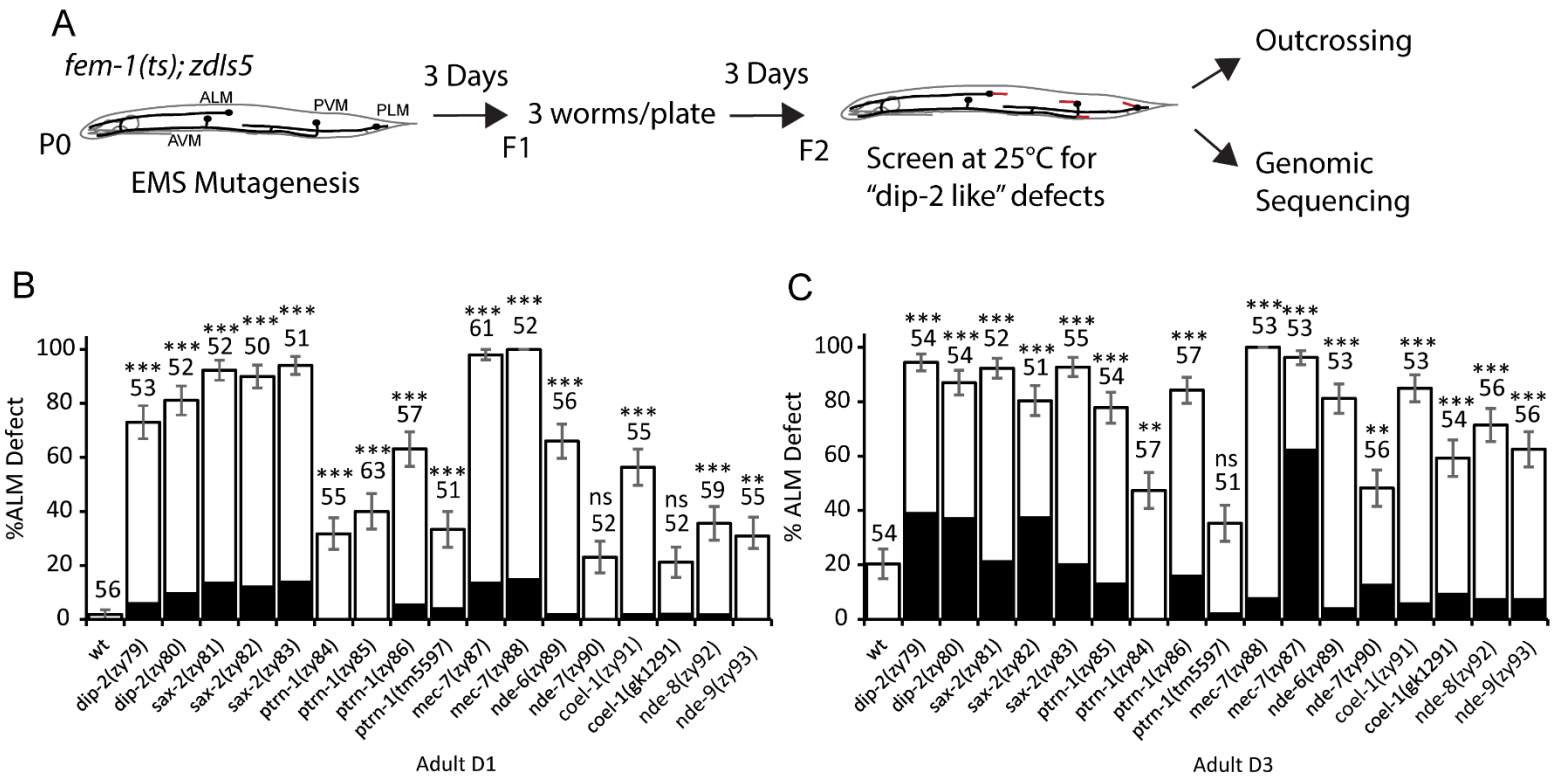

Figure *12: Neurite outgrowth defective (nde) mutants found in a forward mutagenesis screen for dip2-like phenotypes. (A) Schematic of the screen protocol (C) Quantification of ALM ectopic sprouting in nde mutants at 1 day of adulthood (D) Quantification of ALM ectopic sprouting in nde mutants at 3 days of adulthood. The number of worms in each population is indicated above the appropriate bar. Error bars indicate SEs of proportion for the indicated number of animals. Significance compared to wt using a one-way ANOVA with Tukey's test for multiple comparisons. **p<0.01, ***p<0.001.*

Table 1: Summary of the genetic screen for dip-2 like defects.

Gene	Alleles	Human Homolog	Identity
dip-2	2	DIP2A, B, C	Adenylate forming enzyme
sax-2	3	FRY	Furry homolog with HEAT/Armadillo repeats
ptrn-1	3	CAMSAP1, 2, 3	Microtubule minus-end binding protein
coel-1	1	TBCEL	Tubulin destabilizing protein
mec-7	2	TUBB	Beta tubulin
nde-6	1	Unknown	Unknown
nde-7	1	Unknown	Unknown
nde-8	1	Unknown	Unknown
nde-9	1	Unknown	Unknown

SAX-2/Furry and DIP-2 act in parallel to regulate neuronal morphology

Of the novel mutants identified in our genetic screen (Table 1), only mutations in *sax-2* and *mec-7* resulted in ALM morphology defects that were as severe as those in *dip-2* mutants (Figure 12 B and C). Neomorphic mutations have previously been shown to promote ectopic ALM neurite sprouting in *mec-7*/β-tubulin mutants via the incorporation of mutant β-tubulin subunits that result in the formation of hyperstabilized microtubules (Kirszenblat et al., 2013; Zheng et al., 2017). As our *mec-7* alleles are therefore likely to act downstream of DIP-2, we chose to characterize the genetic interaction between *dip-2* and *sax-2*.

SAX-2 and its orthologue Furry in *Drosophila* and mouse are key regulators of neuronal morphology and neurite growth (reviewed in Nagai and Mizuno, 2014). They have been shown to act in a common pathway with the NDR kinases SAX-1 and Tricornered in *C. elegans* and *Drosophila* respectively. To determine if *sax-2* acts in a common pathway with *dip-2* or if it acts in parallel, we made double mutants and assessed the penetrance of ALM morphology defects. If two genes act in a common pathway, the phenotypic penetrance in a double mutant should not be significantly different than that of the most severe single mutant. Conversely, if two genes act in parallel, the double mutant should show a significantly more severe phenotype (Huang and Sternberg, 2006).

We found that *sax-2(ot10); dip-2(gk913988)* double mutants showed significantly more severe ALM morphology defects than either single mutant (Figure 13

B). *sax-2(ot10); dip-2(gk913988)* double mutants displayed a fully penetrant ALM ectopic neurite defect (100%; p<0.01) compared to *dip-2(gk913988*) single mutants (73.6%). We also found that *sax-1(ky491); dip-2(gk913988)* double mutants also showed a significant increase in ALM ectopic neurites (92.2%) compared to *dip-2* (73.6%, P<0.05) or *sax-1* (44.4%, P<0.001) single mutants. These results indicate that a pathway containing *sax-1* and *sax-2* acts in parallel with *dip-2* to ensure the proper morphology of touch neurons

4. Discussion

In contrast to detailed knowledge of how neurons and neuronal circuits develop (Jan and Jan, 2010; Polleux and Snider, 2010; Yogev and Shen, 2017), the mechanisms that maintain neuronal morphology and circuitry over long animal lifespans remain poorly understood. The highly diverse morphologies of neurons contribute to proper connectivity and the processing of electrical signals underlying behaviour and memory (Jan and Jan, 2010; Mainen and Sejnowski, 1996). The maintenance of neuronal morphology during the normal course of aging is therefore of high importance. Indeed, several diseases have been linked with alteration to central nervous system architecture; including neurite growth from amyloid β plaques, ectopic branching in chronic epilepsy and neurite deterioration in Down Syndrome (Becker et al., 1986; Masliah et al., 1993; Represa et al., 1993).

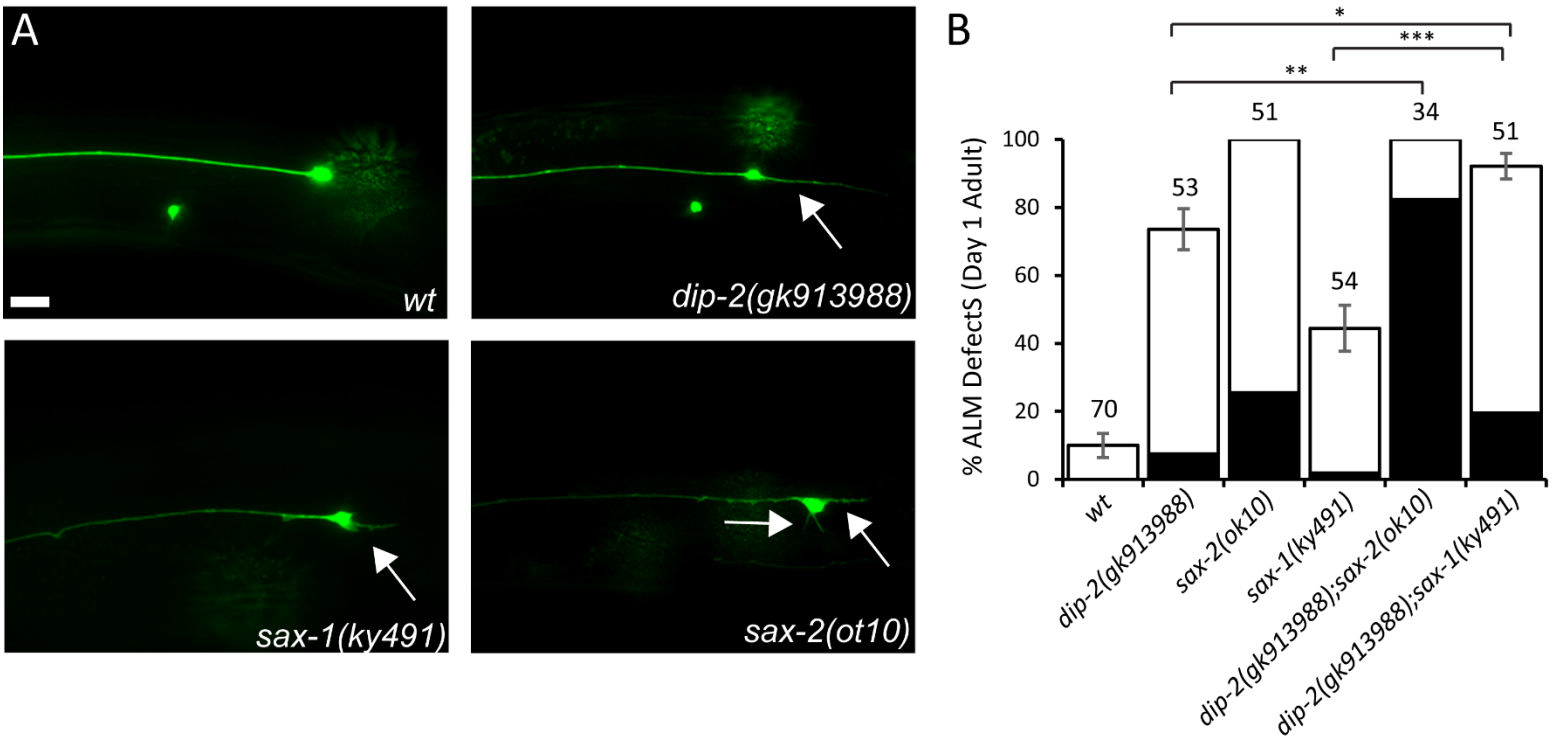

Figure *13: The SAX2/furry functions in parallel to DIP2. (A) ALM defects in sax-2(ot10) and sax-1(ky491) at day 1 resemble those in dip-2(gk913988) (B) Quantification of ALM ectopic sprouting in dip-2(gk913988), sax-2(ot10) and sax-1(ky491) mutants at 1 day of adulthood. The number of worms in each population is indicated above the appropriate bar. Error bars indicate SEs of proportion for the population indicated. Significance compared to dip-2(gk913988) using a one-way ANOVA with Tukey's test for multiple comparisons. *p<0.05, **p<0.01, ***p<0.001. Scale bar = 20um.*

DIP-2 is a conserved regulator of neuronal morphology

We found that *dip-2* mutants display highly penetrant defects in the morphology of several different types of neurons in *C. elegans*. Our work mirrors a recent study that implicated DIP2 in the regulation of stereotypical axon branching in *Drosophila* mushroom body neurons (Nitta et al., 2017). Interestingly, we also found that DIP-2 regulates axon branching in the PVM mechanosensory neuron. The PVM neuron normally extends an axon ventrally to the ventral nerve cord where it turns anteriorly to navigate to the head. Loss of *dip-2* results in the inappropriate bifurcation of the PVM axon at the ventral nerve cord such that axon branches extend both anteriorly and posteriorly. This is strikingly similar to the ectopic axon branching seen in dip2(-) mushroom body neurons (Nitta et al., 2017). These findings suggest a highly conserved role for DIP2 family proteins in the regulation of neuronal morphology.

DIP-2 acts to maintain neuronal morphology in *C. elegans*

Maintenance factors have been typically described in terms of their trophic-like activity to regulate axon and dendritic morphology (Chen et al., 2017; Emoto et al., 2006; Matter et al., 2009). Loss of these proteins, including the morphogen Wnt5a (Chen et al., 2017), the NDR kinase Warts and tumor suppressor Hippo (Emoto et al., 2006), result a failure to maintain proper dendritic arborization patterns. The dendrites of mutants develop normally, but progressively loose branches, during adulthood, leading to a decrease in arbour size and complexity. *dip-2* mutants also show a progressive increase in neuronal morphology defects although we see an increase

rather than a decrease in ectopic branches and neurites. The most prominent defect in *dip-2* mutants, however, is a progressive increase in ectopic neurite formation. Transient expression of DIP-2 in larval animals after most neurons have established normal morphology and connections is able to partially rescue neuronal morphology defects in *dip-2* mutants. This suggests that, post-developmentally, DIP-2 acts continuously to block ectopic neurite formation. Furthermore, overexpression of DIP-2 is able to suppress the normal neuronal morphology defects associated with normal aging. Our findings therefore show that DIP-2 maintains neuronal morphology by suppressing nascent neurite outgrowth in mature neurons; a role which is distinct from trophic-like factors that regulate the complexity of existing neuronal structures in adult animals (Chen et al., 2017; Emoto et al., 2006; Matter et al., 2009).

DIP-2 acts in the cytoplasm in an AFD dependent manner

DIP2 was initially identified in *Drosophila* as a binding partner of *disconnected (disco)*, a transcription factor implicated in axon guidance (Mukhopadhyay et al., 2002). However, there are no obvious *disconnected* homologs in *C. elegans*, and as we found that DIP-2 is not present in the nucleus, neuronal roles for DIP-2 likely do not involve interaction with transcription factors in *C. elegans*. Moreover, we found that DMAP1 binding domain-deleted DIP-2, which would be predicted not to interact with the transcriptional co-repressor DMAP1 and its binding partner DNA methyltransferase (Lee et al., 2010; Rountree et al., 2000), is able to rescue *dip-2(0)* neuronal morphology and migration defects, suggesting that DIP-2 is not involved in the epigenetic regulation of gene expression.

Drosophila and vertebrate DIP2 proteins are broadly expressed in the central nervous system (Zhang et al., 2015; Mukhopadhyay et al., 2002), however their subcellular localization in neurons had not been fully characterized prior to our study. We found that, in neurons, DIP-2 localized to the cytoplasm of axons and cell somas and was mostly excluded from nuclei. In contrast, DIP-2 localized to the membrane of embryonic epidermal cells, consistent with the identification of human DIP2A as a cell surface receptor in endothelial cells (Ouchi et al., 2010). Such differential subcellular localization of DIP-2 suggests that DIP-2 may function via divergent molecular mechanisms in cytosol versus membrane compartments.

In *Drosophila* loss of *DIP2* is associated with a decrease in acyl-CoA levels, consistent with DIP2 acting as an acyl-CoA synthetase (Nitta et al., 2017). AFD-containing enzymes such as acyl-CoA synthetases and luciferases can be cytoplasmic or membrane localized but AFD domains are invariably associated with cytoplasmic compartments (Coleman et al., 2002). Like Nitta et al. (2017) we also found that the AFD domains of DIP-2 were required for normal neuronal morphology. However, while AFD-deleted DIP-2 proteins did not affect localization in neurons, they showed partial or complete loss of membrane localization in embryonic epidermal cells. These findings are consistent with a cytosolic site of action and an AFD-dependent acyl CoA synthetase role for DIP-2 in neuronal development, as proposed in *Drosophila*, versus a possible membrane site of action in non-neuronal cells.

DIP-2 may function alongside the IIS pathway to maintain neuronal morphology

We have shown that ALM ectopic neurite defects in *dip-2* mutants can be supressed by loss-of-function mutations in *daf-2/*IGF receptor or *age-1/*PI3K, key components of the Insulin/IGF-like signaling (IIS) pathway. This suppression results from the upregulation of the transcription factor DAF-16/FOXO, which is normally inhibited by insulin signalling through DAF-2 and AGE-1 (Lapierre and Hansen, 2012). Consistent with this hypothesis, triple mutants bearing a *daf-16* mutation prevent the suppression of *dip-2* ectopic neurite defects in either *age-1* or *daf-2* backgrounds. These results are consistent with previous studies which show that loss of insulin signalling can supress the progressive increase in neuronal morphology defects that spontaneously occur during the normal course of aging (Pan et al., 2011; Tank et al., 2011; Toth et al., 2012).

DAF-16/FOXO proteins are known to regulate a wide range of downstream targets (Kaletsky et al, 2015; Lee et al., 2003; reviewed in Tullet, 2015). Many of these effectors are responsible for the significant role of the IIS pathway in regulating lifespan (Dorman et al., 1995). One possibility is that the suppression of ectopic neurite defects in *daf-2; dip-2* and *age-1; dip-2* double mutants is a non-specific effect of increased lifespans caused by disruption of the IIS pathway. However, neuronal aging has been shown to be genetically separable and independent from aging of the organism as a whole (Libina et al., 2003; Tank et al., 2011). Mirroring these studies, we show that lifespan extension through IIS-independent mechanisms, such as dietary restriction (*eat-2* mutants) or reduced mitochondrial respiration (*isp-1* mutants), is not sufficient to rescue neuronal morphology defects.

DIP-2 may regulate microtubules to maintain neuronal morphology

The results of our genetic screen for mutants with *dip-2*-like ectopic neurite defects identified microtubule (MT) components (MEC-7/β-tubulin), MT binding proteins (COEL-1, PTRN-1/CAMSAP) or known MT regulators (SAX-2/Furry). This suggests that DIP-2 may suppress ectopic neurite formation by regulating MTs. Indeed, regulation of MT dynamics play a key role in neurite outgrowth during development (Schelski and Bradke, 2017). The *mec-7* mutations from our screen are likely to be neomorphic alleles; as these have already been shown to cause ectopic neurite formation as the mutant β-tubulins promote the formation of hyperstabilized MTs (Kirszenblat et al., 2013; Zheng et al., 2017). Loss of the microtubule associated protein COEL-1, which depolymerizes tubulin dimers and promotes their turnover, also leads to an increase in MT stability (Bartolini et al., 2005; Frédéric et al., 2013; Neumann and Hilliard, 2014). MT depolymerisation and regulation of turnover might also act to prevent microtubule array fragmentation, which is known to promote collateral branching during development (Yu et al., 1994). Loss of proteins that promote MT stability can also lead to ectopic neurite growth (Chuang et al., 2014). PTRN-1 acts as a minus-end binding protein that binds and stabilizes microtubules, limiting the amount of dynamic microtubules that are often associated with neurite outgrowth. If DIP-2 acts upstream of MTs to maintain neuronal morphology, its role may be either positive or negative as dysregulation of both microtubule stabilizing and destabilizing proteins can result in ectopic neurite formation.

Our screen also identified *dip-2*-like neuronal morphology defects in *sax-2* mutants. SAX-2, along with the NDR kinase SAX-1, has been previously implicated in

regulating neuronal cell shape, dendritic tiling and neurite initiation and termination in *C. elegans* (Gallegos and Bargmann, 2004; Zallen et al., 2000). SAX-2 and SAX-1 are homologues of Furry and Tricornered in *Drosophila* respectively where they were first shown to regulate neuronal morphology (Emoto et al., 2004). Furry family proteins have been identified as MT regulating factors. For example, in human HeLa cells, Furry has been shown to inhibit spindle microtubule deacetylation by the tubulin deacetylase SIRT2 (Nagai et al., 2013). While the direct effect of acetylation of microtubule stability remains unclear, past studies have demonstrated that tubulin-protein interactions facilitated by acetylation may in fact lead to increased stability (Palazzo et al., 2003; Takemura et al., 1992). We found that *dip-2* and *sax-2* act in parallel to regulate neuronal morphology. Overall, the findings of our genetic screen are consistent with DIP-2 regulating MT dynamics in order to maintain neuronal morphology.

Future Directions

In conclusion, we have described a novel role for the DMAP1 binding and adenylate forming domain (AFD) protein DIP-2 (Disco Interacting Protein 2) in maintaining neuronal morphology in *C. elegans*. *dip-2* mutants display a progressive increase in ectopic neurite sprouting and branching during late larval and adult worms. Our study showed that DIP-2 acts in a DMAP1 binding-domain-independent manner to maintain neuronal morphology post-developmentally, and that DIP-2 overexpression can suppress the normal progressive neuronal sprouting observed in aging worms. Further research however, will be required to determine the molecular mechanisms involved in DIP-2-mediated maintenance of neuronal morphology.

Our genetic screen suggested that DIP-2 may function by regulating MTs. It will therefore be important to compare MT dynamics in wild type and *dip-2* mutants using fluorescently labeled plus end tracking proteins (Garrison et al., 2012). Additionally, the use of a mass spectrometry (MS)-based proteomic approach (reviewed in Smits and Vermeulen, 2016) to identify proteins that interact directly or in proximity to DIP-2 would nicely complement genetic approaches to elucidating DIP-2 signaling pathways. Finally, it will be interesting to determine if mammalian DIP2 family members also play a role in maintaining neuronal morphology in adult animals.

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