NEJIRE, A CBP/P300 FAMILY TRANSCRIPTION FACTOR, REGULATES DENDRITIC DEVELOPMENT BY MODULATING THE LOCALIZATION OF THE KRÜPPEL-LIKE TRANSCRIPTION FACTOR DAR1 IN *DROSOPHILA* SENSORY NEURONS

by

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Nejire, a CBP/p300 Family Transcription Factor, Regulates Dendritic Development by Modulating the Localization of the Krüppel-like Transcription Factor Dar1 in *Drosophila* Sensory Neurons

A thesis submitted in partial fulfillment of the requirements for the degree of Master of Science at George Mason University

By

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Bachelor of Science
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DEDICATION

- Mrs. Thakshayini Shivanandarajah (mummy) who dedicated her whole life raising me.
- My parents Ranjini Rubaharan & Mylvaganam Rubaharan for providing me with opportunities to excel in any shape or form.
- Mr & Mrs Mohanaharan Mylvaganam for providing a stable base to build myself in USA and funding my education at GMU.
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- Vidiyan Jeyanandarajah, Harendra Puviharan and all my cousins.
- Boddu & Sullivan family members for making me part of their family.
- My English teachers Mrs. Valli Somasundaram & Mrs. Shyami Thevathasan
- All my teachers and mentors
- All of my friends

"மறவர்க மாசற்றார் கேண்மை துறவற்க துன்பத்துள் துப்பாயார் நட்பு"-We should not forget the benevolence of those who favored us in trouble and should not forget the friendship of those who were with you in your times of sorrow - Thirukkural 106, Chapter 11: Gratitude.

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ABSTRACT

NEJIRE, A CBP/P300 FAMILY TRANSCRIPTION FACTOR, REGULATES DENDRITIC DEVELOPMENT BY MODULATING THE LOCALIZATION OF THE KRÜPPEL-LIKE TRANSCRIPTION FACTOR DAR1 IN *DROSOPHILA* SENSORY NEURONS

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Dendrite morphogenesis represents a critical process in the establishment, maintenance and modulation of neural connectivity that is the basis of a functional nervous system. Dendrites, as the primary sites of synaptic and/or sensory input largely determine the size and nature of the neuronal receptive field. The *Drosophila melanogaster* peripheral nervous system (PNS) has emerged as an excellent model system for studying molecular mechanisms underlying class specific dendrite development. Dendritic arborization (da) neurons are grouped into four distinct classes (I-IV) based upon increasing orders of dendritic complexity (Grueber et al., 2002). A recent study has identified *dar1*, a Krüppel-like transcription factor, as an essential regulator

involved in controlling dendrite development and growth via microtubule modulation (Ye et al., 2011). Interestingly, at the embryonic stage Dar1 protein exhibits nuclear localization in all da neuron subclasses, however at the third instar larval stage of development, Dar1 exhibits a subcellular shift towards class-specific differential localization. Specifically, Dar1 is primarily nuclear in the morphologically simple class I neurons, in contrast to largely cytoplasmic localization in the highly complex class IV da neurons. This observation led us to investigate putative protein interaction partners of Dar1 that potentially regulate this class specific differential localization, and the result of perturbing this localization. We conducted a pilot RNAi screen of putative Dar1-interacting proteins to investigate their potential mechanistic role(s) in dendrite development and Dar1 differential localization in class IV da neurons. From this pilot screen, we identified the CBP/p300 homolog *nejire* (*nej*) as a novel regulator of dendritic development that also modulates Dar1 subcellular localization. We have conducted detailed structure-function studies using domain-specific deletions of *nej* that have provided further insights into the specific role of different protein domains in mediating distinct aspects of dendritic growth. Furthermore, we have used these domain-specific deletion constructs to elucidate the interaction between Dar1 and Nej. Collectively, these analyses contribute to our understanding of molecular mechanisms of combinatorial transcription factor activity at a class-specific level and how this regulation contributes to specification of distinct neuronal morphologies that underlie the establishment of complex neural networks.

CHAPTER ONE: INTRODUCTION

Dendrites are the primary site of synaptic or sensory input and integration in the developing nervous system. The stereotypic branching pattern of a neuron determines the number and type of synaptic inputs that a neuron can receive and respond to. Moreover, strong neuroanatomical correlates between dendrite abnormalities and neurological disease states have been illustrated in Down's, Rett and Fragile X syndromes, among others (Jan and Jan, 2010). In addition, abnormalities in dendrite spine development and synaptic organization are observed in a broad spectrum of neurodegenerative diseases (Yu and Lu, 2012). Thus, while dendritic field specification is critical in the formation of functional neural networks, much remains unknown regarding the molecular and genetic mechanisms that govern this process. The dendritic arborization (da) sensory neurons of the *Drosophila* PNS fall into four distinct morphological classes (I–IV), that range from simple, restricted dendritic arbors (Class I, II) to complex, space-filling arbors (Class III, IV) thus providing a complete spectrum of dendritic branching complexity (Grueber et al., 2002). Given their accessibility to live imaging and the powerful genetic tools available for high-resolution manipulation of gene function, da neurons provide an

excellent genetic and morphological model system to investigate the molecular mechanisms governing class specific dendrite morphogenesis.

1.1 Transcriptional Regulation of Dendritic Development

Transcription factors have been shown to play critical roles in regulating class specific dendritic morphologies (Jan and Jan, 2010; Tavosanis, 2014; Singhania and Grueber, 2014; Iyer et al., 2013a). Modifications in levels of a transcription factor or its location can alter dendritic arbors of neurons. Moreover, the phenotypic effects are often class specific. For example, the homeodomain protein Cut is one well-studied transcription factor that is differentially expressed in each of the da neuron subclasses leading each class to exhibit distinct patterns of Cut-mediated gene expression and regulation that ultimately impact class specific dendritic morphologies (Grueber et al., 2003; Sulkowski et al., 2011; Iyer et al., 2012; Iyer et al., 2013b). Dar1 (Dendritic arbor reduction 1) is a Krüppel-like transcription factor that controls dendritic growth, promoting microtubule based dendritic branching and growth. Lack of *dar1* function reduces microtubule based dendritic branches in da neurons (Ye et al., 2011; Wang and Ye, 2012).

1.2 Importance of Nejire/CBP in the nervous system

Phosphorylation of CREB (cAMP response element binding protein) recruits CBP (CREB binding protein; encoded by *nejire* in *Drosophila*) or its paralog p300, thereby increasing CREB transcriptional activity (Cortés-Mendoza et al., 2013). In vertebrates,

CREB is associated with controlling neuronal activity dependent dendritic development (Wayman et al., 2006; Redmond et al., 2002). Moreover, CBP interacts with CREST (calcium-responsive transactivator) to mediate calcium-dependent dendritic growth and is physically associated with the neuron-specific BRG1-associated factor (nBAF) which mediates chromatin remodeling complexes involved in specification of distinct neuronal subclasses from neural progenitors (Wu et al., 2007; Jan and Jan, 2010).

CBP is a large multi-domain protein and mutations in CBP have been linked to the development of Rubinstein-Taybi Syndrome in humans (Kumar et al., 2004). Moreover CBP harbors a histone acetyltransferase (HAT) domain and histone acetylation has been shown to have therapeutic potential in treatment of various neurodegenerative disorders (Valor et al., 2013). In *Drosophila*, recent studies demonstrated that Nej promotes dendritic pruning in class IV da neurons as knockdown of *nej* causes severe pruning defects (Kirilly et al., 2011).

1.3 Nejire/CBP functional domains

Nej/CBP encodes a multi-domain protein which includes a nuclear hormone receptor (NHR) domain, multiple zinc finger domains, a KIX domain that binds to CREB, a Bromodomain that binds to acetylated lysine residues and a polyglutamine (polyQ) domain that is associated with transcriptional activation **(Fig.1)**. The histone acetyltransferase (HAT) domain in CBP participates in epigenetic modification and regulation of gene expression and has been linked to neurogenesis (Chatterjee et al., 2013). The polyQ domain serves as a transactivation domain for transcriptional activation

of downstream target molecules. The NHR domain binds to nuclear hormone receptors and promotes CBP function as an integrator of multiple signal transduction pathways within the nucleus (Kamei et al., 1996). The zinc finger protein domains function in DNA recognition, lipid binding, and transcriptional activation/regulation (Laity et al., 2001). Each of these domains is implicated in important roles in signaling and transcription, however their respective functional roles in maintaining dendrite morphology remain poorly understood.

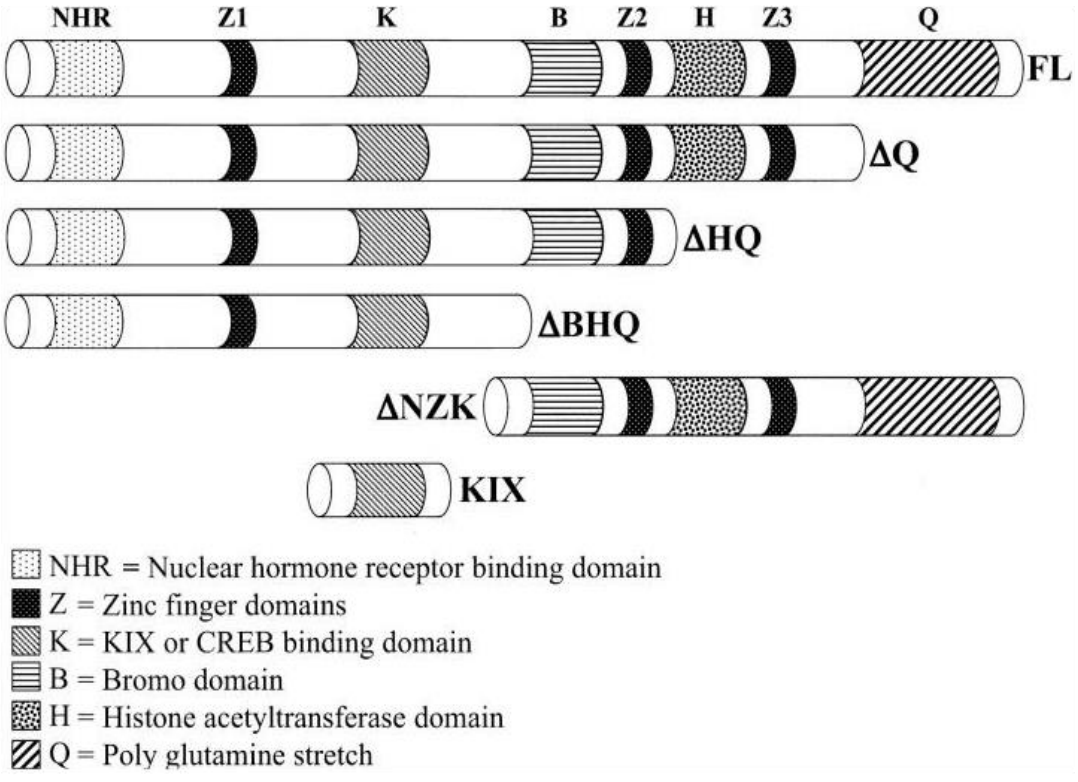

Figure 1: Schematic of Nejire/CBP domain organization and structure-function variants. Nejire variants were expressed using the GAL4/UAS system. Adapted from Kumar et al. (2004). *UAS-CBP ΔH* received from Bloomington Stock Center.

1.4 Significance of studying the *Drosophila* Peripheral Nervous System (PNS)

The PNS da neurons are classified based on the dendritic branching patterns and the targets they innervate as they give rise to multiple dendrites in late embryogenesis. Since these neurons are located below the transparent cuticle, da sensory neurons provide an excellent model system to study neuronal morphogenesis in live preparations. Each larval hemisegment is comprised of 15 da neurons and class-specific reporter strains have been developed to facilitate *in vivo* imaging of dendrite morphogenesis with a high degree of spatial and temporal resolution (**Fig. 2**) via the *GAL4/UAS* binary expression system (**Fig. 3**). *Drosophila* da neurons are classified into four distinct morphological subclasses, Class I-IV which are reflective of the increasing orders of dendritic branching complexity (**Fig. 4**) (Grueber et al., 2002). Class I and II da neurons display relatively simple dendritic branching architecture compared to classes III and IV exhibit complex dendritic arbors and dendritic tiling for receptive field specification/organization. Functional roles and mutations of many genes have been studied using this model to understand dendritic development. Neurodegenerative and neurological disease associated genes and its mechanisms have been identified via the *Drosophila* PNS system (Lu et al., 2009).

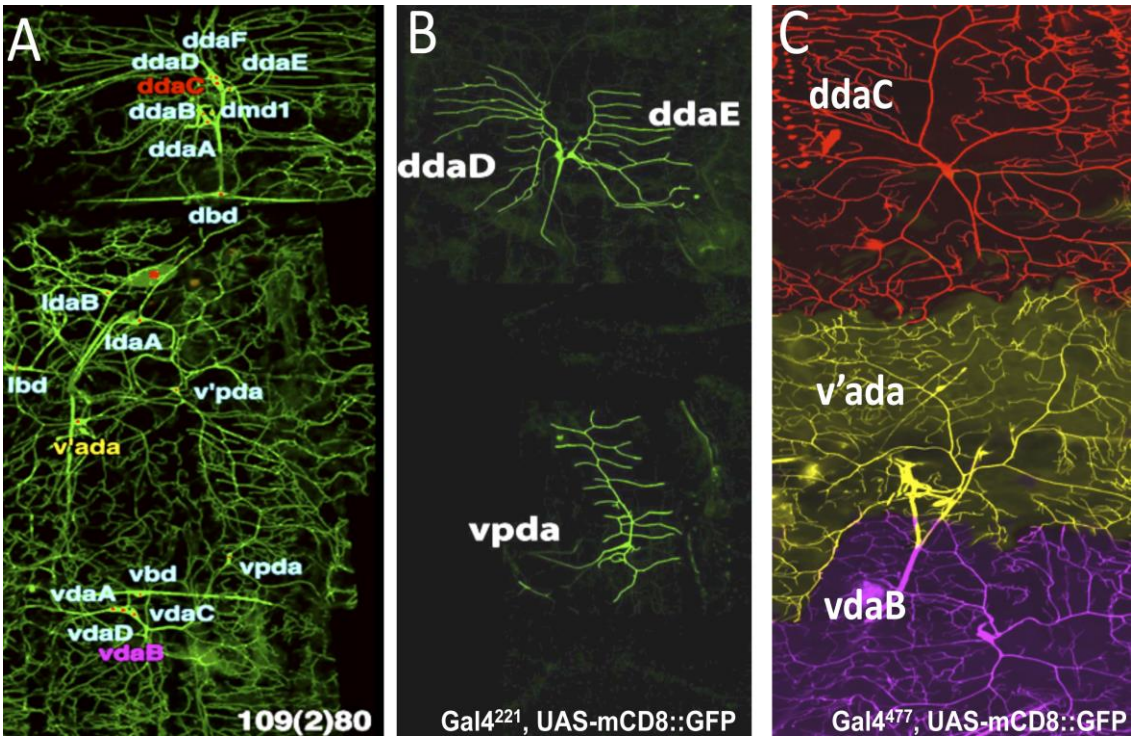

Figure 2: The *Drosophila* PNS as a model to study dendrite development (A) Representative image of the complete set of da neurons driven by *GAL4*$^{109(2)80}$, *UAS-mCD8::GFP* and co-labeled with *UAS-nls-RedStinger* to visualize nuclei. **(B)** Representative image of Class I neurons driven by *GAL4*^{221}, *UAS-mCD8::GFP*. **(C)** Representative image of Class IV neurons driven by *GAL4*^{477}, *UAS-mCD8::GFP* (pseudo-colored to highlight individual neurons). Panel A is adapted from Hughes and Thomas (2007).

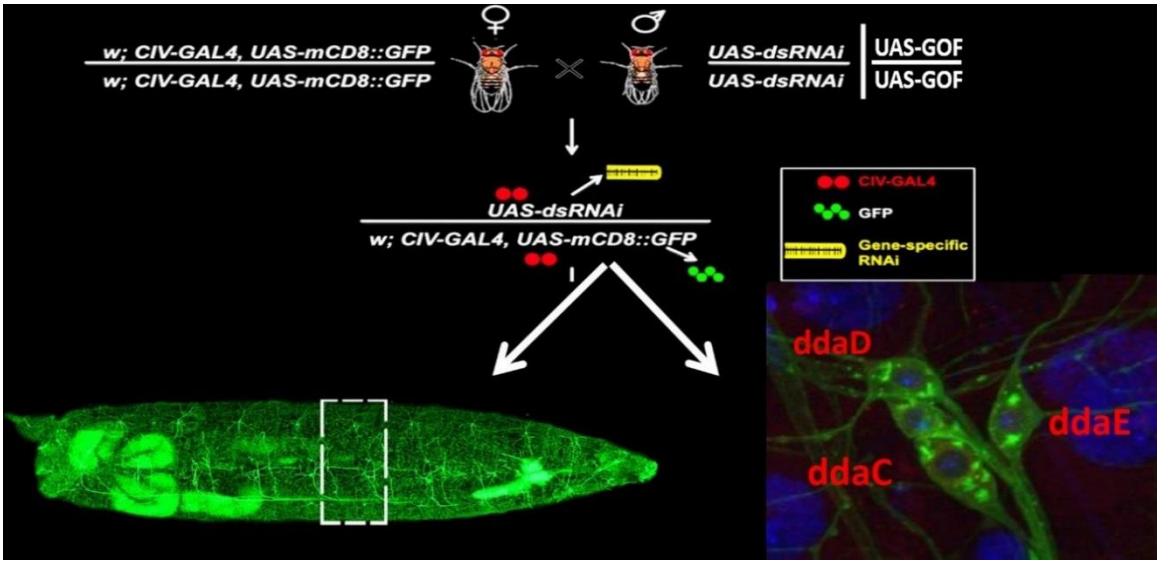

Figure 3: Schematic of *GAL4-UAS* binary expression system in *Drosophila* for high throughput phenotypic analyses of class IV da neuron dendrite morphogenesis. Fly with a *UAS* responder (*e.g. UAS-GFP; UAS-dsRNAi; UAS-GOF*) is mated to a fly carrying a *GAL4* driver resulting in progeny containing both elements of the system. This method of gene expression leads to the activation of the *UAS* responder in a tissue and cell-type specific manner based upon promoter-driven expression of the *GAL4* transcriptional activator. GAL4 drivers used in this study include $GAL4^{221}$which expresses in Class I da neurons (Grueber et al., 2003) and $GAL4^{477}$ / *ppk1.9-GAL4*, which expresses in Class IV da neurons (Iyer et al., 2013a).

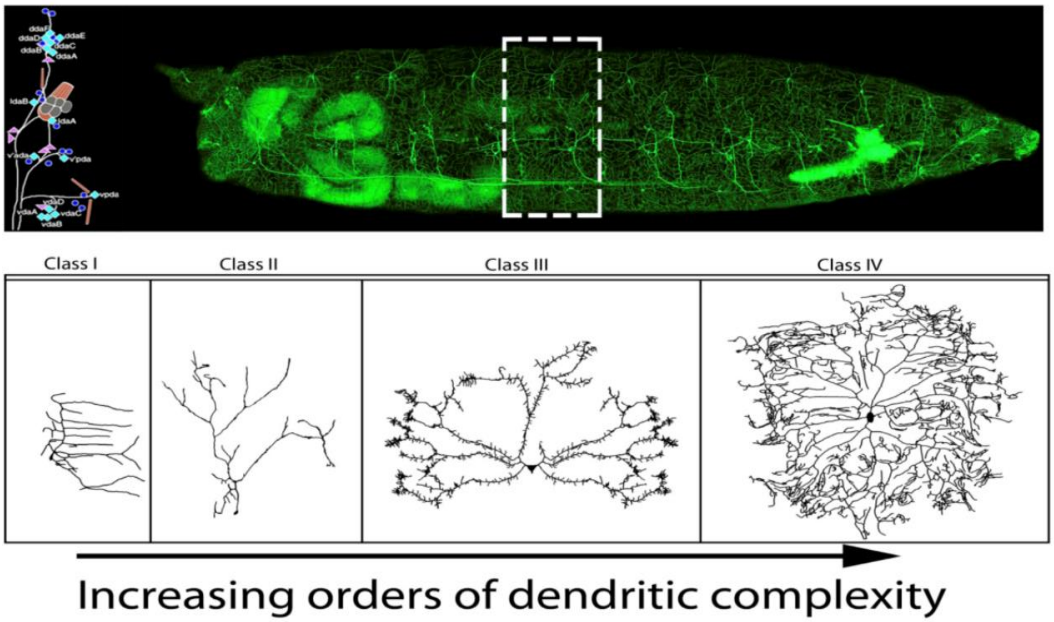

Figure 4. *Drosophila* da neurons. (**Top**) Lateral view of larva with da neurons labeled with GFP. Schematic to the left is depicting a single abdominal hemisegment in which da neurons are represented as diamonds. (**Bottom**) Representative traced images of Class I-IV da neurons showing class-specific characteristic dendritic morphologies. Representative traced images are to scale.

1.5 Preliminary Studies

We conducted a genetic screen to identify potential transcription factors that exert differential control on class-specific dendrite morphogenesis (Iyer et al., 2013a and Cox Lab, unpublished results). From this screen, we identified *CG12029* as a key mediator of class IV dendrite development. While we were conducting our detailed analyses of this

gene, another study was published by Ye et al. (2011) which identified the same gene and named it *dar1* (dendritic arbor reduction 1). This study reported that *dar1* displayed severe defects in dendritic but not axonal growth, and that Dar1 encodes a Krüppel-like transcription factor corresponding to *CG12029*. Moreover, this study demonstrated that Dar1 has a cell autonomous in mediating microtubule-based dendritic growth, but had no effect on actin-based dendritic branches and that Dar1 differentially regulates the cytoskeleton through suppression of the microtubule severing protein Spastin (Ye et al., 2011). Consistent with this study, we likewise observed severe cell autonomous deficits in *dar1* mutant dendrite development via MARCM (Mosaic Analysis with a Repressible Cell Marker) analyses (Wu and Luo, 2006) (**Fig. 5C,E,F**) and observed impaired class IV dendritic growth and branching even in $dar1^{D6}$ heterozygotes (**Fig. 5B**). Moreover, *dar1* MARCM analyses in class I and III da neurons, likewise revealed highly significant disruptions in dendritic branching and growth which are consistent with data previously reported (Ye et al., 2011) (**Fig. 6**) The $dar1^{D6}$ allele carries a nonsense mutation resulting in a premature stop codon after approximately two-thirds of the protein coding sequence, resulting in a truncated protein product that deletes the Dar1 nuclear localization (NLS) sequence and three tandem zinc finger domains which occur at the C-terminal end of the molecule (Ye et al., 2011).

To further explore the role of Dar1 in mediating dendrite morphogenesis, we constructed Dar1 overexpression transgenes. Interestingly, we observed that Dar1 overexpression in class IV neurons revealed a highly significant reduction in both dendritic branching and growth, as well as a qualitative reduction in dendritic field

coverage (**Fig. 5D,G,H**). These findings are in sharp contrast to the previous study which reported that Dar1 overexpression led to dendritic overgrowth in class IV neurons (Ye et al., 2011). In both studies a full length Dar1 cDNA was cloned into a pUAST vector and independent transformant lines were produced. As such, the basis for the phenotypic differences observed between studies with respect to dendritic development upon Dar1 overexpression is unclear, but they could potentially be due to position effect variegation which can impact the level of expressivity for different *UAS-dar1* transgene insertions. Collectively, Loss of Function (LOF) and Gain of Function (GOF) analyses reveal that proper regulation of Dar1 expression is critical for maintaining class specific homeostatic dendritic growth, branching and field coverage.

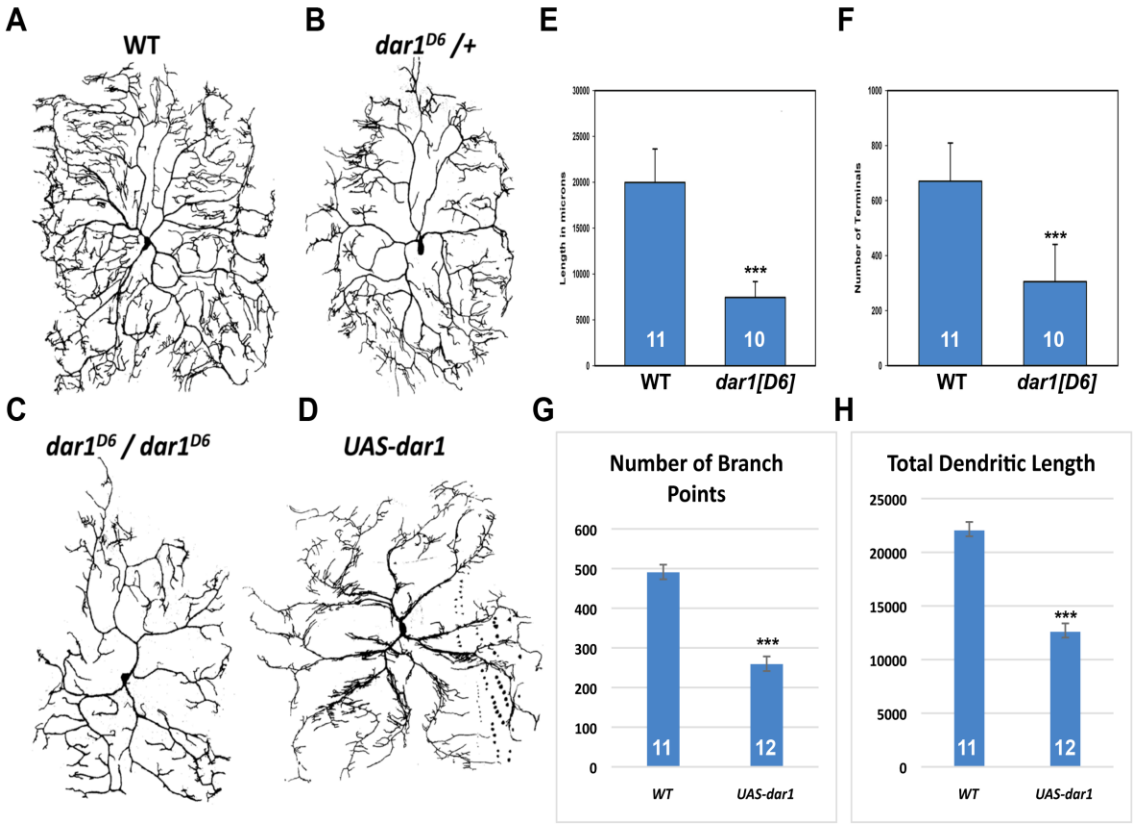

Figure 5: Dar1 is essential for mediating da neuron dendritic growth and branching. (**A-D**) Representative live confocal images of class IV ddaC neurons in various genetic backgrounds. Relative to wild-type MARCM clones (**A**), *dar1*^{D6} heterozygotes (**B**) and homozygous MARCM clones (**C**) exhibit severe reductions in dendritic growth (**E**) and branching complexity (**F**). (**D**) Dar1 overexpression in class IV neurons likewise results in a dramatic reduction in dendritic field coverage, as well as significant impairment in dendritic branching (**G**) and growth (**H**). Quantitative data is presented as mean ± SD (**E,F**) or mean ± SEM (**G,H**), n values are represented on the bar graph and statistical significance is indicated as follows: ***=p<0.001.

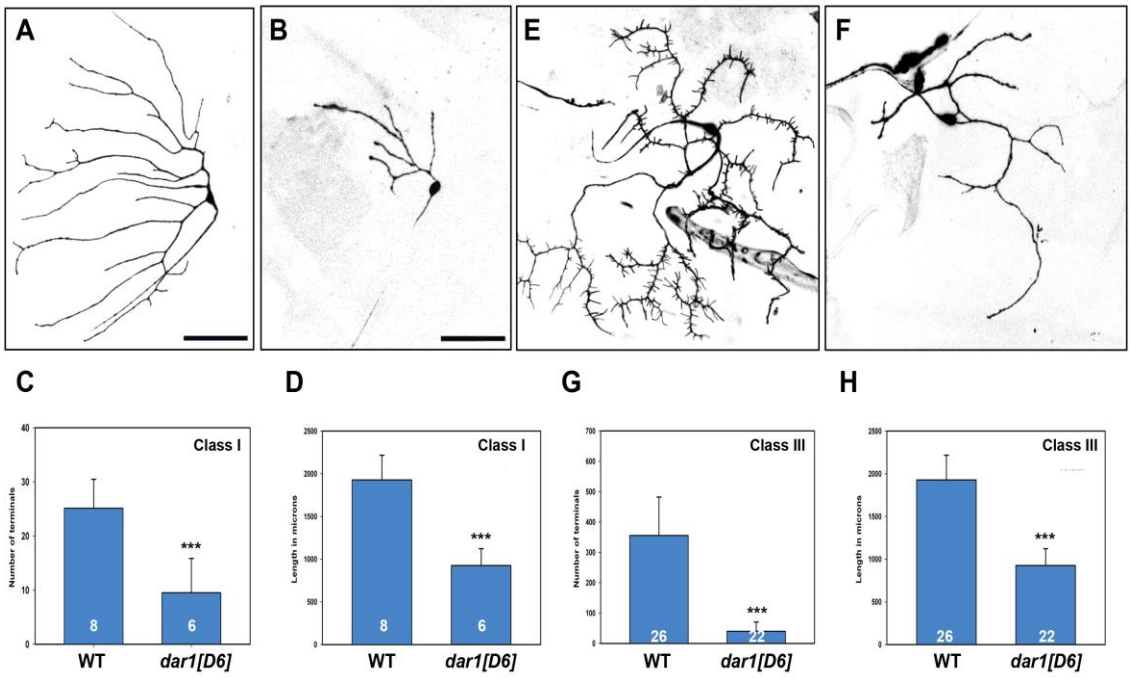

Figure 6: Dar1 functions cell-autonomously to promote dendritic growth and branching. (**A,B,E,F**) Representative live confocal images of class I ddaD (**A,B**) and class III vdaD (**E,F**) MARCM clones in wild-type (**A,E**) and *dar1*^{D6} mutant (**E,F**) genetic backgrounds. Relative to wild-type MARCM clones (**A,E**), *dar1*^{D6} homozygous MARCM clones (**B,F**) exhibit severe reductions in dendritic branching complexity (**C,G**) and growth (**D,H**). Quantitative data is presented as mean ± SD (**C,D,G,H**) with the respective n values presented on the bar graphs and ***=$p<0.001$. Size bar represents 50 microns.

Specific Aims:

AIM I: Investigate putative Dar1 protein interactor Nejire's role in mediating proper da neuron dendrite morphogenesis.

Hypothesis: Based on the preliminary phenotypic analyses, *nej* plays a critical role in mediating class specific dendrite morphogenesis.

Approach: We will use *nejire* targeted loss of function and gain of function genetic screen to study the effect of misexpression or overexpression on dendritic development using class specific *GAL4* drivers. Using confocal microscopy, *in vivo* phenotypic analysis will be conducted by live imaging. The images obtained will be analyzed via a semi-automated pipeline for digital reconstruction and quantitative analyses of dendrite morphology.

AIM II: Investigate the role of Nej in mediating class specific Dar1 differential localization, and the result of perturbing this localization.

Hypothesis: Preliminary studies show Dar1 localization in the nucleus in embryonic stage and in larval stages Dar1 shows differential localization in the cytoplasm in the complex class IV neurons and in the nucleus in the simple class I neurons. We

hypothesize that disruption in Dar1 protein interactors can cause Dar1 localization changes in da neurons.

Approach: We will conduct immunohistochemical experiments on RNAi based knockdown of putative protein interactors crossed with *GAL4* ^{217}, *UAS-mCD8::GFP* to analyze the localization changes to Dar1 in class I and IV da neurons. The images will be analyzed by pixel densitometry of total Dar1/Nej expression vs. nuclear expression.

AIM III: Investigating the role of Nejire functional domains and their contribution to proper dendritic morphogenesis via Nejire-mediated modulation of Dar1 localization.

Hypothesis: Nejire protein domains play different roles in Dar1 localization and disruptions to these domains will lead to defects in dendritic branching and growth.

Approach: We will conduct immunohistochemical experiments on *nejire* structure-function mutants crossed with *GAL4* ^{477}, *UAS-mCD8::GFP* to analyze potential subcellular localization changes to Dar1 and/or Nej in class IV da neurons. The images will be analyzed by pixel densitometry of total Dar1/Nej expression vs. nuclear expression. We will also use *nejire* structure-function mutants to analyze dendritic development using class specific *GAL4/UAS* drivers. Using confocal microscopy, *in vivo* phenotypic analysis will be conducted by live imaging. The images obtained will be

analyzed via a semi-automated pipeline for digital reconstruction and quantitative analyses of dendrite morphology.

CHAPTER 2: EXPERIMENTAL PROCEDURES

Drosophila strains and culture

The *Drosophila* strains used in these studies are as follows:

GAL4 driver stocks include: (1) *yw; +; GAL4^{221},UAS-mCD8::GFP* (Iyer et al.., 2013); (2) *w*; *GAL4^{477},UAS-mCD8::GFP/CyO-GAL80; ppk1.9-GAL4,UASmCD8::GFP*; (3) *GAL4^{477},UAS-mCD8::GFP*; (4) *GAL4$^{21\text{-}7}$,UAS-mCD8::GFP* (Iyer et al.., 2013).

For loss-of-function analyses, *dar1^{D6}* (Ye et al., 2011; Cox Lab unpublished results) and *dar1^{f01014}* were used. For MARCM studies, the *dar1^{D6}* allele was recombined onto the *FRT^{G13}* chromosome and analyses were performed as previously described (Sulkowski et al., 2011). Third instar larvae bearing GFP labeled neurons were subjected to live image confocal microscopy. For overexpression studies, we constructed a *UAS-dar1* transgene by cloning full length *dar1* cDNA into a FLAG-tagged *pUAST* vector. Transformations were performed by BestGene.

For the Dar1-interactor screen the following *UAS-RNAi* lines were used from Bloomington Stock Center (BSC): *Kap-α1* (JF02673, BSC 27523); *fd68A* (JF02827, BSC 27994); *trc* (JF0261, BSC 28326); *cpo* (JF02996, BSC 28360); *Rel* (HM05154, BSC 28943); *Nipped-A* (JF01196, BSC 31255); *Art1* (JF01306, BSC 31348); *ran* (JF01381, BSC 31392); *nej* (HM04037, BSC 31728 and JF02806, BSC 27724);

CG32105 (JF02194, BSC 31905); *CycB3* (HMS02377, BSC 6628); and *Ranbp21/Exportin-5* (GD7541) from Vienna Drosophila Resource Center (VDRC).

To understand the role of *nejire* in controlling dendrite morphogenesis the following lines were used: w *[*] P {w [+mGS] =GSV1} nej[s-20]* (BSC 43487), *nej RNAi* (TriP line: JF02806/HM04037), and *w [*]; P {w [+mC] =UAS-nej.wt-V5}3* (BSC 32573).

To investigate the role of functional domains of *nejire* the following domain deletion lines were used: *UAS-CBP ΔNZK; UAS-CBP ΔQ; UAS-CBP ΔHQ; UAS-CBP ΔBHQ; UAS-CBP KIX* (Kumar et al., 2004) and *UAS-CBP ΔH* (Bloomington stock center)

Oregon-R was used as a wild-type strain (control). *Drosophila* stocks were raised on standard cornmeal-molasses-agar fly food medium at 25℃, unless otherwise noted.

IHC and live image confocal microscopy

Dissection, staining, mounting, and confocal imaging of third instar larval filets were performed as previously described (Sulkowski et al., 2011). Primary antibodies used in these studies include: guinea pig anti-CBP (1:800) (gift of M. Mannervik), rabbit anti-Dar1 (1:200) (gift of J. Kassis); anti-HRP (1:800) (Jackson Immunoresearch, West Grove, PA, USA). Secondary antibodies include donkey anti-rabbit and donkey anti-guinea pig (AlexaFluor 488, 547/555/647; 1:200; Life Technologies). DAPI was used to visualize nuclei at 1:800 (Life Technologies). IHC slides were imaged as previously described (Iyer et al., 2013b). Briefly, IHC slides were mounted in 70% glycerol:PBS or

Mowiol and imaged at room temperature on a Nikon C1 Plus confocal system. Images were recorded using either a 20X (0.75 N.A.), 40X (1.3 N.A.) or 60X (1.4 N.A.) oil immersion objective. For live confocal imaging, third instar larvae were immersed in a few drops of 1:5 (v/v) diethyl ether:halocarbon oil. Three dimensional z-stacks were then volume rendered into a two-dimensional maximum projection and resultant images were processed for quantitative neuronal reconstruction analyses. Image processing subsequent to data acquisition was performed using Adobe Photoshop 6.0. Analyses of protein expression levels were performed by quantifying pixel intensity using the polygon method as previously described (Sulkowski et al., 2011).

Semi-automated digital reconstruction, quantitative analyses of dendritic morphology and bioinformatics

Quantitative analyses were performed via semi-automated digital neuronal reconstruction as previously described (Iyer et al., 2013a). Statistical analyses of neurometric quantitative data, including means ± SEM or SD, were performed by importing data into SigmaPlot (Systat Software) in which pairwise Student's *t*-tests were conducted to determine statistical significance. STRING v9.1 (http://string-db.org) and DroID (http://droidb.org) (Murali et al., 2011; Yu et al., 2008) were used to identify putative Dar1-interacting molecules based upon yeast two hybrid, mass spectrometry, homology evidence, transcription factor networks, genetic interactions, and co-expression data.

CHAPTER 3: RESULTS

Dar1 exhibits class-specific differential localization in da neurons

To further explore the regulatory roles of Dar1 in mediating dendrite development, we performed immunohistochemistry to visualize Dar1 expression and localization. Immunohistochemical (IHC) analyses reveal that Dar1 is localized to the nucleus of all da neuron subclasses at the late embryonic stage of development (Ye et al., 2011, Cox Lab, unpublished results) (**Fig 7A**). Interestingly, as development proceeds to the third instar larval stage Dar1 protein is differentially localized, remaining primarily nuclear in class I, II and III neurons (**Fig. 7B,C**), but shifting to a largely cytoplasmic localization in the highly complex class IV da neurons (**Fig. 7B,C**). Moreover, we confirmed the specificity of the Dar1 antibody by staining $dar1^{f01014}$ mutants which revealed virtually no detectable immunostaining (**Fig. 7D,E**).

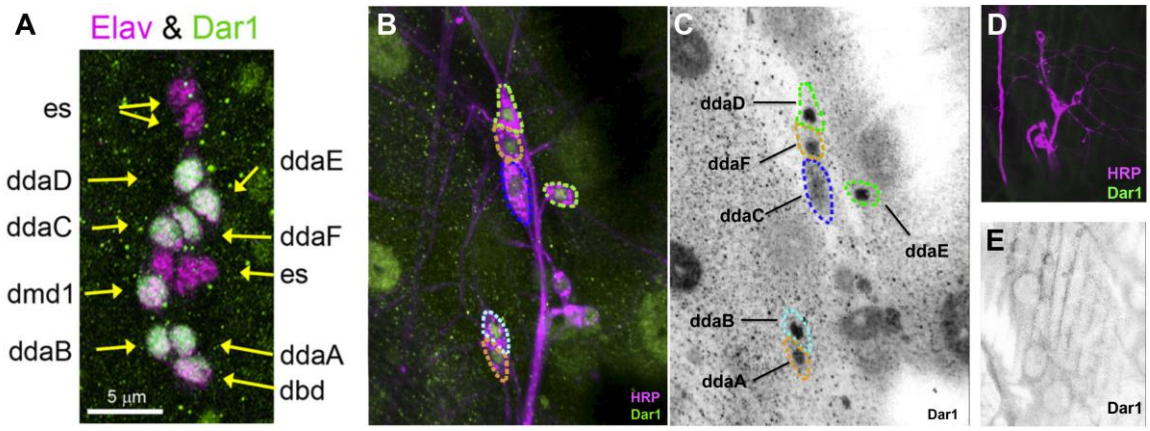

Figure 7: Dar1 exhibits class specific differential localization in da neurons. (**A**) Immunostaining of embryonic dorsal cluster of PNS neurons double labeled for the neuron-specific transcription factor Elav and Dar1. Dar1 is localized to the nucleus of all da neurons (ddaA-F) as well as the dmd1 and dbd neurons, but does not label es neurons which have single dendritic projections (Adapted directly from Ye et al., 2011). (**B,C**) Immunostaining of third instar larval filet with HRP to label PNS neurons and Dar1. Note that Dar1 is predominantly localized to nuclei in all da neuron subclasses with the exception of the class IV ddaC neuron in which Dar1 is predominantly cytoplasmic (**C**). (**D,E**) Immunostaining of *dar1*^{f01014} mutant third instar larval filets with HRP and Dar1. Note that Dar1 signal is virtually undetectable in *dar1*^{f01014} mutants confirming the specificity of the antibody.

Protein interactor screen identifies regulatory factors for class specific Dar1 subcellular localization

This observation led us to investigate the molecular mechanism underlying this class specific Dar1 differential localization and the result of perturbing this localization. To achieve this, we conducted a pilot RNAi screen of 12 putative Dar1-interacting proteins (**Fig. 8, Table 1**). We assessed the effect of RNAi-based knockdown of these putative interacting molecules on the differential cytoplasmic localization of Dar1 in class IV da neurons as compared to nuclear localization in class I neurons as well as the potential phenotypic consequences of aberrant Dar1 localization on class IV dendritic development (**Fig. 9**). These analyses revealed a number of genes which are required for cytoplasmic expression of Dar1 in class IV neurons as disruptions in these genes not only produced phenotypic defects in class IV dendritic growth and branching that resemble those observed with disruptions in Dar1 function, but also significantly shift Dar1 subcellular localization to the nucleus specifically in class IV neurons (**Fig. 10**), but do not disrupt normal nuclear localization in other da neuron subclasses (**Fig. 9**). Moreover, candidate interactor proteins that did not disrupt Dar1 localization in class IV neurons, likewise did not produce defects in class IV dendritogenesis upon RNAi-induced knockdown (**Fig. 9E, J**). Collectively these analyses suggest that select putative Dar1 interactors are essential for maintaining proper Dar1 cytoplasmic localization and therefore promoting normal dendrite morphology in class IV da neurons.

Table 1: Putative Dar1 Interacting Proteins and Functions

Putative Dar1 interactor proteins	Function(s)
CycB3/Cyclin B3	protein binding; cytokinesis after mitosis
Kap-α1/ Karyopherin-alpha1	nuclear transport receptor; targets proteins to the nuclear pore complex
nej/ nejire	protein binding; transcription factor binding; macromolecule modification
fd68A/ forkhead domain 68A	sequence-specific DNA binding transcription factor activity
trc/ tricornered	involved in signaling pathway regulating dendritic branching
cpo/ couch potato	mRNA binding; synaptic transmission; dormancy process; olfactory behavior
Rel/ Relish	protein binding; positive regulation of cellular biosynthetic process
Nipped-A	Protein kinase activity; regulation of transcription
Art1/ Argenine methyltransferase 1	signal transduction; DNA repair; mRNA splicing
ran	GTP- binding protein involved in nucleocytoplasmic transport; protein Import; RNA export
CG32105	sequence specific DNA; transcription facto binding; zinc ion binding
Ranbp21/Exportin5	Ran GTPase binding; intracellular protein transport

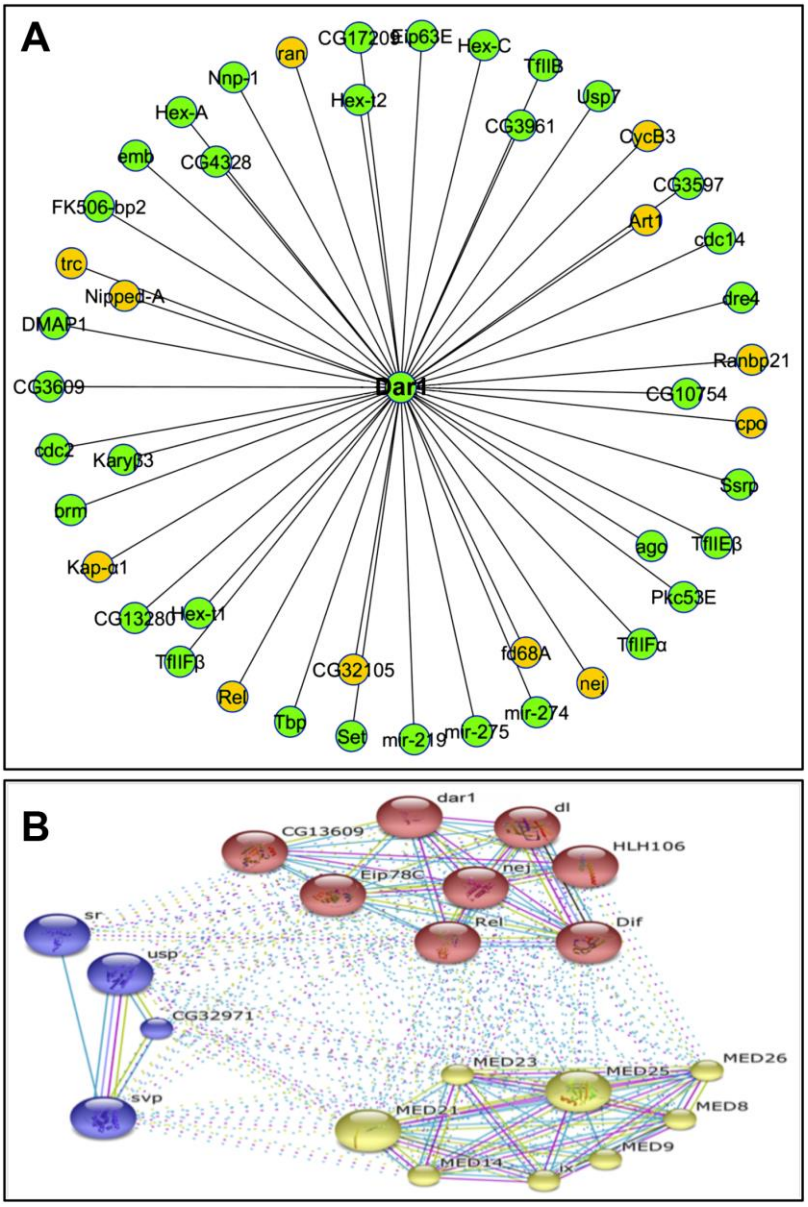

Figure 8: Dar1 protein-interaction maps. (**A**) Predicted Dar1-interacting proteins based upon query of the *Drosophila* Interactions Database (www.droidb.org) (DroID). DroID data is based upon yeast two-hybrid, mass spectrometry, genetic interaction data and homology interaction data from human, yeast, and *C. elegans*. Candidate interactors tested in the pilot screen are highlighted in orange on this map. (**B**) STRING schematic (www.string-db.org) showing predicted interactors of Dar1 (CG12029) based on experimental, database, text mining, and homology evidence. Note that these two interaction databases both predict Nejire as a Dar1-interacting protein and Nejire is the protein with the highest confidence score in the STRING schematic.

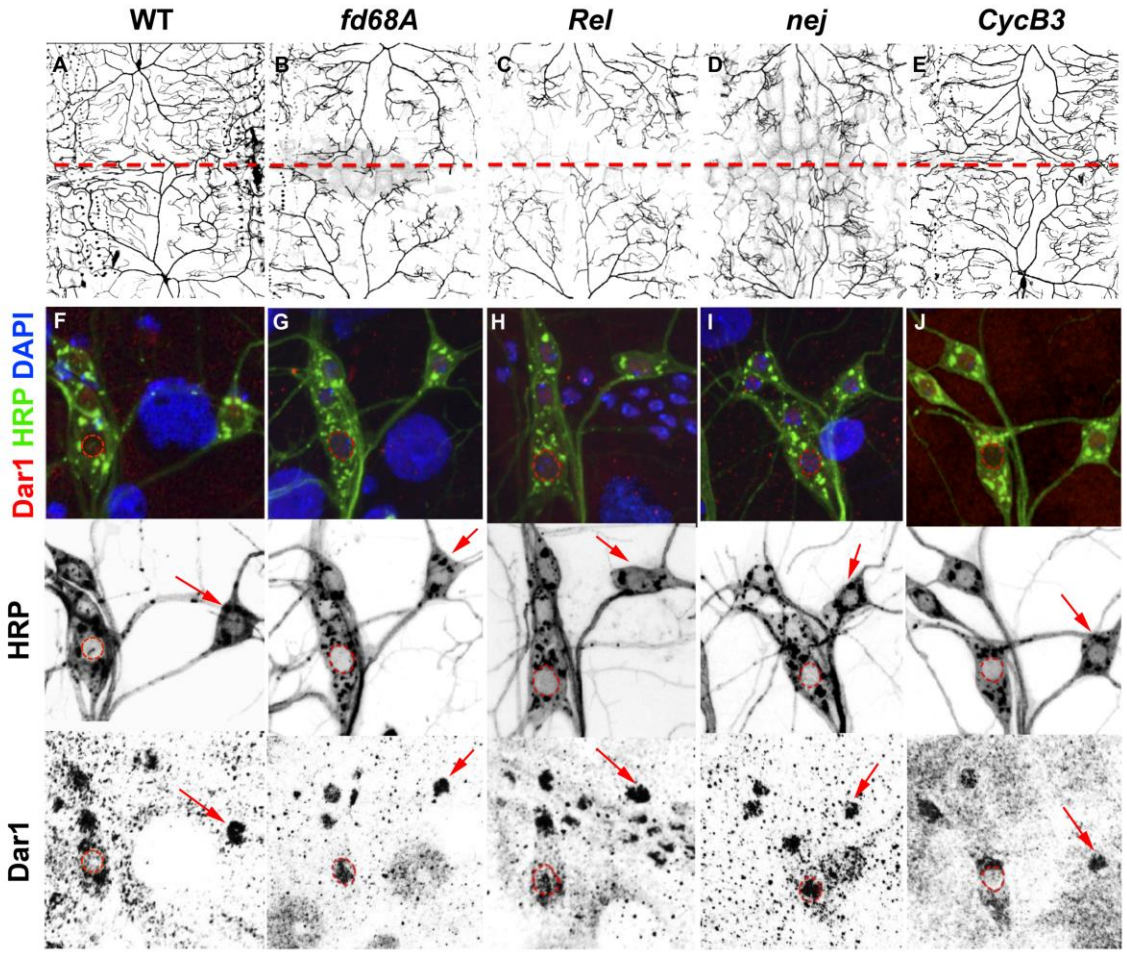

Figure 9: Protein interactor screen identifies regulatory factors for class specific Dar1 subcellular localization. Disruptions in Dar1 localization result in defects in class IV da neuron dendrite development. (**A-E**) Representative images of contra-lateral ddaC class IV da neurons oriented along the dorsal midline (denoted by dashed line**)** collected via live image confocal microscopy. Class IV da neurons are characterized by complex "space-filling" dendritic arbors that completely cover a receptive field as shown in wild-type (WT) (**A**). In contrast to WT, class IV-specific RNAi against the *fd68A* (**B**), *Rel* (**C**) and *nej* (**D**) result in a notable reduction in dendrite

branching complexity and growth, including a failure to cover large segments of the receptive field, similar to the loss-of-function *dar1* phenotype. (**F-J**) Representative images of dorsal da neuron cluster. (**F**) Immunostaining of WT dorsal da neuron cluster triple labeled with Dar1, HRP, and DAPI, reveals Dar1 localization as largely nuclear in the morphologically simple class I neurons (red arrow marks ddaE in all panels F-J), in contrast to largely cytoplasmic localization in the highly complex class IV da neurons (dashed red circle marks class IV ddaC nucleus in all panels F-J). (**G-I**) Dorsal da neuron cluster showing change to predominantly nuclear vs. cytoplasmic Dar1 localization in class IV ddaC neurons (dashed red circle) with pan-da neuron driven (*GAL4*$^{21\text{-}7}$*,UAS-mCD8::GFP*) RNAi knockdown of its putative protein interactors (**G**) *fd68A*, (**H**) *Rel* and (**I**) *nej* indicating a role for these putative interactors in specifically regulating the subcellular localization of Dar1 in the class IV da neurons. Note, despite RNAi knockdown of these genes in all da neurons, defects in Dar1 subcellular localization are only observed in class IV neurons relative to WT controls. RNAi directed against *CycB3* resembles the WT in both morphology (**E**) and Dar1 class IV cytoplasmic subcellular localization (**J**).

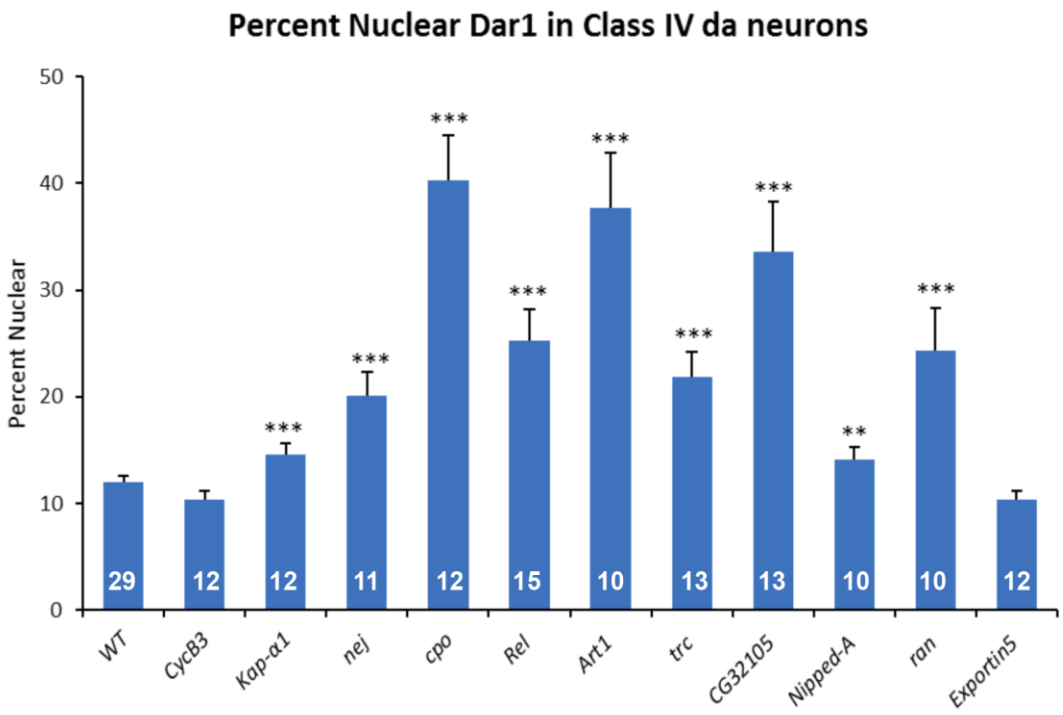

Figure 10: Regulatory effects of Dar1-interactors on class IV subcellular localization. Quantitative analyses of the percent nuclear Dar1 expression observed in class IV da neurons following RNAi-based disruption of putative Dar1-interacting proteins. Data is based upon pixel intensity measures of total Dar1 signal vs. nuclear-specific signal to determine percent nuclear expression. Data is presented as mean ± SEM and n values for each are denoted on the graph. **=p<0.01; ***=p<0.001.

***nejire* is required for higher order dendritic growth and branching in class IV da neurons**

Among the candidate Dar1 interactors, we chose to further analyze the potential functional roles of *nej* in regulating class IV dendritic development based upon the regulatory role of *nej* in mediating Dar1 localization, RNAi-induced phenotypic defects in class IV dendrites (**Figs. 9,10**), as well as protein interaction database findings (**Fig. 8**). Relative to WT class IV neurons, the nej^{RNAi} phenotype is characterized by an overall loss of dendritic branching complexity, growth, and field coverage with a number of "tufted" areas of increased complexity, while the loss-of-function $nej^{S\text{-}20}$ mutant shows an almost complete lack of higher order branching (**Fig. 11A-C,E-F**). Moreover, Nej overexpression likewise results in a dramatic reduction of dendritic branching complexity and reduction of higher order branching indicating a requirement for tight regulatory control of Nej expression in these neurons for homeostatic dendritic development (**Fig. 11D-F**). Collectively, these data suggest that regulated expression of *nejire* is essential for promoting proper higher order dendritic branching, growth, and field coverage, possibly via regulation of Dar1 localization in class IV da neurons.

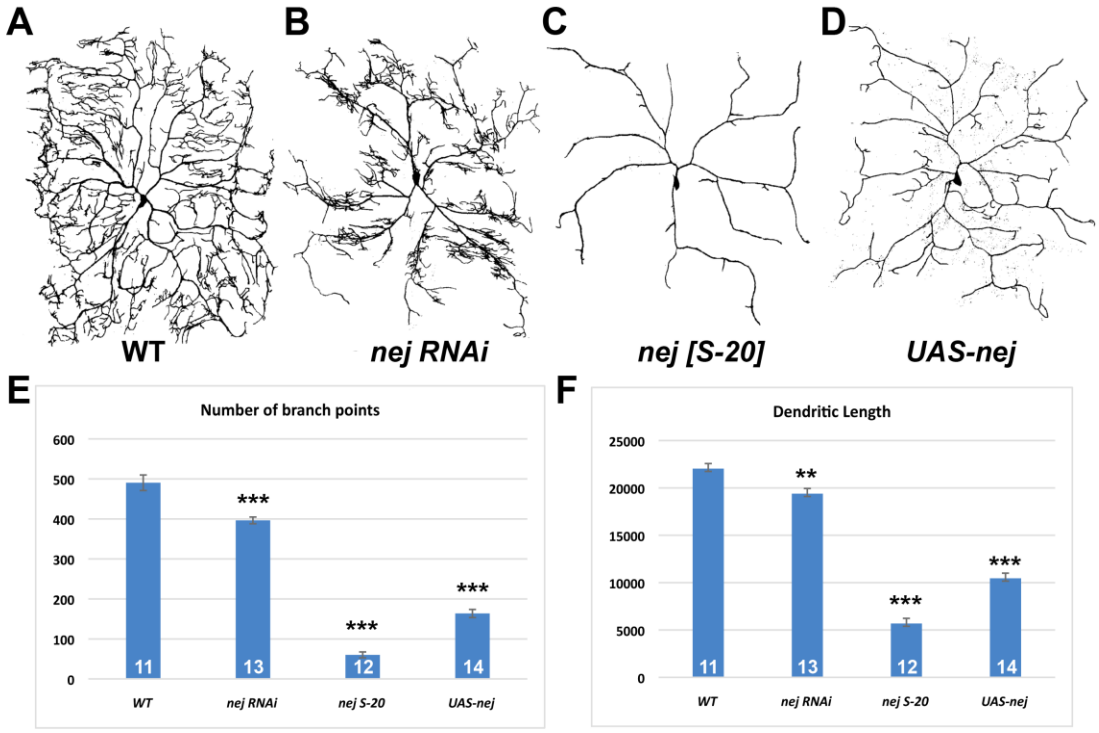

Figure 11: *nejire* is required for higher order dendritic growth and branching in class IV da neurons. (**A-D**) Representative images of dorsal class IV ddaC neurons obtained via live image confocal microscopy. Relative to WT (**A**), disruptions in *nej* function result in aberrant dendritic growth and branching (**B-F**). Quantitative neurometric data is presented as mean ± SEM with n values represented on the bar graphs and *p* values as follows: **=$p<0.01$; ***=$p<0.001$.

***nejire* is required for cytoplasmic Dar1 localization in Class IV da neurons**

Given the class specific differential subcellular localizations observed with Dar1, we investigated whether the same pattern would be observed for its putative interacting protein, Nejire. Immunostaining analyses reveal that both Nej and Dar1 are largely nuclear in the morphologically simple class I neurons, whereas Dar1 is cytoplasmic and Nejire is present in the cytoplasm and the nucleus in the highly complex class IV neurons (**Fig. 12**).

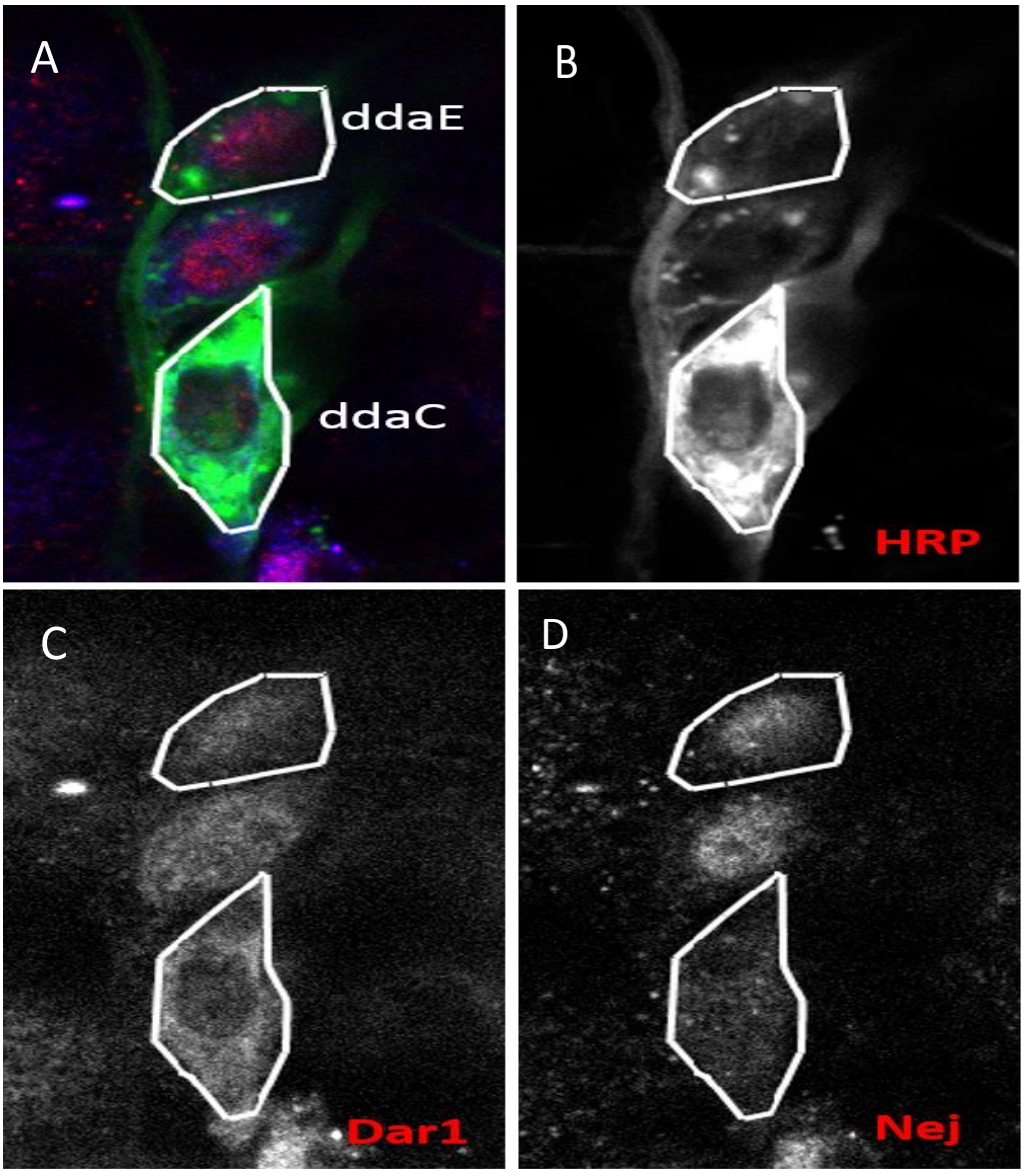

Figure 12: Nejire and Dar1 exhibit differential subcellular localization in da neuron subclasses. (A-D) Representative image of wild-type dorsal da neuron cluster immunostained for HRP, Nej, and Dar1. Nej and Dar1 are predominantly localized to the nucleus in class I ddaE neurons. In class IV ddaC neurons, Dar1 is cytoplasmic and Nejire is present in the cytoplasm and the nucleus. More nuclear Nejire expression is evident.

Qualitative analyses indicate that Nej is required for mediating cytoplasmic Dar1 localization in class IV da neurons (**Fig. 9D,I**). To confirm this regulatory relationship, we performed quantitative analyses on Dar1 subcellular localization following *nej* knockdown which revealed a significant shift from cytoplasmic to nuclear localization in class IV neurons. Interestingly, these analyses revealed no reduction in the overall levels of Dar1 protein expression in these neurons (**Fig. 13**). Collectively, these analyses demonstrate that *nej* is specifically required for maintaining the cytoplasmic localization of Dar1 in class IV neurons.

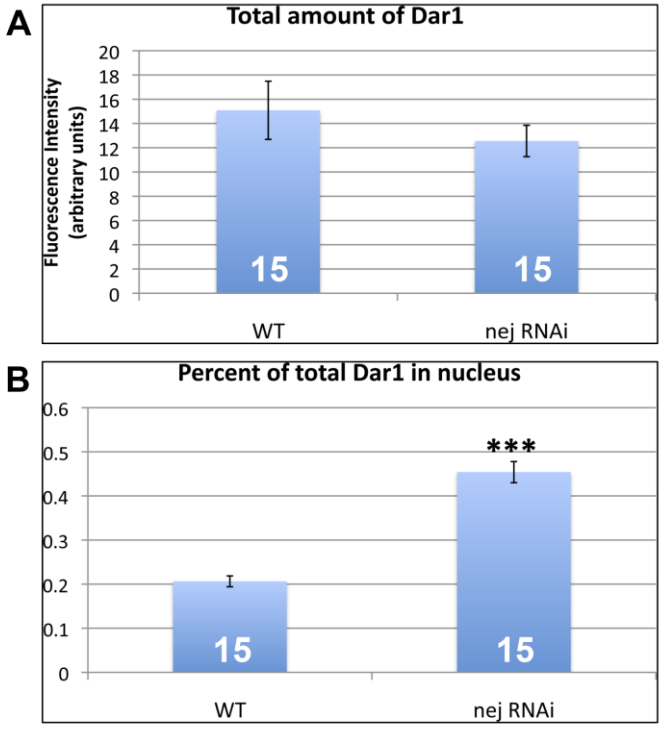

Figure 13: Nejire is required for cytoplasmic Dar1 localization in class IV neurons. Quantification of Dar1 expression in class IV ddaC neurons show no reduction in the total amount of Dar1 present in a nej^{RNAi} genetic background, however the percent of Dar1 localized to the nucleus more than doubles in nej^{RNAi} class IV ddaC neurons relative to control. Data is presented as

mean ± SEM, n values represented on bar graphs, and statistical significance is indicated as follows: ***=p<0.001.

Dar1 overexpression positively regulates Nejire

To further dissect the Nej-Dar1 regulatory relationship, we investigated how Nej overexpression in class IV neurons may impact Dar1 expression and/or subcellular localization. These analyses revealed a moderate but non-significant decrease in Dar1 expression levels, but no change in Dar1 cytoplasmic localization (**Figs. 14,15**), suggesting that the dramatic change in dendritic morphology observed with Nej overexpression (**Fig. 11D)** is likely due to a Dar1-independent mechanism. In contrast, Dar1 overexpression in class IV results in a significant increase in overall Nej expression levels and both Dar1 and Nej become highly nuclear (**Figs. 14,15**). These data suggest that with Dar1 overexpression, Nej may be sequestered in the nucleus, thereby leading to a dendritic defects (**Fig. 5D,** *UAS-dar1*) that are phenotypically similar to those observed with nej^{RNAi} knockdown **(Fig. 11B)**. Even though Nejire increases via ectopic expression of either Nejire or Dar1 only overexpression of Dar1 sequesters Nejire and Dar1 in the nucleus. This relationship indicates that Dar1 regulates the levels and localization of Nejire.

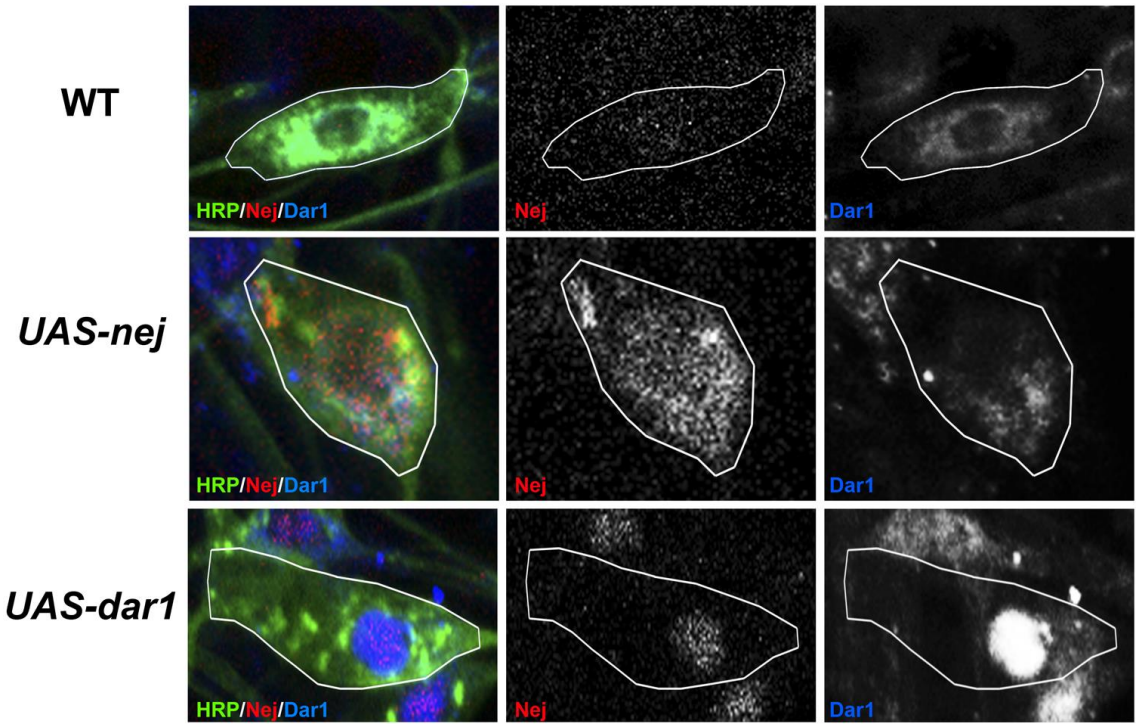

Figure 14: Dar1 overexpression positively regulates, and drives nuclear expression of, Nej. Immunostaining of WT, *UAS-nej* and *UAS-dar1* overexpressing class IV ddaC neurons labeled with HRP, Nej, and Dar1. Relative to WT, Nej overexpression upregulates Nej expression, but does not significantly alter Dar1 expression levels or subcellular localization. In contrast, Dar1 overexpression upregulates Nej expression levels and drives Nej nuclear localization, moreover, Dar1 overexpression also alters Dar1 to a predominant nuclear expression pattern in class IV neurons.

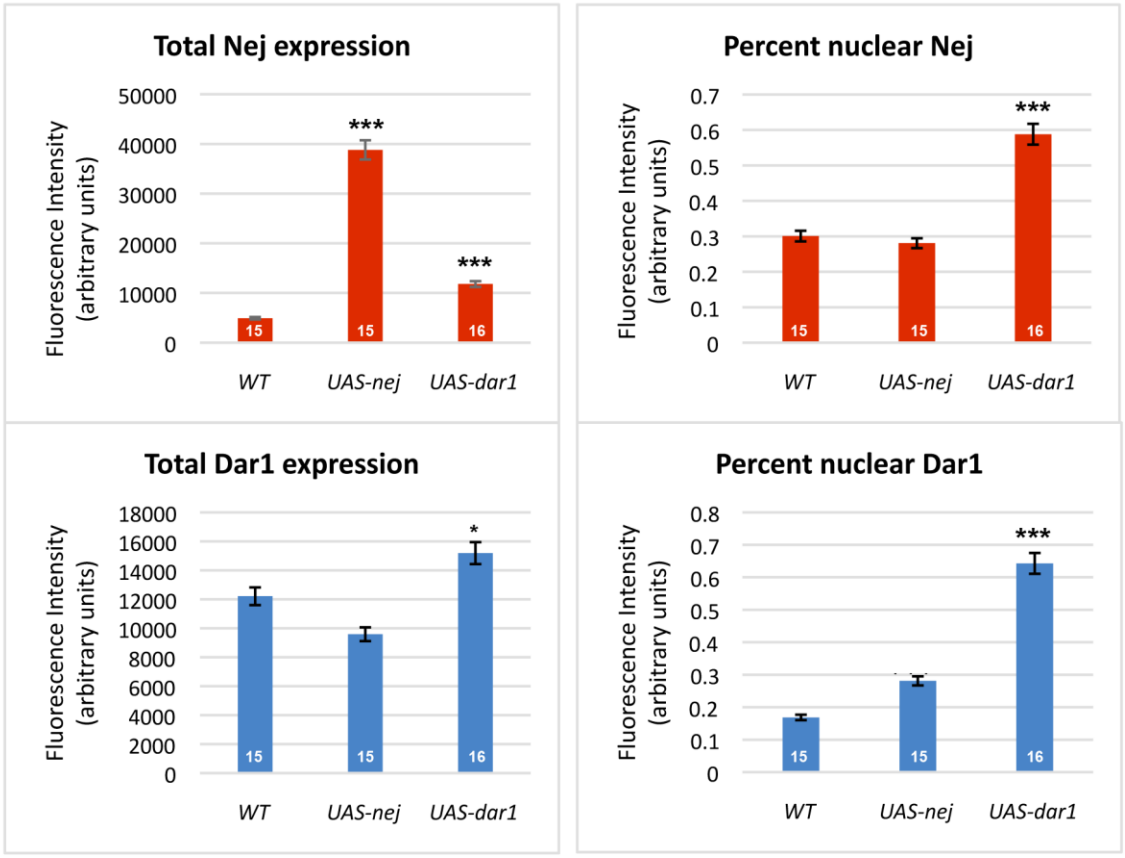

Figure 15: Quantitative analyses of Dar1/Nej expression and localization in class IV neurons. Pixel densitometry analyses of total Nej or Dar1 expression vs. percent nuclear expression in class IV ddaC neurons of WT, *UAS-nej* and *UAS-dar1* genetic backgrounds. Data is presented as mean ± SEM, n values indicated on bar graphs, and statistical values as follows: *=$p<0.05$; ***=p0.001.

Structure-function studies illuminate Nej's role in promoting higher order dendritic branching and growth

To gain molecular insight into the role of Nej in regulating dendrite morphogenesis, we conducted structure-function analyses expressing various domain-specific deletion or overexpression constructs (**Fig. 16A**). To dissect the respective roles of these functional domains, we expressed these mutant transgenes in class IV da neurons and examined their effects on dendritic branching and growth (**Fig. 16**). These analyses revealed that deletion of the polyglutamine (ΔQ) tract at the C-terminus produced severe defect in dendritic growth and branching in which only primary branches remain and all higher order branching is inhibited (**Fig. 16C**). This phenotype strongly resembles that observed in nej^{S-20} mutants (**Fig. 11C**) and indicate that the polyQ stretch is critically important for higher order dendritic branching and growth. Quantitative analyses reveal that expression of the ΔQ variant produces a dramatic 20-fold reduction in dendritic branching and growth (**Fig. 16I,J**). Structure-function analyses further reveal that the HAT domain (ΔH) and BROMO domain (ΔBHQ) also regulate, albeit to a lesser extent, dendritic branching (**Fig. 16D,E,I**) and/or growth (**Fig. 16D,E,J**). Interestingly, simultaneous disruption of the polyQ and HAT domains (ΔHQ) did not itself produce statistically significant effects (**Fig. 16F,I,J**) which may be due to the variable expressivity of the respective transgene variants, as ΔQ is expressed approximately six times more strongly than ΔHQ or ΔBHQ. Moreover, overexpression of the KIX domain alone failed to produce any significant defects in either branching or growth (**Fig. 16H-J**). Finally, N-terminal truncation of the NHR, Zn finger, and KIX domains (ΔNZK)

produced qualitative defects in dendritic field coverage (**Fig. 16G**) and quantitative disruptions in dendritic growth, but not branching (**Fig. 16I,J**). Moreover, ΔNZK also qualitatively altered branching morphology, particularly at termini, which appear 'tufted' with many short, spiky branches similar to those observed in class III da neuron dendritic filopodia (**Fig. 4**). Collectively, these data demonstrate specific roles of different Nejire protein domains and their contribution to establishing proper dendritic branching and field coverage and strongly implicate the C-terminal polyQ tract, which functions in transcriptional activation, as critically important for mediating dendritic branching complexity and growth.

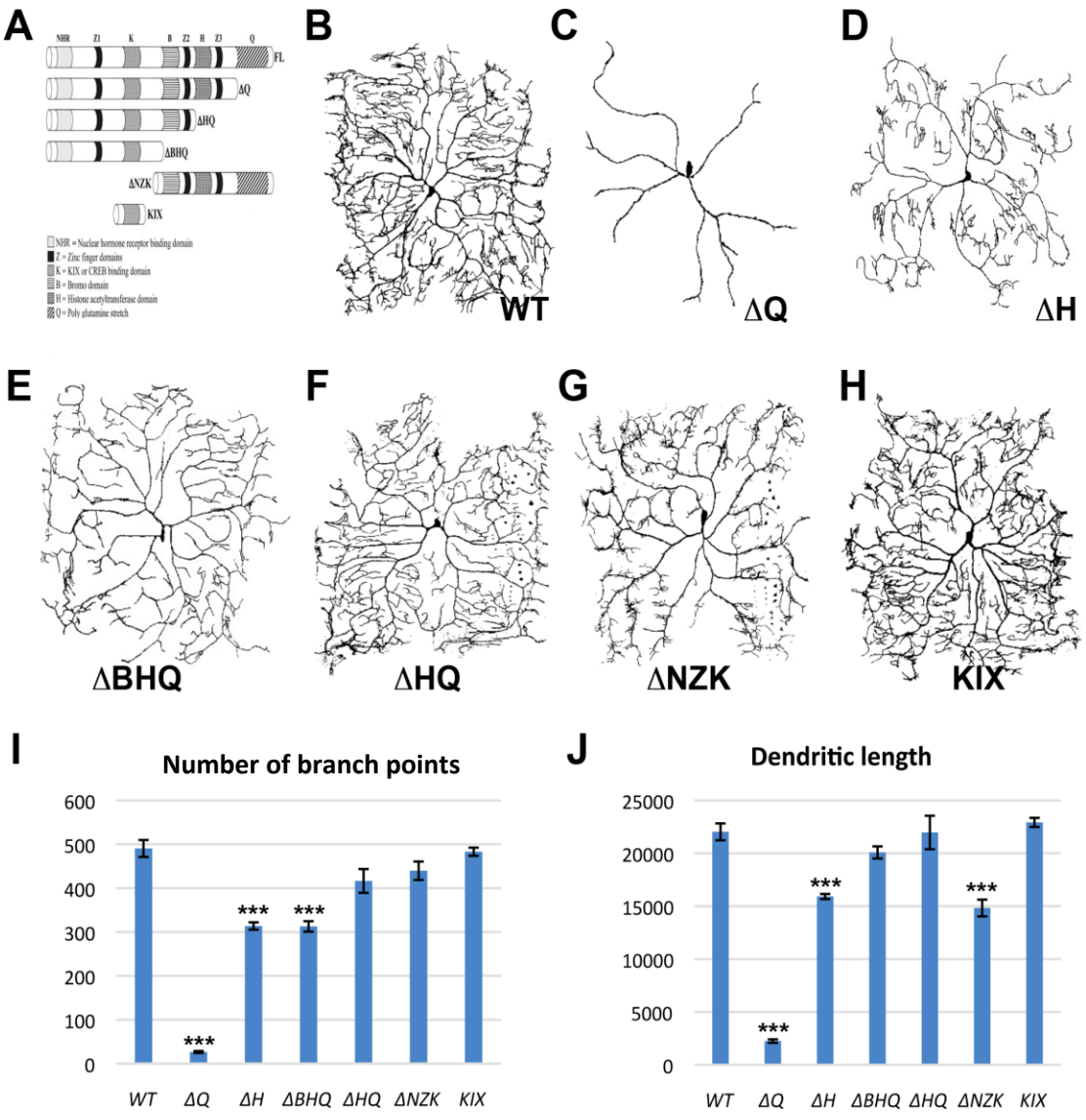

Figure 16: Nej structure-function studies. (**A**) Schematic of Nej domain organization and *UAS* structure-function transgenes (adapted directly from Kumar et al., 2004). (**B-H**) Representative images of WT and Nej variant-expressing class IV ddaC neurons. (**I,J**) Quantitative neurometric analyses of dendritic branching (**I**) and growth (**J**). Data is presented as mean ± SEM and statistically significance as follows: ***=p<0.001. The n

values for the various genotypes are as follows: WT (11); ΔQ (9); ΔH (11); ΔBHQ (12); ΔHQ (10); ΔNZK (9); KIX (10). Note, the ΔH transgene is not presented in the schematic in panel A.

Nej functional domains that regulate dendrite morphogenesis also modulate Dar1 localization

To explore the molecular mechanism by which Nej mediates Dar1 subcellular localization, we examined Dar1 expression in *nej* structure-function mutant backgrounds. Consistent with the range of phenotypic defects observed with deletion of the Nej polyQ (ΔQ), HAT (ΔHQ) and/or BROMO (ΔBHQ) domains (**Fig. 16C-E**), these Nej mutant transgenes also significantly increased nuclear localization of both Nej and Dar1 in class IV neurons (**Fig. 17A-C**). Interestingly, inactivation of the HAT domain alone (ΔH) produced opposite effects with a significant reduction in Nej nuclear localization and a significant increase in Dar1 nuclear localization (**Fig. 17D**). Finally, neither deletion of the NHR/Zn finger/KIX domain (ΔNZK) (**Fig. 17E**) nor overexpression of the Nej KIX domain (**Fig. 17F**) produced any significant effects on modulation of either Nej or Dar1 subcellular localization relative to WT. These results are largely consistent with the phenotypic effects observed with these transgenes in terms of dendrite development (**Fig. 16**) except for ΔHQ which does not show any reduction in dendritic branching or length. Collectively, these data demonstrate that the polyQ tract is required not only for higher

order dendritic branching and growth, but also for proper subcellular localization of both Nej and Dar1.

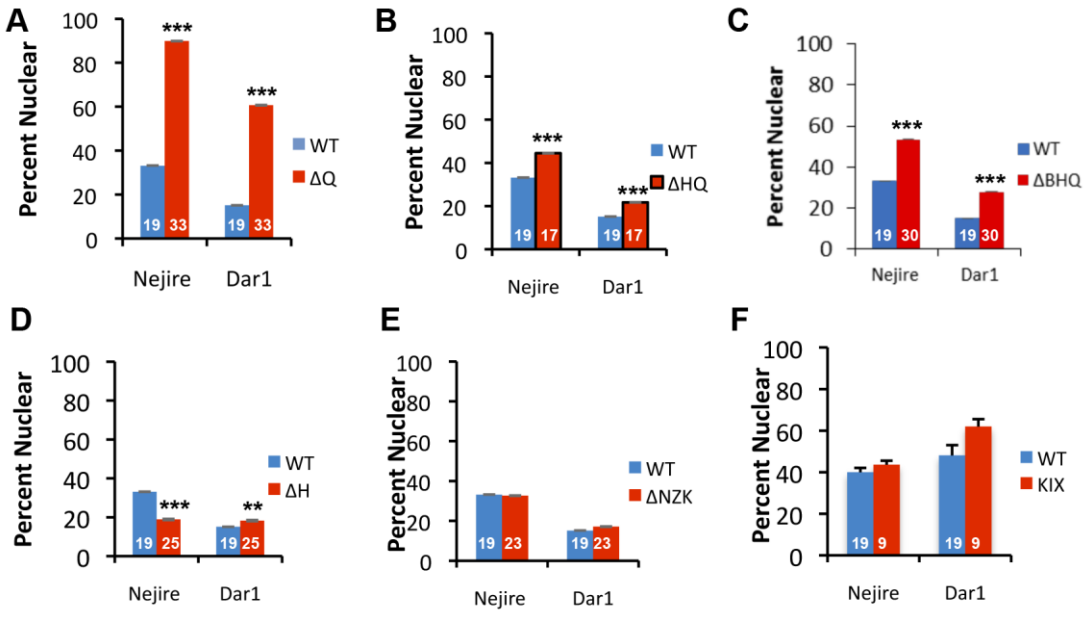

Figure 17: Nejire-mediated modulation of Dar1 localization is essential for proper dendritic morphogenesis. (**A-F**) IHC analyses of Nej and Dar1 nuclear localization in class IV neurons for each of the Nej structure-function variants. Data is presented as mean ± SEM, n values are represented on the bar graphs and *p* values are as follows: $p<0.01$=** and $p<0.001$=***.

CHAPTER 4: DISCUSSION

Complexity-dependent regulatory control of Dar1 localization is mediated via Nejire

Complex transcriptional regulatory programs have been demonstrated to play essential roles in the establishment, maintenance and modulation of dendrite development (Jan and Jan, 2010; Tavosanis, 2014; Singhania and Grueber, 2014; Iyer et al., 2013). Moreover, differential expression of transcription factors has likewise been shown to mediate sub-type specific patterns of dendritic architecture and homeostasis (Grueber et al., 2003; reviewed in Singhania and Grueber, 2014). Here, we identify a functional interaction between the Kruppel-like transcription factor Dar1 and Nejire, the *Drosophila* CBP homolog, with respect to regulating dendritogenesis. Moreover, we demonstrate that Nej functions as a master regulator of higher order dendritic branching in class IV da neurons and that this function is strongly dependent on the polyQ domain of Nej which likewise acts to modulate the differential subcellular localization of Dar1 in these neurons.

Consistent with previous studies of Dar1 (Ye et al., 2011), we demonstrate cell autonomous roles in promoting dendritic growth and branching among each of the four da neuron subclasses. However, in contrast to previous reports, we find that Dar1 overexpression leads to aberrant dendritic growth and branching that result in significant reductions in both neurometric parameters. The basis for this difference is unclear,

however could potentially be due to differential expressivity of the transgenes used between studies. Mechanistically, Dar1 is proposed to promote dendritic growth via downregulation of the microtubule severing protein Spastin (Ye et al., 2011). Given the different phenotypic results between studies, we propose that Dar1 regulates dendritic branching and growth in class IV neurons via mechanisms independent of Spastin regulation and that regulated expression of Dar1 in da neurons is critical to normal homeostatic class-specific dendritogenesis. Intriguingly, we find that Dar1 undergoes a shift in subcellular localization during maturation from late stage embryos, where Dar1 is detected in the nucleus of all da neurons (class I-IV), to third instar larvae, in which Dar1 exhibits complexity-dependent localization which manifests as a nuclear signal in class I-III neurons, but a predominantly cytoplasmic signal in class IV neurons. This shift in localization is required for proper class IV dendrite development, as disruptions in Dar1 cytoplasmic localization lead to defects in dendritic growth and branching in these neurons as revealed by genetic interaction data and Dar1 overexpression results.

In a screen of putative Dar1-interacting proteins, we identified *nejire* which is required for proper complexity-dependent cytoplasmic localization of Dar1 in class IV neurons. Moreover, we demonstrate that *nej* hypomorphs produce severe disruptions in class IV dendritic complexity leading to a complete absence of higher order branching indicative of an essential role for Nej function in this process. Moreover, consistent with the Dar1 mutant and overexpression results, Nej overexpression in class IV neurons results in a similar phenotype to the loss-of-function mutants indicating that control of Nej expression levels is key to proper dendritic development. While Nej is not required

for Dar1 expression, but rather cytoplasmic localization, we find that Dar1 overexpression leads to an increase in Nej levels and a redistribution of Dar1 protein to the nucleus in class IV neurons indicating a putative role for Nej in Dar1 subcellular sequestration potentially through direct protein-protein interactions.

Structure-function analyses implicate Nejire transcriptional activation and chromatin modulation in mediating dendritogenesis and Dar1 subcellular localization

Interestingly, a recent study demonstrated that Nej promotes dendritic pruning in class IV neurons during pupal stages of development (Kirilly et al., 2011), whereas we find that Nej has an essential functional role in promoting higher order dendritic branching and growth in class IV neurons in larval development. These opposing effects indicate that Nej differentially regulates dendritic morphology as a function of developmental metamorphosis. To further understand the regulatory relationship between Nej and Dar1, we conducted structure-function mutant analyses of Nej to explore domain-specific roles for Nej in Dar1 subcellular localization and overall dendritic morphogenesis.

We demonstrate that the C-terminal polyglutamine (polyQ) stretch of Nej, which has been implicated in transcriptional activation, is essential for higher order class IV dendritic branching and growth. Moreover, deletion of the polyQ (ΔQ) domain produces dendritic defects that phenocopy those observed in both $nej^{S\text{-}20}$ hypomorphs and with full-length Nej overexpression. In addition, IHC data reveal that the polyQ domain is

required for cytoplasmic subcellular localization of Dar1 and leads to an increase in Nej nuclear localization. Similarly, deletions of the Bromo and HAT domains (ΔBHQ) also led to defects in both class IV dendritogenesis and Dar1/Nej subcellular localization patterns. These results suggest that in addition to transcriptional regulation, there is Nej-mediated chromatin remodeling. Inactivation of the HAT domain alone (ΔH) also disrupts normal class IV dendrite arborization and shows changes in Nej and Dar1 subcellular localization whereby there is a decrease in Nej nuclear localization and a modest increase in Dar1 nuclear localization. These results suggest that the HAT domain may be required for retaining Nej in the nucleus of class IV neurons. In contrast, deletion of the N-terminal NHR, Zn finger and KIX domains (ΔNZK) causes no change in Dar1 or Nejire subcellular localization patterns, but does lead to a mild decrease in dendritic branching indicating that these domains are not required for Dar1 localization and that the phenotypic effects observed with the deletion of these domains in Dar1-independent. Since the KIX domain is known to mediate binding to CREB, these results indicate that Nej-mediated regulation of Dar1 localization is likely CREB-independent. Moreover, overexpression of KIX domain alone caused no quantifiable defects in either dendrite morphogenesis or Dar1/Nej localization. The phenotypes of ΔNZK and ΔH were also distinct as compared to the phenotypes that emerged from the ΔQ, ΔHQ and ΔBHQ mutants thereby revealing unique domain roles contributing to both Dar1 localization and dendrite morphogenesis. We demonstrate the importance of these domains in influencing Nej and Dar1 localization which subsequently functions in regulating dendritic branching and growth. Collectively, these analyses provide novel insights into the underlying

mechanistic role of Nejire's modulatory interactions with Dar1 to influence its nuclear/cytoplasmic localization in da neurons and how that may contribute to the acquisition of dendritic complexity.

Future Directions

Based on these results we have embarked on transcriptomic, biochemical and cytoskeletal live imaging analyses to further dissect the molecular mechanisms via which Nej modulates dendritogenesis. While both ΔQ and nej^{S-20} mutants strongly disrupt higher order branching/growth in class IV neurons, the molecular mechanisms underlying this regulatory effect remain largely unknown. To gain molecular insight into how Nej regulates gene expression, we propose to conduct RNA-seq analyses on total RNA derived from wild-type, nej^{S-20}, and *nej* (ΔQ) expressing class IV da neurons. Comparative transcriptomics should reveal Nej-mediated transcriptional targets for normal class IV dendritic architecture that will prove valuable in characterizing the molecular machinery that is required for higher order dendritic branching. Moreover, RNA-seq data can also be used to explore potential Nej transcriptional regulation of microRNAs (miRNAs). ChiPBase analyses show that Nej binds to promoter regions for select K box miRNAs (dme-miR-2a, dme-miR-2b and dme-miR-2c). We have recently demonstrated that these K box miRNAs are highly expressed in class IV neurons where they operate to restrict the expression of genes that otherwise restrain dendritic branching and growth (for example, *miR-2b* overexpression increases complexity, whereas knockdown results in reductions in complexity for class IV neurons) (Cox Lab,

unpublished results). Nej may therefore function to promote the expression of these K box miRNAs in complex class IV neurons in order to promote dendritic branching and growth.

To understand the physical interaction between Nejire and Dar1, we can conduct reciprocal co-immunoprecipitation (co-IP) experiments from isolated class IV da neurons in both wild-type backgrounds and in Nej structure-function mutatnt genetic backgrounds to explore where Nej and Dar1 may physically interact. Moreover, through co-IP and mass spectrometry analyses, Nejire domain specific protein partners can likewise be identified. This result will lead to potential identification of novel protein partners responsible for Nejire-Dar1 mediated dendrite morphogenesis.

Since Dar1 is shown to mediate dendritic branching by promoting tubulin (Ye et al., 2011), it is essential to understand the role of Nejire and its domains in regulating actin and tubulin in da neurons. Nejire lines and the domains deletion constructs will be crossed to a multi-fluor reporter strain which allows for live simultaneous visualization of actin and microtubule cytoskeletons in independent channels. Using these cytoskeletal reporters, we can investigate how Nej may modulate cytoskeletal dynamics or localization to modulate dendritic arborization.

Collectively, these above analyses could reveal novel regulatory mechanisms that may aid understanding neurological disorders and syndromes associated with disruptions of Dar1 or Nej homologs in vertebrates, including humans. Dar1 and Nejire (CBP) have been connected to many neurological conditions and syndromes related to aberrations in dendritic branching patterns. KLF5, the closest homolog of Dar1, has been reported to be

expressed in many tissues, including the brain and is downregulated in schizophrenia patients (Ye et al., 2011). Moreover, Rubinstein-Taybi syndrome, which is a condition characterized by short stature, moderate to severe intellectual disability, distinctive facial features, and broad thumbs and first toes (Kumar et al., 2004), is caused by a partial deletion of the short arm of chromosome 16, where CBP is located (Petrif et al, 1995). In addition, Huntingtin, the aberrant protein responsible for Huntington's disease, interferes with CBP-activated transcription by binding CBP into inactive aggregates (Choi et al., 2012). Moreover, CREB dysfunction has been shown to be one of the major causes of learning and memory deficits in Alzheimer's disease, and overexpression of CBP in a mouse model of Alzheimer's is able to improve memory (Caccamo et al., 2010).

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