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The *Drosophila* RNA-binding Protein Nab2 Interacts with the Planar Cell Polarity Pathway to Regulate Neurodevelopment

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The *Drosophila* RNA-binding Protein Nab2 Interacts with the Planar Cell Polarity Pathway to Regulate Neurodevelopment

By

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B.S., University of the Cumberlands, 2014

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Abstract

The *Drosophila* RNA-binding Protein Nab2 Interacts with the Planar Cell Polarity Pathway to Regulate Neurodevelopment
By Edwin B. Corgiat III

RNA-binding proteins (RBPs) play critical roles in post-transcriptional regulation that have profound effects on gene expression. The importance of dysregulation of RNA processing is best illustrated by the numerous tissue-specific diseases associated with mutations in genes encoding RBPs, particularly neurological disorders. Neurodevelopment is highly complex and requires spatiotemporal control of gene expression, largely directed by RBPs. One such RBP is human ZC3H14, a ubiquitously expressed zinc-finger, polyadenosine RBP (ZnF CysCysCysHis #14), which has broad roles in post-transcriptional regulation, that is lost in an inherited form of intellectual disability. To gain insight into ZC3H14 neurological functions in the human brain, we employ the *Drosophila* model to explore the function of this evolutionarily conserved protein in neurodevelopment. *Drosophila Nab2* (nuclear poly(A) binding protein 2), is the sole fly ortholog of ZC3H14. Here, we define roles for Nab2 in controlling the dynamic growth of axons in the developing brain mushroom bodies (MBs), which support olfactory learning and memory, and in regulating abundance of a small fraction of the total brain proteome, which collectively link Nab2 to the processes of brain morphogenesis, neuroblast proliferation, circadian sleep/wake cycles, and synaptic development. Additionally, we show that components of the planar cell polarity (PCP) pathway are enriched in the *Nab2* null brain proteome, that genetic data indicate Nab2 functions in guiding PCP-dependent MB axon projection and growth of larval sensory dendrites by a common, cell-autonomous mechanism, and finally reveal core PCP protein Van Gogh as a potential Nab2 target. In aggregate, these data demonstrate functions for *Nab2* in axonal and dendritic development, provide a window into *Nab2* regulated brain proteome, and identify interactions between Nab2 and components of the planar cell polarity pathway. These data suggest that *Nab2/ZC3H14* may function in neurodevelopment through regulation of the PCP pathway by regulating trafficking and localization of PCP components, possibly identifying a mechanism underlying *ZC3H14*-linked intellectual disability in humans.

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Chapter 1: General introduction

This chapter has been written by Edwin Corgiat specifically for inclusion in this dissertation.

General Introduction

Proper execution of gene expression is critical for multicellular organisms to achieve spatiotemporal control allowing for cell type specific functions and development of multiple tissues (Schieweck, Ninkovic, & Kiebler, 2021). The central dogma of biology, proposed in the 1950s, describes the pipeline of genetic expression as: transcription of DNA into mRNA and the subsequent translation of mRNA into protein (Crick, 1970). The central dogma defines the primary steps of gene expression, but, since the 1950s, the scientific community has gained a great appreciation for the precise post-transcriptional regulation of mRNA that occurs. In all eukaryotes, mRNA must undergo several critical processing and quality control steps (Moore, 2005). These steps are coordinated by a group of RNA-binding proteins (RBPs). RBPs function, often in cooperation with one another, through recognition of transcripts, performance of a processing event, and release to the translation machinery for translation (Schieweck et al., 2021). The functions of these RBPs have a profound effect on gene expression that is best illustrated by the numerous diseases associated with mutations in genes encoding RBPs (Lukong, Chang, Khandjian, & Richard, 2008). Particularly interesting, is the high frequency of RBP mutations that lead to neurological diseases such as intellectual disability.

One example of a gene linked to neurological disease is the human *ZC3H14* gene, which encodes a ubiquitously expressed polyadenosine RNA-binding (Bienkowski et al., 2017; Corgiat, List, Rounds, Corbett, & Moberg, 2021; Jalloh et al., 2020; S. K. Jones et al., 2020; Kelly et al., 2016; W. H. Lee et al., 2020; Pak et al., 2011; Rha et al., 2017; Rounds et al., 2021). Mutations in the gene encoding *ZC3H14* are linked to a non-syndromic, monogenic intellectual disability (Pak et al., 2011). RBPs play an important role in nervous system development due to the fact that neurons require spatially and temporally controlled gene expression (Holt, Martin, & Schuman,

2019; Maday, Twelvetrees, Moughamian, & Holzbaur, 2014). Neurons extend over great distances and require rapid response to events occurring at the distal end of axons and dendrites, far from the nucleus (Holt et al., 2019; Hörnberg & Holt, 2013; Maday et al., 2014; Stoeckli, 2018). This dynamic response is crucial for proper formation of the neuronal architecture in the brain during neurodevelopment.

A variety of model systems have been used to provide insight into the molecular mechanisms that link steps in gene expression to neurological disease. The fruit fly, *Drosophila melanogaster*, with a simplified brain and vast genetic toolbox, provides an excellent genetic model to probe questions of neurodevelopment. This dissertation investigates the specific role of *Drosophila* Nab2, the ortholog of human *ZC3H14*, in neurodevelopment. Here, I provide evidence that Nab2 regulates a subsection of the brain proteome, is required for proper dendritic branching and projection, and genetically interacts with the planar cell polarity pathway during axo-dendritic development.

1.1 Post-transcriptional gene regulation

Gene expression events begin with transcription of the DNA template to generate the mRNA molecules that will eventually be translated, but there are a vast number of processing events that must occur between transcription and translation, which in aggregate are referred to as post-transcriptional processing. Post-transcriptional processing occurs in both the nucleus and cytoplasm with mRNA processing events including: capping, splicing, cleavage, 3’ end processing, and polyadenylation in the nucleus; nucleocytoplasmic transport and export through the nuclear pore; and lastly, localization, translation, and turnover of the mRNA in the cytoplasm

(Figure 1-1) (Alberts et al., 2008; Lodish et al., 2013; Rasmussen & Lis, 1993; Salditt-Georgieff, Harpold, Chen-Kiang, & Darnell, 1980; Zhao, Hyman, & Moore, 1999).

1.1.a Nucleus

The nuclear post-transcriptional processing events begin with capping of the emerging mRNA. This capping event occurs to the RNA as transcription continues by RNA polymerase II. As the first 25-30 nucleotides of the nascent mRNA become accessible, a methylguanosine cap is added to the 5' end of the mRNA (Figure 1-1) (Rasmussen & Lis, 1993; Salditt-Georgieff et al., 1980). Failure to be capped results in an unstable RNA molecule. A prevalent RNA degradation pathway requires removal of the cap prior to exonuclease activity (Losh & Van Hoof, 2015; Masuda et al., 2005; Meyer, Temme, & Wahle, 2004). Therefore, having a 5' cap adds stability, giving time for transcript to undergo further processing including continued transcription, pre-mRNA processing, quality control allowing for nuclear export, and eventually translation through direct interaction between the 5' cap and eIF4e translation initiation factor (Pestova et al., 2001).

Splicing of pre-mRNA molecules into mature mRNA is critical for eukaryotic genes that contain large sections of intervening, non-coding introns (Figure 1-1). These introns are removed, or spliced out, by the spliceosome allowing for the flanking regions of coding exons to be ligated together to form the mature mRNA (Collins & Guthrie, 2000). The spliceosome achieves this splicing through trans-acting activity via recognition of cis-acting elements within the pre-mRNA, such as the 5' and 3' splice sites (Collins & Guthrie, 2000). Splicing allows for proper export and translation to occur but additionally, alternative splicing can occur, which greatly increases the protein diversity that a cell can generate (K. Chen, Dai, & Wu, 2015). Alternative splicing refers to the differential inclusion or exclusion of certain exons, thus generating distinct mRNA isoforms

from a single gene (Brinegar & Cooper, 2016). These distinct mRNA variants allow for greater protein diversity, which is beneficial for adapting to different environmental conditions or developmental requirements.

Similar to the critical processing steps that occur at the 5’ end of the mRNA, the 3’ end of the mRNA also undergoes a series of maturation steps. The 3’ end processing occurs in two coupled steps consisting of cleavage and polyadenylation (Figure 1-1). Cleavage occurs in proximity to a conserved sequence, a polyadenylation signal, found within the 3’UTR. Sequences upstream and downstream of the polyadenylation site, such as AAUAAA, recruit cleavage and polyadenylation factors that enhance efficiency of endonuclease cleavage and polyadenosine tail addition (Alberts et al., 2008; Lodish et al., 2013). Polyadenylation is a crucial for many proteins to recognize and act on the RNA. For example, poly(A) tails serve as signals for proteins involved in quality control of nuclear export. Beyond the life of an RNA inside the nucleus, poly(A) tails remain important for many RBPs to recognize the RNA and direct nucleocytoplasmic transport and cytoplasmic processing. Defects here can disrupt nucleocytoplasmic transport and decrease efficiency of translation. Polyadenylation occurs through addition of a non-templated poly(A) tail to the 3’ UTR cleavage site by poly(A) polymerase (Figure 1-1) (Van Dijk et al., 2002). Even though polyadenylation plays such a crucial role in regulating transcript fate, there is much about the tail that is still disputed or poorly understood. For example, poly(A) tails have a median length of 70 nucleotides in budding yeast but can easily be found in human cells as long as 250-300 nucleotides, and the distribution of these lengths throughout the lifetime of an mRNA is unclear (Collins & Guthrie, 2000).

1.1.b Nucleocytoplasmic transport

In eukaryotes, transcription and translation are spatially separated by the nuclear envelope (Figure 1-1) (Tapley & Starr, 2013). RNAs are transcribed in the nucleus and once processed into mature transcripts are packaged into ribonucleoprotein (RNP) complexes (covered in detail in 1.2.a) for export from the nucleus (Balagopal & Parker, 2009). Although there is incredible complexity in the interactions with the nuclear pore complex that facilitates shuttling of complexes in and out of the nucleus, there are two general modes of nucleocytoplasmic transport: export and import (Balagopal & Parker, 2009). Export of RNA generally includes binding of RNP factors, interaction with the nuclear pore complexes, movement through the pore, and release of RNP export factors (Brodsky & Silver, 2000; Elbarbary & Maquat, 2016; Terry & Wente, 2007). Import most typically refers to proteins, which need to access the nucleus and generally includes recognition of nuclear localization sequences by nuclear transport receptors, interaction with nuclear pore complexes, movement through the pore, and release of import factors (Boruc et al., 2015; Terry & Wente, 2007). Taken together the functions included in export and import contribute to the complex nature of interactions with the nuclear pore.

Many of the factors involved in both nucleocytoplasmic export and import can shuttle between the nucleus and cytoplasm, and all shuttling proteins must pass through the nuclear pore complex (NPC). The NPC directs nucleocytoplasmic transport of RNPs into the cytoplasm and of the non-RNA bound RBPs back into the nucleus (Figure 1-1). The NPC is a large macromolecular complex of ~120 MDa that acts as both a pathway to and gatekeeper between the nucleus and cytoplasm (Tran & Wente, 2006). It is composed of several classes of nucleoporins (Nups) that provide structure for the pore and allow for a dynamic, non-static, complex to adjust to various stresses and signals (Köhler & Hurt, 2007; Tran & Wente, 2006). The changing composition of

the NPC allows for a high degree of control over nucleocytoplasmic transport without disrupting the functional exchange of materials across the nuclear envelope (Chook & Süel, 2011; Tran, King, & Corbett, 2014). The final translocation of the RNP through the nuclear pore serves as a final quality control checkpoint before the mRNA gains access to the cytoplasm where the translation machinery is located. Several nuclear pore proteins, such as Nup60, face inward from the nuclear basket and serve RNP surveillance functions preventing the release of pre-mRNA into the nucleus (Fasken & Corbett, 2009; Palancade & Doye, 2008). Export of RNPs through the nuclear pore is mediated by interactions with the nuclear basket and cytoplasmic filaments, which extend into the cytoplasm and provide binding sites (Figure 1-1) (Tran & Wente, 2006). Once an RNP reaches the cytoplasm, new proteins can join the complex and proteins involved in export can be released. Nuclear import allows these export proteins to cycle back into the nucleus now that they have been released from the exported RNP complex, which allows for subsequent rounds of mRNAs to export from the nucleus (Figure 1-1).

1.1.c Cytoplasm

While there are some export factors that shuttle from the nucleus into the cytoplasm, there are many other transport factors that regulate RNPs in the cytoplasm allowing for proper spatiotemporal control of translation. Translation can occur near the nucleus or at distal ends of the cell. The large and small subunits of the ribosome join together on the new mRNA near the 5’ end and initiate protein synthesis (Lodish et al., 2013). Protein synthesis occurs as the ribosome processes the mRNA, reading codons and using tRNAs as adaptors to add new sequence to the polypeptide chain. Control of when and where translation occurs is regulated by a host of proteins including eukaryotic initiation factors (eIFs), elongation factors (EFs), and RBPs such as the

poly(A)-binding protein (PABP) which binds the poly(A) tail (Alberts et al., 2008; Lodish et al., 2013). Turnover and degradation of mRNA occurs in the cytoplasm and can serve as a quality control step. Damaged or aberrantly processed RNAs can be targeted for degradation by various ribonucleases, including the RNA exosome complex (Alberts et al., 2008). While translation and turnover occur in all cells, there are some cells, such as neurons, which require RNPs to undergo local translation (Kandel, Schwartz, Jessell, Siegelbaum, & Hudspeth, 2013). Localization of RNPs to the distal ends of neurons and regulation of translation of the mRNA are critical steps in post-transcriptional regulation of gene expression in neurons (Hörnberg & Holt, 2013). Thousands of mRNAs are transported into axons and dendrites that require regulation but allow for spatial control of gene expression (Holt et al., 2019). RNPs are trafficked via microtubules and are typically guided by RNA binding proteins that interact with cis-acting sequences within the 3' and 5'UTRs of the specific mRNA (Alberts et al., 2008). Once in the distal axons and dendrites, the RNPs are joined by ribosomes and Golgi outposts that are scattered throughout the cytoplasm, which allows for local translation to occur (Holt et al., 2019; Kandel et al., 2013). Furthermore, poly(A) tails can function as a master regulator of gene expression in the cytoplasm (Passmore & Coller, 2021). Interaction of the poly(A) tail with the 5'-cap can stimulate translation, while RBPs can repress translation by blocking ribosome access to the mRNA until translation is required (Hörnberg & Holt, 2013; Passmore & Coller, 2021). RBPs, such as Pumilio, bind the poly(A) tail to regulate the rate of mRNA turnover and thus reduce the available mRNA and amount of translation (Passmore & Coller, 2021).

1.2 Importance of RBPs

As noted in the post-transcriptional processing of mRNAs, RBPs play many critical roles. One particularly interesting role is in tissue-specific regulation of gene expression, which is particularly critical in specific tissues such as the brain, nervous system, and muscle (Cutler et al., 2017; Hörnberg & Holt, 2013; Wigington, Morris, Newman, & Corbett, 2016; Wigington, Williams, Meers, Bassell, & Corbett, 2014). Spatiotemporal control provided by RBPs (see section 1.1.c above) allows for specific subcellular control and evolution of specialized cells as exemplified by highly polarized neurons. Nucleocytoplasmic transport, localization, and local translation are critical steps in regulating gene expression that depend on numerous RBPs. The importance of RBPs in control of gene expression is highlighted by the numerous diseases linked to mutations in RBPs, especially in neurologic diseases.

1.2.a RBPs in mRNA processing

Localization of RNA to distinct sub-cellular locations and the restriction/activation of translation allows for spatiotemporal control of gene expression (Heraud-Farlow & Kiebler, 2014). RBPs bind to mRNAs using RNA-binding domains (RBDs) often in conjunction with other proteins (Heraud-Farlow & Kiebler, 2014). RBPs have four primary categories based on domain: RNA recognition motif (RRM), zinc finger domain (ZnF), double-stranded RNA-binding domain (dsRBD), and heterogeneous nuclear RNP (HNRNP) K-homology domain (KH) (Daubner, Cléry, & Allain, 2013; Gleghorn & Maquat, 2014; Nguyen et al., 2011; Nicastro, Taylor, & Ramos, 2015; Ravanidis, Kattan, & Doxakis, 2018). RBDs allow for RBP specificity as seen with *Staufen* (*Stau*), a dsRBD RBP that functions in mRNA localization, which binds approximately 1200 of the 2500+ mRNAs localized in the brain (Cajigas et al., 2012; Heraud-Farlow & Kiebler, 2014; Ravanidis et

al., 2018). Loss of *Stau* alone alters the steady state level of only 38 of the 1200 mRNAs that it binds, which illustrates that binding of an RBP does not necessarily mean a functional change, in part due to the redundancy of multiple RBPs regulating the same transcript (Heraud-Farlow & Kiebler, 2014; Ravanidis et al., 2018). RBPs bind mRNA targets during transcription to facilitate pre-mRNA processing and the formation of RNP granules using the mRNA target as a scaffold to bring multiple proteins into complex together (Ravanidis et al., 2018). RNPs are dynamic allowing for adaptation to the local cellular environment with RNP proteins changing throughout the lifespan of a specific RNP (Ravanidis et al., 2018). This dynamic response is led by nucleating RBPs such as the eIF3 complex and PABP (Protter & Parker, 2016; Ravanidis et al., 2018).

Localization of mRNA greatly impacts cells with complex architectures. Because neurons have complex architecture with dendritic and axonal projections, these cells serve as an excellent example cell type to illustrate RBP function (Ainsley, Drane, Jacobs, Kittelberger, & Reijmers, 2014; Ravanidis et al., 2018; Shigeoka et al., 2016; Zappulo et al., 2017). Once RNPs have assembled in neurons, mRNA can be transported to the distal neurite projections. Transport is facilitated by RNP RBPs that act as adaptors for molecular motor kinesins and dyneins allowing fast bidirectional transport of RNPs along the neuronal microtubule network (Jia et al., 2015; Kanai, Dohmae, & Hirokawa, 2004; Ravanidis et al., 2018; Schuldt et al., 1998; Song et al., 2015). One important function of RNP RBPs during transport is to restrict translation until proper localization is achieved. This translational repression can occur in a variety of ways with some RBPs blocking translation initiation (e.g., 4E-BP family proteins bind eIF4E blocking initiation), while other RBPs can stall ribosomes or shorten poly(A) tails preventing PABP binding (Jennifer C. Darnell et al., 2011; J. H. Kim & Richter, 2006; Ravanidis et al., 2018). Proper localization and translational de-repression are typically signaled via intracellular signaling cascades that modify

associated RBPs and release them as exemplified by phosphorylation of zip code binding protein 1 (ZBP1) by mammalian target or rapamycin (mTOR) (Urbanska et al., 2017) and fragile X mental retardation protein (FMRP) dephosphorylation/rephosphorylation by protein phosphatase 2A (PP2A) / mTOR (Narayanan et al., 2007; Ravanidis et al., 2018). Such regulation allows dynamic control of translation providing both spatial and temporal control of gene expression.

1.2.b RBPs in disease

Although RBPs play important roles in processing and expression of all eukaryotic RNAs and many are ubiquitously expressed, mutations in genes encoding RBPs often result in tissue-specific diseases (Brinegar & Cooper, 2016). RBP functions are particularly important in the brain and nervous system (as noted in 1.1.c) and result in tissue-specific diseases as seen with mutations in the genes encoding the fragile X mental retardation protein (FMRP) resulting in intellectual disability, survival motor neuron protein (SMN) resulting in spinal muscular atrophy, and three RBPs: TAR DNA binding protein 43, Fused in Sarcoma, and RNA-binding motif 45 resulting in amyotrophic lateral sclerosis and fronto-temporal lobar dementia (Agrawal et al., 2019; Bienkowski et al., 2017; J. C. Darnell & Richter, 2012; Hörnberg & Holt, 2013).

A group of RBPs, poly(A) RNA binding proteins (Pabs), bind to poly(A) RNA with high affinity (Kelly et al., 2010). Conventional Pab proteins use RNA recognition motifs (RRMs) to recognize RNA and regulate mRNA stability, polyadenylation, and translation in the nucleus and cytoplasm (Mangus, Evans, & Jacobson, 2003; Maris, Dominguez, & Allain, 2005; Smith, Blee, & Gray, 2014; Soucek et al., 2016b). Mutations in Pab genes often result in tissue-specific diseases. Defects in polyadenylate-binding nuclear protein 1 (PABPN1) cause muscle specific defects and result in Oculopharyngeal Muscular Dystrophy (Banerjee, Apponi, Pavlath, & Corbett, 2013). The

tissue-specificity of the pathology caused by defects in genes encoding RBPs is particularly interesting given that many are ubiquitously expressed and suggests that the tissues where defects arise are sensitized to alterations in spatiotemporal control of gene expression.

The close association of RBP mutation with disease, specifically neurological disease, are exemplified by mutation in *Pumilio* (*Pum*), *Stau*, and *FMR1* (Ravanidis et al., 2018). Pum is an RBP with a Pumilio homology domain (Pum-HD) that binds a consensus sequence (i.e., UGUANAUA) in mRNA (Gerber, Luschnig, Krasnow, Brown, & Herschlag, 2006; Ravanidis et al., 2018; Weidmann et al., 2016; Zamore, Bartel, Lehmann, & Williamson, 1999). Stau functions to repress translation during transport via antagonization of eIF4E (Cao, Padmanabhan, & Richter, 2010; Menon et al., 2004; Ravanidis et al., 2018; Vessey et al., 2006). In mouse models, Pum loss has been linked to progressive motor dysfunction and spinocerebellar ataxia type 1 like neurodegeneration (Gennarino et al., 2015; Ravanidis et al., 2018). In *Drosophila*, Pum functions in memory formation, olfactory learning, and dendrite morphogenesis (Dubnau et al., 2003; Ravanidis et al., 2018; Ye et al., 2004). Stau is an RBP with a double stranded RBD which can enhance translation or promote RNA decay via interactions with the 3'-UTR (Dugré-Brisson et al., 2005; Y. K. Kim, Furic, DesGroseillers, & Maquat, 2005; Park, Gleghorn, & Maquat, 2013; Ravanidis et al., 2018). Stau regulates dendritic spine and arbor morphology, long-term potentiation, and synaptic plasticity (Goetze et al., 2006; Lebeau et al., 2008; Ravanidis et al., 2018; Vessey et al., 2008). FMRP is a largely cytoplasmic brain RBP with two KH domains and an RGG-type RBD (Eberhart, Malter, Feng, & Warren, 1996; Ramos, Hollingworth, & Pastore, 2003; Ravanidis et al., 2018). FMRP functions in mRNA transport to repress translation of mRNA targets via ribosomal stalling and mRNA trapping (Jennifer C. Darnell et al., 2011; Mazroui et al., 2002; Ravanidis et al., 2018). Mutations in *FMR1* led to fragile-X syndrome (FXS), which is the

most common form of inherited intellectual disability (Higuchi et al., 1997; Ravanidis et al., 2018). FMRP regulates dendritic spine morphology and synaptic function (e.g., modulates Ca^{+2} signaling and neurotransmitter release) (Cruz-Martín, Crespo, & Portera-Cailliau, 2010; Deng et al., 2013; Grossman, Aldridge, Weiler, & Greenough, 2006; Pan, Zhang, Woodruff, & Broadie, 2004; Ravanidis et al., 2018; Suresh & Dunaevsky, 2017). These examples illustrate how defects in various stages of RNA post-transcriptional processing can cause disease that impact neurological function. Continued work is required to define precisely what molecular defects underlie RBP-linked neurological pathology.

1.2.c Regulation of RNA in the brain

Regulation of gene expression via post-transcriptional mechanisms is particularly important in the brain due to the highly complex neuronal architecture required for proper function and the unique, polarized structure of neurons that leave them particularly reliant on spatiotemporal control of gene expression. The sensitivity of nervous system tissue to RBP loss is linked to enhanced reliance on post-transcriptional mechanisms, including mRNA localization and local translation, and is supported by the presence of brain-specific 3'-UTRs (Engel, Arora, Goering, Lo, & Taliaferro, 2020; Thelen & Kye, 2020). During development of the brain, neurons create complex architectures that form interconnected cellular networks allowing for transmission of signals among thousands of neurons. The polarized morphology of neurons is particularly unique with each cell containing axonal and dendritic projections that extend enormous distances relative to cell size (A. Lee et al., 2003). The growth and guidance of neuronal projections is largely directed through the growth cone, which responds to guidance cues to steer the neuronal projection (Holt et al., 2019; Stoeckli, 2018). Neuronal guidance is regulated in part by predelivery of mRNAs

to the growth cone and local translation at the growth cone, allowing for rapid shifts in translation to respond to extracellular cues that would otherwise be lost due to the slow speed of intracellular transport and the large distance from the nucleus to the distal end of the projecting neuron (Holt et al., 2019; Hörnberg & Holt, 2013; Maday et al., 2014; Stoeckli, 2018).

Mechanisms regulating axo-dendritic growth and guidance are vastly complex. Axonal development is guided by long range cues, such as netrin and sonic hedgehog signaling, and by short range cues such as the repulsive signaling pathways Slit-Robo and Sema3B-Nnpn-2 (Stoeckli, 2018). These developmental cues also have a substantial amount of cross talk between them as with Slit-Robo and Sema3B-Nnpn-2 in midline crossing of commissural axons. Dendritic development is controlled by pathways including PI3K-mTOR, Hippo, and Slit-Robo (Puram & Bonni, 2013). While there are mechanisms unique to the growth of each cellular compartment, there are shared pathways, such as Slit-Robo, that regulate the growth in each subcellular compartment. Disruptions in neurodevelopment can occur through defects in proliferation, migration, guidance, branching, or synaptogenesis leading to a wide range of neuromorphological defects such as microencephaly (reduced brain size), lissencephaly (smooth brain surface), and cortical dysplasia/heterotopia (neuronal mislocalization) (Sans, Ezan, Morreau, & Montcouquiol, 2016). Ultimately, these disruptions to neurodevelopment alter the structure or function of the brain and result in cognitive disorders such as autism spectrum disorder (ASD), intellectual disability (ID), attention-deficit hyperactivity disorder (ADHD), and many more neurobehavioral and neuropsychiatric disorders (Huguet & Bourgeron, 2016; Sans et al., 2016; Shen & Gong, 2016). Proper neuronal architecture and function is achieved in large part through precise spatiotemporal control of gene expression by RBPs in both the axonal and dendritic neuronal subcellular compartments (F. B. Gao & Bogert, 2003; Stoeckli, 2018).

1.3 Drosophila as a model – of RBP function and disease

Given the complexity of neurodevelopmental pathways, model systems have been critical in defining critical players and interactions. Use of model systems is facilitated by the conservation of both the pathways and factors required to control gene expression. *Drosophila melanogaster* provides many advantages as a genetic model system. The generation time of *Drosophila* is quick at 10 days from egg laying to adult eclosion (Figure 1-2). The rapid generation time allows for efficient screening of candidate alleles for genetic interaction. Flies also have a well-established set of genetic tools, such as the yeast-derived *UAS-Gal4* system, optogenetic control of specific neurons via light-sensitive ion channels, or the labeling of single cells using mosaic analysis with a repressible cell marker (MARCM), which allow for spatiotemporal control of gene expression.

1.3.a The Drosophila brain

Drosophila provide an excellent genetic model for studying the genetics and molecular biology underlying neurodevelopment (Spindler & Hartenstein, 2010). Importantly all animal brains work in largely similar ways, therefore, the smaller more simplistic fly brain provides a good, genetically tractable model to test human brain function and development (Scheffer et al., 2020). Compared to a human brain, the fly provides a simplified brain with the fly brain consisting of ~100,000 neurons while the human brain consists of ~86 billion neurons (Scheffer et al., 2020). A particular advantage to studying *Drosophila* neurodevelopment is due to the clonal nature of the brain (Spindler & Hartenstein, 2010). *Drosophila* neurons are grouped into lineage units that allow for the study of sibling neurons originating from single neuroblast parents and are bundled together during development (e.g., mushroom body Kenyon cells). Clonal sibling neurons provide a greater

resolution, relative to the mammalian brain where little lineage information is available, to investigate aspects of neurodevelopment such as axon guidance, branch formation, and circuit formation (Spindler & Hartenstein, 2010). The *Drosophila* brain forms from approximately 100 neuroblasts derived from the early procephalic (head) neuroectoderm (T. Kunz, Kraft, Technau, & Urbach, 2012). These initial neuroblasts form ~1500 neurons that make up the beginning of the fly brain during embryonic development (Spindler & Hartenstein, 2010). A portion of the neurons born during embryonic development undergo a restructuring and reorganization during both larval development and pupal metamorphosis into the adult fly (Heisenberg & Technau, 1982; T. Kunz et al., 2012; T. Lee, Lee, & Luo, 1999; Marin, Watts, Tanaka, Ito, & Luo, 2005; Williams & Truman, 2004). The neuroblasts active during embryonic development undergo mitotic quiescence and then reactivation during larval and pupal development with nearly all of the original 100 neuroblasts reactivating postembryonically (Cardona et al., 2010; Thomas Kunz, Kraft, Technau, & Urbach, 2012; Pereanu & Hartenstein, 2006; Yu, Chen, Shi, Huang, & Lee, 2009; Yu et al., 2010; Yu & Lee, 2007). These periods of developmental reorganization of the fly brain provide multiple timepoints (e.g., embryonic, larval, pupal, and adult) and neuronal structures (e.g., mushroom bodies, fan-shaped body, ellipsoid body, protocerebral bridge, noduli, etc.) to investigate (Thomas Kunz et al., 2012; Spindler & Hartenstein, 2010; Wolff & Rubin, 2018). The variety of neuronal structures and the well-established developmental time courses of these neuronal structures provide a model to further understand the intricacies of *Drosophila* neurodevelopment and neurological disease.

1.3.b Mushroom Bodies

The *Drosophila* mushroom bodies (MBs) are twin neuropil structures that mirror across the brain midline and required for olfactory learning and memory (Figure 1-4 A) (Davis, 2011; Heisenberg, 2003; Thomas Kunz et al., 2012). The MBs are formed from approximately 2000 Kenyon cell neurons that have dendritic clusters forming the calyx around the cell bodies and axon bundles that project to form dorsal and medial lobes. Five distinct lobes are formed, with two ventral α- and α'-lobes and medial β-, β'-, and γ-lobes (Figure 1-4 A,B,C). Kenyon cells form from four neuroblasts termed the mushroom body neuroblasts (MBNBs). Initially, the MB ventral and medial structures are all formed from γ-neurons until mid-larval stage when reorganization occurs with the γ-neurons being pruned back and the lobes of the MB reforming (Aso, Hattori, et al., 2014; T. Lee et al., 1999). During late larval stage, γ-neuron reprojection from the neuroblast pools form new medial lobes and α'/β'-neurons project down from the neuroblast pools, bifurcating at the anterior end of the pedunculus to form a ventral α'-lobe and β'-lobe (Figure 1-3 D) (T. Lee et al., 1999). During early pupal development (24 through 72hrs after puparium formation), α/β-neurons project from the neuroblast pools to make the last of the five MB lobes. As with α'/β'-neurons, α/β-neurons bifurcate to form the ventral α-lobe and medial β-lobe, with the α/β -lobes sitting in front of the α'/β'-lobes (Figure 1-3 B,C) (Aso, Hattori, et al., 2014). These MB axon bundles form from the outside inward with the newest axonal projections residing at the core of the bundle. The parallel fibers formed by the bundles of α/β-, α'/β'-, and γ-neurons (approximately 2000 total neurons) form synapses with a surprisingly small number of outputs termed MB output neurons (MBONs) (Aso, Sitaraman, et al., 2014). A total of 21 MBONs have dendrites that innervate along stereotyped sub-regions of the MB axon bundles and are responsible for the output of signals to neuropils outside the MB (Aso, Sitaraman, et al., 2014). The convergence of learned

responses outputting through 21 MBONs to five specific neuropils emphasize the necessity for proper neuronal architecture.

1.3.c Dendritic arborization neurons

The central nervous system (CNS) of *Drosophila* is primarily unipolar unlike the multipolar neurons of the mammalian CNS, but in the peripheral nervous system (PNS) of the fly many neurons are multipolar as in the mammalian CNS (F. B. Gao & Bogert, 2003). Complex neuronal architecture requires proper axo-dendritic guidance, and the complexity of dendritic arbors relies on a variety of cues to guide growth and projection (F. B. Gao & Bogert, 2003; Jan & Jan, 2001; Scott & Luo, 2001). Dendritic arborization (da) neurons, a subgroup of multiple dendritic (md) neurons, are segmentally stereotyped PNS neurons that have elaborate dendritic arbors that spread along the epidermis (Brown et al., 2017; F. B. Gao & Bogert, 2003; Grueber, Jan, & Jan, 2002; W. K. Yang & Chien, 2019). These da neurons tile the body wall of the larvae and sit between the basal muscle and apical epidermal cells (Figure 1-4 A,C,D) (W. K. Yang & Chien, 2019). The da neuron dendritic arbor is attached to epidermal extracellular matrix by dendritic integrin and epidermal laminin on the extracellular matrix, with a portion of the dendritic arbor sitting within the epidermal cell (Figure 1-4 D) (W. K. Yang & Chien, 2019). The morphology of da neurons is used to categorize them into four classes, classes I through IV, with class IV having the most complex and elaborate arbor (Grueber et al., 2002; W. K. Yang & Chien, 2019). There are 15 da neurons per segment, each with a different notation (Figure 1-4 B), that function in proprioception, muscle contraction, & nociception (Grueber et al., 2002; W. K. Yang & Chien, 2019). Dendritic arbors of da neurons express mechano-transducers Painless (Tracey, Wilson, Laurent, & Benzer, 2003), Pickpocket (Zhong, Hwang, & Tracey, 2010), and Piezo, but

Pickpocket (*ppk*), a Degenerin/Epithelial Sodium Channel subunit, is confined to class IV da neurons (Brown et al., 2017; W. K. Yang & Chien, 2019). The dorsal-most class IV neurons, termed ddaC neurons, extend to both the anterior and posterior body segment boundaries as well as from the dorsal midline to the lateral cluster neurons (F. B. Gao & Bogert, 2003). Using a *ppk*>Gal4 driving CD8:GFP expression, the neuromorphology of ddaC neurons dendritic arbors can be examined *in vivo*, making this an ideal system to probe perturbations in dendritic architecture.

1.4 Intellectual disability, ZC3H14, and Nab2

1.4.a Intellectual disabilities

Intellectual disability (ID) is a developmental disorder characterized by deficits in cognitive function and adaptive behavior (Association, 2013; Schwartz & Boccuto, 2016). Deficits in cognitive function are typically defined by an intellectual quotient (IQ) of less than 70, while deficits in adaptive behavior are identified qualitatively by difficulty with conceptual, social, and practical skills. If deficits in cognitive function and adaptive behavior are identified in a patient during development (i.e., before age 18), the individual may be diagnosed with ID. Due to deficits in adaptive functioning, ID patients can struggle with tasks such as driving a car or putting on clothing, and therefore require ongoing care and support, which the CDC estimates costs $1-2 million in lifetime support (NCBDDD (National Center on Birth Defects and Developmental Disabilities), 2017). ID is a relatively common disorder with a worldwide prevalence of 2-3% in the general population (Perou et al., 2013; Schwartz & Boccuto, 2016), which suggests a large health, social and economic impact.

ID is caused by a wide range of genetic factors, including both monogenic (e.g., FMRP mutation resulting in Fragile X Syndrome) and polygenic (e.g., multiple synergistic gene mutations resulting in ID), and environmental factors (e.g., alcohol consumption during fetal development resulting in Fetal Alcohol Syndrome). Over 700 genes have been linked to ID (Srivastava & Schwartz, 2014), but ~60% of ID cases have no clear cause (Rauch et al., 2006). Genetic approaches with whole exome and genome sequencing have led to the identification of many of these 700+ ID-linked gene mutations (Saillour & Chelly, 2016; Srivastava & Schwartz, 2014), which has opened new avenues to greater understand the molecular mechanisms underlying ID. While the genetic causes of ID are vast, the impact of ID-linked gene mutations converge on a far smaller number of the cellular and molecular pathways, often those affecting neuromorphogenesis or synaptic function. ID can present with malformation in cortical development (i.e., neuromorphogenesis), which can show up on MRI brain scans or can present without apparent malformations suggesting a defect in synaptic connectivity or function (Saillour & Chelly, 2016). Monogenic causes of ID are particularly valuable for understanding the mechanisms behind ID because they provide a more genetically tractable system to identify disrupted cellular and molecular mechanisms that may more broadly underlie IDs.

1.4.b Mammalian *ZC3H14*

The human *ZC3H14* gene encodes a ubiquitously expressed zinc-finger, polyadenosine RBP (ZnF CysCysCysHis #14) that is lost in an inherited form of intellectual disability (Pak et al., 2011). The *ZC3H14* mutations were identified in a cohort of over 200 consanguineous Iranian families through a large-scale autozygosity mapping and linkage analysis that was performed to identify molecular causes of non-syndromic, autosomal-recessive ID (NS-ARID) (Pak et al., 2011;

A. Walter, Masoud, Kimia, Andreas, & Farkhondeh, 2011). Mammalian ZC3H14 protein contains three structural domains. The N-terminal domain contains a conserved Proline-Tryptophan-Isoleucine (PWI) fold, a protein-protein interaction domain, followed by two predicted classic nuclear localization signals (cNLSs), and then the C-terminus containing five tandem zinc fingers (ZnFs) (Figure 1-5) (Fasken, Corbett, & Stewart, 2019). Three different patient *ZC3H14* mutations have been identified, an R154X creating an early stop codon in exon 6, a 25-bp deletion located 16-bp downstream of the 3'-end boundary of exon 16, and an N309fs creating a frameshift (Al-Nabhani et al., 2018; Pak et al., 2011; A. Walter et al., 2011) (Figure 1-5). While ZC3H14 is ubiquitously expressed, mutations cause tissue-specific consequences, suggesting that the role of ZC3H14 is particularly important in neurons.

A *Zc3h14* knockout mouse model (*Zc3h14*$^{\Delta ex13/\Delta ex13}$) was generated to explore the function of ZC3H14 in mammals (Rha et al., 2017). The mouse model removes exon 13, which encodes part of the essential zinc finger RNA binding domain and produces a frameshift. Knockout of the murine ortholog *Zc3h14* reveals that ZC3H14 is not essential in mice, but *Zc3h14* loss does lead to altered dendritic spine and axon morphology among hippocampal neurons, and impairs working memory (S. K. Jones et al., 2020; Rha et al., 2017). Proteomic analysis of P0 *Zc3h14*$^{\Delta ex13/\Delta ex13}$ hippocampi identified several proteins involved in synaptic development and function that change in abundance upon ZC3H14 loss (Rha et al., 2017). These studies in mouse provide a mammalian model to study the consequences of loss of ZC3H14.

1.4.c Drosophila Nab2

As mentioned above (section 1.3.a), *Drosophila* provide an ideal model for studying the genetics of neurodevelopment. A *Drosophila melanogaster* model of *Nab2* (nuclear poly(A)

binding protein 2), the sole fly *ZC3H14* ortholog, was generated so that the genetic, cellular, and molecular functions of ZC3H14 could be further understood through examination of the fly ortholog (Bienkowski et al., 2017; Corgiat & List et al., 2021; Jalloh et al., 2020; Kelly et al., 2016; W. H. Lee et al., 2020; Pak et al., 2011; Rounds et al., 2021). While Nab2 only shares 41% amino acid identity with ZC3H14, the conservation of structure is high within the functionally important domains, the N-terminal PWI-like domain, predicted NLS, and five tandem CCCH-type Zinc finger RNA-binding domains shared between the fly and human orthologs (Pak et al., 2011). The *Nab2* fly model was generated via imprecise excision of a P-element (*EY08422*) (Pak et al., 2011), located at the end of 5' end of the *Nab2* locus (Bellen et al., 2004), which removes a large portion of the gene, including the transcriptional start site, generating an RNA and protein null (Pak et al., 2011). Functional studies of Nab2 reveal that Nab2 is required for adult viability, locomotor function, and regulation of poly(A) tail length, and is required cell-autonomously to support olfactory memory and axonal branching and projection into the brain MB. Significantly, transgenic expression of human ZC3H14 only in fly neurons is sufficient to rescue a variety of *Nab2* null phenotypes (Kelly et al., 2016; Pak et al., 2011), supporting a model in which Nab2 and ZC3H14 share critical molecular roles and mRNA targets.

1.4.d Planar cell polarity pathway based intellectual disability

Many tissues and cell types exhibit polarization including the vertebrate brain. Planar cell polarity refers to the coordinated orientation (i.e., polarization) of cells across a plane of tissue (Adler, 2012; Adler & Wallingford, 2017; Goodrich & Strutt, 2011; M. Mlodzik, 2020; Peng & Axelrod, 2012a; Sans et al., 2016). There are many types of polarization that occur within cells and tissues, but polarization of tissue across the plane orthogonal to the apical-basolateral axis is

regulated by the planar cell polarity (PCP) pathway. PCP was originally investigated in *Drosophila* but was subsequently defined in vertebrates, including *Xenopus* and *Danio rerio*, as well as in mammals, including mouse and human. The PCP pathway is now understood as the noncanonical branch of Wnt signaling and functions in a variety of tissues. In *Drosophila*, PCP signals are based on asymmetric distribution of two apically localized transmembrane complexes, the Stan-Vang-Pk complex (Starry Night, also Flamingo-Van Gogh-Prickle) and the Stan-Fz-Dsh-Dgo complex (Frizzled-Disheveled-Diego), which are intracellularly antagonistic but intercellularly attractive, leading to apical polarization across an epithelial plane (Figure 1-6) (Adler, 2012; Adler & Wallingford, 2017; Boutros & Mlodzik, 1999; Goodrich & Strutt, 2011; M. Mlodzik, 2020; Peng & Axelrod, 2012b; Taylor, Abramova, Charlton, & Adler, 1998; Vladar, Antic, & Axelrod, 2009).

The core components of the PCP pathway and its functions are largely conserved from flies to mammals. The *Drosophila* Stan-Vang-Pk complex has mammalian orthologs for Stan of cadherin/epidermal growth factor/lamininG seven-pass G-type receptors 1, 2, and 3 (Celsr1/2/3), for Vang of Van Gogh-like 1 and 2 (Vangl1/2), and Pk of Prickle 1 and 2 (Pk1/2) (Ezan & Montcouquiol, 2013; Sans et al., 2016). The Stan-Fz-Dsh-Dgo has mammalian orthologs for Fz of Frizzled 1, 2, 3, and 6 (Fz1/2/3/6), for Dsh of Dishevelled 1,2, and 3 (Dvl1/2/3), and for Dgo of Diversin/Ankrd6 (Ezan & Montcouquiol, 2013; Sans et al., 2016). In flies, wing hair and ommatidial orientation are regulated by PCP. In the wing cells, PCP drives the turning of the cells through asymmetric localization of the Fz and Vang complexes, but if any member of the complex is defective, the orientation of the wing hairs become disrupted. Disruption of PCP pathway function by loss of one member is in part due to the intracellular antagonism of the Fz and Vang complexes. If the function of a single member of either the Fz or Vang complex is lost, asymmetry of the complexes and thus PCP function can be disrupted. Similar to the effects of PCP loss found

in fly wings, mammalian PCP regulates development of tissues including orientation of the mouse cochlea and closure of the neural tuba via convergent extension (CE) (Chacon-Heszele & Chen, 2009; Fenstermaker et al., 2010; B. Gao, 2012; Rida & Chen, 2009).

Core PCP components signal to downstream effector molecules (Adler, 2012; Courbard, Djiane, Wu, & Mlodzik, 2009; Fagan et al., 2014; Gombos et al., 2015; Soldano et al., 2013) that exert localized effects on the F-actin cytoskeleton that, in turn, guide epithelial traits like proximal-distal wing hair orientation in *Drosophila* and sensory hair cell polarity in the mouse cochlea (M. S. and M. Mlodzik, 2010; Qian et al., 2007; J. Wang et al., 2005).While PCP signaling has primarily been explored in epithelial tissue, the discovery of CE defect in mammals has led to an increased appreciation for PCP function in neuronal tissues (B. Gao, 2012). PCP has been identified in both fly and mouse neurodevelopment having functions ranging from axon guidance to synaptogenesis and from dendritic development to cognitive function. In the fly, loss of the core PCP components *stan*, *Vang*, *pk*, *fz*, or *dsh* individually disrupts α and β axon projection into the MBs (Ng, 2012; Shimizu, Sato, & Tabata, 2011). Intriguingly, loss of *stan* or its LIM-domain adaptor *espinas* (*esn*) also disrupts dendritic self-avoidance among the class IV da neurons (Matsubara, Horiuchi, Shimono, Usui, & Uemura, 2011), demonstrating a requirement for PCP factors in both axon and dendrite morphogenesis in specific sets of neurons in the CNS and PNS. In the mouse, all core PCP orthologs are expressed during development of the brain and CNS (Rock, Schrauth, & Gessler, 2005; Sans et al., 2016; Tissir & Goffinet, 2006), and loss of *Vangl2* leads to defects in axon guidance of spinal cord commissural axons (Shafer, Onishi, Lo, Colakoglu, & Zou, 2011).

PCP gene mutations have been linked to many human pathologies including neural tube closure defects, craniorachischisis, polycystic kidney disease, but have also been linked to defects

in hearing, balance, vision, skeletal development, and cardiovascular function (Greene & Copp, 2014; Juriloff & Harris, 2012; Papakrivopoulou, Dean, Copp, & Long, 2014; Sadeqzadeh, Bock, & Thorne, 2013; Sans et al., 2016; Yates & Dean, 2011). Interestingly, in mouse models, mutations in nearly all core PCP components (*Celsr1/2, Dvl1/2/3, Fz3/6,* and *Vangl1/2*) have documented cases of craniorachischisis, a congenital malformation of the nervous system that affects about 1 in 1000 human children (Sans et al., 2016). The molecular basis of craniorachischisis is still poorly understood but appears to have some link to the PCP pathway. PCP gene mutations have been increasingly linked to developmental delay, intellectual disabilities, and autism spectrum disorder (ASD)(Sans et al., 2016). Many genes have been implicated through mouse models, but mutations in human patients have been identified linking *FZD4, WNT5A, DVL1/2,* and *ANKRD6* to IDs, *DVL2, PRICKLE1/2, WNT2, FZD5, VANGL1,* and *ANKRD6* to ASD, *WNT5A* and *DVL1* to Robinow syndrome, and *VANGL2* to neural tube defects (Sans et al., 2016). The multitude of PCP gene mutations linked to human disease highlight the significance of the PCP pathway during development and also suggests that using model systems to study PCP pathway function could provide more understanding of human diseases such as neural tube closure defects and intellectual disabilities.

There are also plenty of PCP downstream effectors that are linked to neurodevelopmental disorders, but the human *DLG3* (Synapse-Associated protein 102 *Drosophila* discs large tumor suppressor protein) gene is particularly interesting. *DLG3* functions in the trafficking of PCP receptors to and from synapses as well as trafficking of NMDA receptor subunits, and *DLG3* has been linked to IDs in Finnish families with severe deficits in cognitive function and adaptive behavior (Sans et al., 2016). Growing evidence indicates that PCP gene mutations increase susceptibility to IDs and ASD through detrimental disruption of synapse neurotransmission,

synaptic long-term potentiation, and synaptic long-term depression. These links to disease and neurodevelopmental function suggest that more effectors of the PCP pathway will be found, and the network of PCP component interactions will grow.

1.5 Scope of dissertation

Post-transcriptional regulation of gene expression by RBPs, especially in neurodevelopment is a growing and active area of research. Nab2/ZC3H14 RBPs are required for proper post-transcriptional regulation of mRNA with evidence for roles in polyadenylation (Pak et al., 2011), alternative splicing (Jalloh et al., 2020), and local translation (Bienkowski et al., 2017) with impacts on neurodevelopment (Corgiat & List et al., 2021; Kelly et al., 2016; Pak et al., 2011) and cognitive function in flies and mammals (Kelly et al., 2016; Pak et al., 2011; Rha et al., 2017). The *Drosophila melanogaster* model of Nab2 has provided evidence for conserved functions from flies to mammals, but, despite these findings, few insights into the functional targets of Nab2 or the particular importance of Nab2 function in neurodevelopment have been made.

The overarching goal of this research is to address the question, what is the function of Nab2, and why is it particularly critical in neurons? In this dissertation, we probe the basis of Nab2 function in the brain, and we provide the first evidence for Nab2 function in *Drosophila* dendritic development. In **Chapter 2**, we address what the function of Nab2 in the brain is by revealing changes to the *Nab2* null brain proteome during development. We identify roles for Nab2 in controlling the dynamic growth of axons in the developing brain mushroom bodies (MBs), which support olfactory learning and memory, and in regulating the abundance of a small fraction of the total brain proteome. Collectively, these proteomic changes link Nab2 to the processes of brain morphogenesis, neuroblast proliferation, circadian sleep/wake cycles, and synaptic development.

The analyses from **Chapter 2** suggest a role for Nab2 in control of a subset of the brain proteome that is critical for proper neurodevelopment and may be conserved from *Drosophila* Nab2 to mammalian ZC3H14. In **Chapter 3**, we investigate the role of Nab2 in two neuronal contexts, CNS axons of the pupal MB and PNS dendrites of larval class IV dorsal dendritic arbor C neurons. We determine that Nab2 acts cell autonomously to guide axonal and dendritic development, and additionally genetically link Nab2 and the planar cell polarity (PCP) pathway in both axonal and dendritic development. Collectively, these genetic and cell biological data demonstrate the requirement of Nab2 for axonal and dendritic development by a PCP-linked mechanism and identify the Nab2 RBP as required for the accumulation of Vang protein into distal axonal compartments. Experiments presented in this dissertation provide the first evidence for Nab2 regulating *Drosophila* dendritic development, provide a window into the impact of Nab2 on the brain proteome, and suggest a PCP-linked mechanism for Nab2 function in neurodevelopment. Finally, to end, in **Chapter 4** we discuss the findings in this dissertation, providing an overarching context and discuss future directions for studies moving forward.

1.6 Figures

Figure 1-1

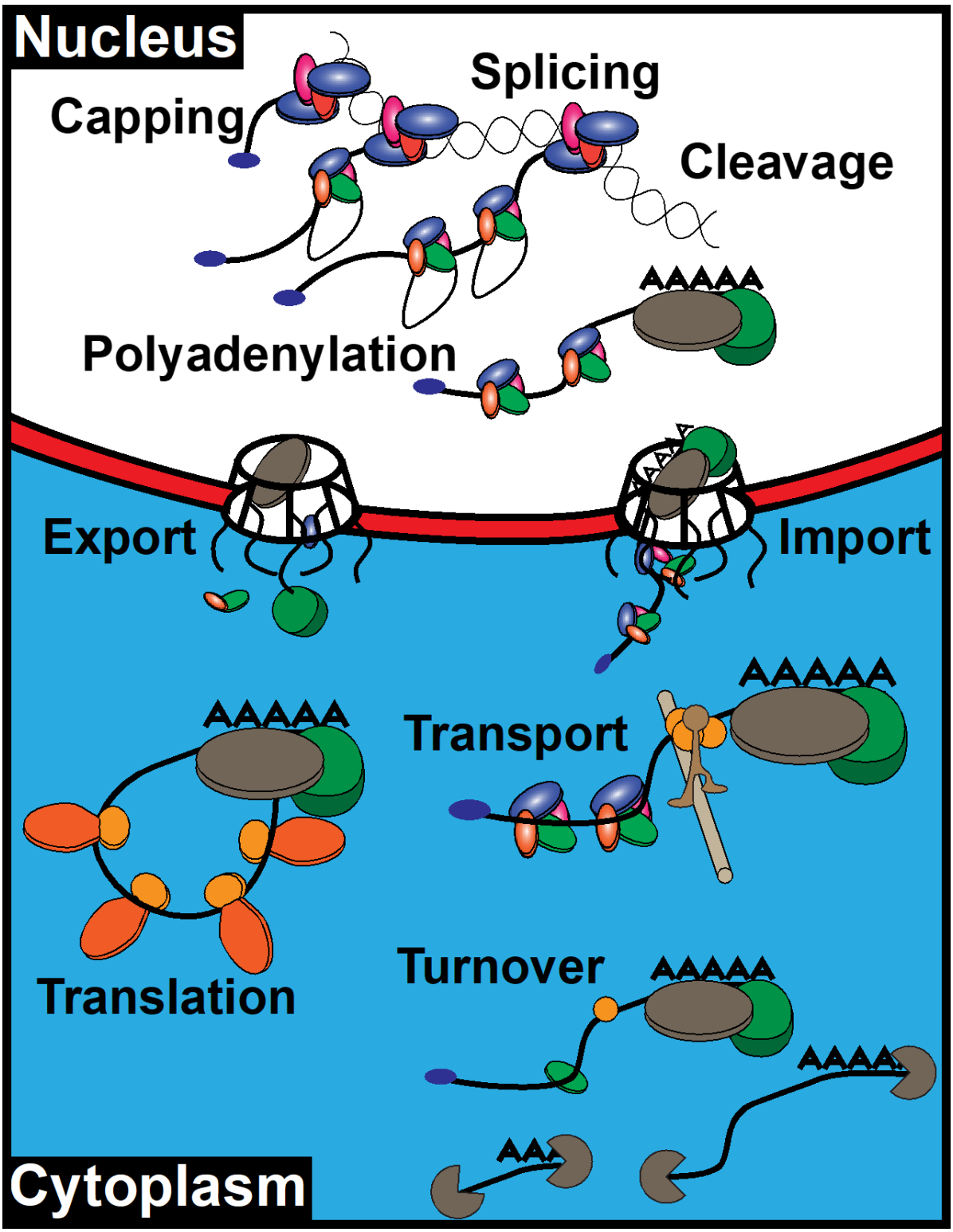
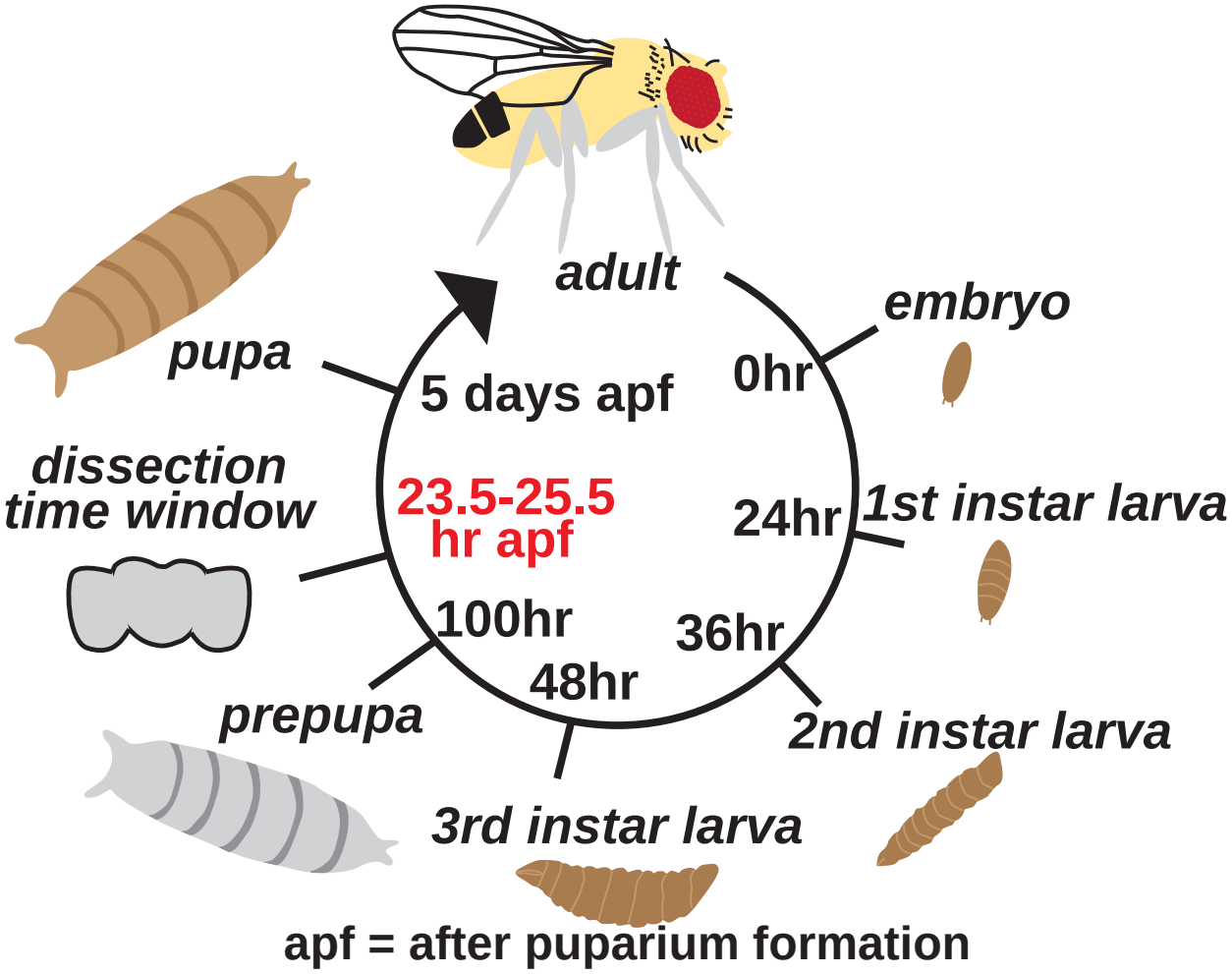


Figure 1-1: Post-transcription gene regulation.

Post-transcription processing of mRNA occurs in both the nucleus and cytoplasm. Within the nucleus, the pre-mRNA undergoes capping, splicing, cleavage, 3’ end processing, and polyadenylation. Mature mRNA undergoes nucleocytoplasmic transport with export and import occurring through the nuclear pore. Within the cytoplasm, mRNA undergoes localization, translation, and turnover of the mRNA. Nucleus denoted by white background with cytoplasm indicated by blue background. Red bar indicates nuclear envelop with cage structures indicating nuclear pore.

Figure 1-2


adult
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24hr
2nd instar larva
36hr
3rd instar larva
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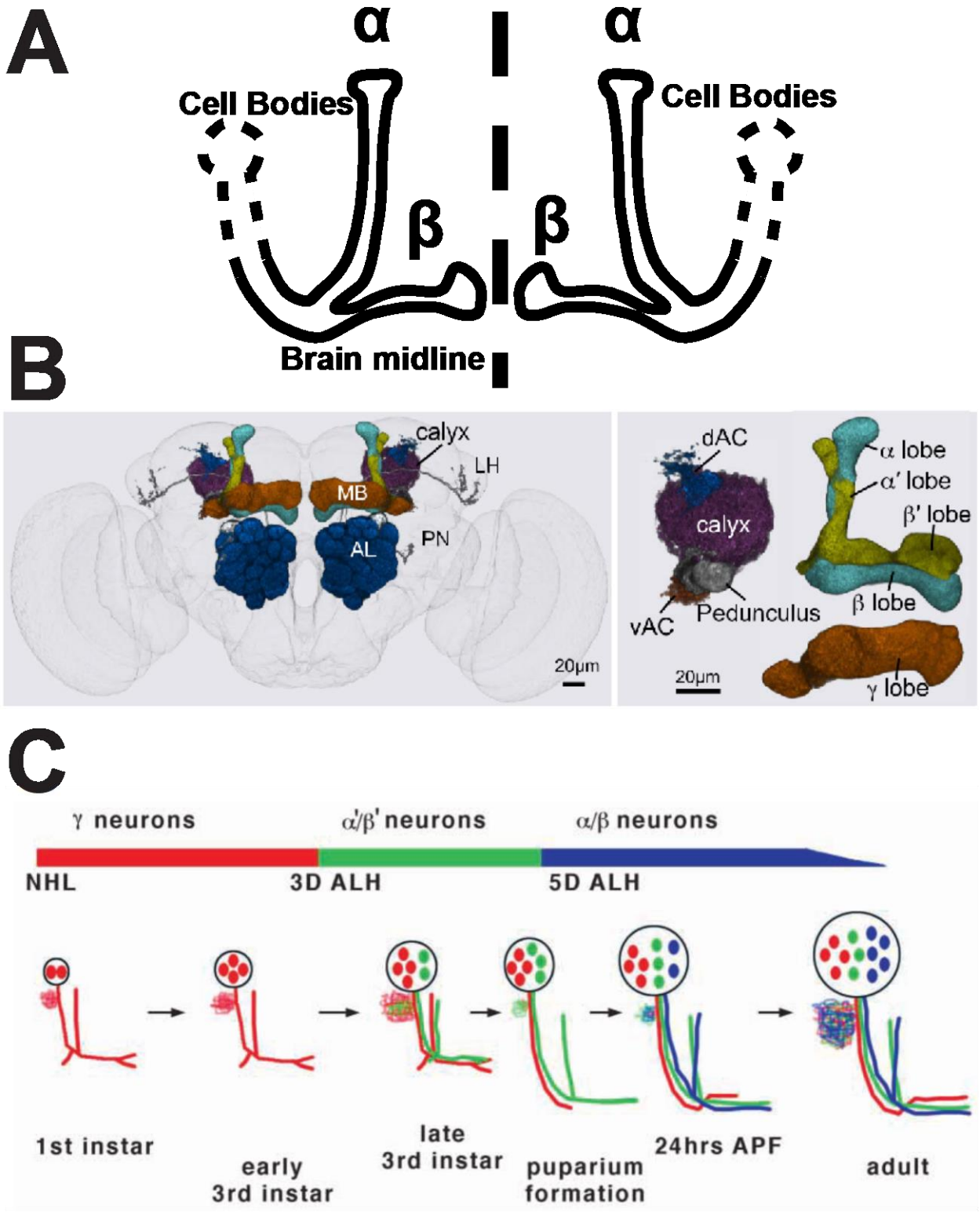
prepupa
100hr
dissection
time window
pupa
5 days apf
23.5-25.5
hr apf
apf = after puparium formation

Figure 1-2: The *Drosophila* life cycle.

The *Drosophila* life cycle with developmental stage and hours of development depicted. *Drosophila* have a ten day life cycle consisting of three developmental stages (embryo, larvae, and pupae) before reaching the adult stage. The time window (23.25-25.5 hr apf) in **red** is highlighted as a midpoint in mushroom body development; also denotes the dissection time window used for experiments in Chapter 2. apf = after puparium formation.

Figure 1-3


A
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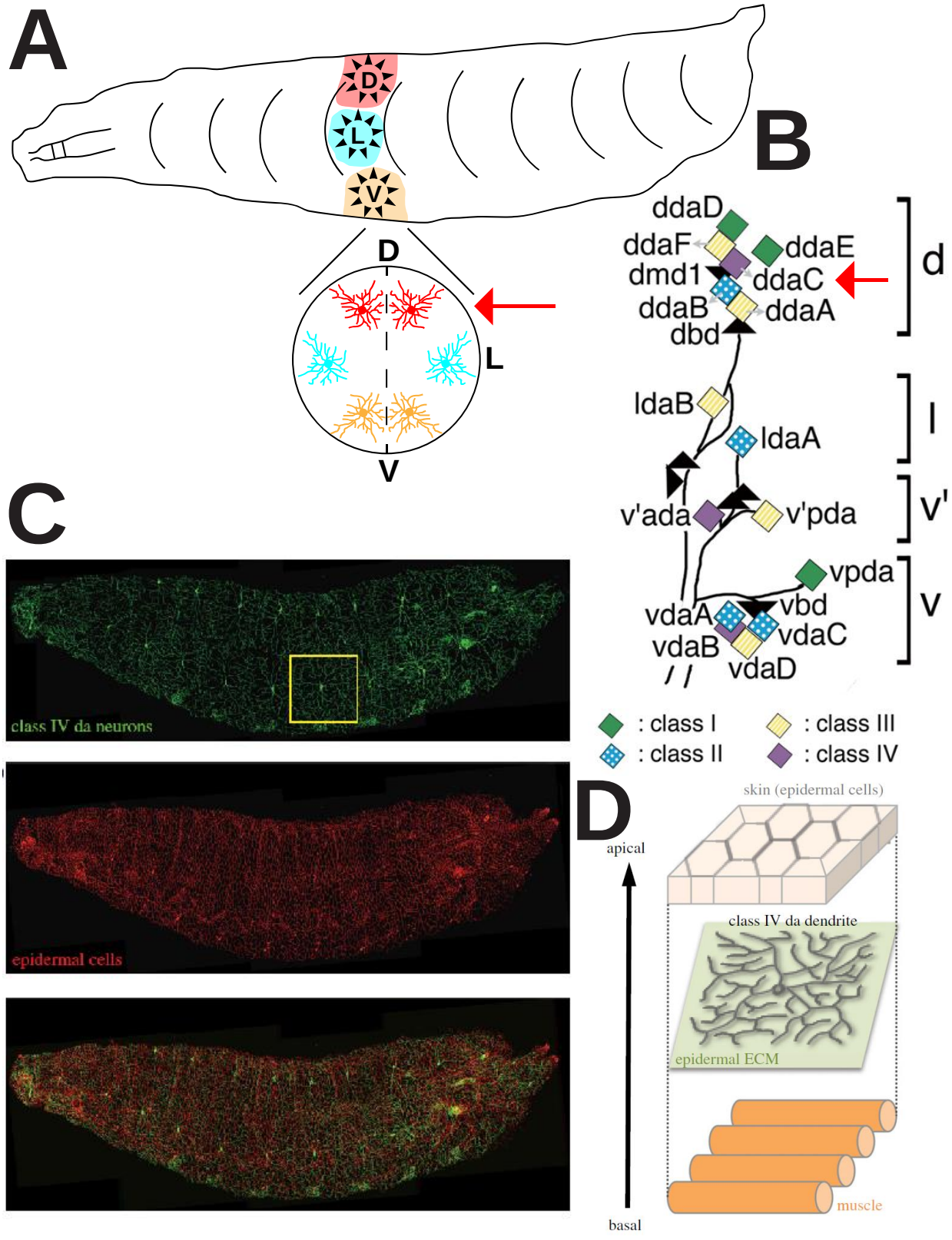
NHL
3D ALH
5D ALH
1st instar
early 3rd instar
late 3rd instar
puparium formation
24hrs APF
adult

Figure 1-3: *Drosophila* mushroom body neuroanatomy.

The *Drosophila* mushroom bodies (MBs) are centers of olfactory learning and memory. (A) MBs are formed from four neuroblast pools that project axons down from cell bodies to form the α-, β-, and γ-lobe structures. The MBs are twin neuropil structures that mirror across the midline of the brain (dashed line). MBs are visualized by fasciclin II staining which labels the Drosophila orthologue of neural cell adhesion molecule (Mao & Freeman, 2009), and allows for visualization of α- and β-lobes. (B) Left – Digital image of the *Drosophila* brain constructed using FluoRender rendering software. Image shows MBs and antennal lobes (AL) structures in color. MB calyx and lateral horn (LH) indicated; AL projection neurons (PN) indicated. Right – Detailed subregions of the MBs. Subregions are separated from the typical position shown in B so that more detailed structure can be seen without overlap. Calyx (purple) with dorsal accessory calyx (dAC) (navy blue) and ventral accessory calyx (vAC) (magenta), and pedunculus (gray) represent the ventral region of the MBs which contain the Kenyon cell bodies and dendritic clusters. There are three groups of MB lobes formed from axon projections, the γ-lobes (orange) which generate one medial projecting lobe in the pupal and adult brain, αβ-lobes (cyan) which bifurcate as the project forming the medial projecting β-lobe and dorsal projecting α-lobe, and α'β'-lobes (green) which bifurcate like the αβ-lobes. Figure is adapted from (Aso, Hattori, et al., 2014). (C) Overview of MB developmental timeline. There is a well-defined temporal order to MB development. γ-lobe neurons (red) are born before the mid-3rd instar stage, α′β′ neurons (green) are born between the mid-3rd instar stage and puparium formation, and αβ neurons (blue) are born after puparium formation. In early larval development, γ-lobe neurons form dorsal and medial projection but early in pupal development have partial degeneration and pruning of the axons and reformation of only

medial projecting lobes. In late larval development, α′β′ neurons project with αβ neurons projecting early in puparium formation. Figure is adapted from (T. Lee et al., 1999).

Figure 1-4


A
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vdaC
vdaB
vdaD
v
C
class IV da neurons
epidermal cells
: class I
: class II
: class III
: class IV
D
apical
basal
skin (epidermal cells)
class IV da dendrite
epidermal ECM
muscle

Figure 1-4: *Drosophila* ddaC neuroanatomy.

Drosophila dendritic arborization (da) neurons provide an excellent system to study *in vivo* dendritic development and morphology. (A) Schematic of a *Drosophila* L3 larvae with dorsal - **red**, lateral - **blue**, and ventral - **yellow** da neurons indicated. The work presented in this thesis focused on specifically dorsal dendritic arbor C (ddaC) neurons marked with **red** arrow. (B) There are 15 da neurons per segment, each with a different notation. ddaC neurons have some of the largest and most elaborate arbors; marked with **red** arrow. Figure is adapted from (Grueber et al., 2002). (C) da neurons cover the body wall of the larvae with neurons of each adjacent body wall tiling against the other; da neurons marked in **green** with mCD8:GFP; epidermal cells marked in red with CD4-tdTomato. Figure is adapted from (W. K. Yang & Chien, 2019). (D) Schematic of the arrangement of da neuron dendritic arbors between the epidermis and muscle. Green plane represents extracellular matrix to which the dendrites attach. Figure is adapted from (W. K. Yang & Chien, 2019).

Figure 1-5

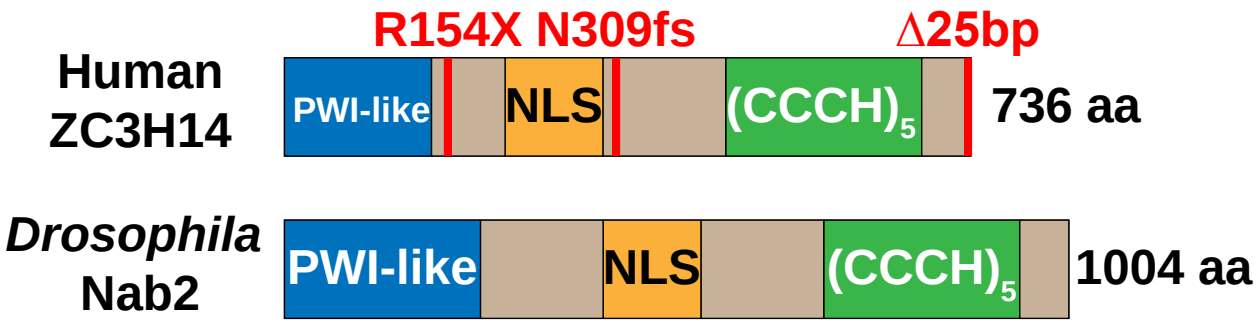
R154X N309fs
Δ25bp
Human
ZC3H14
PWI-like
NLS
(CCCH)5
736 aa
Drosophila
Nab2
PWI-like
NLS
(CCCH)5
1004 aa

Figure 1-5: ZC3H14/Nab2 domain structure.

The mammalian ZC3H14 protein contains three structural domains. The N-terminal domain contains a conserved Proline-Tryptophan-Isoleucine (PWI) fold, a protein-protein interaction domain, followed by two predicted classic nuclear localization signals (cNLSs), and then the C-terminus containing five tandem zinc fingers (ZnFs). Three patient mutations are indicated in **red**; R154X creating an early stop codon, 25-bp deletion located 16-bp downstream of the 3'-end boundary of exon 16, and N309fs creating a frameshift. *Drosophila* Nab2 shares 41 % amino acid identity with human ZC3H14 and shares the three major structures indicated.

Figure 1-6

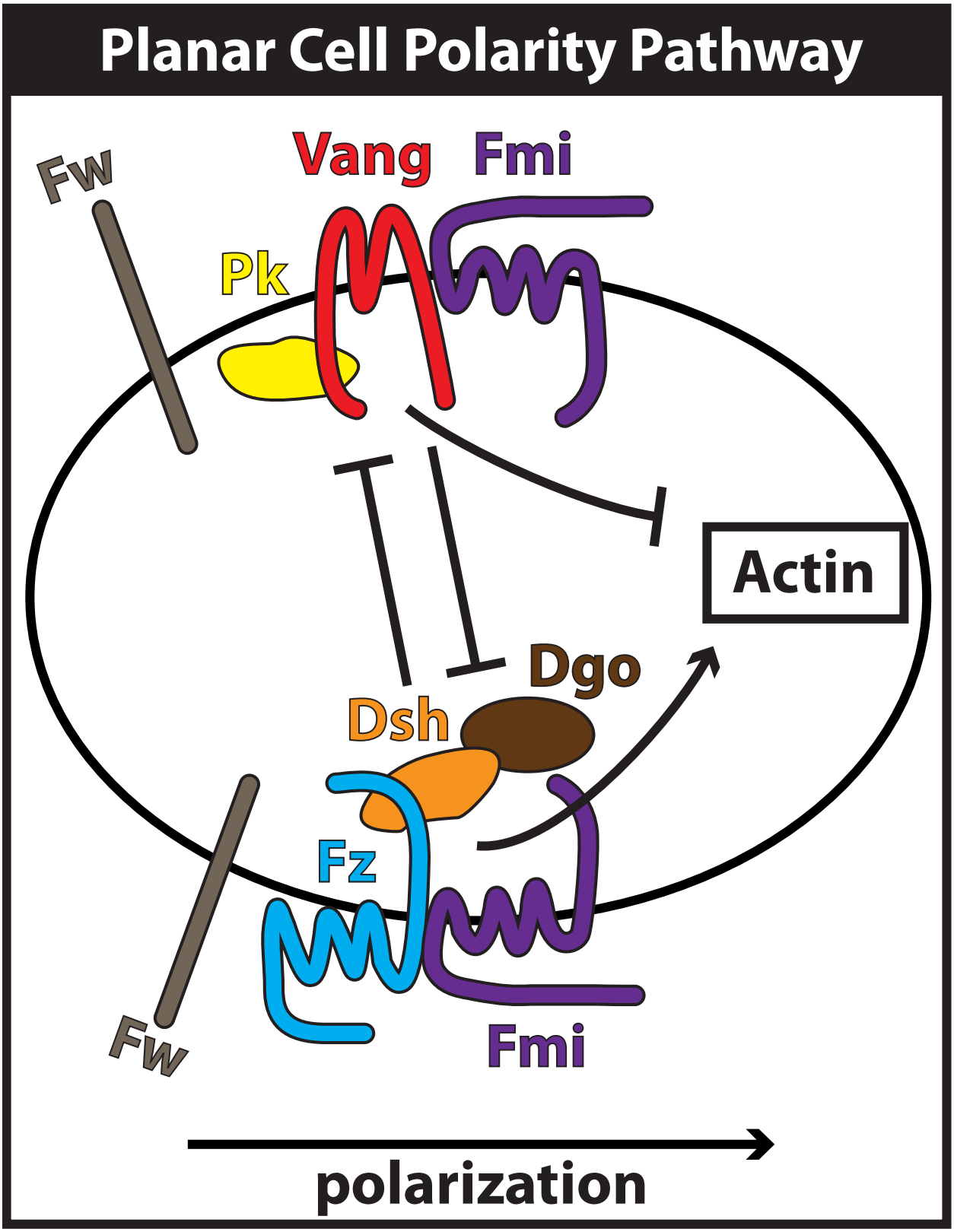
Planar Cell Polarity Pathway
Fw
Vang
Fmi
Pk
Actin
Dgo
Dsh
Fz
Fw
Fmi
polarization

Figure 1-6: Planar cell polarity pathway.

The planar cell polarity (PCP) pathway consists of two asymmetrically localized protein complexes Stan-Vang-Pk complex (Starry Night, also Flamingo-Van Gogh-Prickle) and the Stan-Fz-Dsh-Dgo complex (Frizzled-Disheveled-Diego). PCP complexes are intracellularly antagonistic but intercellularly attractive, leading to apical polarization across an epithelial plane. Core PCP components signal to downstream effector molecules that exert localized effects on the F-actin cytoskeleton.

Chapter 2: The RNA-binding protein Nab2 regulates the proteome of the developing *Drosophila* brain

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The RNA-binding protein Nab2 regulates the proteome of the developing Drosophila brain

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Please Note:

In the current chapter, Edwin Corgiat served as the lead researcher and co-first author on the contents of the chapter. He performed genetic, biochemical, and bioinformatic experiments and analyses as described within. He alone performed the experiments and analyses for Figures 2-2, 2-7, and 2-S1, and conducted the wet-lab side of the proteomic experiments. In pair with Dr. Sara M. List, they performed analyses for Figures 2-3, 2-4, 2-5, 2-6, and Table 1. Edwin authored the text of this chapter in conjunction with Drs. Kenneth H. Moberg and Anita H. Corbett.

The global proteomic experiments (LC-MS/MS) were conducted at the Emory Integrated Proteomics Core (EIPC); Edwin Corgiat performed all preceding experiments and sample collection and coordinated the project with the core.

Dr. J. Christopher Rounds provided critical intellectual discussion and contribution to the development of this project; Drs. Kenneth H. Moberg and Anita H. Corbett mentored Edwin Corgiat and provided essential intellectual contribution, guidance, and financial support.

Abstract

The human *ZC3H14* gene, which encodes a ubiquitously expressed polyadenosine zinc finger RNA binding protein, is mutated in an inherited form of autosomal recessive, non-syndromic intellectual disability. To gain insight into ZC3H14 neurological functions, we previously developed a *Drosophila melanogaster* model of ZC3H14 loss by deleting the fly ortholog, Nab2. Studies in this invertebrate model reveal that Nab2 controls final patterns of neuron projection within fully developed adult brains. Here, we examine a developmental role for Nab2. We identify roles for Nab2 in controlling the dynamic growth of axons in the developing brain mushroom bodies (MBs), which support olfactory learning and memory, and in regulating abundance of a small fraction of the total brain proteome. The group of Nab2-regulated brain proteins, identified by quantitative proteomic analysis, includes the microtubule binding protein Futsch, the neuronal Ig-family transmembrane protein Turtle, the glial:neuron adhesion protein Contactin, the RacGAP Tumbleweed, and the planar cell polarity factor Van Gogh, which collectively link Nab2 to the processes of brain morphogenesis, neuroblast proliferation, circadian sleep/wake cycles, and synaptic development. Overall, these data indicate that Nab2 controls the abundance of a subset of brain proteins during the active process of wiring the pupal brain mushroom body, and thus provide a window into potentially conserved functions of the Nab2/ZC3H14 RNA binding proteins in neurodevelopment.

Introduction

Neurons develop complex architectures that allow them to function within massive interconnected networks that transmit electrochemical signals among thousands of other neurons in a shared circuit. The polarized morphology of neurons is particularly unique, with each cell containing axon and dendrite projections that can extend over enormous distances relative to the size of the cell body. Axonal growth and guidance is largely directed through the growth cone, which responds to guidance cues to steer the axon (Holt et al., 2019; Stoeckli, 2018). This axonal guidance is regulated in part by local translation of mRNAs within the growth cone that modifies the local proteome. This process of local translation, which relies on pre-delivery of mRNAs to the axon tip, facilitates rapid shifts in translation in response to extracellular cues that would otherwise be limited by distance from the nucleus and relatively slow speed of intracellular transport (Holt et al., 2019; Hörnberg & Holt, 2013; Maday et al., 2014; Stoeckli, 2018). The local translation of mRNAs in distal neuronal projections is critical for proper development of the nervous system (Holt et al., 2019; Maday et al., 2014) but poses many biological challenges, including the need to maintain mRNAs in a translationally repressed state during transport from the nuclear periphery to distal sites where regulated translation must occur (Hörnberg & Holt, 2013; Stoeckli, 2018). RNA binding proteins (RBPs) play a major role in this process (Hörnberg & Holt, 2013).

RBPs play critical roles in regulating temporal and spatial expression of numerous mRNAs that encode proteins with roles in neuronal function (Moore, 2005). Although RBPs play broadly important roles in regulating multiple steps in gene expression shared by all cell types, mutations in genes encoding RBPs often result in tissue- or cell-type specific diseases (Cooper, Wan, & Dreyfuss, 2009; Hörnberg & Holt, 2013; Jung, Yoon, & Holt, 2012; Lepelletier et al., 2017;

Preitner, Quan, Li, Nielsen, & Flanagan, 2016; Stoeckli, 2018; Welshhans & Bassell, 2011). A large number of these RBP-linked diseases include significant neurologic impairments, which likely reflects an enhanced reliance on post-transcriptional mechanisms to pattern spatiotemporal gene expression over the long distances that neurons extend (Agrawal et al., 2019; Castello, Fischer, Hentze, & Preiss, 2013; Holt et al., 2019). This dependence on RBP-based mechanisms of gene expression is exemplified by disease-causing mutations in the genes (Hörnberg & Holt, 2013) encoding the fragile X mental retardation protein (FMRP) (C. Gross, Berry-Kravis, & Bassell, 2012), survival of motor neuron protein (SMN) (Edens, Ajroud-Driss, Ma, & Ma, 2015), and TAR DNA binding protein 43 (TDP-43) (Agrawal et al., 2019). Mutations in the *ZC3H14* gene, which encodes a zinc finger RBP (zinc finger CysCysCysHis [CCCH]-type 14), cause neurological defects that broadly resemble those associated with these more extensively characterized RBPs (Hörnberg & Holt, 2013; Pak et al., 2011).

The human *ZC3H14* gene encodes a ubiquitously expressed polyadenosine RNA binding protein that is lost in a heritable non-syndromic form of intellectual disability (Pak et al., 2011). The *Drosophila* ZC3H14 homolog, Nab2, has provided an excellent model to probe the function of ZC3H14/Nab2 in neurons (Kelly et al., 2016, 2012; Kelly, Leung, Pak, Banerjee, & Moberg, 2014). *Nab2* deletion in flies results in defects in locomotion and neuromorphology that are rescued by neuronal specific re-expression of Nab2 (Kelly et al., 2016). Neuronal specific expression of human ZC3H14 partially rescues many of the *Nab2* null phenotypes, demonstrating a high level of functional conservation between ZC3H14 and Nab2 (Fasken et al., 2019; Kelly et al., 2016, 2012).

Nab2 and its orthologs are found primarily in the nucleus at steady-state (Bienkowski et al., 2017; Green et al., 2002; Leung et al., 2009; Morris & Corbett, 2018; Rha et al., 2017; Wigington

et al., 2016), but evidence shows that these proteins can shuttle between the nucleus and cytoplasm (Morris & Corbett, 2018; van den Bogaart, Meinema, Krasnikov, Veenhoff, & Poolman, 2009; Wigington et al., 2016). Within neurons, small pools of Nab2 are detected within axons and dendrites (Bienkowski et al., 2017; Kelly et al., 2016; Kelly, Leung, Pak, Banerjee, & Moberg, 2014; Pak et al., 2011; Rha et al., 2017) raising the possibility that Nab2 has both nuclear and cytoplasmic roles in this cell type. Multiple studies in a variety of model organisms have defined key roles for Nab2 in pre-mRNA processing events within the nucleus, including regulation of splicing events (Fasken et al., 2019; Jalloh et al., 2020; Soucek et al., 2016a), transcript termination (Alpert, Straube, Carrillo Oesterreich, & Neugebauer, 2020; Fasken et al., 2019), and control of poly(A) tail length (Fasken et al., 2019; Kelly, Leung, Pak, Banerjee, Moberg, et al., 2014). Additional studies localize Nab2 within cytoplasmic mRNA ribonucleoprotein particles and imply roles in translational repression, likely mediated in part through interactions with FMRP (Bienkowski et al., 2017; Rha et al., 2017; Soucek et al., 2016a; Wigington et al., 2016). Ultimately, all of these post-transcriptional regulatory events are likely to alter levels of key proteins that are critical for proper neuronal function.

At a morphological level, zygotic deficiency for Nab2 produces structural defects in the adult *Drosophila* brain mushroom bodies (MBs) (Kelly et al., 2016), twin neuropil structures that mirror across the brain midline and are required for olfactory learning and memory (Kang et al., 2019; Kelly et al., 2016; Strausfeld, Hansen, Li, Gomez, & Ito, 1998). The MBs are formed of five lobes: γ, α, α', β, and β' (**Figure 1A**) (Thomas Kunz et al., 2012; T. Lee et al., 1999). In the fully formed adult brain, *Nab2* null neurons fail to project axons into the α-lobe and β-lobe axons inappropriately cross the midline into the contralateral hemisphere (Bienkowski et al., 2017; Kelly et al., 2016). These findings implicate Nab2 in developmental control of axonogenesis and growth

cone guidance. MB development begins in the larval stage with neuroblast pools that project axons into nascent γ-lobes (Armstrong, de Belle, Wang, & Kaiser, 1998; Jefferis, Marin, Watts, & Luo, 2002; Thomas Kunz et al., 2012; Kurusu et al., 2002; T. Lee et al., 1999). During the subsequent pupal stage, these γ-lobes are pruned back, and α and β-axons begin to project into their corresponding tracks (Armstrong et al., 1998; Jefferis et al., 2002; Thomas Kunz et al., 2012; Kurusu et al., 2002; T. Lee et al., 1999). By 24 hours after pupal formation (APF), α and β-lobes have formed their initial structure and are being thickened by new axons that project through the core of the bundle. This process continues through ~72 hours APF, when the α and β-lobes are fully formed (Kurusu et al., 2002; T. Lee et al., 1999). The effect of *Nab2* alleles on final α and β-lobe structure in the adult brain implies a role for the Nab2 RBP in axon projection and guidance during early pupal stages (Armstrong et al., 1998; Bienkowski et al., 2017; Kelly et al., 2016; Thomas Kunz et al., 2012; Kurusu et al., 2002).

Here, we exploit the predictable time course of brain development in *Drosophila* to perform temporally coupled analysis of the effect of Nab2 loss on the pupal brain proteome and the process of axon projection into the forming pupal MBs. We find that Nab2 loss disrupts α and β-axon projection in the pupal MBs coincident with significant increases in the steady-state abundance of proteins that are enriched for roles in neurodevelopment, neuronal and glial metabolism, axon guidance, and trans-synaptic signaling. Complementary analysis of neuronal specific Nab2-overexpressing brains confirms that a subset of these proteins also change abundance in response to excess Nab2. In sum, this paired morphological-proteomic analysis provides strong evidence that Nab2 is required to control the abundance of proteins with critical roles in *Drosophila* neurons that may play conserved roles in humans.

Results

Nab2 loss disrupts axon projection into the forming pupal mushroom bodies

Our prior finding that loss of Nab2 impairs mushroom body (MB) neuromorphology in the mature adult *Drosophila* brain (Cooper et al., 2009; Hörnberg & Holt, 2013; Welshhans & Bassell, 2011) suggests a role for Nab2 in MB morphogenesis in the preceding pupal phase. Consistent with this idea, serial optical sectioning of α-FasII-stained $Nab2^{ex3}$ (i.e. zygotic null) and *control* brains 48-72 hours after pupal formation (APF) reveals thinning or missing α-lobes and β-lobes that project and fuse across the midline that are not present to the same extent in *control* brains (**Figure 1A-B**). The 48-72 hr APF time window coincides with a midpoint in projection and guidance of α and β-lobes. At this stage, *control* brains show incompletely formed α and β-lobes with a low degree of defects (13% and 18%, respectively) while $Nab2^{ex3}$ brains already display a high rate of missing/thinning α-lobes and fused/missing β-lobes (both 85%) (**Figure 1C**). These data indicate that *Nab2* is required during pupal projection and guidance of the mushroom body axons, raising the question of how loss of the Nab2 RBP affects the pupal brain proteome.

Quantitative proteomic analysis of developmentally timed pupal brains

Nab2 has been identified as a component of cytoplasmic ribonucleoprotein particles (RNPs) linked to mRNA trafficking and translation (Bienkowski et al., 2017; Green et al., 2002; Morris & Corbett, 2018), and as a nuclear component of post-transcriptional complexes (Bienkowski et al., 2017; Rha et al., 2017) that control mRNA splicing (Jalloh et al., 2020; Soucek et al., 2016a; Wigington et al., 2016), transcription termination (Alpert et al., 2020), and polyadenylation (Kelly, Leung, Pak, Banerjee, & Moberg, 2014). To explore how *Drosophila* Nab2 affects the mRNA-derived proteome in the developing pupal brain, global label-free LC-MS/MS was performed on

dissected 24hr APF brains of *control* (*C155>Gal4, w^{1118}*), mutant *$Nab2^{ex3}$* (*C155>Gal4;;$Nab2^{ex3}$*), and neuronal-specific *Nab2* overexpression (*Nab2 oe*) (*C155>Gal4;$Nab2^{EP3716}$;$Nab2^{ex3}$*) animals as illustrated in **Figure 2**. We employed 24h APF brains for proteomic analysis to capture the developmental window during which MB defects were observed in the absence of Nab2.

Mass spectrometry was carried out for ten biological replicates for each of the three genotypes (*control*, *$Nab2^{ex3}$*, *Nab2 oe*), with five male samples and five female samples analyzed separately. Across brain samples, a total of 4302 proteins were detected. Unbiased principal component analysis (PCA), which was performed using summed peptide intensities across all 30 samples per protein, per genotype, reveals three distinct clusters (**Figure 3A**). The thirty plotted samples form three distinct clusters by genotype, indicating high similarity between male and female samples within a given genotype. Subsequent simple linear regression modelling of the data obtained indicated that male and female samples could be combined for analyses adding power. These combined datasets (n=10 per genotype) were used for subsequent analyses.

Proteomic analysis identifies proteins that change in abundance when Nab2 levels are altered

We first analyzed differences between each experimental and control. Differentially expressed proteins were then identified for *$Nab2^{ex3}$* and *Nab2 oe* genotypes by comparing each to the *control* dataset (*$Nab2^{ex3}$* vs. *control* and *Nab2 oe* vs. *control*) with protein abundance change thresholds of $\log_2$(experimental/control) $\geq$0.32 or $\leq$-0.32 and a significance threshold of -$\log_{10}$(p-value) $\geq$1.3.

***$Nab2^{ex3}$* vs. *control*:** Of the 4302 total proteins detected by LC-MS/MS across all three groups, 346 proteins (~8% of total proteins detected) are differentially expressed in the *$Nab2^{ex3}$* brains vs *control* brains (**Figure 3B**) (**Supplementary Table 1**) (full dataset available at ProteomeXchange

Consortium via PRIDE under the accession #PXD022984). Within this group, 158 proteins score ≥0.32 log_2 fold change increase (five most elevated: CG1910, Got1, Ida, Mtp, and Wwox) and 188 proteins score ≤-0.32 log_2 fold change decrease , with Nab2 among the top five most decreased (Nab2, Pglym78, Mkk4, Cortactin, and Psa) (**Figure 3B**).

***Nab2 oe* vs. *control*:** Of 4302 total proteins detected, 514 proteins are differentially expressed in *Nab2 oe* brains relative to *control* brains (approximately 12% of total proteins detected). Within this group, 229 proteins score ≥0.32 log_2 fold change increase (five most elevated: CG1910, Ccp84Ae, Ida, Ccp84Ag, and Alien) and 285 proteins scored ≤-0.32 log_2 fold change decrease (five most decreased: Pglym, Mkk4, Cortactin, Gnmt, and CG34280) (**Figure 3C**) (**Supplementary Table 1**). Nab2 itself was the 32nd most elevated protein among the 229 proteins increased in abundance in *Nab2 oe* relative to control, confirming the effectiveness of the neuronal specific expression of the *C155>Gal4;Nab2^{EP3716}* genotype.

Gene ontology analysis supports a role for Nab2 in neurodevelopment

Looking beyond individual protein changes can provide a broader understanding of the effects of disrupting Nab2. Therefore, gene ontology analysis for biological process enrichment was performed with FlyEnrichr by analyzing the differentially expressed (*Nab2^{ex3}* vs. *control* and *Nab2 oe* vs. *control*) protein datasets. This FlyEnrichr analysis reveals that proteins increased in the *Nab2^{ex3}* differentially expressed dataset represent biological processes involved in genome maintenance (e.g., DNA replication initiation, G_2 DNA damage checkpoint, centromere complex assembly) and development (e.g., female germline stem cell) (**Figure 3D**), while proteins increased in the *Nab2^{oe}* differentially expressed dataset represent processes related to development (e.g., striated muscle development, cuticle development) and muscle organization (e.g., sarcomere

organization, myosin filament assembly) (**Figure 3E**). Proteins decreased in the *Nab2*^{ex3} differentially expressed and *Nab2*^{oe} differentially expressed datasets are strongly enriched for processes linked to neurodevelopment, synaptic function, and brain maintenance (**Figure 3F**, **G**). Within the *Nab2*^{ex3} differentially expressed dataset, decreased proteins are enriched for the processes of neuroblast proliferation, circadian sleep/wake cycle, and axonal transport (**Figure 3F**). Within the *Nab2*^{oe} differentially expressed dataset, decreased proteins are enriched for the processes of axon injury response, circadian sleep/wake cycle, and neurotransmitter transport (**Figure 3G**).

Comparison of individual protein changes and FlyEnrichr GO terms between *Nab2*^{ex3} differentially expressed (346 proteins) and *Nab2*^{oe} differentially expressed (514 proteins) datasets provides some significant insights (**Figure 4A-C**). There are individual protein changes and GO terms that are shared between *Nab2*^{ex3}-DE and *Nab2*^{oe}-DE, and there are changes that are exclusive to one or the other dataset (**Figure 4A**). Of the total differentially expressed proteins in both datasets, 23% are unique to *Nab2*^{ex3}, 47% are unique to *Nab2 oe*, and 30% are shared between the two genotypes (referred to as "shared DE changes") (**Figure 4A**). Among the last category, in addition to protein identity, there is significant correlation in protein expression between *Nab2*^{ex3} and *Nab2 oe* shared DE changes (**Figure 4B**). A total of 195 proteins accounted for the shared differentially expressed changes between *Nab2*^{ex3} and *Nab2 oe* brains (**Figure 4A**), and these shared changes are highly correlated with one another ($R=0.86$, $p<2.2^{-16}$; **Figure 4B**). Of the 195 shared proteins, a large fraction (184 out of 195, approximately 94%) change abundance in *Nab2*^{ex3} differentially expressed and *Nab2*^{oe} differentially expressed datasets in the same direction (**Figure 4B**). However, a subset of 11 shared differentially expressed proteins are altered in opposing directions e.g., increased in *Nab2*^{ex3} differentially expressed and decreased in *Nab2*^{oe} differentially

expressed or *vice versa* (**Table 1**). Nab2 itself is one of these 11 shared proteins (**Figure 4B, Table 1)**. Nab2 is decreased relative to control in $Nab2^{ex3}$ brains ($\log_2(-8.36)$) and increased relative to control in *Nab2 oe* brains ($\log_2(3.94)$) (**Figure 4B, Nab2 labeled data point).** Finally, the $Nab2^{ex3}$ differentially expressed and $Nab2^{oe}$ differentially expressed datasets each have unique proteins that may provide insight into previously observed phenotypes in *Nab2* mutants or overexpression systems (Bienkowski et al., 2017; Jalloh et al., 2020; Kelly et al., 2016; W. H. Lee et al., 2020; Pak et al., 2011; Rounds et al., 2021). There are 152 proteins changed exclusively in $Nab2^{ex3}$ brains relative to control, and 311 proteins changed exclusively in the *Nab2 oe* brains relative to control (**Figure 4A)**. As general overexpression of *Nab2* is more lethal than zygotic *Nab2* loss (Pak et al., 2011), the 311 changes unique to *Nab2 oe* may represent dominant effects of excess Nab2. However, the 152 proteins that are significantly changed only in $Nab2^{ex3}$ brains, and not in the *Nab2 oe* genotype (which is in the $Nab2^{ex3}$ background), are thus rescued by re-expression of wildtype Nab2 in $Nab2^{ex3}$ brain neurons. These differences in $Nab2^{ex3}$ and *Nab2 oe* differentially expressed proteins are also reflected in the FlyEnrichr gene ontology analysis, which reveals 172 terms unique to $Nab2^{ex3}$ and 999 unique to *Nab2 oe* (**Figure 4C**). Differences between $Nab2^{ex3}$ and *Nab2 oe* have the potential to provide insight into the neuroanatomical defects observed in $Nab2^{ex3}$ pupal brains (**Figure 1B**).

As previous studies suggest Nab2 can function as a translational repressor (Bienkowski et al., 2017; Rha et al., 2017), the most direct Nab2 targets could be expected to increase in abundance upon loss of Nab2 function ($Nab2^{ex3}$). However, factors that decrease in protein abundance, whether due to direct or indirect effects of Nab2, may also be phenotypically significant in the $Nab2^{ex3}$ genotype. To parse these effects, the unique and shared changes in the $Nab2^{ex3}$-DE and $Nab2^{oe}$-DE datasets were further divided into *increased* and *decreased* groups, and then subjected

to FlyEnrichr analysis (**Figure 4C**). Protein increases unique to the *Nab2^{ex3}* differentially expressed dataset represent processes involved in metabolism (**Figure 5A**), while increases unique to the *Nab2^{oe}* differentially dataset represent processes involved in tissue development and organization (**Figure 5B**). The increases common to both *Nab2^{ex3}* differentially expressed and *Nab2^{oe}* differentially expressed datasets are enriched in processes involved in genome maintenance and development (**Figure 5C**). A chord plot of biological process GO terms relating to RNA processing and neurodevelopment highlights proteins *increased* in both datasets (**Figure 5D**). Among these are the glial-neuronal adhesion protein Contactin (Cont), the planar cell polarity (PCP) accessory protein A-kinase anchor protein 200 (Akap200), the condensin subunit Gluon (Glu), and the neuroblast regulator Polo (**Figure 5D**).

A similar analysis of shared and exclusive *decreased* proteins between the *Nab2^{ex3}* differentially expressed and *Nab2^{oe}* differentially expressed datasets (**Figure 6A-D**) reveals that decreases unique to *Nab2^{ex3}* are enriched for the processes of neuroblast proliferation, taste perception, and brain morphogenesis (**Figure 6A**), while unique *Nab2 oe* decreases are enriched for the processes of post-synapse assembly, synaptic vesicle recycling, and sodium ion transport (**Figure 6B**). The shared *decreases* between *Nab2^{ex3}* and *Nab2 oe* represent processes involved in neurodevelopment and brain function (**Figure 6C**). A chord plot of biological process GO terms relating to neurodevelopment, behavior, and brain function highlights proteins *decreased* in both datasets (**Figure 6D**). Among these are the microtubule associated protein Futsch, the neuronal Ig-family transmembrane protein Turtle, the axon guidance and PCP component Vang, and the Rho GEF Trio (**Figure 6D**). The proteomic changes revealed here resulting from disruption of the RBP Nab2, likely correspond in part to changes in mRNA regulation.

Shared protein changes between Nab2^{ex3} flies and ZC3H14$^{\Delta ex13/\Delta ex13}$ mice

Comparing the differentially expressed proteins from *Nab2^{ex3}* brains to a previously reported proteomic dataset generated from hippocampi of P0 *Zc3h14* knockout (*Zc3h14$^{\Delta ex13/\Delta ex13}$*) mice (Rha et al., 2017), reveals six proteomic changes shared between flies and mice (**Figure 7A,B**). These conserved changes may give insight into conserved targets of Nab2/ZC3H14. The transcripts, of these conserved protein changes, may represent targets of Nab2/ZC3H14 and thus may share a sequence motif recognized by Nab2/ZC3H14. To test for shared motifs among this set of conserved candidate target RNAs, sequence analysis was performed using multiple EM for motif elicitation (MEME) (Bailey et al., 2009; Bailey & Elkan, 1994). The transcripts representing the twelve shared proteins, six from flies and six from mice, were used as input for MEME analysis (**Figure 7A,B**). MEME discovers novel, ungapped motifs and identified a 29-bp long, internal-A-rich motif as the most enriched among the transcripts **Figure 7C**). This 29-bp motif (log likelihood ratio 370, E-value 9.0e-37) is overrepresented in these transcripts relative to the random chance expected across the transcriptome. The shared motif across these conserved targets suggests this could be a binding sequence common to fly Nab2 and mouse ZC3H14. The location of this 29-bp motif varies among the transcripts analyzed (**Figure 7D, Supplementary Figure 1**).

Discussion

Here, we examine the role of a conserved RNA binding protein in neurodevelopment by exploiting a *Drosophila* model. Using carefully timed brain collections, we find that axon projection and development of MB α and β-lobes structure are severely perturbed in pupal brains, and that coincident with these defects in axonal trajectories, we detect clear changes in a small fraction (~8%) of the brain proteome. This restricted effect on a subset of brain proteins is

consistent with our recent finding that Nab2 loss has specific effects on the brain transcriptome (Jalloh et al., 2020), and supports the hypothesis that Nab2 regulates expression of a subset of neuronal mRNAs and proteins that are involved in various neurodevelopmental processes, including axon growth and guidance in the MBs.

Bioinformatic analysis of differentially expressed proteins in *Nab2^{ex3}* mutant brains relative to control samples indicates that Nab2-regulated proteins are enriched in functional classes corresponding to axonal development, but also suggest a potential role in dendrites. The former link to axonogenesis matches the observed MB α- and β-lobe defects, but the latter link to dendritic proteins is more novel and may be conserved. The murine Nab2 homolog, ZC3H14, localizes to dendritic shafts and spines and controls dendritic spine morphology in cultured neurons (S. K. Jones et al., 2020; Rha et al., 2017). Nab2-regulated proteins identified in the current study that have predicted dendritic roles include the planar cell polarity factor Vang, the adhesion protein Cortactin, the netrin receptor Frazzled, the neuronal Ig-family transmembrane protein Turtle, the Fragile-X mental retardation homolog Fmr1, the Rho GEF Trio, the RNA binding protein Alan Shepherd/RBMS3, and the microtubule associated protein Futsch (MAP1ß). Significantly, a proteomic dataset generated from hippocampi of P0 *Zc3h14* knockout mice (Rha et al., 2017) also shows enrichment for the Vang homolog Vangl2, in addition to five other neurodevelopmental proteins that are also detected here as differentially expressed in *Nab2^{ex3}* pupal brains: the oxioreductase Wwox, the PDZ-domain protein X11Lβ/Apba1, the DnaJ protein CG6693/Dnajc9, the ARF-GEF factor Sec71/Psd3, and the endosomal protein Asrij/Ociad1 (**Table 1**).

Human ZC3H14 expressed in neurons of *Nab2^{ex3}* flies rescues many of the *Nab2* null phenotypes (Pak et al., 2011). This finding suggests that there should be shared function and RNA targets between mammalian ZC3H14 and fly Nab2. The 29-bp, A-rich motif identified in the

transcripts represented by these conserved protein changes between fly and mouse (**Figure 7C**) may represent a target binding motif for Nab2/ZC3H14. The potential for this A-rich motif to be a Nab2 binding site is supported by the previous definition of a Nab2 binding motif in *Saccharomyces cerevisiae* (A11G and A12) (Aibara, Gordon, Riesterer, McLaughlin, & Stewart, 2017; Fasken et al., 2019; Guisbert, Duncan, Li, & Guthrie, 2005). This A-rich motif identified in the present study by examining conserved proteomics changes between *Nab2*^{ex3} fly brains and *ZC3H14*$^{\Delta ex13/\Delta ex13}$ mouse hippocampi is similar to a recently identified A-rich motif defined via RNA-IP of fly Nab2 (Rounds et al., 2021).

The evidence to suggest conserved target RNAs suggests that Nab2/ZC3H14 may have a shared role in regulating key RNAs involved in neuronal development and signaling. Of note, fly Nab2 physically and functionally interacts with the *Drosophila* Fragile-X mental retardation protein (Fmr1) (Bienkowski et al., 2017), which has a key role in post-synaptic, activity-dependent local mRNA translation and is required for normal dendritic morphology (C. Gross et al., 2012).

Our comparison of the effects of Nab2 dosage reveals that almost one-third of proteomic changes (29%) that occur in Nab2-deficient pupal brains are shared in brains with neuronal overexpression of Nab2. Of 195 proteins that change in abundance in the *Nab2*^{ex3} and *Nab2 oe* datasets, only 11 of these are inverse changes (i.e., increased in *Nab2*^{ex3} and decreased in *Nab2 oe* or *vice versa*) while the other 184 proteins change in the same direction between these two genotypes (i.e., increased or decreased in both *Nab2*^{ex3} and *Nab2 oe*). A simplistic model would predict that loss and gain of Nab2 would have the opposite effect on targets, but these data suggest that excess Nab2 can generate a dominant-negative effect on some candidate target RNAs, perhaps by sequestering Nab2-interacting proteins or blocking access of other RBPs to sites on RNAs. The 184 shared protein changes that occur in the same direction can be explained either by a dominant

negative effect of Nab2 overexpression or by the nature of the experiment where the *Nab2 oe* is performed in a background of *Nab2 ex3* flies. As the *Nab2 ex3* is a zygotic allele (Pak et al., 2011) and *Nab2 oe* is driven by a neuron-specific promoter (*C155>Gal4*), the shared proteomic changes could reflect changes in non-neuronal cell types. Indeed, the 11 proteins that show inverse changes in the $Nab2^{ex3}$ and *Nab2 oe* datasets could represent a subset of targets that respond in a linear fashion to Nab2 dose in neurons. One possibility is that the mRNAs encoding these proteins represent direct targets of the Nab2 RNA binding protein. Our analysis detects 152 significantly changed proteins in $Nab2^{ex3}$ brains that are rescued back to normal levels in *Nab2 oe* brains, which parallels the morphological rescue of $Nab2^{ex3}$ by *Nab2 oe* documented in prior studies (Kelly et al., 2016; Kelly, Leung, Pak, Banerjee, Moberg, et al., 2014; Pak et al., 2011). Among the proteins in this group is Tumbleweed (Tum), which is homologous to human RacGap1 and required for normal MB development (Goldstein, Jan, & Luo, 2005). This putative link from Nab2 to Tum-based control of MB patterning warrants further study.

Evidence of interactions between *Nab2* and elements of the microRNA (miRNA) machinery (e.g., *argonaute*) and ncRNA processing factors (e.g., *Rm62*) detected in our prior work (Bienkowski et al., 2017; Kelly et al., 2016) are also supported by this proteomic analyses. Seven gene ontology (GO) terms relating to miRNA/ncRNA are enriched in the $Nab2^{ex3}$ dataset including *pre-miRNA processing*, *production of small RNA involved in gene silencing by RNA* and *ncRNA 3'-end processing*. As miRNAs and ncRNAs can regulate gene expression (Catalanotto, Cogoni, & Zardo, 2016), some observed effects of *Nab2* alleles on the brain proteome could be indirect, rather than changes to direct (i.e., bound) Nab2 target RNAs. This model aligns with our prior work showing that Nab2 physically associates with Fmr1 and coregulates some mRNAs (Bienkowski et al., 2017). In the adult brain, depletion of Nab2 derepresses a *CamKII* translation

but Nab2 depletion has no effect on *futsch* (Bienkowski et al., 2017). In the current study of pupal brains, Futsch protein is decreased in *Nab2*^{ex3} brains ($\log_2$(*Nab2*^{ex3}/Cont) = -0.38) while CamKII protein levels are not significantly changed. These stage-specific effects on the brain proteome raise the possibility that Nab2 interactions are not only target-specific (e.g., as in the case of alternative splicing) (Jalloh et al., 2020) but can also vary across developmental stages.

As noted above, the planar cell polarity (PCP) component Vang and the Vang murine homolog Vang like-2 (Vangl2) are among a small group of proteins that are differentially expressed in both *Drosophila Nab2*^{ex3} pupal brains and in P0 hippocampi dissected from *Zc3h14* knockout mice (Rha et al., 2017) (**Table 1**). This finding is particularly significant given the strong genetic interactions detected between an eye-specific *Nab2* overexpression system (*GMR-Nab2*) and multiple PCP alleles, including an allele of *Vang* (W. H. Lee et al., 2020). The PCP pathway plays a conserved role in regulating axon projection and guidance in multiple higher eukaryotic species (Fenstermaker et al., 2010; C. Jones & Chen, 2007; Tissir, Bar, Jossin, & Goffinet, 2005; Y. Wang, Chang, & Nathans, 2010; Zou, 2004a), including in the *Drosophila* MBs (Hakanen, Ruiz-Reig, & Tissir, 2019; He, Liao, & Pan, 2018; Ng, 2012; Shimizu et al., 2011; Zou, 2012). Thus, the change in levels of Vang, a core PCP component (Axelrod, 2002; Bastock, Strutt, & Strutt, 2003; Butler & Wallingford, 2017; Taylor et al., 1998; Y. Yang & Mlodzik, 2015), in *Nab2*^{ex3} brains could provide an additional, direct link from Nab2 to a pathway that guides neurodevelopment including the MB α and β-lobes.

In aggregate, these data provide a comprehensive view of the role Nab2 plays in regulating abundance of a specific cohort of proteins in the developing pupal brain, some of which are likely to correspond to mRNAs that are bound and regulated by Nab2 in brain neurons. Furthermore,

this set of proteins is enriched for neurodevelopmental factors that could represent evolutionarily conserved targets of this class of Zinc finger RBPs.

Experimental Procedures

***Drosophila* genetics.** All crosses were maintained in humidified incubators at 25ºC with 12hr light-dark cycles unless otherwise noted. The *Nab2*^{ex3} loss of function mutant has been described previously (Pak et al., 2011). Alleles and transgenes: *Nab2*^{EP3716} (Bloomington (BL) #17159) and *P{GawB}elav*^{C155} (BL #458), and *w*^{1118} (‘*control’*; BL #3605).

Brain imaging, statistical analysis, and visualization. Brain dissections were performed as previously described (Kelly et al., 2016). Briefly, 48-72 hours after puparium formation (APF) brains were dissected in PBS (1xPBS) at 4ºC, fixed in 4% paraformaldehyde at RT, washed 3x in PBS, and permeabilized in 0.3% PBS-T (1xPBS, 0.3% TritonX-100). Following blocking for 1hr (0.1% PBS-T, 5% normal goat serum), brains were stained o/n in block+primary antibodies. After 5x washes in PBS-T (1xPBS, 0.3% TritonX-100), brains were incubated in block for 1hr, moved into block+secondary antibody for 3hrs, then washed 5x in PBS-T and mounted in Vectashield (Vector Labs). The anti-FasII monoclonal antibody 1D4 (Developmental Studies Hybridoma Bank) was used at 1:20 dilution. Whole brain anti-FasII images were captured on a Nikon AR1 HD25 confocal microscope using NIS-Elements C Imaging software v5.20.01, and maximum intensity projections were generated in ImageJ Fiji. Mushroom body morphological defects were called as α-lobe thinning or missing and β-lobe fusion or missing for *control* (α-lobe = 11 biological and 22 technical replicates; β-lobe = 11 biological and technical replicates) and *Nab2*^{ex3} (α-lobe = 17 biological and 34 technical replicates; β-lobe = 17 biological and technical replicates). Quantitation of MB phenotypes was performed as previously described (Kelly et al., 2016).

Global proteomics

Sample collection: Five biological replicates of control, *Nab2^{ex3}*, and *Nab2 oe* for both female and male brains were collected at 23.25 – 25.5hr APF (5 pools per condition, 20 brains per pool), lysed in urea buffer (8M urea, 100mM NaHPO4, pH 8.5) with HALT protease and phosphatase inhibitor (Pierce) and processed at the Emory Proteomics Core.

LC-MS/MS: Data acquisition by LC-MS/MS was adapted from a previously published procedure (Ping et al., 2018). Derived peptides were resuspended in 20µL of loading buffer (0.1% trifluoroacetic acid, TFA). Peptide mixtures (2µL) were separated on a self-packed C18 (1.9µm, Dr. Maisch, Germany) fused silica column (25 cm x 75 µM internal diameter (ID); New Objective, Woburn, MA) and were monitored on an Orbitrap Fusion Tribrid Mass Spectrometer (ThermoFisher Scientific). Samples were run in 30 technical replicates of five biological replicates per condition. Elution was performed over a 130-minute gradient at 250nL/min with buffer B ranging from 3% to 99% (buffer A: 0.1% formic acid in water, buffer B: 0.1% formic acid in acetonitrile). The mass spectrometer duty cycle was programmed to collect at top speed with 3s cycles. The full MS scans (300–1500 m/z range, 50ms maximum injection time) were collected at a nominal resolution of 120,000 at 200 m/z and AGC target of 200,000 ion counts in profile mode. Subsequently, the most intense ions above an intensity threshold of 5,000 were selected for higher-energy collision dissociation (HCD) (0.7 m/z isolation window with no offset, 30% collision energy, 10,000 AGC target, and 35ms maximum injection time) and the MS/MS spectra were acquired in the ion trap. Dynamic exclusion was set to exclude previously sequenced precursor ions for 30s within a 10ppm window. Precursor ions with charge states 2–7 were included.

MaxQuant protein identification: Label-free quantification analysis was adapted from a previously published procedure (Seyfried et al., 2017). Data files for the samples were analyzed using

MaxQuant v1.5.2.8 with Thermo Foundation 2.0 for RAW file reading capability. Spectra were searched using the search engine Andromeda and integrated into MaxQuant against the *Drosophila melanogaster* Uniprot database (43,836 target sequences, downloaded February 2018). The Andromeda score measures how an acquired spectrum matches the theoretical fragment masses and is defined as the -10 logarithmic probability of observing the given number of matches or more by chance (Tyanova, Temu, & Cox, 2016). Methionine oxidation (+15.9949 Da), asparagine and glutamine deamidation (+0.9840 Da), and protein N-terminal acetylation (+42.0106 Da) were variable modifications (up to 5 allowed per peptide); cysteine was assigned as a fixed carbamidomethyl modification (+57.0215 Da). Only fully tryptic peptides were considered with up to 2 missed cleavages in the database search. A precursor mass tolerance of ±20 ppm was applied prior to mass accuracy calibration and ±4.5 ppm after internal MaxQuant calibration. Other search settings included a maximum peptide mass of 6,000Da, a minimum peptide length of 6 residues, 0.6 Da tolerance for ion trap MS/MS scans. Co-fragmented peptide search was enabled to deconvolute multiplex spectra. The false discovery rate (FDR) for peptide spectral matches, proteins, and site decoy fraction were all set to 1%. Quantification settings were as follows: re-quantify with a second peak finding attempt after protein identification has completed; match MS1 peaks between runs; a 0.7 min retention time match window was used after an alignment function was found with a 20 minute RT search space. Quantitation of proteins was performed using summed peptide intensities given by MaxQuant. The quantitation method only considered razor plus unique peptides for protein level quantitation.

Statistical analysis and data visualization: Statistical analyses were performed in either RStudio v1.1.453 (Vienna, Austria) or GraphPad Prism 8 (Sand Diego, CA). Statistical analyses for MB phenotypes and plotting were performed using GraphPad. Significance determined using student's

t-test. Graphs reported either quartile ranks or error bars representing standard deviation. Significance scores indicated on graphs are * = $p \leq 0.05$, ** = $p \leq 0.01$, and *** = $p \leq 0.001$. Statistical analyses for the proteomics, including differential expression analysis, linear regression modelling, and comparison across genotypes of protein and GO term differences, were performed using RStudio v1.1.453 (Team, 2018), custom in-house scripts, and the following packages: ggpubr v0.2 (Alboukadel, 2018), cluster v2.1.0 (Maechler et al., 2016), and GOplot v1.0.2 (W. Walter, Sánchez-Cabo, & Ricote, 2015). Five biological replicates of control, *Nab2*^{ex3}, and *Nab2 oe* for both female and male brains were collected as 5 pools per condition with 20 brains per pool (each pool meets the needed amount of protein for detection on Orbitrap Fusion Tribrid mass spectrometer). Simple linear regression modelling was performed to test variability across biological replicates including covariates of: genotype, sample ID, and sex. The results of the models did not support the null hypothesis that the LFQ value of the biological replicates was dependent on sample ID ($F=0.0888$) or sex ($F=0.2135$). Linear modeling was performed in RStudio using lm (default stats package v3.5.1) (Chambers & Hatie, 1992; Wilkinson & Rogers, 1973). Based on modelling results, no samples were removed but male and female samples were combined based on genotype (n=10 per genotype). Subsequent analyses consist of ten biological replicates per genotype (20 brains per pooled biological replicate) with 30 technical replicates in total. By applying Benjamini-Hochberg false-discovery-rate (FDR) correction to group-wise ANOVA p-values, significant differentially expressed proteins were determined. Thresholds for significance of differentially expressed proteins set at $\log_2$(protein abundance change genotype 1 / protein abundance change genotype 2) ≥ 0.32 or ≤ -0.32 and $-\log_{10}$(p-value) ≥ 1.3 (equivalent to individual protein adj p-val<0.05) which were based on power calculation and instrumental detection limits. Protein abundance ratios use LFQ values. Additionally, for quality control, all

proteins with fewer than eight peptide reads were not considered for further analysis. Principal component analysis (PCA) was performed in RStudio using prcomp (default stats package v3.5.1), and summed peptide intensities were used as input(Becker, JM, & Wilks, 1988; Fox & Weisberg, 2011; Mardia, Kent, & Bibby, 1979; Venables & Ripley, 2002). Input data came from 24hr after pupal formation *Drosophila* brains from ten biological replicates of *control*, *Nab2^{ex3}*, and *Nab2 oe* flies (*control = C155>Gal4, w^{1118}; Nab2^{ex3} = C155>Gal4;;Nab2^{ex3}*; *Nab2 oe = C155>Gal4;Nab2^{EP3716};Nab2^{ex3}*). Prcomp PCA was conducted (k = 3) with mapping of normal confidence ellipses and posthoc genotype labeling. Ellipses indicate significance of clusters; Prcomp default ellipse assumes a multivariate t-distribution. Gene ontology analyses were performed using FlyEnrichr (FlyEnrichr:amp.pharm.mssm.edu/FlyEnrichr/ ; accessed June 2020) (E. Chen et al., 2013; Kuleshov et al., 2019, 2016). FlyEnrichr is a *Drosophila* specific gene ontology enrichment analysis package. Input data were differentially expressed proteins (*Nab2^{ex3}* relative to *control*; *Nab2 oe* relative to *control*). FlyEnrichr analyses were performed under default conditions with following term databases used: Coexpression Predicted GO Biological Process 2018, GO Biological Process AutoRIF Predicted zscore, and GO Biological Process AutoRIF. Significance of terms were determined using c-scores (c-score = ln(adj p-val) * z-score) in each dataset and a threshold of adj. p-val<0.05. C-score is the combined score of the p-value computed using Fisher's exact test and the z-score computed to assess the deviation from the expected rank (E. Chen et al., 2013; Kuleshov et al., 2019, 2016). FlyEnrichr corrects for multiple hypotheses using the Benjamini-Hochberg procedure with a threshold of 0.05. Multiple EM for Motif Elicitation (MEME) analysis conducted with OOPS (exactly one site per sequence) motif site distribution, with minimum motif width of six and maximum motif width of fifty. Threshold of significance: E-value < 0.05. E-value estimates the number of motifs given the log likelihood ratio,

accounting for with and site count, that one would find in a set of random sequences. Where appropriate, additional analysis parameters used default settings. Analysis performed under MEME version 5.3.2 (release date: 02/06/2021) (Bailey et al., 2009; Bailey & Elkan, 1994).

Data availability: The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE (Vizcaíno et al., 2016) partner repository with the dataset identifier PXD022984. All remaining data are contained within the article.

Supporting information:

This article contains supporting information.

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Table 1. *Nab2*^{ex3} and *Nab2 oe* shared proteins that change in different directions

Protein Symbol	**log_2(*Nab2*^{ex3}/Cont)**	**-log10(p-value)**	**Log_2(*Nab2oe*/Cont)**	**-log10(p-value)**
Nab2	-8.4	8.3	3.9	6.2
Hml	-1.1	2.0	1.0	3.3
Mhc	-0.8	1.8	0.3	4.0
LamC	0.3	2.2	-1.8	1.6
CG15369	0.4	2.3	-0.5	2.1
Sgs7	0.8	1.9	-1.7	2.2
Sgs5	0.8	3.1	-1.2	3.1
Sgs3	0.8	1.3	-5.3	2.7
Sgs8	0.9	2.2	-1.5	3.0
Eig71Ed	1.9	2.2	-7.3	2.0
sls	2.4	6.8	-2.6	1.3

Figures

Figure 2-1

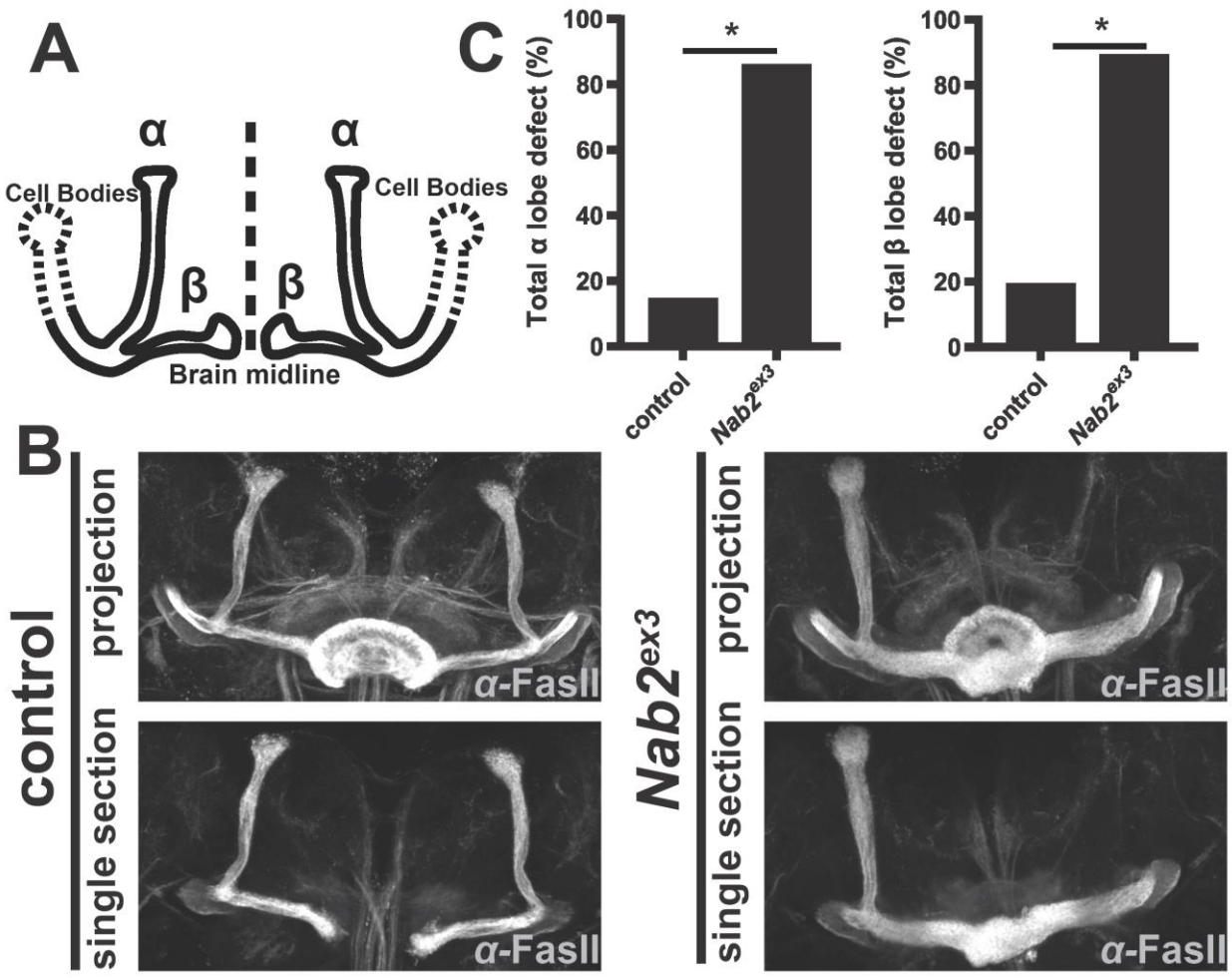

Figure 2-1. Nab2 is required during pupal development for proper neuro-morphological patterning of the mushroom bodies.

(A) Diagram of the *Drosophila* mushroom body depicting cell bodies (dashed lines) projecting axons that bundle to make the dorsal (α) and medial (β) lobes that are mirrored across the brain midline (dashed line). **(B)** Fasciclin II (FasII) antibody staining of *control* (*C155>Gal4, w^{1118}*) and *$Nab2^{ex3}$* (*C155>Gal4;;$Nab2^{ex3}$*) 48-72hr after pupal formation brains. Confocal images show maximum intensity Z-stack projections (projection) which display full mushroom bodies and single transverse plane sections (single section) which display midline crossing of β-lobe axons. Imaging reveals that *control* rarely shows defects in α and β-lobes while *$Nab2^{ex3}$* brains often have thinning or loss of the α-lobes and β-lobes that project across the midline into the contralateral hemisphere resulting in fusion of the lobes or occasionally loss of β-lobes. The ellipsoid body (donut shaped structure at the brain midline) is visible in maximum intensity projection images which masks the β-lobe status, so single section images are included for clarity. **(C)** Quantification of the frequency of *control* and *$Nab2^{ex3}$* (left) total α-lobe defect (thinning or missing α-lobe) or (right) total β-lobe defect (fusion or missing β-lobes) using the scoring system as described in Experimental Procedures. *Control* (α-lobe = 11 biological and 22 technical replicates; β-lobe = 11 biological and technical replicates) and *$Nab2^{ex3}$* (α-lobe = 17 biological and 34 technical replicates; β-lobe = 17 biological and technical replicates). * indicates $p<0.05$; α- lobe $p = 0.002$; β-lobe $p = 0.007$.

Figure 2-2

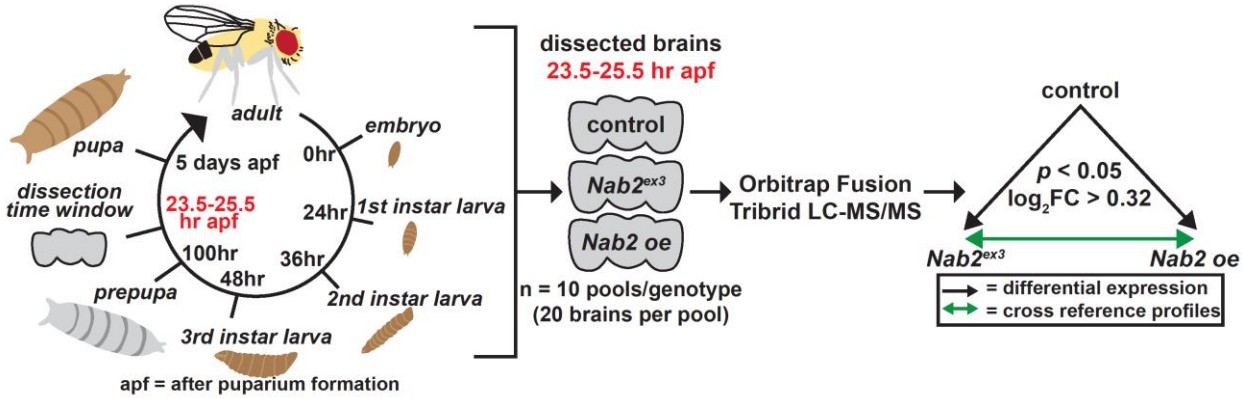
adult
embryo
0hr
pupa
5 days apf
dissection
time window
23.5-25.5
hr apf
24hr
1st instar larva
100hr
36hr
48hr
prepupa
2nd instar larva
3rd instar larva
apf = after puparium formation
dissected brains
23.5-25.5 hr apf
control
Nab2ex3
Nab2 oe
n = 10 pools/genotype
(20 brains per pool)
Orbitrap Fusion
Tribrid LC-MS/MS
control
p < 0.05
log2FC > 0.32
Nab2ex3
Nab2 oe
= differential expression
= cross reference profiles

Figure 2-2. Study design and analytic approach for quantitative proteomic analysis of *Drosophila* pupal brains.

A workflow summary showing dissection window, experimental design, and analysis. The *Drosophila* life cycle with developmental stage and hours of development depicted with the dissection time window (23.25-25.5 hr APF) in **red, left**. There were 600 developmentally timed brain samples which were pooled by genotype: *control* (*C155>Gal4, w^{1118}*); *Nab2* zygotic null (*$Nab2^{ex3}$* = *C155>Gal4;;$Nab2^{ex3}$*); *Nab2 overexpression in neurons* (*Nab2 oe* = *C155>Gal4;$Nab2^{EP3716}$;$Nab2^{ex3}$*) and by sex resulting in 30 individual pools**, center**. Each sample pool was processed, analyzed using an Orbitrap Fusion Tribrid Mass Spectrometer, and quantified using MaxQuant against the *D. melanogaster* Uniprot database**, center**. Arrows depict the performed analyses. Differential protein abundance of *$Nab2^{ex3}$* and *Nab2 oe* brains was calculated with an FDR adjusted p-value (**black** arrows), and then second-degree analyses cross referencing the *$Nab2^{ex3}$* and *Nab2 oe* proteomic profiles (**green** arrows)**, right**.

Figure 2-3

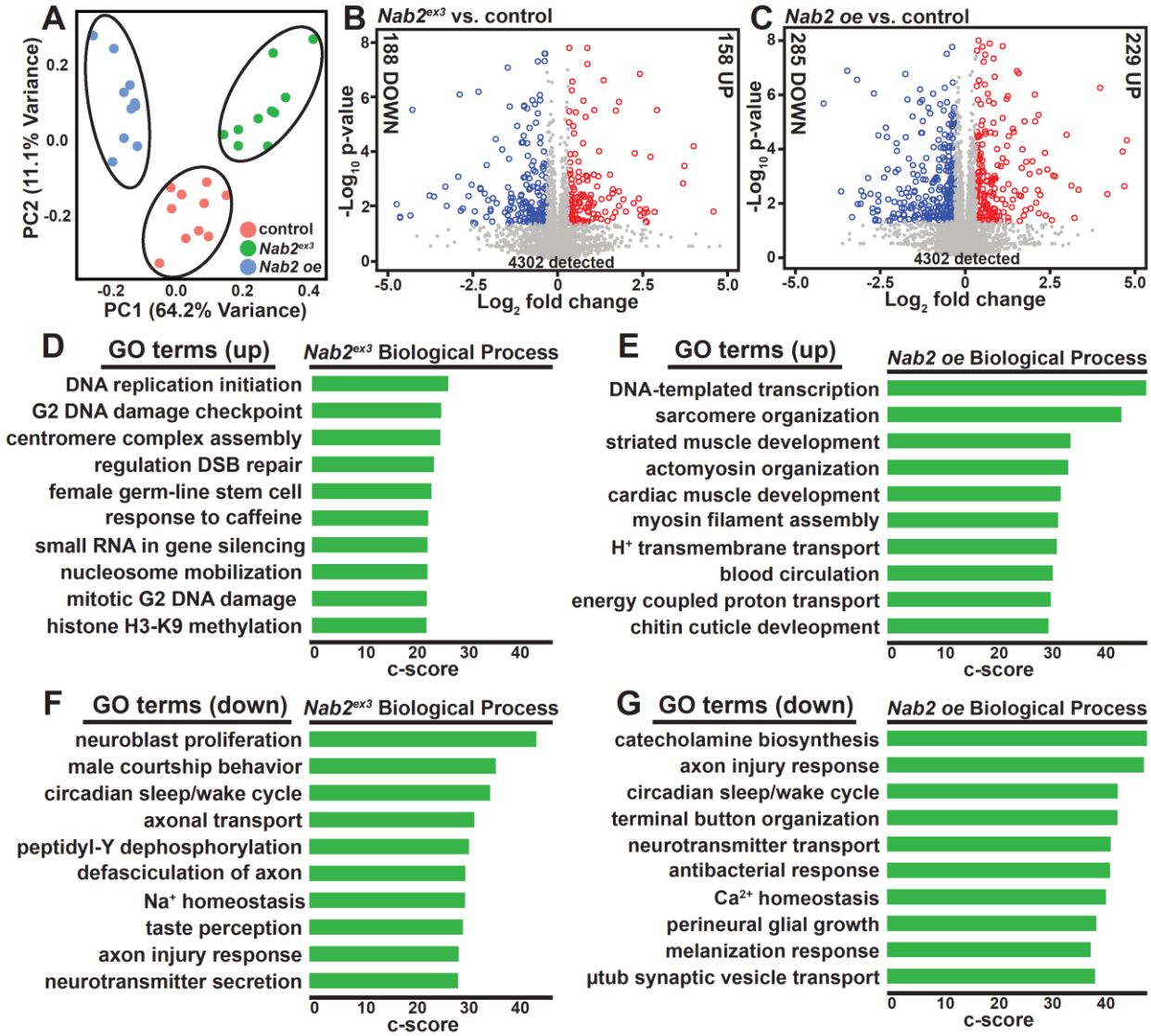
A
PC2 (11.1% Variance)
PC1 (64.2% Variance)
control
Nab2ex3
Nab2 oe
B
Nab2ex3 vs. control
188 DOWN
158 UP
4302 detected
-Log10 p-value
Log2 fold change
C
Nab2 oe vs. control
285 DOWN
229 UP
4302 detected
-Log10 p-value
Log2 fold change
D
GO terms (up)
Nab2ex3 Biological Process
DNA replication initiation
G2 DNA damage checkpoint
centromere complex assembly
regulation DSB repair
female germ-line stem cell
response to caffeine
small RNA in gene silencing
nucleosome mobilization
mitotic G2 DNA damage
histone H3-K9 methylation
c-score
E
GO terms (up)
Nab2 oe Biological Process
DNA-templated transcription
sarcomere organization
striated muscle development
actomyosin organization
cardiac muscle development
myosin filament assembly
H+ transmembrane transport
blood circulation
energy coupled proton transport
chitin cuticle devleopment
c-score
F
GO terms (down)
Nab2ex3 Biological Process
neuroblast proliferation
male courtship behavior
circadian sleep/wake cycle
axonal transport
peptidyl-Y dephosphorylation
defasciculation of axon
Na+ homeostasis
taste perception
axon injury response
neurotransmitter secretion
c-score
G
GO terms (down)
Nab2 oe Biological Process
catecholamine biosynthesis
axon injury response
circadian sleep/wake cycle
terminal button organization
neurotransmitter transport
antibacterial response
Ca2+ homeostasis
perineural glial growth
melanization response
µtub synaptic vesicle transport
c-score

Figure 2-3. Quantitative proteomic analysis of developmentally timed pupal brains reveals a role for Nab2 in neurodevelopment.

(A) Principal component analysis (PCA) of proteomic data from 24hr after pupal formation *Drosophila* brains from ten biological replicates of *control*, *Nab2*^{ex3}, and *Nab2 oe* flies (*control* = *C155>Gal4, w*^{1118}*; Nab2*^{ex3} = *C155>Gal4;;Nab2*^{ex3}; *Nab2 oe* =*C155>Gal4;Nab2*^{EP3716}*;Nab2*^{ex3}) show results cluster based on genotype and that *Nab2*^{ex3} and *Nab2 oe* are distinct from *control* and each other. PCA was performed in RStudio using prcomp (default stats package v3.5.1), and summed peptide intensities were used as input. **(B,C)** Volcano plots show proteins differentially expressed in each *Nab2* genotype compared to *control* [**(B)** *Nab2*^{ex3} (346; 188 down and 158 up) and **(C)** *Nab2 oe* compared to *control* (514; 285 down and 229 up)]. Ten biological replicates (n=10) per genotype (20 brains per pooled biological replicate) with 30 technical replicates in total. Significance thresholds: $\log_2(\geq 0.32$ and $\leq -0.32)$ and $-\log_{10}$(p-value) ≥ 1.3); thresholds based on power calculation and instrumental limits. Protein abundance change (Down or Up) indicated on each side of the plot ($\log_2$ *Nab2*^{ex3}/Cont or *Nab2 oe*/Cont: **grey** = n.s., **blue** ≤ -0.32, **red** ≥ 0.32). Number of differentially expressed proteins to total detected proteins show atop graph; 346 out of 4302 *Nab2*^{ex3} proteins are differentially expressed **(B)** and 514 out of 4302 *Nab2 oe* proteins are differentially expressed **(C)**. **(D-G)** The enriched gene ontology terms from FlyEnrichr database for Biological Process are shown for proteins increased $\log_2$(*Nab2*^{ex3}/Cont) ≥ 0.32 in **(D)** *Nab2*^{ex3} and $\log_2$(*Nab2 oe*/Cont) in **(E)** *Nab2 oe* and decreased $\log_2$(*Nab2*^{ex3}/Cont) ≤ -0.32 in **(F)** *Nab2*^{ex3} and $\log_2$(*Nab2 oe*/Cont) in **(G)** *Nab2 oe*. The bars shown correspond to the top ten c-scores (c-score = ln(adj p-val) * z-score) in each dataset (adj. p-val<0.05) (E. Chen et al., 2013; Kuleshov et al., 2019, 2016).

Figure 2-4

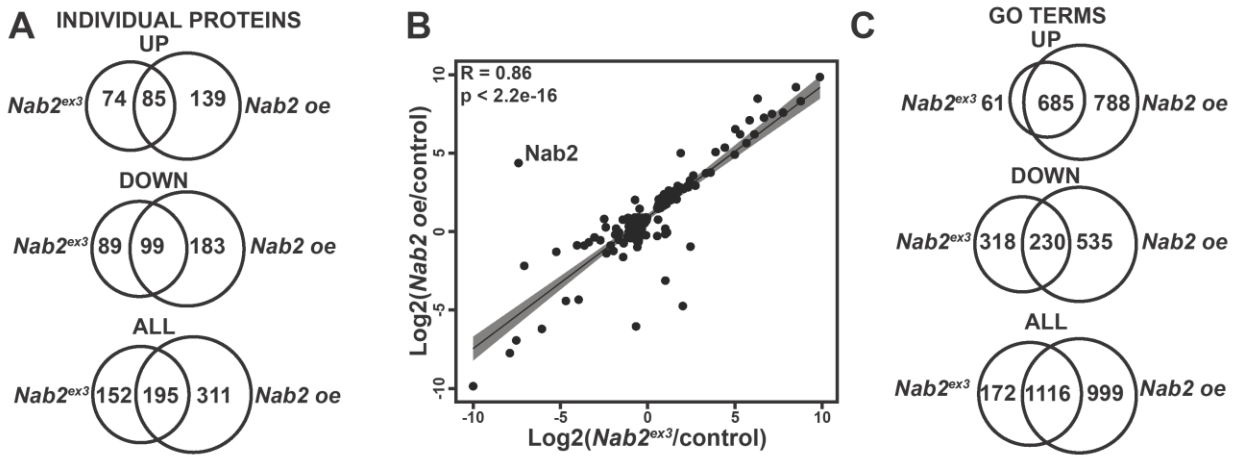
A
INDIVIDUAL PROTEINS
UP
Nab2ex3
74
85
139
Nab2 oe
DOWN
Nab2ex3
89
99
183
Nab2 oe
ALL
Nab2ex3
152
195
311
Nab2 oe
B
R = 0.86
p < 2.2e-16
Nab2
Log2(Nab2 oe/control)
Log2(Nab2ex3/control)
10
5
0
-5
-10
C
GO TERMS
UP
Nab2ex3
61
685
788
Nab2 oe
DOWN
Nab2ex3
318
230
535
Nab2 oe
ALL
Nab2ex3
172
1116
999
Nab2 oe

Figure 2-4. *Nab2*^{ex3} and *Nab2 oe* brains display distinct sets of differentially expressed proteins but have similar changes among shared proteins.

(A) Venn diagrams illustrating the number of individual, differentially expressed proteins, which are shared or unique to *Nab2*^{ex3} and *Nab2 oe* that (top) increase or (middle) decrease in protein abundance (*Nab2*^{ex3} relative to *control* and *Nab2 oe* relative to control) or (bottom) all abundance changes. **(B)** A correlation curve comparing the changes in protein abundance for proteins changed in both *Nab2*^{ex3} and *Nab2 oe* relative to *control* was produced by plotting on a logarithmic scale. Results show that the shared changes (195) observed are highly correlated ($R = 0.86$, $p < 2.2e\text{-}16$, Pearson coefficient) in magnitude and direction. Regression line plotted in **black** with 95% confidence interval depicted by **grey** shading. Nab2 is expected to change in direction and magnitude between *Nab2*^{ex3} and *Nab2 oe* and is annotated on the plot. **(C)** Venn diagrams illustrating the number of GO Biological Process terms enriched in *Nab2*^{ex3} and *Nab2 oe* that are shared or unique based on the subset of proteins that (top) increase or (middle) decrease protein abundance or (bottom) all abundance changes.

Figure 2-5

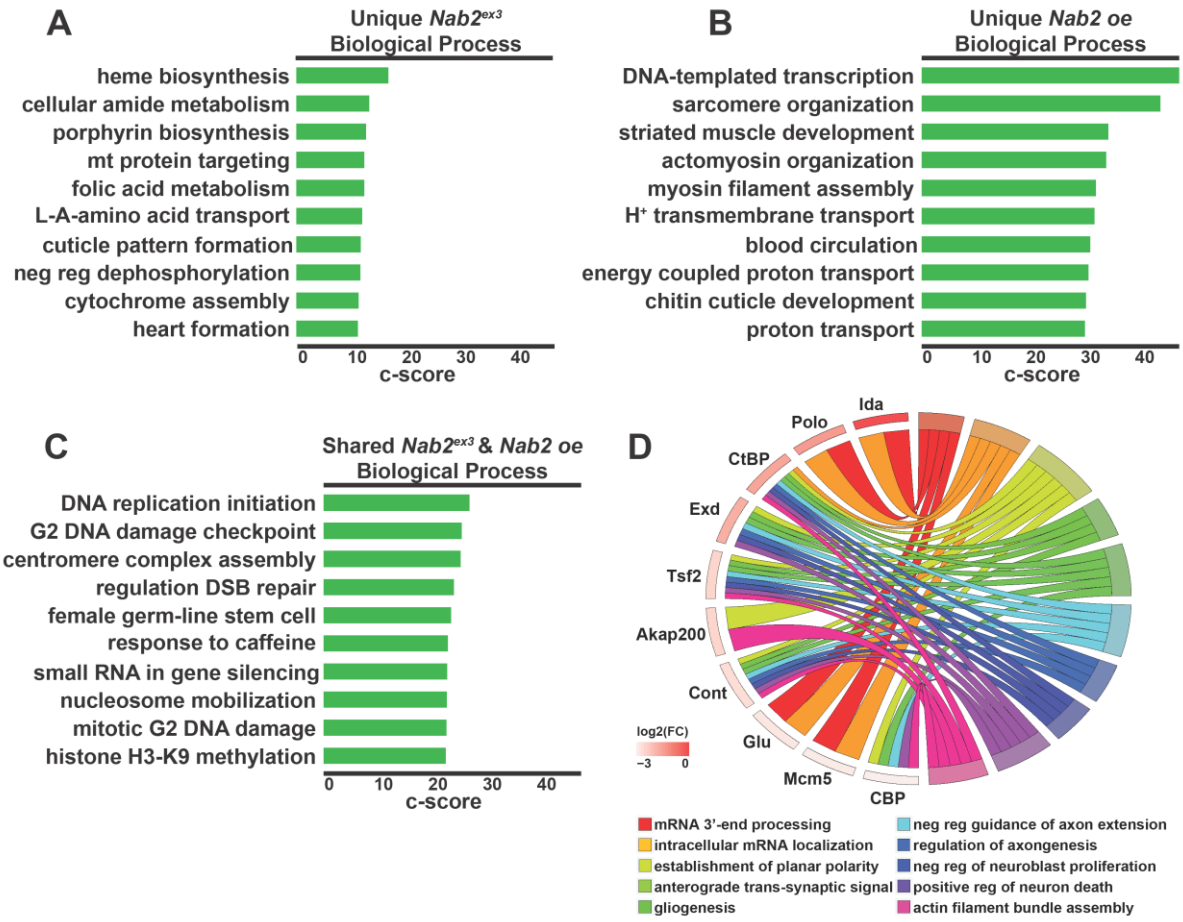
A
Unique Nab2ex3
Biological Process
heme biosynthesis
cellular amide metabolism
porphyrin biosynthesis
mt protein targeting
folic acid metabolism
L-A-amino acid transport
cuticle pattern formation
neg reg dephosphorylation
cytochrome assembly
heart formation
0 10 20 30 40
c-score
B
Unique Nab2 oe
Biological Process
DNA-templated transcription
sarcomere organization
striated muscle development
actomyosin organization
myosin filament assembly
H+ transmembrane transport
blood circulation
energy coupled proton transport
chitin cuticle development
proton transport
0 10 20 30 40
c-score
C
Shared Nab2ex3 & Nab2 oe
Biological Process
DNA replication initiation
G2 DNA damage checkpoint
centromere complex assembly
regulation DSB repair
female germ-line stem cell
response to caffeine
small RNA in gene silencing
nucleosome mobilization
mitotic G2 DNA damage
histone H3-K9 methylation
0 10 20 30 40
c-score
D
Ida
Polo
CtBP
Exd
Tsf2
Akap200
Cont
Glu
Mcm5
CBP
log2(FC)
−3 0
mRNA 3'-end processing
intracellular mRNA localization
establishment of planar polarity
anterograde trans-synaptic signal
gliogenesis
neg reg guidance of axon extension
regulation of axongenesis
neg reg of neuroblast proliferation
positive reg of neuron death
actin filament bundle assembly

Figure 2-5. Proteins increased in abundance in *Nab2^{ex3}* and *Nab2 oe* brains are enriched for processes including RNA processing and neurodevelopment.

(A-C) The enriched terms from FlyEnrichr database for Biological Process resulting from the subset of proteins increased in abundance that are **(A)** unique to *Nab2^{ex3}*, **(B)** unique to *Nab2 oe*, and **(C)** shared between *Nab2^{ex3}* and *Nab2 oe*. The bars shown correspond to the top ten c-scores (c-score = ln(adj p-val) * z-score) in each dataset (adj. p-val<0.05) (E. Chen et al., 2013; Kuleshov et al., 2019, 2016). **(D)** A chord plot showing how proteins are represented in multiple GO Biological Process terms enriched from the subset of proteins increased in abundance in both *Nab2^{ex3}* and *Nab2 oe* relative to control. The selected terms are shown on the right of the plot and are color coded according to the legend, with the chords extending to the left of the plot showing which proteins are represented in each term. The $\log_2$ (*Nab2^{ex3}*/Cont) in *Nab2^{ex3}* is represented by color change (white to **red**) next to each protein annotation.

Figure 2-6

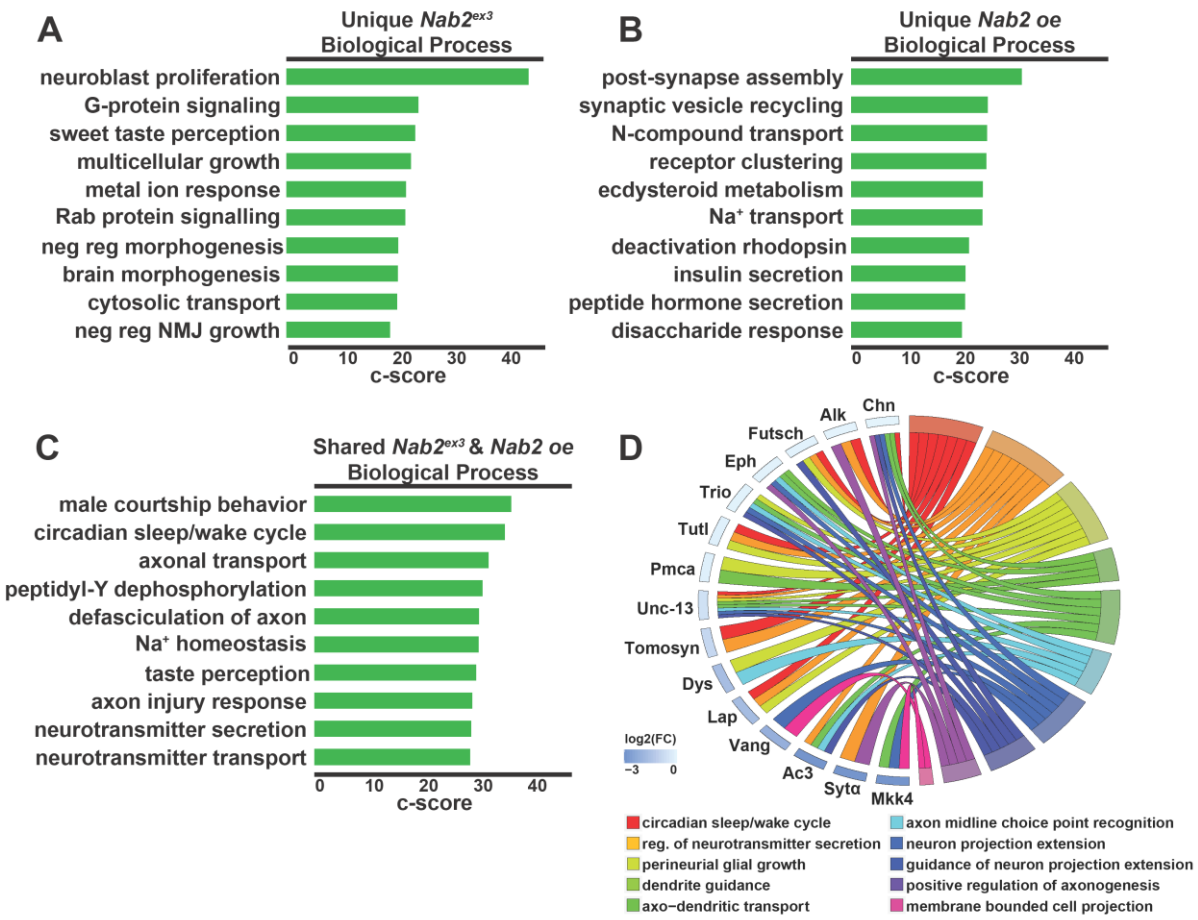
A
Unique Nab2ex3
Biological Process
neuroblast proliferation
G-protein signaling
sweet taste perception
multicellular growth
metal ion response
Rab protein signalling
neg reg morphogenesis
brain morphogenesis
cytosolic transport
neg reg NMJ growth
0 10 20 30 40
c-score
B
Unique Nab2 oe
Biological Process
post-synapse assembly
synaptic vesicle recycling
N-compound transport
receptor clustering
ecdysteroid metabolism
Na+ transport
deactivation rhodopsin
insulin secretion
peptide hormone secretion
disaccharide response
0 10 20 30 40
c-score
C
Shared Nab2ex3 & Nab2 oe
Biological Process
male courtship behavior
circadian sleep/wake cycle
axonal transport
peptidyl-Y dephosphorylation
defasciculation of axon
Na+ homeostasis
taste perception
axon injury response
neurotransmitter secretion
neurotransmitter transport
0 10 20 30 40
c-score
D
Alk
Chn
Futsch
Eph
Trio
Tutl
Pmca
Unc-13
Tomosyn
Dys
Lap
Vang
Ac3
Sytα
Mkk4
log2(FC)
−3 0
circadian sleep/wake cycle
reg. of neurotransmitter secretion
perineurial glial growth
dendrite guidance
axo−dendritic transport
axon midline choice point recognition
neuron projection extension
guidance of neuron projection extension
positive regulation of axonogenesis
membrane bounded cell projection

Figure 2-6. Proteins reduced in abundance in *Nab2^{ex3}* and *Nab2 oe* are enriched for neurological roles.

(A-C) The enriched terms from FlyEnrichr database for Biological Process resulting from the subset of proteins decreased in abundance that are **(A)** unique to *Nab2^{ex3}*, **(B)** unique to *Nab2 oe*, and **(C)** shared between *Nab2^{ex3}* and *Nab2 oe*. The bars shown correspond to the top ten c-scores (c-score = ln(adj p-val) * z-score) in each dataset (adj. p-val<0.05)(E. Chen et al., 2013; Kuleshov et al., 2019, 2016). **(D)** A chord plot showing how proteins are represented in multiple GO Biological Process terms enriched from the subset of proteins decreased in abundance in both *Nab2^{ex3}* and *Nab2 oe* relative to control. The selected terms are shown on the right of the plot and color coded according to the legend, with the chords extending to the left of the plot showing which proteins are represented in each term. The $\log_2$(*Nab2^{ex3}*/Cont) in *Nab2^{ex3}* is represented by color change (white to **blue**) next to each protein annotation.

Figure 2-7

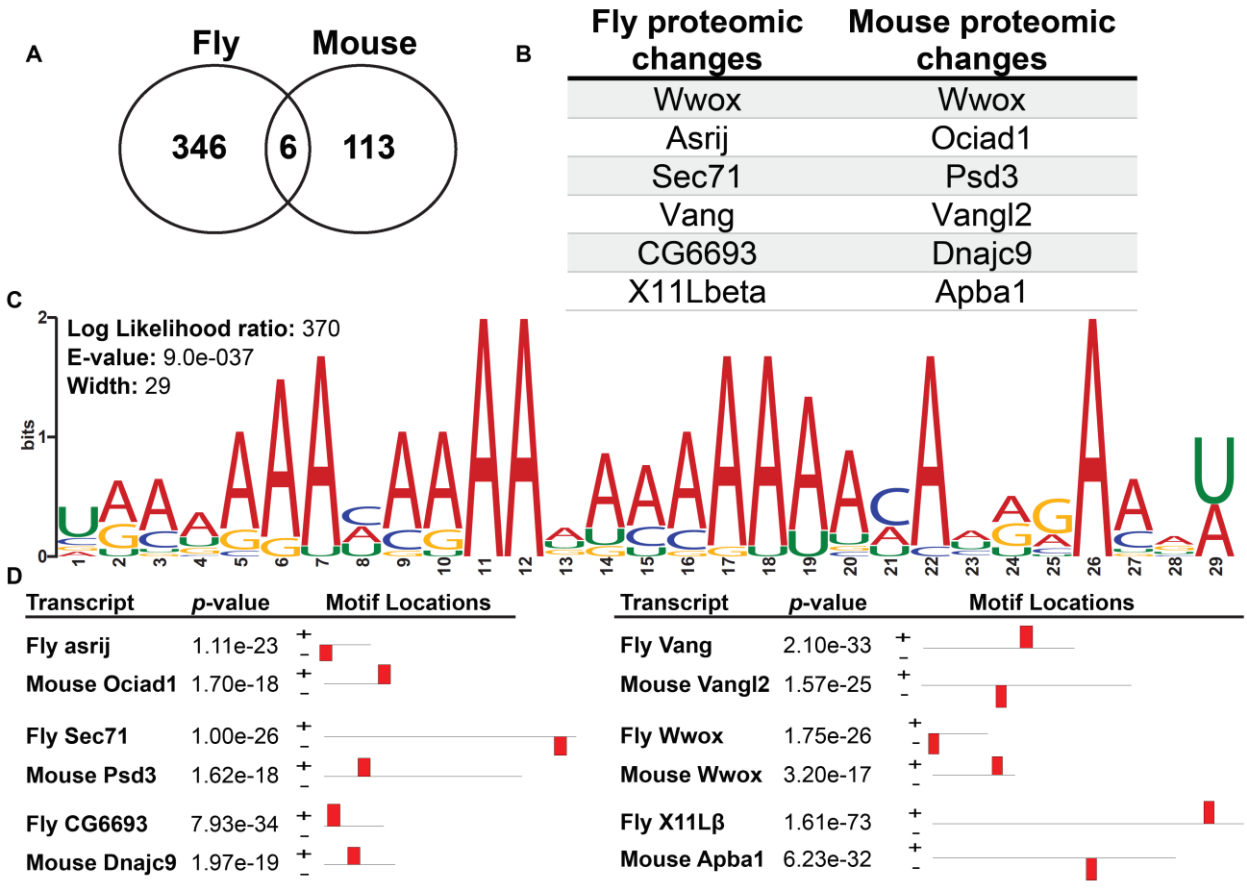
A
Fly
Mouse
346
6
113
B
Fly proteomic changes
Mouse proteomic changes
Wwox
Wwox
Asrij
Ociad1
Sec71
Psd3
Vang
Vangl2
CG6693
Dnajc9
X11Lbeta
Apba1
C
Log Likelihood ratio: 370
E-value: 9.0e-037
Width: 29
bits
D
Transcript
p-value
Motif Locations
Fly asrij 1.11e-23
Mouse Ociad1 1.70e-18
Fly Sec71 1.00e-26
Mouse Psd3 1.62e-18
Fly CG6693 7.93e-34
Mouse Dnajc9 1.97e-19
Fly Vang 2.10e-33
Mouse Vangl2 1.57e-25
Fly Wwox 1.75e-26
Mouse Wwox 3.20e-17
Fly X11Lβ 1.61e-73
Mouse Apba1 6.23e-32

Figure 2-7. Six protein changes are shared between Nab2^{ex3} flies and ZC3H14$^{\Delta ex13/\Delta ex13}$ mice and contain a shared A-rich motif.

(A) Venn diagram showing total number of differentially expressed proteins in *Nab2^{ex3}* pupal brain (346 proteins) and *Zc3h14$^{\Delta ex13/\Delta ex13}$* P0 hippocampi (113 protein) (Rha et al., 2017) with six shared protein changes. **(B)** List showing the six shared differentially expressed proteins between *Nab2^{ex3}* pupal brain and *Zc3h14$^{\Delta ex13/\Delta ex13}$* P0 hippocampi. This study found proteomic changes ($\log_2$(*Nab2^{ex3}*/Cont)) of: Wwox $\log_2$(7.5); Asrij $\log_2$(0.6); Sec71 $\log_2$(-1.3); Vang $\log_2$(-2.3); CG6693 $\log_2$(-2.8); X11Lβ $\log_2$(-4.7). **(C)** MEME logo of A-rich motif identified in the twelve transcripts encoding the six fly proteins and the six mouse proteins. MEME (Multiple EM for Motif Elicitation) conducted with OOPS (exactly one site per sequence) motif site distribution, with minimum motif width of six and maximum motif width of fifty. Analysis performed under MEME version 5.3.2 (release date: 02/06/2021) (Bailey et al., 2009; Bailey & Elkan, 1994). This motif was the most enriched among the transcripts with a log likelihood ratio of 370, E-value of 9.0e-37, and width of 29. Threshold of significance: E-value <0.05. **(D)** A-rich motif location shown within the transcripts corresponding to the differentially expressed proteins. Fly and mouse transcript pairs are shown with transcript name, the p-value significance of motif, and motif location within the transcript (indicated by **red** bar).

Figure 2-S1

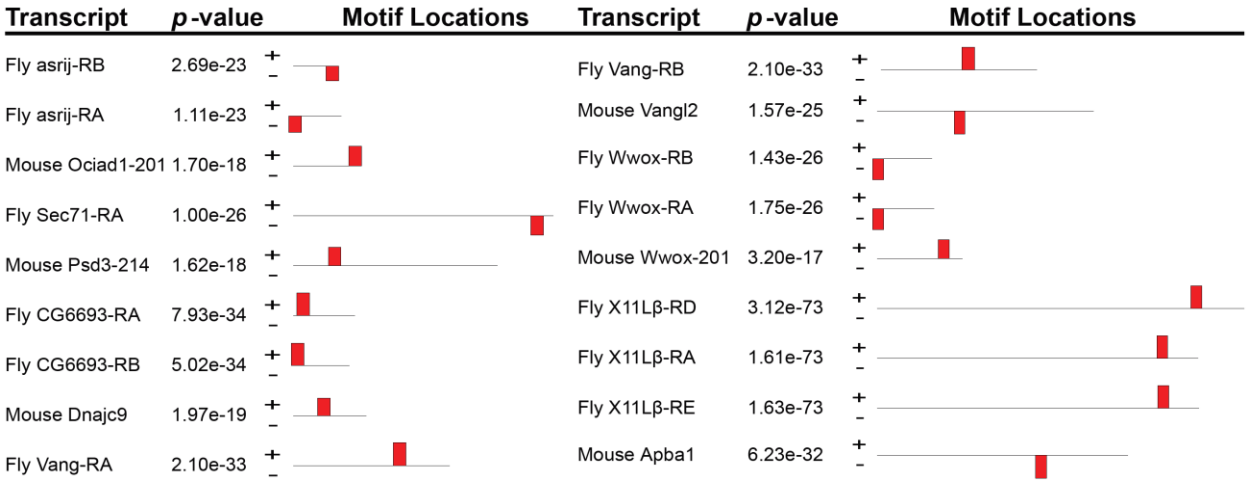
Transcript
p-value
Motif Locations
Fly asrij-RB 2.69e-23
Fly asrij-RA 1.11e-23
Mouse Ociad1-201 1.70e-18
Fly Sec71-RA 1.00e-26
Mouse Psd3-214 1.62e-18
Fly CG6693-RA 7.93e-34
Fly CG6693-RB 5.02e-34
Mouse Dnajc9 1.97e-19
Fly Vang-RA 2.10e-33
Transcript
p-value
Motif Locations
Fly Vang-RB 2.10e-33
Mouse Vangl2 1.57e-25
Fly Wwox-RB 1.43e-26
Fly Wwox-RA 1.75e-26
Mouse Wwox-201 3.20e-17
Fly X11Lβ-RD 3.12e-73
Fly X11Lβ-RA 1.61e-73
Fly X11Lβ-RE 1.63e-73
Mouse Apba1 6.23e-32

Figure 2-S1. A novel A-rich motif is shared among all transcripts corresponding to shared protein changes shared between Nab2^{ex3} flies and ZC3H14$^{\Delta ex13/\Delta ex13}$ mice.

(A) Novel A-rich motif location shown with the 18 transcripts corresponding to the differentially expressed proteins. Fly and mouse transcript pairs are shown with transcript name, the p-value significance of motif, and motif location within the transcript (indicated by **red** bar). MEME (Multiple EM for Motif Elicitation) conducted with OOPS (exactly one site per sequence) motif site distribution, with minimum motif width of six and maximum motif width of fifty. Analysis performed under MEME version 5.3.2 (release date: 02/06/2021) (Bailey et al., 2009; Bailey & Elkan, 1994). Threshold of significance: p-value <0.05.

Chapter 3: The RNA binding protein Nab2 patterns dendritic arbors and axons via the planar cell polarity pathway

This chapter has been submitted to *Current Biology* for publication consideration. This manuscript, on which Edwin Corgiat is first author, was submitted as:

The RNA binding protein Nab2 patterns dendritic arbors and axons via the planar cell polarity pathway

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Please Note:

In the current chapter, Edwin Corgiat served as the lead researcher and first author on the contents of the chapter. He performed genetic, biochemical, and bioinformatic experiments and analyses as described within. He alone performed the experiments for Figures 3-1, 3-2, 3-3, 3-4, 3-5, 3-6, 3-S1, 3-S2, 3-S3, and S-4, and analyses for Figure 3-2. In pair with Dr. Sara M. List, they performed analyses for Figures 3-1, 3-2, 3-3, 3-4, 3-5, 3-6, 3-S1, 3-S2, 3-S3, and S-4, and Tabl2 1. Dehong Yu and Ping Chen performed experiments and analyses for Figure 3-7. Edwin authored the text of this chapter in conjunction with Drs. Kenneth H. Moberg and Anita H. Corbett.

The global proteomic experiments (LC-MS/MS) were conducted at the Emory Integrated Proteomics Core (EIPC); Edwin Corgiat performed all preceding experiments and sample collection and coordinated the project with the core.

Drs. Kenneth H. Moberg and Anita H. Corbett mentored Edwin Corgiat and provided essential intellectual contribution, guidance, and financial support.

Summary

RNA binding proteins contribute to neurodevelopment by modulating numerous steps in post-transcriptional regulation, including splicing, export, translation, and turnover of mRNAs that can traffic into axons and dendrites. One such RBP is ZC3H14, which is lost in an inherited intellectual disability. The *Drosophila melanogaster* ZC3H14 ortholog, Nab2, localizes to neuronal nuclei and cytoplasmic ribonucleoprotein granules, and is required for olfactory memory and proper axon projection into brain mushroom bodies (MBs). Nab2 can act as a translational repressor in conjunction with the Fragile-X mental retardation protein homolog Fmr1 and shares target RNAs with the Fmr1-interacting RBP Ataxin-2. However, neuronal signaling pathways regulated by Nab2 and their potential roles outside of MB axons remain undefined. Here, we demonstrate that Nab2 has a role in restricting branching and projection of larval sensory dendrites via the planar cell polarity (PCP) pathway, and that this Nab2-PCP link may provide an evolutionarily conserved mechanism through which Nab2/ZC3H14 could modulate projection of both axons and dendrites. PCP proteins are enriched in a Nab2-regulated brain proteomic dataset. Complementary genetic data indicate that Nab2 guides PCP-dependent projection of larval sensory dendrites and adult MB axons. Analysis of the core PCP protein Vang, which is depleted in the *Nab2* mutant whole-brain proteome, uncovers selective and dramatic loss of Vang within axon/dendrite-enriched brain neuropil relative to brain regions containing cell bodies. Collectively, these data demonstrate that Nab2 regulates dendritic arbors and axon projection by a PCP-linked mechanism and identify Nab2 as required for accumulation of the core PCP factor Vang in distal neuronal projections.

Introduction

While many key developmental events are triggered by extracellular factors that signal through cytoplasmic cascades to alter nuclear gene transcription, other key events are triggered by shifts in posttranscriptional processing or localization of mRNAs that guide cell fates and differentiation. Importantly, the fidelity of these mRNA-based developmental mechanisms relies on RNA binding proteins (RBPs) that associate with nascent RNAs and regulate splicing, export, stability, localization, and translation (Schieweck et al., 2021). These key regulatory mechanisms are particularly evident in the developing nervous system, where mutations in genes encoding RBPs are often linked to human diseases. Examples of this linkage include Fragile X mental retardation protein (FMRP) (C. Gross et al., 2012), the survival of motor neuron protein (SMN) (Edens et al., 2015), and the TAR DNA binding protein 43 (TDP-43) (Agrawal et al., 2019; Gebauer, Schwarzl, Valcárcel, & Hentze, 2021). Sensitivity of the central and peripheral nervous systems to loss of RBPs has been attributed to the importance of post-transcriptional mechanisms, such as local translation of mRNAs and brain-specific extension of 3'UTRs (Engel et al., 2020; Mattioli et al., 2016; Thelen & Kye, 2020) that enable fine-tuned spatiotemporal control of neuronal gene expression. This spatiotemporal control of mRNA processing and translation plays an important role in forming complex dendritic architectures and the uniquely polarized morphology of neurons (A. Lee et al., 2003). Accordingly, neurological diseases caused by mutations in genes encoding RBPs often include defects in axonal or dendritic morphology (Holt et al., 2019; Hörnberg & Holt, 2013; Jung et al., 2012), and in some cases, these axonal and dendritic defects can be traced to defective post-transcriptional control of one or a few mRNAs normally bound by the corresponding RBP.

The human *ZC3H14* gene encodes a ubiquitously expressed zinc-finger, polyadenosine RBP (ZnF CysCysCysHis #14) that is lost in an inherited form of intellectual disability (Pak et al., 2011). Studies in multiple model organisms have begun to define functions for ZC3H14 in guiding neuronal morphogenesis. Analysis of the sole *Drosophila* ZC3H14 homolog, Nab2 (nuclear poly(A) binding protein 2 (Anderson, Wilson, Datar, & Swanson, 1993)), detects cell-autonomous requirements in Kenyon cells (KCs) for olfactory memory as well as axonal branching and projection into the brain mushroom bodies (MBs) (Bienkowski et al., 2017; Kelly et al., 2016), twin neuropil structures that are the center for associative olfactory learning in insects (Hige, Aso, Rubin, & Turner, 2015). Significantly, transgenic expression of human ZC3H14 only in fly neurons is sufficient to rescue a variety of *Nab2* null phenotypes (Kelly et al., 2016; Kelly, Leung, Pak, Banerjee, & Moberg, 2014; Pak et al., 2011), supporting a model in which Nab2 and ZC3H14 share critical molecular roles and mRNA targets. The *Zc3h14* gene is not essential in mice but its loss results in defects in working memory (Rha et al., 2017) and dendritic spine morphology (S. K. Jones et al., 2020). An accompanying proteomic analysis of *Zc3h14* knockout hippocampi identified several proteins involved in synaptic development and function that change in abundance upon ZC3H14 loss (Rha et al., 2017), and which are thus candidates to contribute to *Zc3h14* mutant phenotypes. Intriguingly, the homologs of some of these ZC3H14-regulated proteins in the mouse hippocampus are also sensitive to Nab2 loss in the developing *Drosophila* pupal brain (Corgiat & List et al., 2021), suggesting conserved links between Nab2/ZC3H14 and neurodevelopmental pathways.

A variety of intercellular signaling mechanisms play required roles in sensing extracellular cues that guide the complex axonal and dendritic structures that characterize specific areas of the central and peripheral nervous system (CNS and PNS). These cascades can respond to long-range

directional cues, such as Netrin signaling, or to short-range directional cues from the Slit-Robo, Abl-Ena, and Semaphorin pathways (Puram & Bonni, 2013; Stoeckli, 2018). One pathway with an emerging role in both axonal and dendritic development is the planar cell polarity (PCP)-noncanonical Wnt pathway (Andre et al., 2012; Gombos et al., 2015; Misra et al., 2016; Zou, 2004a, 2012). PCP signals are based on asymmetric distribution of two apically localized transmembrane complexes, which in *Drosophila* correspond to the Stan-Vang-Pk complex (Starry Night aka Flamingo-Van Gogh-Prickle) and the Stan-Fz-Dsh-Dgo complex (Frizzled-Disheveled-Diego); these complexes are intracellularly antagonistic but intercellularly attractive, leading to apical polarization across an epithelial plane (Adler, 2012; Adler & Wallingford, 2017; Boutros & Mlodzik, 1999; Goodrich & Strutt, 2011; M. Mlodzik, 2020; Peng & Axelrod, 2012a; Taylor et al., 1998; Vladar et al., 2009). Core PCP components signal to downstream effector molecules (Adler, 2012; Courbard et al., 2009; Fagan et al., 2014; Gombos et al., 2015; Soldano et al., 2013) that exert localized effects on the F-actin cytoskeleton that, in turn, guide epithelial traits like proximal-distal wing hair orientation in *Drosophila* and sensory hair cell polarity in the mouse cochlea (Chacon-Heszele & Chen, 2009; C. Jones & Chen, 2007; M. S. and M. Mlodzik, 2010; Qian et al., 2007; Rida & Chen, 2009). One such effector is encoded by *β amyloid protein precursor-like* (*Appl*) and modulates the PCP pathway during axonal and dendritic outgrowth (Liu et al., 2021; Soldano et al., 2013). Importantly, PCP is required for axon guidance in specific groups of neurons in *Drosophila*, *C. elegans*, mice, and chick, and for dendritic branching of murine hippocampal neurons and *Drosophila* body wall sensory neurons (Cang & Feldheim, 2013; Hagiwara, Yasumura, Hida, Inoue, & Ohtsuka, 2014; Hindges, McLaughlin, Genoud, Henkemeyer, & O'Leary, 2002; McLaughlin & O'Leary, 2005; Schmitt et al., 2006; Shafer et al., 2011). For example, loss of the murine *Vang* homolog *Vangl2* leads to defects in axon guidance

of spinal cord commissural axons (Shafer et al., 2011), and *dsh* mutants in *C. elegans* cause neuronal projection and morphology defects (Zheng, Diaz-Cuadros, & Chalfie, 2015). In *Drosophila*, loss of the core PCP components *stan*, *Vang*, *pk*, *fz*, or *dsh* individually disrupt α and β axon projection into the MBs (Ng, 2012; Shimizu et al., 2011). Intriguingly, loss of *stan* or its LIM-domain adaptor *espinas* (*esn*) also disrupts dendritic self-avoidance among the class IV dendritic arborization (da) neurons (Matsubara et al., 2011), demonstrating a requirement for PCP factors in both axon and dendrite morphogenesis in specific sets of neurons in the central (CNS) and peripheral (PNS) nervous systems.

Integrating data from two of our recent studies provides evidence for pathways through which the Nab2 RBP could guide axonal and dendritic projections. These analyses, one a genetic modifier screen based on a *GMR-Nab2* rough eye phenotype (W. H. Lee et al., 2020) and the other a proteomic analysis of *Nab2* null pupal brains (Corgiat & List et al., 2021), each suggest a link between Nab2 and the PCP pathway in neurons. The *GMR-Nab2* modifier screen identified alleles of PCP components, both core components and downstream effectors (e.g., *Vang, dsh, fz, stan, pk, Appl,* and the formin *DAAM*), as dominant modifiers of *Nab2* overexpression phenotypes in the retinal field (W. H. Lee et al., 2020). In parallel, gene ontology (GO) analysis of proteomic changes in *Nab2* null brains detected enrichment in dendrite guidance and axodendritic transport GO terms (Corgiat & List et al., 2021), including specific protein changes of the core PCP factor Vang and the PCP accessory factor A-kinase anchor protein 200 (Akap200). Significantly, *Drosophila* Vang and its murine homolog Vangl2 are one of six pairs of homologs whose levels change significantly in *Nab2* null fly brains and *Zc3h14* knockout mouse hippocampi (Corgiat & List et al., 2021; Rha et al., 2017), suggesting a conserved relationship between Nab2/ZC3H14 and the PCP pathway in the metazoan central nervous system (CNS).

In light of observations outlined above, we have investigated interactions between Nab2 and PCP factors in two neuronal contexts - CNS axons of the *Drosophila* pupal MB α- and β-lobes, and in larval dendrites of class IV dorsal dendritic arbor C (ddaC) neurons - which provide complementary settings to analyze the Nab2-PCP link in pre- and post-synaptic compartments. We detect enrichment for PCP factors among brain-enriched proteins affected by Nab2 loss and a pattern of genetic interactions between *Nab2* and multiple PCP alleles in both MB axons and ddaC dendrites that are consistent with Nab2 regulating axon and dendrite outgrowth by a common PCP-linked mechanism. However, differences in how individual PCP alleles modify axonal vs dendritic *Nab2* mutant phenotypes suggest that the Nab2-PCP relationship may vary between neuronal subtypes (i.e., pupal Kenyon cells vs. larval ddaC neurons). Cell type-specific RNAi indicates that Nab2 acts cell autonomously to guide PCP-dependent axon and dendrite growth, implying a potentially direct link between Nab2 and one or more PCP components within Kenyon cells and ddaC neurons. Based on the drop in Vang levels detected by proteomic analysis of *Nab2* null brains (Corgiat & List et al., 2021), we analyze the levels and distribution of a fluorescently tagged Vang protein in adult fly brains. Consistent with prior bulk proteomic data, overall Vang-GFP fluorescence is reduced in *Nab2* null brains compared to control; significantly, this drop is accompanied by an unexpected and selective loss of Vang protein in neuropil regions enriched in axons compared to regions enriched in cell bodies. Collectively, these data demonstrate that Nab2 is required to regulate axonal and dendritic growth through a PCP-linked mechanism and identify the Nab2 RBP as required for the accumulation of Vang protein into distal axonal compartments.

Results

Nab2 loss alters levels of planar cell polarity pathway proteins in the *Drosophila* brain.

Our recent study comparing proteomic changes in *Drosophila* pupal brains lacking Nab2 identified *planar cell polarity* gene ontology (GO) terms as one category of significantly altered factors (Corgiat & List et al., 2021) (**Fig. 1A**). A deeper analysis of this protein dataset detects enrichment of five PCP-related GO terms (*establishment of planar polarity*, *establishment of epithelial cell planar polarity*, *establishment of body hair or bristle planar polarity*, *protein localization involved in planar polarity*, *regulation of establishment of planar polarity*) (**Fig. 1B**) extracted from 17 PCP-annotated proteins, including the core PCP component Van Gogh (Vang), and five putative PCP effectors: the Tumbleweed GTPase activating protein (GAP) (W. M. Jones, Chao, Zavortink, Saint, & Bejsovec, 2010; Sotillos & Campuzano, 2000), the neuron-specific PCP modulator Appl (Liu et al., 2021; Singh & Mlodzik, 2012; Soldano et al., 2013), the anchoring protein Akap200 (Bala Tannan et al., 2018; Jackson & Berg, 2002; Weber, Gault, Olguin, Serysheva, & Mlodzik, 2012), the endocytic regulator X11Lβ (G. G. Gross, Mohiddin Lone, Leung, Hartenstein, & Guo, 2013), and the muscle LIM-domain protein at 84B (Mlp84B) (Weber et al., 2012). Together these factors represent 6.4% of the total differentially expressed proteins in $Nab2^{ex3}$ pupal brains relative to control (346 proteins in total; see ref. (Corgiat & List et al., 2021) (**Table S1**). The Vang protein (decreased 5-fold in $Nab2^{ex3}$ vs control) and Appl protein (increased 1.5-fold in $Nab2^{ex3}$ vs control) are particularly notable because alleles of these genes dominantly modify phenotypes produced by *GMR-Gal4* driven Nab2 overexpression in the developing retinal field (W. H. Lee et al., 2020).

Planar cell polarity components dominantly modify Nab2 axonal phenotypes.

To pursue the Nab2-PCP link in the developing CNS, we tested whether axon projection defects in MBs lacking Nab2 (homozygous for the *Nab2^{ex3}* null allele (Pak et al., 2011)) are sensitive to subtle modulation of PCP pathway activity using single copies of loss-of-function alleles of PCP components. Our previous work established genetic interactions between Nab2 and an array of PCP/Wnt alleles in the adult *Drosophila* eye (W. H. Lee et al., 2020). Here, we focused on three of these factors: the core PCP/Wnt factor Vang, which proteomic data indicate is reduced 5-fold in *Nab2^{ex3}* brains (Corgiat & List et al., 2021), the accessory factor Appl (Amyloid precursor protein-like), which is a proposed PCP/Wnt co-receptor and has established links to neurological disease (Liu et al., 2021; Singh & Mlodzik, 2012; Soldano et al., 2013), and the PCP/Wnt cytoplasmic adaptor Dsh, which also genetically interacts with *Nab2* in the wing to control hair polarity (W. H. Lee et al., 2020). As has been observed in *Nab2^{ex3}* adult brains (Bienkowski et al., 2017; Corgiat & List et al., 2021; Kelly et al., 2016), *Nab2^{ex3}* mutant pupal brain at 48-72h APF (after puparium formation) display highly penetrant defects in structure of the α-lobes (85% thinned or missing) and β-lobes (88% fused or missing) as detected by anti-Fas2 staining (**Fig. 2A-D,Q,R**). Both the *Vang^{stbm6}* and *Appl^{d}* loss-of-function alleles have no effect on MB structure in an otherwise wildtype background but suppress the frequency of *Nab2^{ex3}* α-lobe defects from 85% to 49% in a *Vang^{stbm6}*/+ heterozygous background and to 62% in a *Appl^{d}*/+ heterozygous background; the frequency of *Nab2^{ex3}* β-lobe defects drops from 88% to 33% in *Vang^{stbm6}*/+ heterozygous background and to 35% in *Appl^{d}*/+ heterozygous background (**Fig. 2E-F,I-J,M-N**). The PCP-specific allele *dsh^{1}* (Gombos et al., 2015; Theisen et al., 1994) lowers *Nab2^{ex3}* α-lobe defects from 85% to 63% but has no effect on the frequency or severity of *Nab2^{ex3}* β-lobe defects

(**Fig. 2Q,R**) (**Fig. S1**). Intriguingly, animals with single copies of *Vang*^{stbm6}, *Appl*^{d}, and *dsh*^{1} in the *Nab2*^{ex3} homozygous background also develop a MB phenotype not observed in any single mutant: a bulbous, Fas2-positive lobe at the point at which the peduncle splits into the five lobes (α,α',β,β',γ) (arrowhead in **Fig. 2G,K,O**). The basis of this bulbous phenotype is unclear but may indicate that lowering levels of PCP factors in Kenyon cells that also lack Nab2 leads to a novel axon guidance defect among α/β axons. In sum, these data reveal a pattern of dose-sensitive genetic interactions between endogenous Nab2 and PCP factors that are consistent with Nab2 modulating PCP-mediated control of MB axon projection.

Nab2 is required to restrict dendritic branching and projection

Loss of murine *Zc3h14* causes defects in dendritic spine morphology among hippocampal neurons (S. K. Jones et al., 2020) prompted us to test whether Nab2-PCP interactions in axons are also conserved in developing dendrites. For this approach, we visualized dendrites of *Drosophila* class IV dorsal dendritic arbor C (ddaC) neurons located in the larval body wall using a *pickpocket (ppk)-Gal4,UAS-GFP* system and quantified branching using Sholl intersection analysis (Cuntz, Forstner, Borst, & Häusser, 2010). In L3 larvae, complete loss of Nab2 leads to increased dendritic branch complexity measured by the number of Scholl intersections relative to control (median 200 intersections in *ppk>GFP* vs. median 252 in *Nab2*^{ex3}; **Fig. 3A-B,F**) which is phenocopied by Nab2 RNAi depletion in ddaC neurons (median 250 intersections in *ppk>Nab2*^{RNAi}; **Fig. 3C,F**). Nab2 overexpression in ddaC neurons using the *Nab2*^{EP3716} transgene has the inverse effect of decreasing Scholl intersections (median 179 in *ppk>Nab2*; **Fig. 3E,F**). Significantly, RNAi depletion of the Wnt/PCP receptor *frizzled 2* in ddaC neurons also increases Scholl intersections (median 216

intersections in *ppk>fz2^{RNAi}*; **Fig. 3D,F**), confirming prior work that Wnt/PCP signaling is involved in ddaC dendritic development (Misra et al., 2016).

The data above confirm that Nab2 and the PCP pathway are each required within ddaC neurons to guide the degree of dendritic branching. To further assess whether modulation of PCP pathway activity affects this newly defined *Nab2* dendritic role, we exploited the Matlab TREES toolbox and custom scripts to simultaneously quantify multiple dendritic phenotypes in *Nab2^{ex3}* homozygous larvae (**Fig. 4E**) (Cuntz et al., 2010). This approach confirmed that *Nab2* loss elevates the total number of branches compared to control (**Fig. 4A,B,D**), but also revealed an extension of overall cable length (**Fig. 4A,B,C**) that is indicative of increased total dendritic projections. A further breakdown of *Nab2^{ex3}* branching defects shows an increase in maximum branch order (# of branch points along a given branch from soma to distal tip) (**Fig. 4D,F**) and coupled decrease in mean branch length (distance between consecutive branches) (**Fig. 4F**). Thus, *Nab2^{ex3}* ddaC arbors project and branch significantly more than control across multiple parameters (**Fig. 4F**). Due to the increased branching, *Nab2^{ex3}* ddaC arbors exhibit reduced mean path length (-4%), smaller mean branch angles (-9%), and smaller mean branch lengths (-22%) compared to control (**Fig. 4F**). Significantly, these effects of Nab2 loss on branch/length metrics increase with distance from the cell body (**Fig. S2A-B**), suggesting that the role of Nab2 in restricting dendritic branching and projection becomes more significant with increasing distance from the cell soma.

Planar cell polarity components dominantly modify Nab2 dendritic phenotypes.

Having established that Nab2 loss elicits a spectrum of ddaC branching and projection defects, we proceeded to test whether genetic modulation of PCP activity could affect one or more of these parameters. While *Nab2^{ex3}* homozygotes show an increase in arborization compared to

controls (**Fig. 5A-B**), single copies of the *Vang*^{stbm6} and *Appl*^{d} alleles (i.e., as heterozygotes) each have no significant effects on ddaC arbors in an otherwise wildtype background. In contrast, *dsh*^{1} heterozygosity results in increased branch points, Sholl intersections, and total cable length compared to controls. When placed into the *Nab2*^{ex3} background, single copies of *Vang*^{stbm6} and *Appl*^{d} alleles dominantly modify *Nab2*^{ex3} phenotypes in opposite directions: *Vang*^{stbm6} enhances the severity of *Nab2*^{ex3} ddaC branching and length phenotypes while *Appl*^{d} suppresses many of the same phenotypes (e.g., total cable length and maximum branch order; **Fig. 5I-K**). The *dsh*^{1} allele enhances *Nab2*^{ex3} phenotypes (**Fig. 5I-K**), although the presence of ddaC defects in *dsh*^{1} heterozygotes suggests that this could be an additive effect. Intriguingly, use of Matlab TREES to assess branching defects as a function of distance from the cell body indicates that complexity changes induced by the *Vang*^{stbm6} allele are primarily in *Nab2*^{ex3} proximal arbors, while those associated with *Appl*^{d} are primarily in distal areas of *Nab2*^{ex3} arbors (**Fig. S2C**). Collectively, these genetic and quantitative data argue that Nab2 and PCP components are each individually required for control of ddaC arbors, and that loss of *Nab2* sensitizes ddaC development to the dosage of the core PCP component Vang and the accessory component Appl.

Nab2 is required for proper Vang localization in the central complex of the brain.

The pattern of genetic interactions between *Nab2* and *Vang* alleles across the axon-dendrite axis parallel the tandem mass-spectrometry (MS-MS) detection of reduced levels of Vang protein in *Nab2*^{ex3} fly brains (**Fig. 1**; see also (Corgiat & List et al., 2021)) and altered levels of Vangl2 protein in *Zc3h14* knockout murine brains (Rha et al., 2017). Given these data, we analyzed Vang protein distribution in control and *Nab2*^{ex3} brains using a *P[acman]* genomic fragment containing the complete *Vang* locus with an *eGFP* inserted at the C-terminus of the coding sequence and

retaining endogenous 5' and 3'UTRs (*Vang*$^{eGFP.C}$). The *Vang*$^{eGFP.C}$ transgene rescues *Vang* loss-of-function phenotypes and thus provides a reliable readout of Vang expression patterns (Strutt, Gamage, & Strutt, 2016). Developmentally timed pupal brains were analyzed for Vang-eGFP (anti-GFP) and Bruchpilot (Brp), a presynaptic active zone protein that is highly enriched in the neuropil (Christiansen et al., 2011; Damulewicz & Pyza, 2011; Laissue et al., 1999; Owald et al., 2010). In control brains, Vang-eGFP fluorescence is distributed in cell bodies at the brain cortical surface as well throughout the Brp-positive central complex brain neuropil, which likely represents Vang in axons, dendrites, and glial processes (**Fig. 6A-B, D-E**). In contrast, Vang-eGFP is absent in Brp-positive neuropil regions of *Nab2*^{ex3} brains (**Fig. 6D,F**) but is present in cortical surface cell bodies and other areas of the brain, including the intersection of the lateral anterior optic tubercle and medulla layer (Krzeptowski, Walkowicz, Plonczynska, & Górska-Andrzejak, 2018; Nériec & Desplan, 2016; Tai, Chin, & Chiang, 2021) (**Fig. S4B,E**) and ventral cortical surface adjacent to the antennal lobes (Wolff, Iyer, & Rubin, 2015; Wolff & Rubin, 2018) (**Fig. S4D-G**). Quantification of Vang-eGFP signal intensity in Brp-positive central neuropil ('n' box region in **Fig. 6B**,**E**) and a region of the dorsal cortical surface ('c' box in **Fig. 6B**,**E**) reveals substantial loss of neuropil-localized Vang-eGFP in *Nab2*^{ex3} brains relative to controls, with relatively little effect on the level of cortical surface-localized Vang-eGFP in cell bodies (**Fig 6G-H**). Given that Brp-positive neuropil regions are enriched in axons, dendrites, and glial processes, these localization data indicate that Nab2 is required for Vang-eGFP protein to accumulate in distal neuronal and glial processes, and that the overall drop in Vang protein levels detected in MS-MS analysis of *Nab2*^{ex3} brains (**Fig. 1B**) is accompanied by a change in steady-state Vang-eGFP localization that may deprive distal axon-dendritic compartments of factors required for normal PCP signaling.

***Zc3h14* deficient mice show PCP-like defects in the cochlea.**

Many phenotypes are conserved from *Nab2*^{ex3} flies to *Zc3h14*$^{\Delta 13/\Delta 13}$ mice including defects in working memory (Bienkowski et al., 2017; Kelly et al., 2016; Rha et al., 2017), a subset of proteomic changes in the brain (Corgiat & List et al., 2021; Rha et al., 2017), and defects in dendritic morphology (S. K. Jones et al., 2020). Nab2 has strong genetic interactions with PCP components, as shown here, but also has PCP-like defects in orientation of the fly wing hair bristles, a classic PCP phenotype (Adler, 2012; W. H. Lee et al., 2020; Olofsson & Axelrod, 2014). Given that mammalian ZC3H14 can rescue a variety of phenotypes when expressed in the neurons of *Nab2* mutant *Drosophila* (Bienkowski et al., 2017; Kelly et al., 2016; Pak et al., 2011), we assessed *Zc3h14*$^{\Delta 13/\Delta 13}$ mice for evidence of PCP defects, with a focus on elements of the sensory nervous system. Development of the organ of Corti within the cochlea is well established as a PCP-regulated process in the mouse (Chacon-Heszele & Chen, 2009; C. Jones & Chen, 2007; Qian et al., 2007; Rida & Chen, 2009). The organ of Corti is formed by sensory cells, known as hair cells, that are patterned in one row of inner cells, and three rows of outer cells (Chacon-Heszele & Chen, 2009). Mutations in murine PCP genes result in altered orientation and patterning of these hair cells, due in part to the requirement for PCP in the process of convergent extension (Qian et al., 2007). To test whether a Nab2-PCP functional link is conserved in the mouse cochlea, we analyzed *Zc3h14*$^{\Delta 13/\Delta 13}$ mutant cochlea for PCP-like phenotypes. Phalloidin staining the organ of Corti from E14.5 *Zc3h14*$^{\Delta 13/\Delta 13}$ embryos revealed additional rows of outer hair cells (OHCs) in both the basal and middle regions compared to control (**Fig. 7A**). There are occasional inner hair cells (IHCs) patterning defects in the middle region (**Fig. 7A**). Quantification of extra cells per cochlea confirmed significant OHC patterning defects (**Fig. 7B**) in *Zc3h14*$^{\Delta 13/\Delta 13}$ mice with no significant defects in IHC patterning (**Fig. 7C**). These data suggest that PCP-like phenotypes are

shared from *Nab2*^{ex3} flies to *Zc3h14*$^{\Delta 13/\Delta 13}$ mice and that Nab2 interactions with PCP components may be conserved in ZC3H14.

Discussion

Here, we uncover a role for *Drosophila* Nab2, an evolutionarily conserved RBP with links to human inherited intellectual disability, in control of dendrite branching and projection among ddaC body wall sensory neurons via a mechanism that is linked to the Nab2 role in axon projection and branching via shared dependence on the PCP pathway. Loss of Nab2 increases dendrite branching and projection while overexpression of Nab2 has the opposite effect of restricting dendrite growth. Using proteomic data collected from *Nab2* null developing fly brains (21), we uncover an enrichment for planar cell polarity factors among proteins whose steady-state levels are affected by Nab2 loss, and define a pattern of genetic interactions that are consistent with Nab2 regulating projection and branching of ddaC dendrites and MB axons by a common PCP-linked mechanism. Cell type-specific RNAi indicates that Nab2 acts cell autonomously to guide PCP-dependent axon and dendrite growth, implying a direct link between Nab2 and one or more PCP components within ddaC neurons and MB Kenyon cells. Based on reduction in levels of the PCP component Vang detected in proteomic analysis (Corgiat & List et al., 2021), we analyze the levels and distribution of a fluorescently-tagged Vang protein (Vang-eGFP) in adult brains. The overall drop in Vang-eGFP levels detected by proteomics is also evident in optical sections of whole brains and is unexpectedly accompanied by selective loss of Vang protein in axon/dendrite-enriched neuropil regions relative to brain regions containing nuclei and cell bodies. Analysis of a *Zc3h14* mutant mouse (19) reveals PCP phenotypes within the sensory nervous system, suggesting that the functional link between Nab2/ZC3H14 and the PCP pathway is evolutionarily conserved. Collectively, these data demonstrate that Nab2 is required to regulate axonal and dendritic growth by a PCP-linked mechanism and identify the Nab2 RBP as required for the steady-state accumulation of Vang protein in distal neuronal compartments.

RBPs shape axon and dendrite architecture by modulating steps in post-transcriptional regulation of key neuronal mRNAs, including their export, trafficking, stability, and translation (Cha et al., 2020; Ravanidis et al., 2018; Schieweck et al., 2021). Of note, the analysis presented here shows that effects of Nab2 on dendritic morphology are exaggerated in regions of neurons more distal from the soma as compared to more proximal regions (**Fig. S2A-B**).This enhanced effect of Nab2 loss on distal branching of ddaC arbors implies that Nab2 controls expression of an mRNA or mRNAs that has/have a specific role in more distal dendrites. While neuronal Nab2 protein is primarily nuclear (13, 19), the protein is detected in cytoplasmic messenger ribonucleoprotein (mRNP) granules (Bienkowski et al., 2017) and is linked to translational repression (16), suggesting that cytoplasmic Nab2 could inhibit translation of an mRNA that traffics to distal dendrites and promotes branching/projection. Core PCP proteins localize to membranes at distal tips of some neuronal growth cones (28, 79) and multiple *Drosophila* Wnt/PCP factors act autonomously in ddaC cells to control dendritic growth (e.g., *fz2* in this study and see 28, 55). Considering these observations, Nab2 might inhibit translation of one or more PCP mRNAs, perhaps as it is trafficked for subsequent expression at distal tips of axons and dendrites. The disrupted spatial localization of Vang-eGFP from the axon/dendrite enriched brain neuropil (**Figs. 6** and **S4**) is consistent with this model; the drop in overall Vang protein could be a consequence of precocious translation in the soma and subsequent turnover, or it be indicative of a Nab2 role in promoting its translation. In sum, these data provide first evidence that *Drosophila* Nab2 may aid in trafficking neurodevelopmental factors into distal dendrites, and that this may be coupled with a role in regulating mRNA translation.

Within brain neurons, Nab2 loss depletes Vang-eGFP from neuropil, which in enriched in axons, dendrites, and glial processes and depleted of soma/nuclei (**Figs. 6** and **S4**). One

parsimonious model to explain this observation is that the *Vang-eGFP* mRNA is regulated by Nab2 and that Vang-eGFP depletion from $Nab2^{ex3}$ brain neuropil is thus due to a defect in *Vang-eGFP* mRNA localization and/or local translation. In this model, Nab2 could either bind directly to the *Vang* mRNA or indirectly regulate *Vang* mRNA via an intermediary factor. As the $Vang^{eGFP.C}$ allele retains the single *Vang* intron and intact 5' and 3' UTRs, post-transcriptional regulation of the *Vang* mRNA by Nab2 should be mirrored by effects on endogenous Vang protein, which indeed drops in abundance in *Nab2* null brains. Intriguingly, Vang protein is expressed in core axons of the a and b MB lobes far from their originating Kenyon cell bodies (54) and is required to pattern these distal axons as shown by the disruptive effect of *vang* loss on a/b lobe structure (53, 54). Thus, the interactions between *Nab2* and *Vang* alleles in MB axons may also reflect a specific role for both factors in controlling projection and branching of distal neuronal processes that mirrors their relationship in ddaC dendrites.

The genetic interactions between *Nab2* and alleles of PCP components in MB axons imply a degree of context-dependence to the Nab2-PCP interaction between ddaCs and MBs, and even between the two distinct axon compartments represented by the MB a- and b-lobes. While $Vang^{sbm6}$ heterozygosity enhances $Nab2^{ex3}$ ddaC defects, this allele selectively suppresses $Nab2^{ex3}$ MB a-lobe defects but not b-lobe defects. Given the broad drop in Vang-eGFP levels observed in $Nab2^{ex3}$ brains (**Fig. 6**), and the requirement for *Vang* in Kenyon cells (KCs) for normal development of both the a and b-lobes (Ng, 2012; Shimizu et al., 2011), the a-lobe-specific *Nab2-Vang* genetic interaction could be regarded as unexpected. However, very similar a-lobe-specific genetic interactions occur between *Nab2* and alleles of two other RBP genes, *fmr1* and *Atx2* (16, 82), implying that Nab2 has distinct interacting pathways in these two different axonal sub-compartments. As noted, suppression of $Nab2^{ex3}$ MB defects by $Vang^{stbm6}$ is the inverse of how

this same allele affects *Nab2*^{ex3} ddaC phenotypes. The relationship could arise if PCP signals are exchanged between MB axons and the surrounding neuro-substrate, which could invert a relationship between Nab2 in Kenyon cells and Wnt/PCP signals emanating from surrounding cells. For example, the receptor *derailed* is expressed in the dorsomedial lineage neuropil and binds Wnt5 for presentation to repulsive *derailed2* receptors on a-lobe axons, thus non-autonomously guiding a-lobe projection (M. Montcouquiol, Jones, & Sans, 2008; Mireille Montcouquiol, Crenshaw, & Kelley, 2006). In addition, the projection paths of individual *vang*^{stbm6} mutant a and b-axon tracts can be rescued by adjacent cells with normal Vang level, indicating that Wnt/PCP control of a and b-axon branching is not strictly cell-autonomous (Ng, 2012; Shimizu et al., 2011). These complex signaling relationships within MB axons, and the potential for extra-cellular Wnt/PCP guidance cues emanating from surrounding dorsomedial cells, are both potential explanations for context-specific genetic interactions between *Nab2* and *Vang* in ddaCs and Kenyon cells. In contrast to *Vang* alleles, partial loss of Appl (*Appl*^{d}) consistently suppresses both *Nab2*^{ex3} dendritic and axonal phenotypes (**Fig. S3**) which parallels the increase in Appl protein detected in brain proteomics in *Nab2* mutant brains (**Fig. 1B, Table S1**). Appl acts as a downstream neuronal-specific effector of the PCP pathway (Liu et al., 2021; Soldano et al., 2013) and elevated Appl protein in response to Nab2 loss could be an indirect consequence of altered core PCP pathway activity or evidence of direct regulation of the *Appl* transcript. These differing interactions illustrate the complexity of RBP function across a neuron with specific interactions affecting sub-cellular compartments in different ways.

An additional question that arises from analysis of Nab2-PCP interactions in the MBs is why *Nab2* mutant α-lobe defects are rescued by *Vang*, *Appl*, and *dsh* alleles to a greater degree than are b-lobe defects? As noted above, alleles of the Nab2-interacting factors *fmr1* and *Atx2* also

specifically suppress *Nab2*^{ex3} α-axon defects but not b-axon defects (Bienkowski et al., 2017; Rounds et al., 2021), implying that these gene may define a Nab2-dependent α-lobe Nab2-Fmr1-Atx2 regulatory network that also includes PCP factors. Indeed, *fmr1* also shares some functional features with *Nab2* in MBs and ddaCs: Fmr1 controls both α- and b-lobe development (83) and limits ddaC dendrite development, in part through an interaction the mRNA encoding the PCP-effector Rac1 (9, 84). Significantly, the normal development of a and β-axons has been proposed to rely on a lobe-specific PCP mechanism involving the formin DAAM (Dsh associated activator of morphogenesis) interacting with Wg/Wnt receptor Frizzled (Fz) in the α-lobes and Vang in the β-lobes (Gombos et al., 2015). A similar type of mechanism could occur for the Nab2-PCP interaction, with Nab2 either regulating different PCP components in a vs. b lobes, or regulating components that themselves have lobe-specific roles e.g., DAAM or the Derailed-Wnt5 receptor ligand pair (Reynaud et al., 2015). In sum, it seems likely that future studies will identify other mechanisms and pathways through which Nab2 regulates axon-dendrite development in opposition to or cooperation with the Wnt/PCP pathway, including for example mechanisms involving the Fmr1 and Atx2 RBPs interacting with Nab2 to regulate expression of co-bound RNAs.

We extended the data from *Drosophila* to mouse by taking advantage of a mouse model lacking functional ZC3H14/Nab2 protein (19). This analysis reveals that *zc3h14* mutant mice show phenotypes in orientation of the hair cell stereociliary bundles within the cochlea that are similar to multiple PCP mutants, including *Vangl2* (85). Future studies could employ mouse models to explore whether genetic interactions identified in Drosophila extend to mammals, but this conserved PCP phenotype argues for a conserved link between ZC3H14/Nab2 and the PCP pathway.

In aggregate, these data reveal Nab2 interactions with the PCP pathway and provide the first evidence that Nab2 is required for dendritic development. These interactions between Nab2 and PCP factors in control of dendritic complexity and MB axon projection appear to be cell-autonomous and, at least in ddaC arbors, more dramatic in distal projections. Changes in expression level and localization of Vang protein in fly brains lacking Nab2 highlight the *Vang* mRNA as a potential target of post-transcriptional control by Nab2 both in axons and dendrites. Given that loss of the Nab2 ortholog in mice, *Zc3h14*, also alters levels of the Vangl2 PCP protein in the adult hippocampus, and that mutations in PCP genes including *Vangl2* are linked to intellectual disabilities, severe neural tube closure defects, and microencephaly in humans (Hilal et al., 2017; M. Wang, Marco, Capra, & Kibar, 2019) dysregulation of the levels and localization of PCP components in neurons is one potential mechanism to explain axonal and dendritic phenotypes in *Zc3h14* mutant mice (S. K. Jones et al., 2020) and cognitive defects in human patients lacking ZC3H14 (Pak et al., 2011).

Experimental Procedures:

***Drosophila* genetics.** All crosses were maintained in humidified incubators at 25ºC with 12hr light-dark cycles unless otherwise noted. The *Nab2^{ex3}* loss of function mutant was described previously (Pak et al., 2011). Alleles and transgenes: *Nab2^{EP3716}* (referred to as "*Nab2 oe*"; Bloomington (BL) #17159), *UAS-Nab2^{RNAi}* (Vienna *Drosophila* Research Center (VDRC), #27487), *UAS-fz2^{RNAi}* (BL #27568), *appl^{d}* (BL #43632), *dsh^{1}* (BL #5298), *Vang^{stbm-6}* (BL #6918), *pk$^{pk-sple-13}$* (BL #41790), *Vang$^{EGFP.C}$* ('*Vang-eGFP')* (gift of D. Strutt), *ppk-Gal4;UAS-mCD8::GFP* (gift of D. Cox), and *w^{1118}* ('*control*').

***Drosophila* brain dissection, immunohistochemistry, visualization, and statistical analysis.** Brain dissections were performed essentially as previously described (Kelly et al., 2016). Briefly, 48-72 hours after puparium formation (APF) brains were dissected in PBS (1xPBS) at 4ºC, fixed in 4% paraformaldehyde at RT, washed 3x in PBS, and then permeabilized in 0.3% PBS-T (1xPBS, 0.3% TritonX-100). Following blocking for 1hr (0.1% PBS-T, 5% normal goat serum), brains were stained o/n in block+primary antibodies. After 5x washes in PBS-T (1xPBS, 0.3% TritonX-100), brains were incubated in block for 1hr, moved into block+secondary antibody for 3hrs, then washed 5x in PBS-T and mounted in Vectashield (Vector Labs). Antibodies used: anti-FasII 1D4 (Developmental Studies Hybridoma Bank) at 1:50 dilution, anti-GFP polyclonal (ThermoFisher Catalog# A-11122) at a 1:200 dilution, and anti-nc82 (Developmental Studies Hybridoma Bank) at 1:50 dilution. Whole brain images were captured on a Nikon AR1 HD25 confocal microscope using NIS-Elements C Imaging software v5.20.01, and maximum intensity projections were generated in ImageJ Fiji. Mushroom body morphological defects were called as α-lobe thinning or missing and β-lobe fusion or missing for *control*, *Nab2^{ex3}*, and control and experimental PCP alleles (e.g., *Vang^{stbm-6}*/+, *appl^{d}*/+, and *dsh^{1}*/+ paired with *control* or *Nab2^{ex3}*).

Statistical analyses for MB phenotypes and plotting performed using GraphPad Prism8™. Significance determined using student's t-test or ANOVA as indicated in figure legends. Error bars representing standard deviation. Significance scores indicated are *$p \leq 0.05$, **$p \leq 0.01$, and ***$p \leq 0.001$. Vang-eGFP fluorescence intensity was quantified using two isolated regions of interest (ROI). One ROI located at right hemisphere dorsal cortical surface approximately near α-lobe (referred to as cortical surface ROI) and a second ROI located at left hemisphere central complex neuropil approximately near β-lobe and ellipsoid body (referred to as central neuropil ROI). The fluorescence intensity of each ROI was measured in *control* (n=9) and *Nab2*^{ex3} (n=9) brains. Significance determined using student's t-test; significances scores indicated by * = $p<0.05$.

***Drosophila* neuron live imaging confocal microscopy, neuronal reconstruction, data analyses, and statistical analysis.** Live imaging of class IV dorsal dendritic arbor C (ddaC) neurons was performed essentially as described in Prasad et al. (2013) (see text for detailed explanation) . Briefly, 3rd instar *ppk-Gal4,mCD8::GFP* labelled larvae were mounted in 1:5 (v/v) diethyl ether:halocarbon oil under an imaging bridge of two 22x22mm glass coverslips topped with a 22x50mm glass coverslip. ddaC images were captured on an Olympus FV 1000 BX61WI upright microscope using Olympus Fluoview software v4.2. Maximum intensity projections were generated with ImageJ Fiji. Neuronal reconstruction was performed as in (65; see text for detailed explanation) with the TREES toolbox. MathWorks Matlab R2010a v7.10.0.499 (Natick, MA) was used to process 2D stacks with local brightness thresholding, skeletonization, and sparsening to leave carrier points (Cuntz et al., 2010). Dendritic roots were defined at the soma and used to create synthetic dendritic arbors. Reconstruction parameters were equivalent across neurons. Various morphological metrics were obtained using the TREES toolbox including: Sholl analysis, total

cable length, maximum path length, number of branch points, mean path/Euclidean distance, maximum branch order, mean branch order, mean branch angle, mean path length, mean branch order, field height/width, center of mass x, and center of mass y. These metrics were extracted in batch processing using in-house custom scripts and exported into RStudio v1.1.453 (Vienna, Austria), where quantification was visualized using other in-house custom scripts. Statistical analyses for ddaC phenotypes and plotting were performed using RStudio and Matlab. Balloon plots showing phenotypic data generated using either ddaC measurements generated in Matlab or MB defect counts. Balloon plots generated using RStudio v1.1.453 ggpubr v0.2 (Alboukadel, 2018; Team, 2018).

Global proteomics

MS/MS-LC data previously described (see full text for more detail) (Corgiat & List et al., 2021). Briefly, ten biological replicates of 24 hr apf control (w^{1118}) or $Nab2^{ex3}$ pupal brains (60 brains per replicate) were lysed in urea buffer (8 M urea, 100 mM NaHPO4, pH 8.5) with HALT protease and phosphatase inhibitor (Pierce) and processed at the Emory Proteomics Core. Separate samples were prepared for male and female brains. Label-free quantification analysis was adapted from a previously published procedure (Seyfried et al., 2017). Data was analyzed using MaxQuant v1.5.2.8 with Thermo Foundation 2.0 for RAW file reading capability. Spectra were searched using the search engine Andromeda and integrated into MaxQuant against the *Drosophila melanogaster* Uniprot database (43,836 target sequences). Analyses presented here used RStudio v1.1.453 (Team, 2018), custom in-house scripts, and the following packages: ggpubr v0.2 (Alboukadel, 2018), cluster v2.1.0 (Maechler et al., 2016), and GOplot v1.0.2 (W. Walter et al., 2015), to examine ‘planar cell polarity’ annotated proteins. Gene ontology analyses were

performed using FlyEnrichr (FlyEnrichr:amp.pharm.mssm.edu/FlyEnrichr/) (E. Chen et al., 2013; Kuleshov et al., 2019, 2016), a *Drosophila* specific gene ontology enrichment analysis package.

Mouse strain, animal care, and histologic analysis of inner ear tissues

Animals used are *Zc3h14*$^{\Delta ex13/\Delta ex13}$ mouse line (referred to as *Zc3h14*$^{\Delta 13/\Delta 13}$ or Δ*13/*Δ*13*) (Rha et al., 2017). *Zc3h14*$^{\Delta ex13/+}$ were mated to generate *Zc3h14*$^{\Delta ex13/\Delta ex13}$ for at least four generations. *C*ontrol *Zc3h14*$^{+/+}$ were maintained in the colony as control counterparts from heterozygous *Zc3h14*$^{\Delta ex13/+}$ breeders. Generations F4-F8 of *Zc3h14*$^{\Delta ex13/\Delta ex13}$ and *Zc3h14*$^{+/+}$ were used for experiments. All procedures involving mice were done in accordance with the HIH guideline for use and care of live animals and were approved by the Emory University Institutional Animal Care and Use Committee. Inner ear dissection, sectioning, and immunostaining were described previously (Radde-Gallwitz et al., 2004). Cochlea dissected from E14.5 embryos of *Zc3h14*$^{\Delta ex13/\Delta ex13}$ and *Zc3h14*$^{+/+}$. For PCP analysis, hole mount preparation of the organs of Corti were prepared and stained with FITC-conjugated phalloidin to label the stereocilia (Qian et al., 2007; J. Wang et al., 2005). Samples were analyzed and imaged using Zeiss LSM510. Cochlear morphological defects were called as extra based on a three OHC layers and one ICH layer separated by pillar cell region. Significance determined using student's t-test; significances scores indicated by *$p<0.05$

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Figures

Figure 3-1

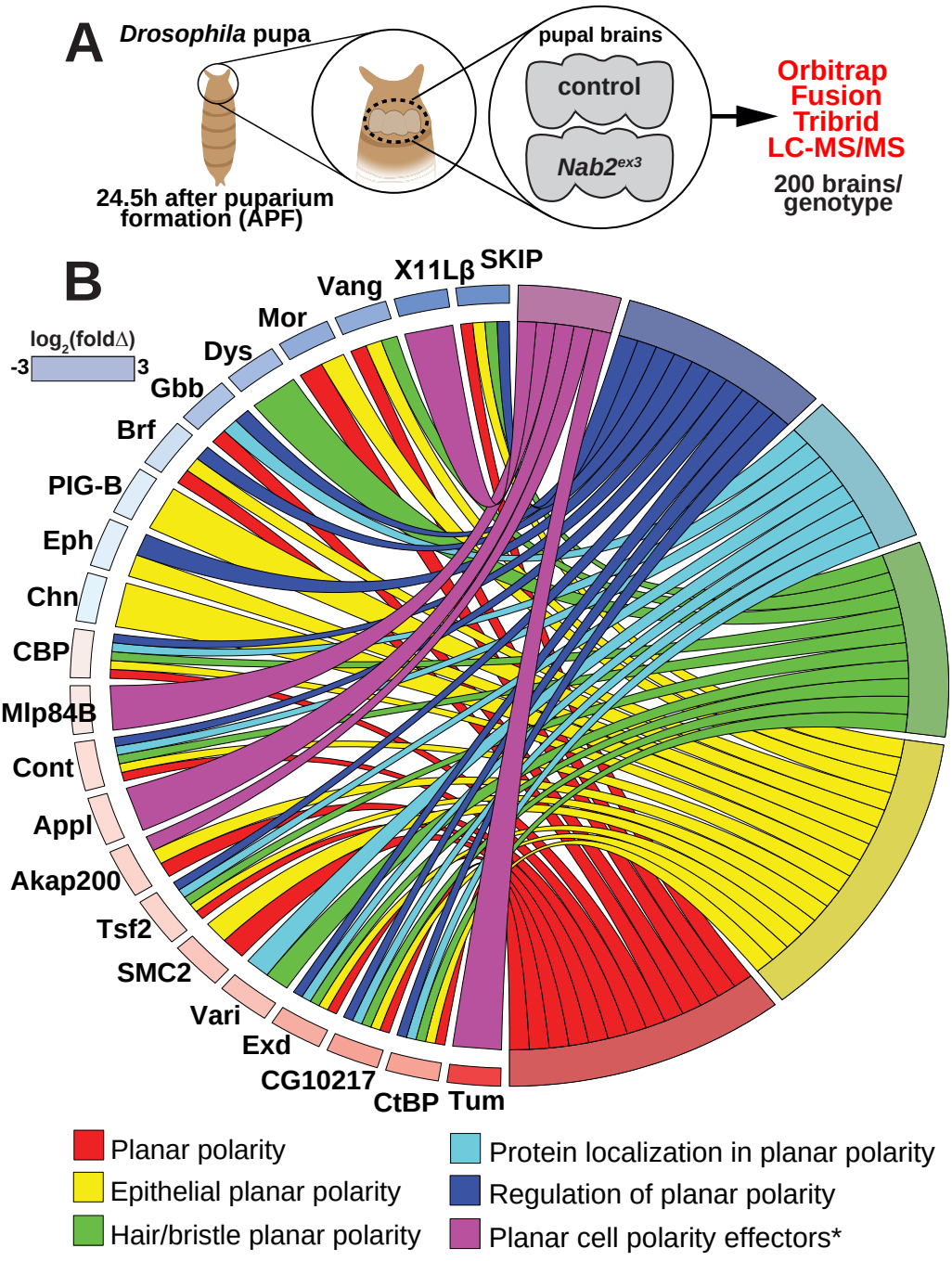
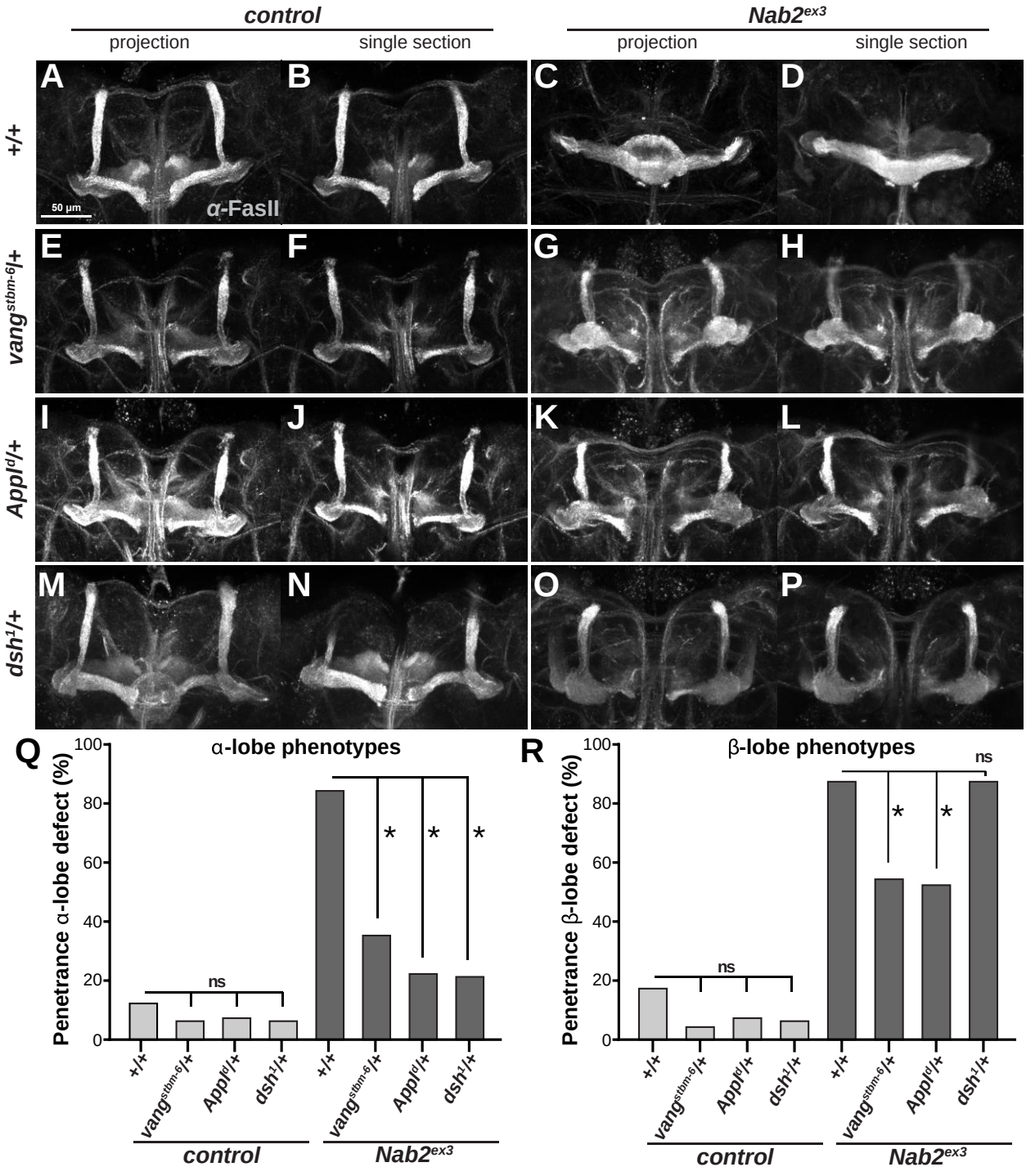


Figure 3-1: Nab2 loss alters levels of planar cell polarity pathway proteins in the *Drosophila* brain.

(A) Schematic summary of quantitative proteomic analysis of *Nab2*^{ex3} pupal brains dissected from *control* or *Nab2*^{ex3} pupa 24.5 hours after puparium formation. Ten samples per genotype, each composed of 20 brains (i.e., 200 *control* brains and 200 *Nab2*^{ex3} brains) were processed and analyzed using an Orbitrap Fusion Tribrid Mass Spectrometer (Corgiat & List et al., 2021) and data was quantified using MaxQuant against the *Drosophila melanogaster* Uniprot database. **(B)** Chord plot analysis of protein abundance changes in *Nab2*^{ex3} relative to *control* for selected color-coded planar cell polarity ontology terms. Heat map indicates fold-change in abundance of each protein ($\log_2$(*Nab2*^{ex3}/*control*)); blue=decreased, red=increased.

Figure 3-2


control
Nab2ex3
projection
single section
projection
single section
A
B
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+/+
50 µm
α-FasII
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vangstbm-6/+
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K
L
Appld/+
M
N
O
P
dsh1/+
Q
α-lobe phenotypes
Penetrance α-lobe defect (%)
100
80
60
40
20
0
ns
*
*
*
+/+
vangstbm-6/+
Appld/+
dsh1/+
+/+
vangstbm-6/+
Appld/+
dsh1/+
control
Nab2ex3
R
β-lobe phenotypes
Penetrance β-lobe defect (%)
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*
+/+
vangstbm-6/+
Appld/+
dsh1/+
+/+
vangstbm-6/+
Appld/+
dsh1/+
control
Nab2ex3

Figure 3-2: Planar cell polarity components dominantly modify Nab2 axonal phenotypes.

Paired maximum intensity Z-stack projections images and single transverse sections of anti-Fasciclin II (FasII) stained 48-72hr pupal brains from **(A-B)** *control* or (**C-D**) $Nab2^{ex3}$ animals, or each of these genotypes combined with (**E-H**) $Vang^{stbm6}$/+, (**I-L**) $Appl^{d}$/+, or (**M-P**) dsh^{1}/+. Frequencies of **(Q)** α-lobe or **(R)** β-lobe structure defects in these genotypes using the scoring system as described in Experimental Procedures. $Nab2^{ex3}$ brains show high penetrance thinning/loss of α-lobes (85%) and fusion/missing of β-lobe (88%) which are dominantly suppressed by $Vang^{stbm-6}$ (49% α-lobe and 33% β-lobe defects), $Appl^{d}$ (62% α-lobe and 35% β-lobe defects). dsh^{1} selectively suppress $Nab2^{ex3}$ α-lobe defects to 63%.

Figure 3-3

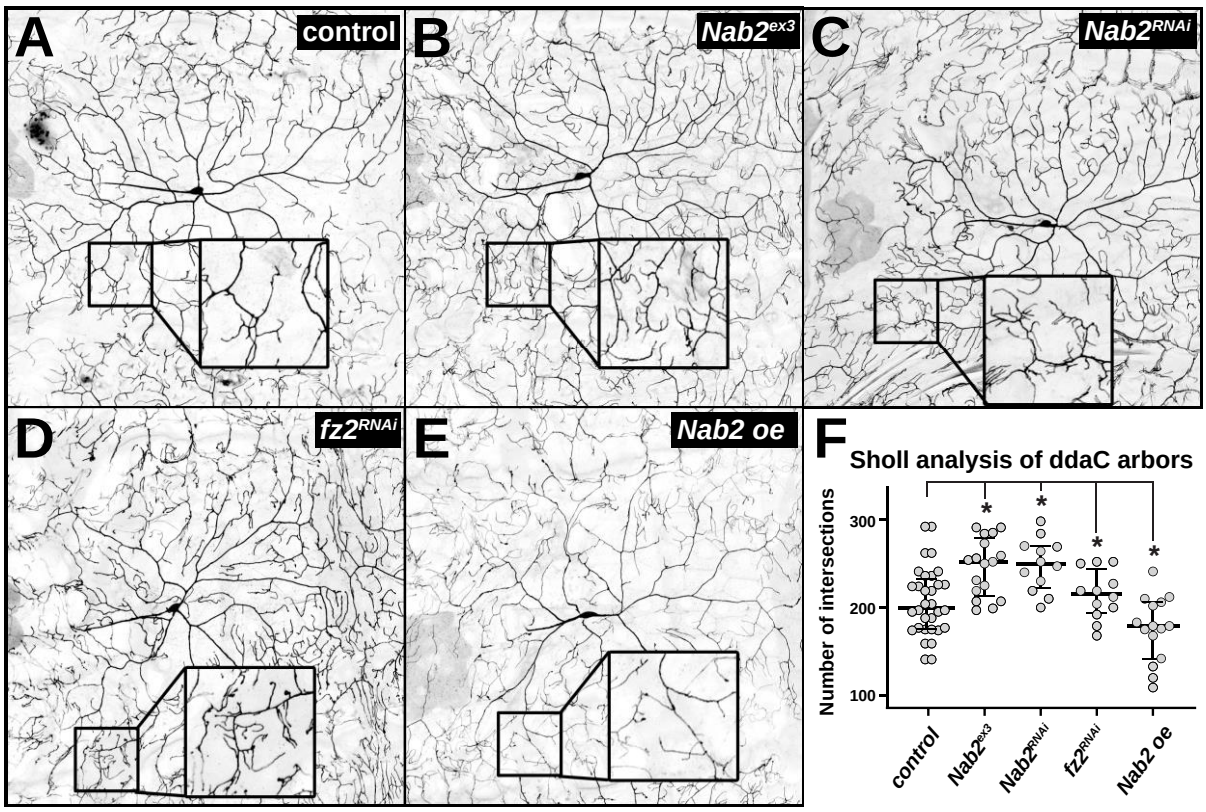
A
control
B
Nab2ex3
C
Nab2RNAi
D
fz2RNAi
E
Nab2 oe
F
Sholl analysis of ddaC arbors
Number of intersections
300
200
100
control
Nab2ex3
Nab2RNAi
fz2RNAi
Nab2 oe

Figure 3-3: Nab2 is required for proper dendritic development.

Inverted intensity images of *Drosophila* class IV dorsal dendritic arbor C (ddaC) neurons from (**A**) *pickpocket (ppk)-Gal4,UAS-GFP*, (**B**) $Nab2^{ex3}$, (**C**) *ppk-Gal4,UAS-GFP,*$Nab2^{RNAi}$, (**D**) *ppk-Gal4,UAS-GFP,*$fz2^{RNAi}$, and (**E**) *ppk-Gal4,UAS-GFP,*$Nab2^{oe}$ L3 larvae. Inset black boxes show high magnification views of dendritic arbors. (**F)** Quantification of branching complexity by Sholl analysis of total intersections across dendritic arbor; bars represent median and upper/lower quartile, * $p<0.05$. Median Sholl intersection values are 200 in *ppk-Gal4,UAS-GFP* (n=32), 252 in $Nab2^{ex3}$ (n=17), 250 in *ppk-Gal4,UAS-GFP,*$Nab2^{RNAi}$ (n=12), 216 in *ppk-Gal4,UAS-*fz^{RNAi} (n=12), and 179 in *ppk-Gal4,UAS-*$Nab2^{oe}$ (n=15).

Figure 3-4

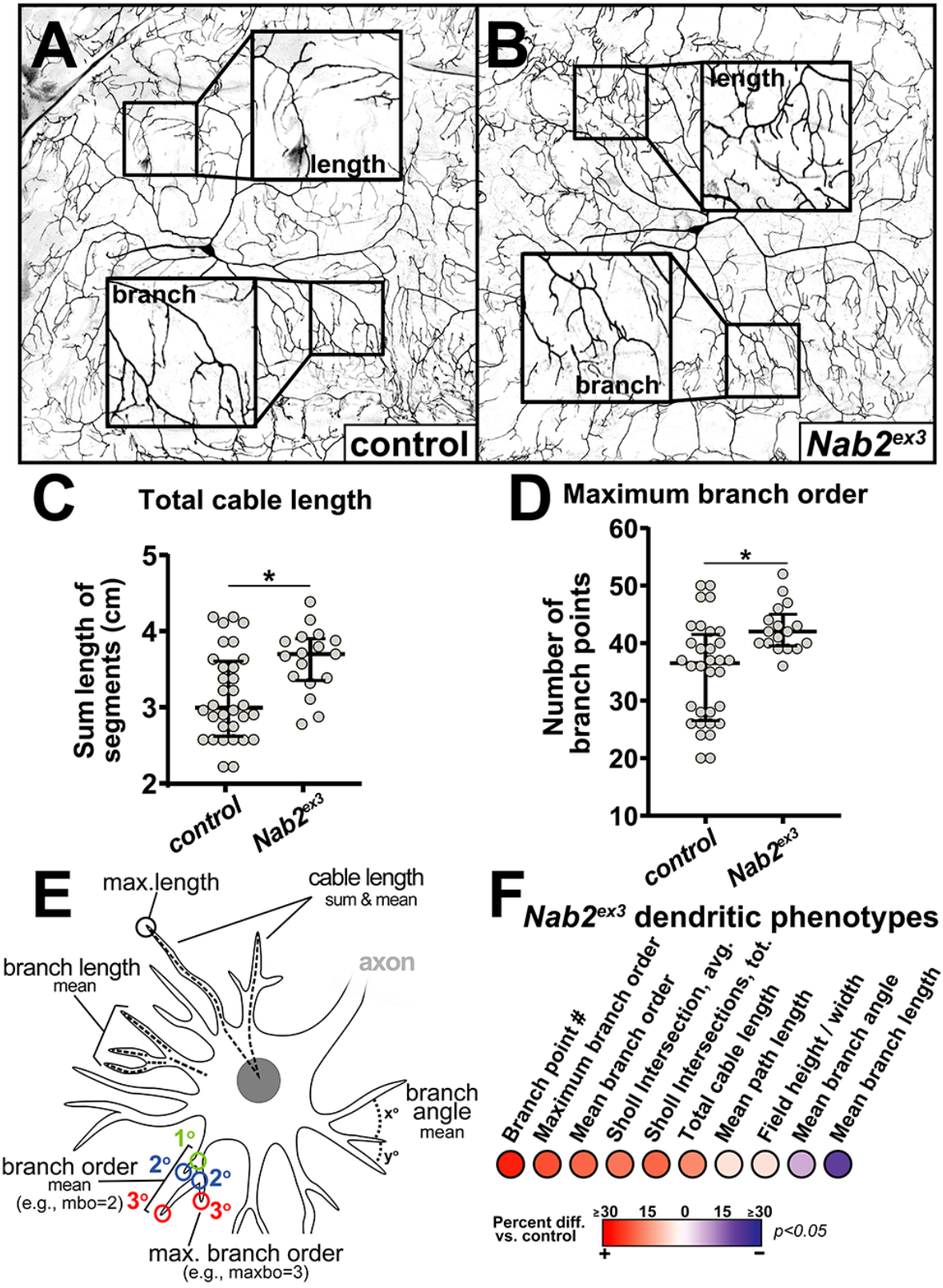
A
length
branch
control
B
length
branch
Nab2ex3
C
Total cable length
Sum length of segments (cm)
5
4
3
2
*
control
Nab2ex3
D
Maximum branch order
Number of branch points
60
50
40
30
20
10
*
control
Nab2ex3
E
max.length
cable length
sum & mean
branch length
mean
axon
branch angle
mean
x°
y°
1°
branch order
mean
(e.g., mbo=2)
2°
2°
3°
3°
max. branch order
(e.g., maxbo=3)
F
Nab2ex3 dendritic phenotypes
Branch point #
Maximum branch order
Mean branch order
Sholl Intersection, avg.
Sholl Intersections, tot.
Total cable length
Mean path length
Field height / width
Mean branch angle
Mean branch length
Percent diff. vs. control
≥30
15
0
15
≥30
+
–
p<0.05

Figure 3-4: Nab2 restricts dendritic branching and projection.

Inverted intensity images of *Drosophila* class IV dorsal dendritic arbor C (ddaC) neurons from (**A**) *control* +/+, (**B**) *Nab2*^{ex3} larvae. Inset black boxes show high magnification views of dendritic arbors. Quantification of **(C)** total cable length and **(D)** maximum branch order for *control* (n=32) and *Nab2*^{ex3} (n=17); bars represent median and upper/lower quartile, * $p<0.05$. **(E)** Schematic depicting measured dendritic parameters using Matlab TREES toolbox and custom scripts (Cuntz et al., 2010). **(F)** Balloon plot depicting ten measurements of the *Nab2*^{ex3} dendritic arbor. Heat map shows change percent changes in *Nab2*^{ex3} vs *control*.

Figure 3-5

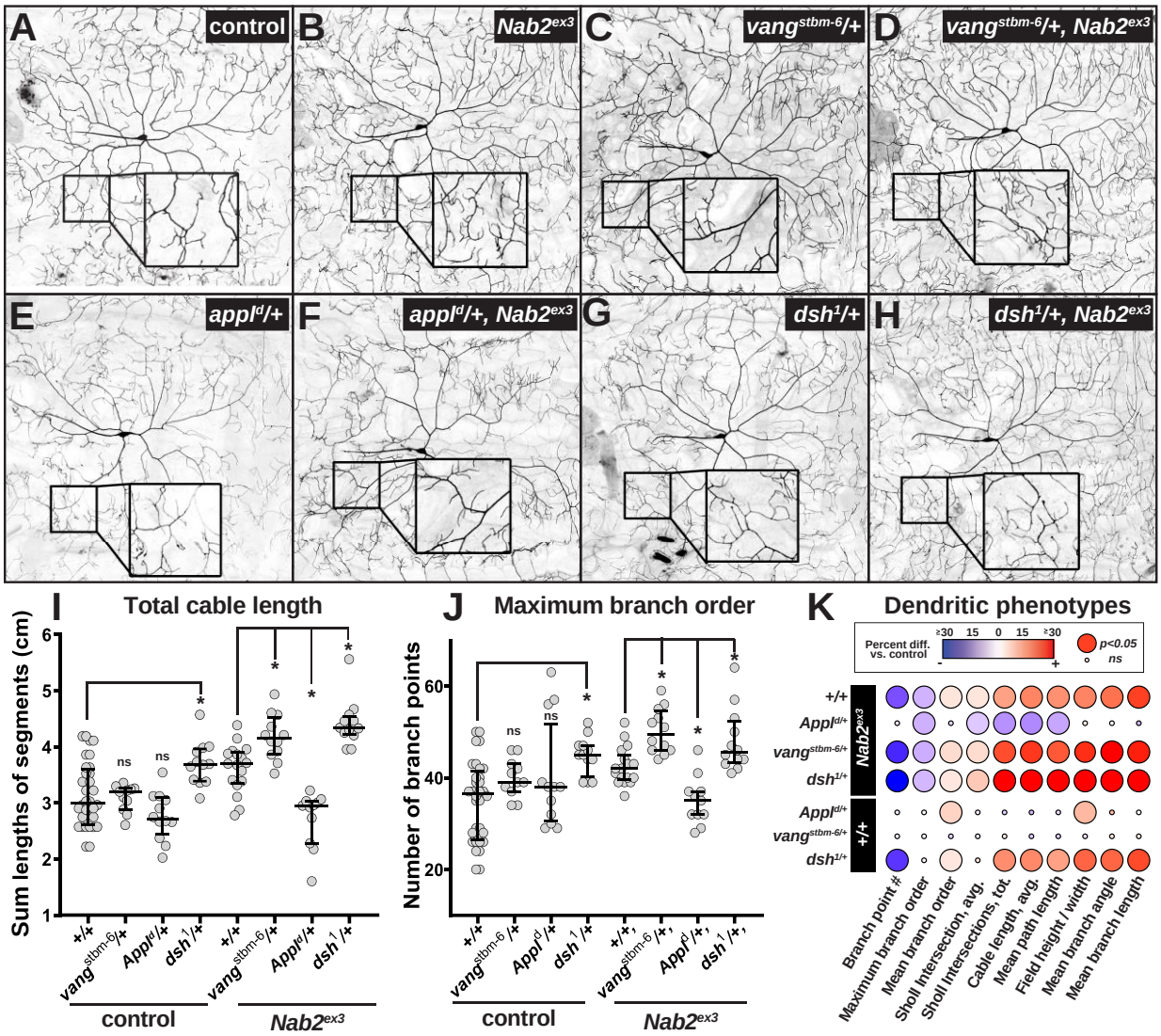
A control
B Nab2ex3
C vangstbm-6/+
D vangstbm-6/+, Nab2ex3
E applD/+
F applD/+, Nab2ex3
G dsh1/+
H dsh1/+, Nab2ex3
I Total cable length
Sum lengths of segments (cm)
J Maximum branch order
Number of branch points
K Dendritic phenotypes
Percent diff. vs. control
p<0.05
ns
control
Nab2ex3
Branch point #
Maximum branch order
Mean branch order
Sholl Intersection, avg.
Sholl Intersections, tot.
Cable length, avg.
Mean path length
Field height / width
Mean branch angle
Mean branch length

Figure 3-5: Planar cell polarity components dominantly modify Nab2 dendritic phenotypes. Inverted intensity images of *Drosophila* class IV dorsal dendritic arbor C (ddaC) neurons from (**A**) *control* +/+ or **(B)** $Nab2^{ex3}$ larvae alone, or in combination with **(C-D)** $Vang^{stbm6}$/+, **(E-F)** $Appl^{d}$/+, **(G-H)** dsh^{1}/+. Inset black boxes show high magnification views of dendritic arbors. **(I-J)** Quantification of **(I)** total cable length and **(J)** maximum branch order in the indicated genotypes; errors bars represent median and upper/lower quartile, *$p<0.05$. **(K)** Balloon plot analysis of 10 arbor parameters in the indicated genotypes. Heat map shows change percent changes in $Nab2^{ex3}$ vs *control*. Significance depicted by balloon size (large balloon = $p<0.05$, small balloon = ns).

Figure 3-6

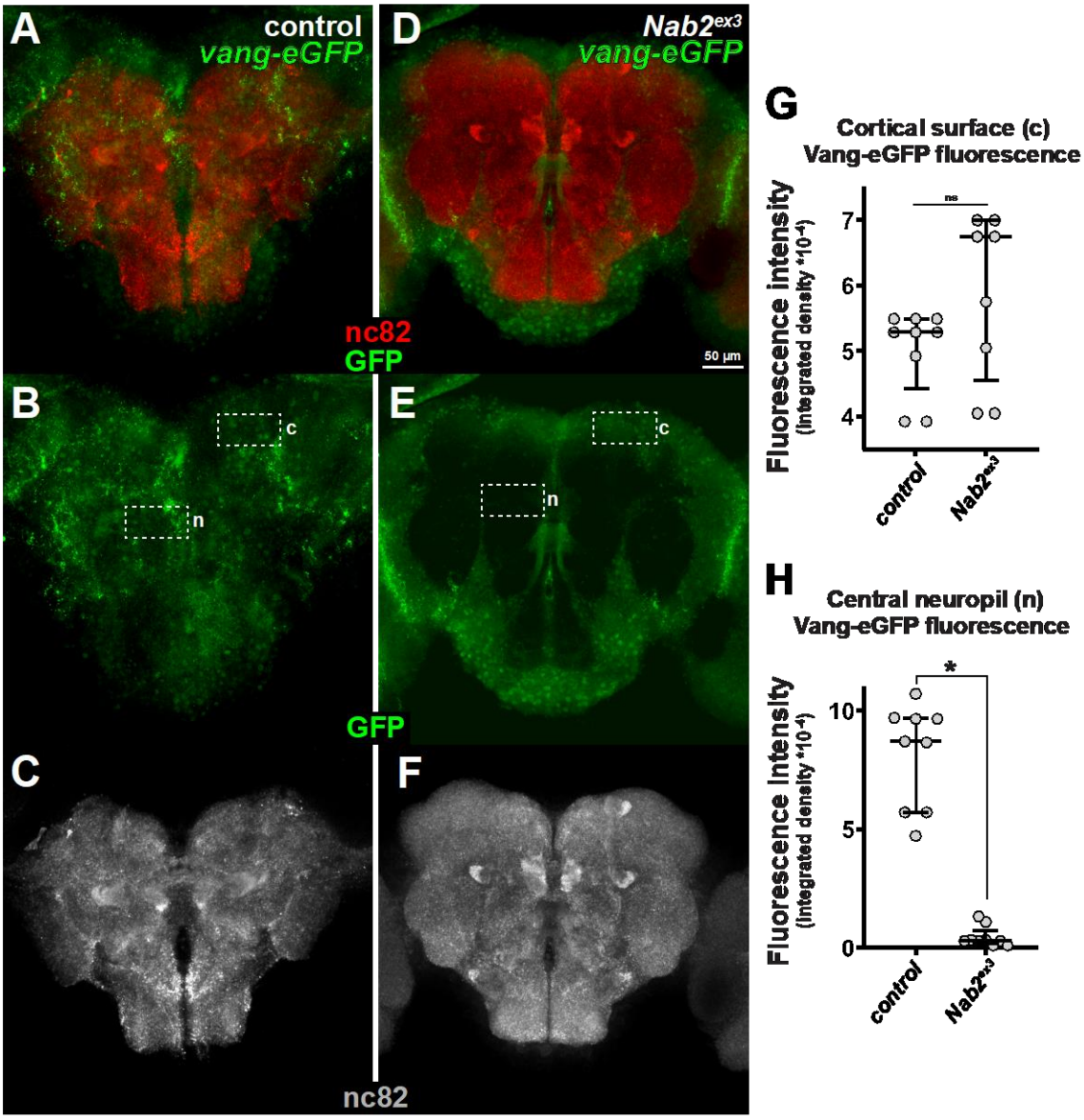
A
control
vang-eGFP
D
Nab2ex3
vang-eGFP
nc82
GFP
50 µm
B
c
n
E
c
n
GFP
C
F
nc82
G
Cortical surface (c)
Vang-eGFP fluorescence
Fluorescence intensity
(Integrated density *10-4)
ns
7
6
5
4
control
Nab2ex3
H
Central neuropil (n)
Vang-eGFP fluorescence
Fluorescence Intensity
(Integrated density *10-4)
*
10
5
0
control
Nab2ex3

Figure 3-6: Nab2 is required for proper Vang localization in the central complex of the brain.

Visualization of brains from 48-72hr $vang^{EGFP.C}$ (**A-C**) and $Nab2^{ex3};vang^{EGFP.C}$ (**D-F**) pupae co-stained with anti-GFP (green) and the nc82 mAb (red) to detect Vang-eGFP and Brp, which marks presynaptic actives zones. Dashed boxes indicate regions used for quantifying fluorescence in **c** (cortical surface) and **n** (central neuropil) regions. (**G-H**) Quantification of Vang-eGFP fluorescence intensity in the (**G**) cortical surface and (**H**) central neuropil regions of $vang^{EGFP.C}$ (n=9) and $Nab2^{ex3};vang^{EGFP.C}$ (n=9) pupae. Bars represent median and upper/lower quartile, * $p<0.05$.

.

Figure 3-7

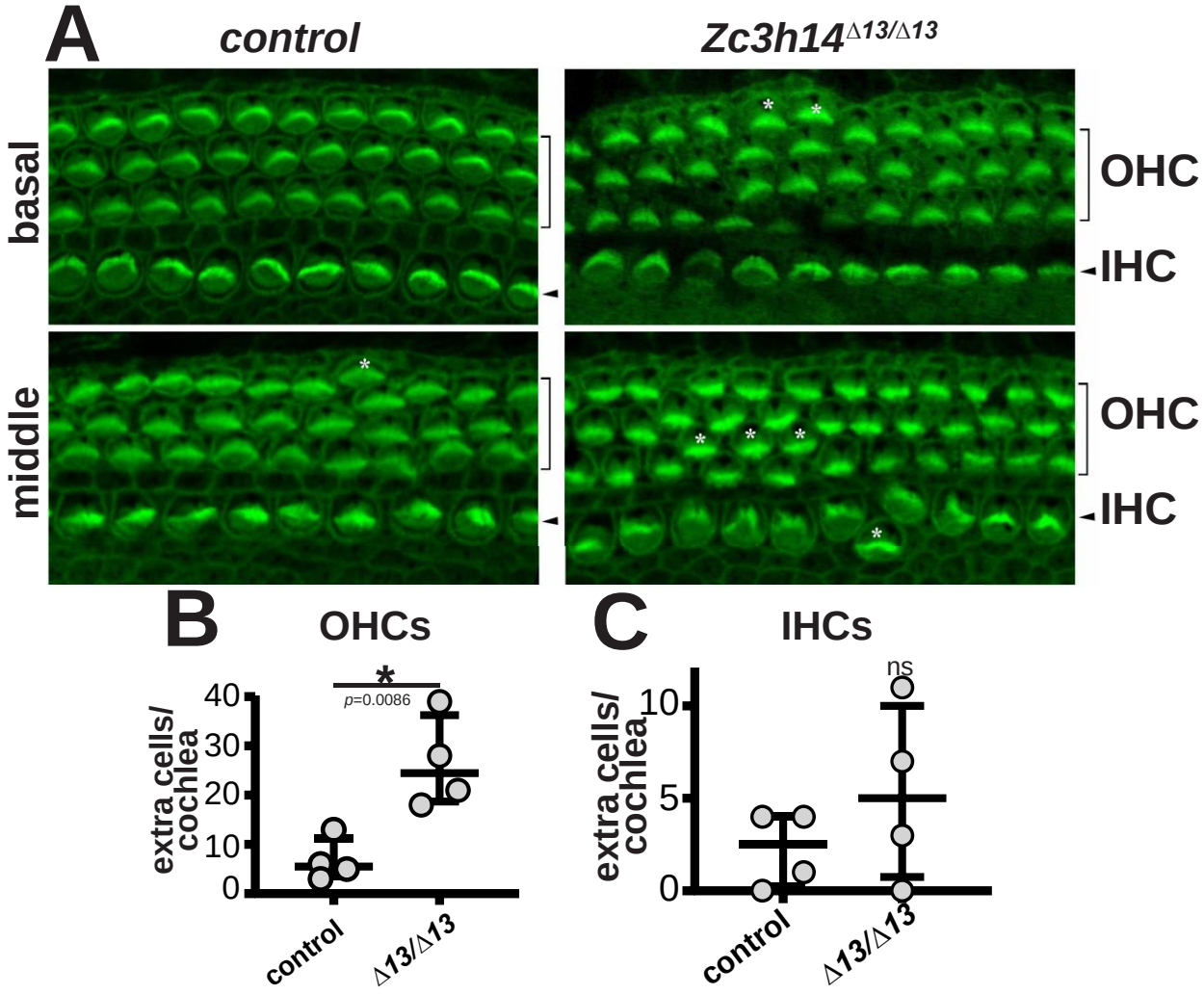
A
control
Zc3h14Δ13/Δ13
basal
middle
OHC
IHC
OHC
IHC
B
OHCs
extra cells/ cochlea
40
30
20
10
0
*
p=0.0086
control
Δ13/Δ13
C
IHCs
ns
extra cells/ cochlea
10
5
0
control
Δ13/Δ13

Figure 3-7: *Zc3h14*$^{\Delta 13/\Delta 13}$ mice have PCP-like cochlear defects.

(**A**) The cochlea from *control* and *Zc3h14*$^{\Delta 13/\Delta 13}$ E14.5 embryos showing basal and middle regions. Stereocilia are visualized by phalloidin staining. Brackets indicate outer hair cells (OHC) and arrowheads indicate inner hair cells (IHC). Staining reveals normal orientation and hair cell layers for *control* but extra OHC and some orientation defects around the pillar cell region for *Zc3h14*$^{\Delta 13/\Delta 13}$. (**B-C**) Quantification of extra cells per cochlea in the (**B**) OHC and (**C**) IHC; bars represent median and upper/lower quartile, * indicates $p<0.05$. *control* $n=4$, *Zc3h14*$^{\Delta 13/\Delta 13}$ $n=4$.

Figure 3-S1

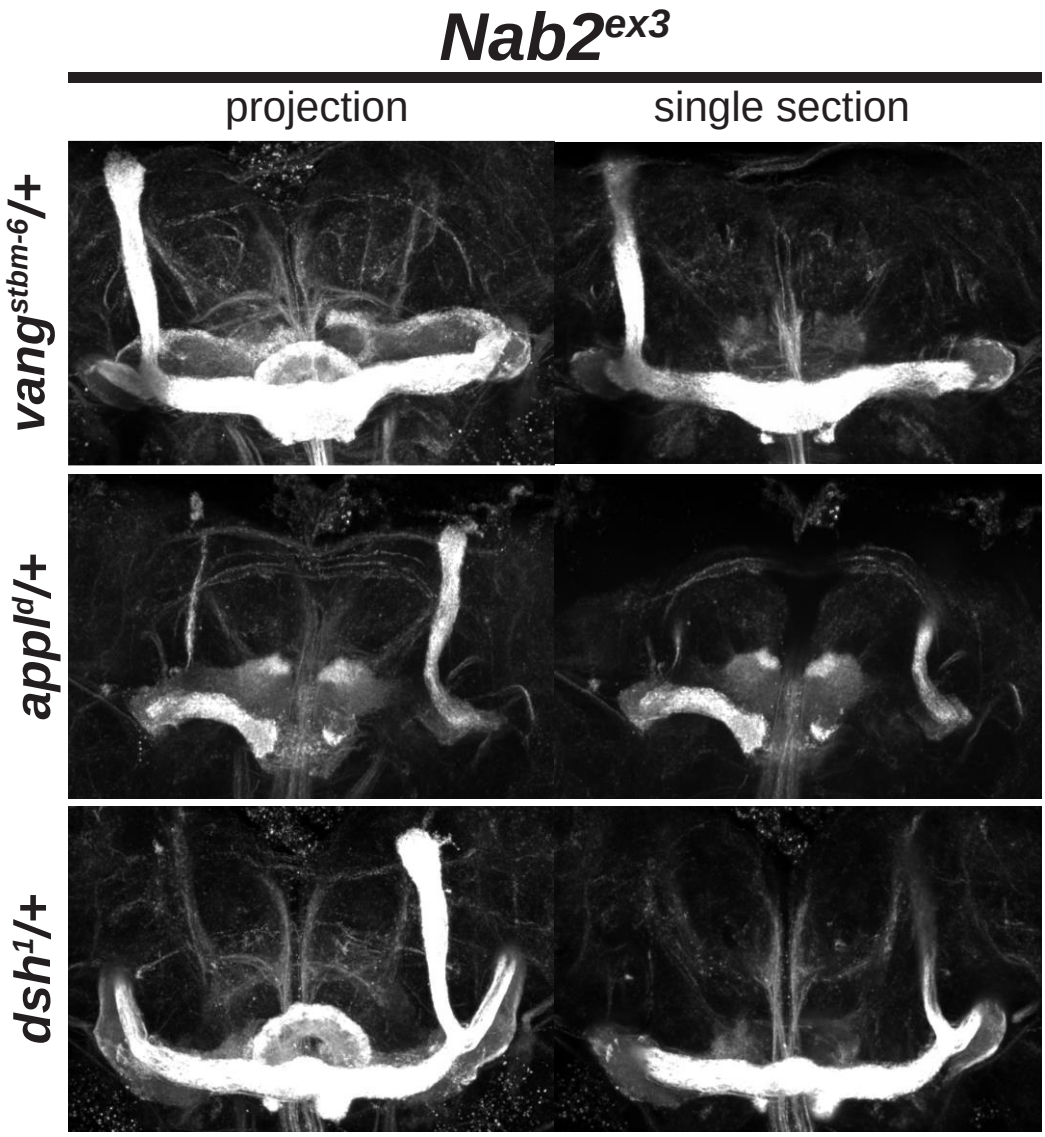

Figure 3-S1: Variance in mushroom body morphological defects with PCP modifying alleles.

Confocal images of Fasciclin II (FasII) antibody staining of 48-72hr after pupal formation brain show maximum intensity Z-stack projections (projection) which show full mushroom body structure and single transverse plan section (single section) which display midline crossing of β-lobe axons. Dominant modification of $Nab2^{ex3}$ by single copy alleles of $Vang^{stbm-6}$, $Appl^{d}$, and dsh^{1} have variable presentation. Typical phenotypes are shown in Figure 2 G,H,K,L,O,P. Alternative phenotypes are shown here.

Figure 3-S2

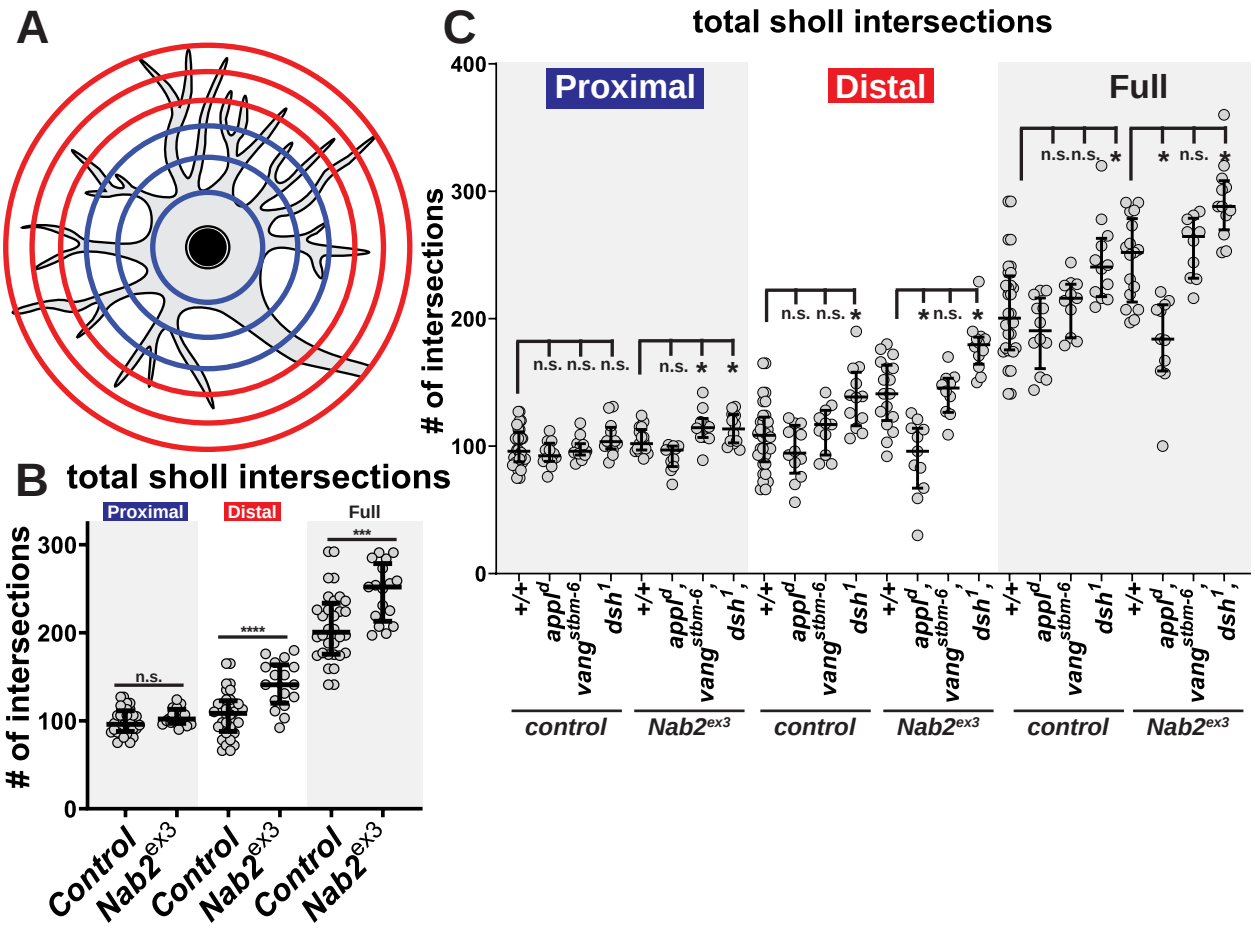
A
B
total sholl intersections
Proximal
Distal
Full
# of intersections
300
200
100
0
n.s.
****
***
Control
Nab2ex3
C
total sholl intersections
Proximal
Distal
Full
# of intersections
400
300
200
100
0
n.s. n.s. n.s.
n.s. * *
n.s. n.s. *
* n.s. *
n.s.n.s. *
* n.s. *
+/+
appld
vangstbm-6
dsh1
appld, vangstbm-6, dsh1,
control
Nab2ex3

Figure 3-S2: Proximal-distal effect on dendritic arbor complexity.

(A) Diagram depicting the concentric rings used to perform Sholl analysis overlaid on the dendritic arbor of a neuron. The half of the rings proximal to the soma labeled in **blue**; the half of the rings distal to the soma labeled in **red**. (B-C) Quantification of branching complexity by Sholl analysis using total Sholl intersections split across the proximal half, distal half, or full dendritic arbor. (B) Proximal-distal effect on total Sholl intersections of *control* compared to $Nab2^{ex3}$. (C) Proximal-distal effect on total Sholl intersections of *control* compared to single copies of $Vang^{stbm-6}$, $Appl^{d}$, and dsh^{1}; and of $Nab2^{ex3}$ compared to single copies of $Vang^{stbm-6}$, $Appl^{d}$, and dsh^{1} in the background of $Nab2^{ex3}$. Bars represent median and upper/lower quartile, * indicates $p<0.05$, **** indicates $p<0.001$. *Control* $n=32$, $Nab2^{ex3}$ $n=17$.

Figure 3-S3

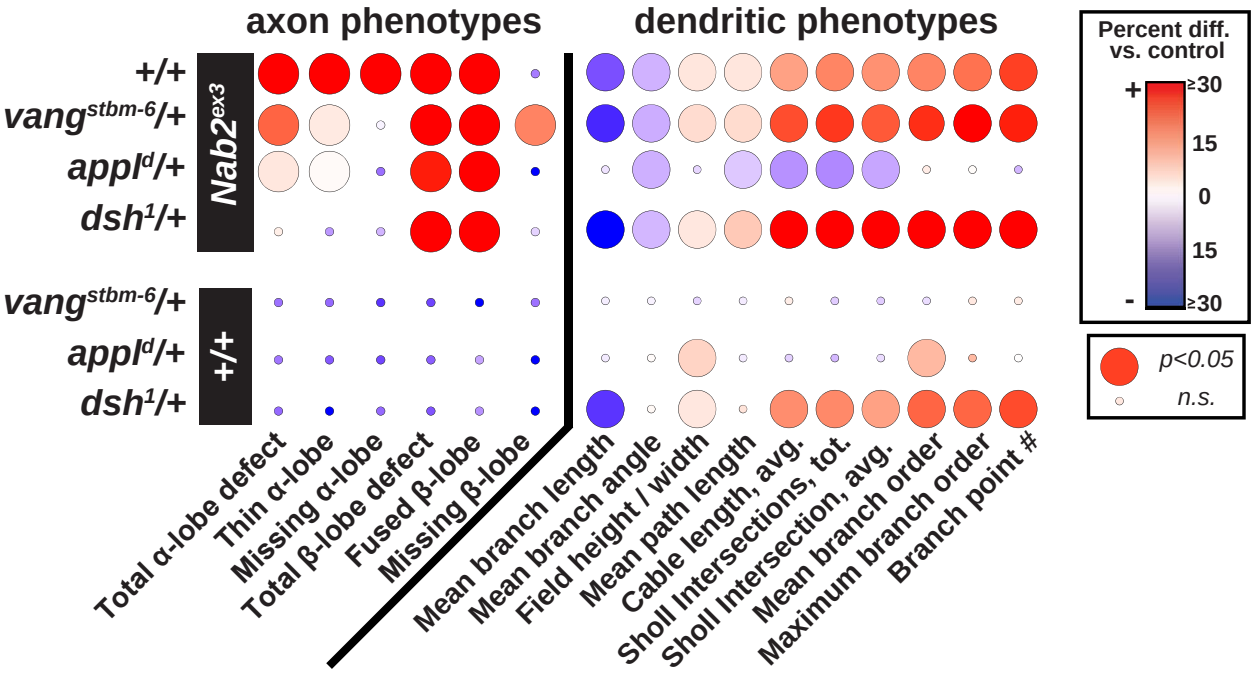
axon phenotypes
dendritic phenotypes
Nab2ex3
+/+
vangstbm-6/+
applᵈ/+
dsh¹/+
+/+
vangstbm-6/+
applᵈ/+
dsh¹/+
Total α-lobe defect
Thin α-lobe
Missing α-lobe
Total β-lobe defect
Fused β-lobe
Missing β-lobe
Mean branch length
Mean branch angle
Field height / width
Mean path length
Cable length, avg.
Sholl Intersections, tot.
Sholl Intersection, avg.
Mean branch order
Maximum branch order
Branch point #
Percent diff. vs. control
+
-
≥30
15
0
15
≥30
p<0.05
n.s.

Figure 3-S3: Overview of dominant modification of *Nab2^{ex3}* phenotypes by PCP component alleles.

Balloon plot depicting ten measurements of the ddaC neuron dendritic phenotypes and six measurements of the MB axon phenotypes. Change is shown as percent difference vs *control*; percent change ranges from increased in red to decreased in blue; significance depicted by balloon size (large balloon = $p<0.05$, small balloon = ns). The top four rows represent alleles in the *Nab2^{ex3}* background; the bottom three rows represent alleles in *control* background.

Figure 3-S4

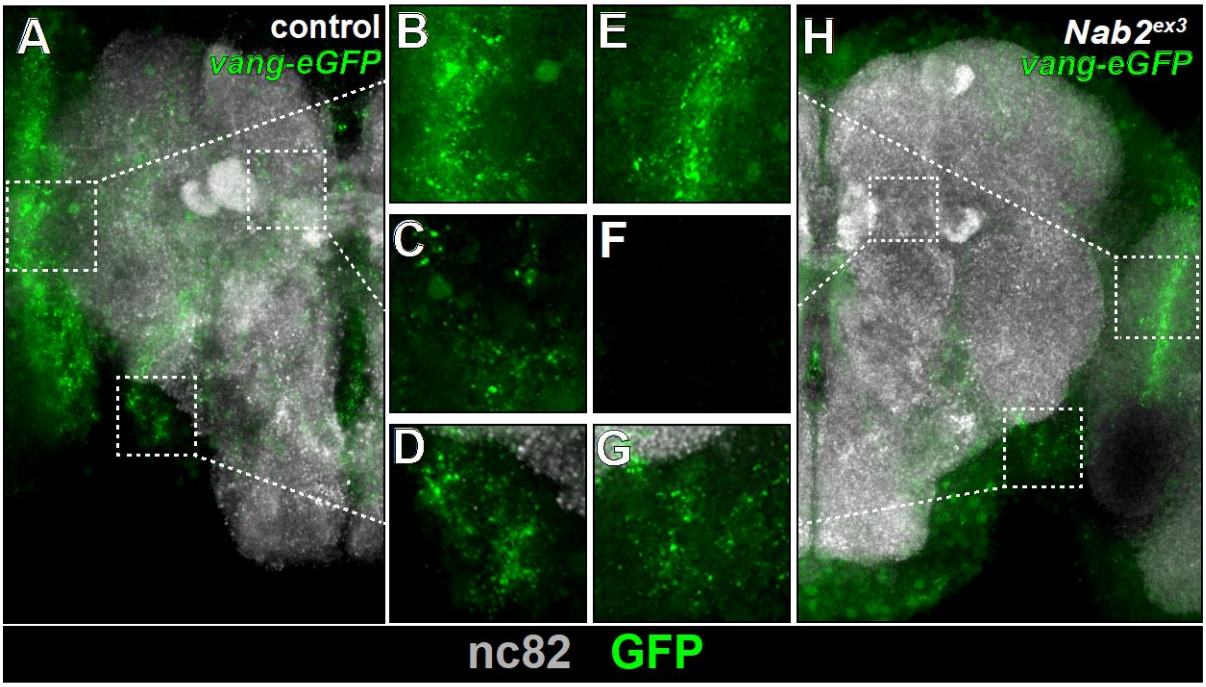

Figure 3-S4: Expanded view of Vang-eGFP localization.

(A-G) Visualization of brains (48-72hr after puparium formation) co-stained for GFP and nc82 (Bruchpilot; Brp). GFP staining indicates Vang localization using a *Vang-eGFP* construct (*vang*$^{EGFP.C}$) and nc82 indicates Brp which marks presynaptic actives zones. (A-D) *control* brain showing (A) left hemisphere with highlights at the (B) intersection of the lateral anterior optic tubercle and medulla layer (Krzeptowski et al., 2018; Nériec & Desplan, 2016; Tai et al., 2021), (C) central neuropil dorsal to the MB β-lobes near the midline, and (D) ventral cortical surface adjacent to the antennal lobes (Wolff & Rubin, 2018). (E-H) *Nab2*^{ex3} brain showing (H) right hemisphere with highlights at the (E) intersection of the lateral anterior optic tubercle and medulla layer (Krzeptowski et al., 2018; Morante & Desplan, 2008; Tai et al., 2021), (F) central neuropil dorsal to the MB β-lobes near the midline, and (G) ventral cortical surface adjacent to the antennal lobes (Wolff & Rubin, 2018). GFP labeled in **green**; nc82 labeled in **gray**. Vang-eGFP is present throughout the *control* brain (A-D). Vang-eGFP is present throughout most of the cortical surface of the *Nab2*^{ex3} brain (H,E,G) but specifically absent in the Brp-positive (F) neuropil regions of central complex.

Data availability: Proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE (78) partner repository with the dataset identifier PXD022984. All remaining data are contained within the article.

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The authors declare no competing financial interests.

Chapter 4: Discussion and conclusions

This chapter has been written by Edwin Corgiat specifically for inclusion in this dissertation.

4.1 Discussion

4.1.a Neurodevelopment requires highly tuned post-transcriptional regulation.

Neurodevelopment is a vastly complex process, requiring precise spatiotemporal control of gene expression for proper formation of overall neuronal architecture (Holt et al., 2019; Hörnberg & Holt, 2013; Maday et al., 2014; Stoeckli, 2018). To account for the unique cellular architecture of neurons, where axo-dendritic projections can extend over enormous distances relative to the size of the cell body, RBPs aid in control of gene expression using mRNA trafficking and local translation to provide spatiotemporal control (Agrawal et al., 2019; Castello et al., 2013; Holt et al., 2019; Jung et al., 2012). Because of the nature of RBP dysfunction to impact regulation of spatiotemporal control of gene expression (section 1.2.a & 1.2.b), there are many diseases that arise from mutations in RBP genes (section 1.2.b & 1.2.c). Understanding how dysregulation of post-transcriptional mechanisms alters proper neurodevelopment and brain function will give insight into numerous genetic diseases, including intellectual disabilities, autism spectrum disorders, encephalopathies, among other diseases. Insights into these post-transcriptional mechanisms are important because many of the mutations that lead to these neurological disorders converge on similar biological processes and pathways.

4.1.b Overview of main findings

The experiments presented in this dissertation use the *Drosophila melanogaster* model system of Nab2 RBP loss to address three main questions: first, W*hat occurs to the proteome of a developing Drosophila brain lacking Nab?*; second, *What impact does Nab2 loss have on development of axonal and dendritic projections?;* and third, *How does Nab2 interact with the planar cell polarity (PCP) pathway in neurodevelopment of both axons and dendrites?* Here, we

show that Nab2 controls the abundance of a subset of brain proteins during the active process of wiring the pupal brain mushroom body, and thus provide a window into potentially conserved functions of the Nab2/ZC3H14 RNA binding proteins in neurodevelopment (**Chapter 2**). We identify roles for Nab2 in regulating abundance of a small fraction of the total brain proteome (~8%), including the microtubule binding protein Futsch, the neuronal Ig-family transmembrane protein Turtle, the glial:neuron adhesion protein Contactin, the RacGAP Tumbleweed, and the core planar cell polarity factor Van Gogh (Vang). Gene ontology analyses collectively link Nab2 to the processes of brain morphogenesis, neuroblast proliferation, circadian sleep/wake cycles, and synaptic development (**Chapter 2**). Additionally, we combine proteomic, genetic, and cellular approaches to elucidate a role for Drosophila Nab2 in dendritic development, characterize an interaction between Nab2 and the planar cell polarity (PCP) pathway in axonal and dendritic growth, and identify PCP component Vang as a potential Nab2 target (**Chapter 3**). These genetic data, collected in mushroom body (MB) axons and class IV dorsal dendritic arbor C (ddaC) neurons, suggest that there is a Nab2-PCP interaction in both axonal and dendritic sub-cellular neuronal compartments (**Chapter 3**). Furthermore, these data suggest that Nab2 is required to regulate axonal and dendritic growth by a PCP-linked mechanism, possibly through localization of Vang protein in distal neuronal projections (**Chapter 3**). This Nab2-PCP interaction finding is particularly interesting when considering the six proteomic changes shared between whole *Nab2* null fly brain and *Zc3h14* null mouse hippocampi because three of the six have PCP function (core PCP component Vang, PDZ-domain protein X11Lβ, and oxioreductase Wwox) (**Chapter 3**) (Corgiat & List et al., 2021; Rha et al., 2017). In aggregate, these studies have leveraged our *Drosophila* model using biochemical and genetic approaches to further elucidate the cellular and

molecular functions of Nab2/ZC3H14 in neurodevelopment, potentially providing insight into the broader mechanisms underlying mutant-RBP pathology and human intellectual disabilities.

4.2 Implications of Nab2 findings

The overarching question of this research has been, *What is the function of Nab2, and why is it particularly critical in neurons?* Parts of this question have been addressed in previous studies of the functions of Nab2 (Pak et al., 2011) and mouse ZC3H14 (Rha et al., 2017) in neurodevelopment (Kelly et al., 2016) that have revealed Nab2 cooperatively functions in neurodevelopment with the RBPs Fmr1 and Atx2 (Bienkowski et al., 2017; Rounds et al., 2021), a potentially conserved role in translational repression (Bienkowski et al., 2017; Corgiat & List et al., 2021; S. K. Jones et al., 2020) and transcript-specific effects on mRNA splicing and N6-methylation (m^6A) modulation (Bienkowski et al., 2017; Corgiat & List et al., 2021; Jalloh et al., 2020; Rounds et al., 2021). These data follow foundational work examining the molecular functions of the yeast homolog, also termed Nab2 (Anderson et al., 1993; Brockmann et al., 2012; Fasken et al., 2019; Green et al., 2002; Hector et al., 2002; Kelly et al., 2010; Kelly, Leung, Pak, Banerjee, Moberg, et al., 2014; Soucek et al., 2016a). Cumulatively, these studies have contributed to an expanded understanding of Nab2 function in the brain, from the effect on Nab2 loss on the neuronal proteome, to identifying neurodevelopmental factors that genetically interact with Nab2 in patterning brain axon projection, and finally uncovering a cell autonomous role of Nab2 in controlling branching and projection of distal axonal and dendritic processes. While the work presented in this dissertation provided many conclusions relating to Nab2 function, speculating on the implications of these data may provide further insight and direction for future experimentation (section 4.2.a & 4.2.b).

4.2.a Implications of Nab2 regulation of the brain proteome

Although we have yet to pinpoint the full range of molecular mechanisms employed by *Drosophila* Nab2 during neurodevelopment, our global proteomic analysis on the whole brain of *Nab2* null flies have given us insight into the phenotypically relevant changes, thus providing insight into both direct and indirect impacts on the fly brain and potential targets for future experiments (**Chapter 2**). In **Chapter 2**, we discussed insights around Nab2 specificity (e.g., Nab2 loss alters only ~8% of the brain proteome suggesting that Nab2 does not regulate all mRNAs) and broadly which protein changes seem the most important (i.e., Vang, Tum, Trio, Cortactin, and Wwox) based on proteomic analyses alone (**Chapter 2**) (Corgiat & List et al., 2021). Further examination of the Nab2 brain proteome data can yield implications of a speculative nature that were not included in-depth in the **Chapter 2** discussion.

Exploration of the *Nab2* null brain proteome revealed gene ontology terms relating to *brain morphogenesis*, *neuroblast proliferation*, *circadian sleep/wake cycles*, and *synaptic development* (**Chapter 2**). The way these terms relate to existing Nab2 phenotypes can provide some insight into the potential mechanisms of Nab2 regulated neurodevelopment. Proteins decreased in abundance are enriched for terms including *axon mid-point recognition*, *neuron projection extension*, *positive regulation of axonogenesis*, which are all logically linked to the observed *Nab2* null MB defects (i.e., α-lobe thinning/missing and β-fusion/missing). Protein changes included in the term *neuron projection extension* are particularly interesting because they can include proteins involved in positive (e.g., Dscam1 (Sawaya et al., 2008)) and negative (e.g., Netrin-A (Kang et al., 2019)) control of neuron extension. If a protein involved in positive *control of neuron extension* (i.e., promotes neuron extension) is decreased, the neuron would not appropriately extend and grow, and this failure to project is one potential reason why we see the thinning and

loss of MB α-lobes. Alternatively, if proteins involved in negative *regulation of neuron extension* (i.e., inhibits neuron extension) is decreased, the neuron would inappropriately overextend and possibly overgrow into other regions of the brain, which could explain the fusion of the β-lobes across the mid-line. In addition to neuron projection extension, decreased proteins that function in *axon mid-point recognition* could easily provide a mechanism leading to the *Nab2* null β-lobe fusion phenotype. For example, if there is a failure to recognize mid-point signals, the axon could over project and fuse into the contralateral β-lobe. The *Nab2* null phenotype of missing α- and β-lobes could be due to the decrease in abundance of proteins involved in positive regulation of axonogenesis. During late larval development, the MB structures are pruned back and then start to reform during early pupal development. The MB lobes, including the α- and β-lobes, form from four neuroblast pools, and one could envision a scenario where these new *Nab2* null Kenyon cells have reduced signaling for axonogenesis that could lead to failure of the axon to grow and project, resulting in the observed thinning and missing of the α- and β-lobes. All of these protein changes in the Nab2 null proteome could be the result of direct binding of Nab2 to the respective mRNAs and control of gene expression, or could occur through indirect interactions (i.e., Nab2 regulates a protein that regulates other proteins), but regardless of how direct or indirect the effect is, these protein changes are likely to be functionally relevant to the observed *Nab2* null MB phenotypes.

In addition to providing potential explanations for the *Nab2* null MB phenotypes, our proteomic analyses have also provided insight into which neuronal sub-compartments Nab2 may function in as well as hinting at potential neurodevelopmental and molecular mechanisms. The biological process term *dendrite guidance* was enriched in the *Nab2* null brain proteome, along with other dendritic development related terms, which suggests that Nab2 can function in dendritic as well as axonal development. This hypothesis that Nab2 functions in dendritic development

derived from our proteomic analyses is supported by evidence from mouse showing that ZC3H14 functions in dendritic spine development (S. K. Jones et al., 2020) and is co-localizes with PSD-95 in neurite projections (Bienkowski et al., 2017) (covered in section 1.4.b), and is further supported by the work presented in **Chapter 3**. The proteins and potential pathways regulated by Nab2 may overlap but interact functionally with Nab2 in different ways between axonal and dendritic compartments. Further analyses of the proteins annotated in dendritic development could yield targets for future investigation. Potentially most interesting are the terms that relate to function within neurons including, for example, *axo-dendritic transport* and *anterograde trans-synaptic signaling*. These two terms highlight two potential mechanisms for Nab2 to function in neurodevelopment or cognitive function. Axo-dendritic transport proteins were down regulated in the absence of Nab2. This reduction in axo-dendritic transport proteins could occur for multiple reasons. Speculatively, one possible reason is that the proteins involved in transport of Nab2 and the associated transcripts are not needed and thus under produced. In this scenario, Nab2 RNPs normally make it to the cytoplasm, are picked up by the axo-dendritic transport machinery, and transported to the distal ends of the neuron where local translation can take place. When Nab2 is lost, Nab2 RNPs never form or make it the axo-dendritic transport machinery, resulting in less need for those transport proteins and subsequently less production of the axo-dendritic transport proteins. The reasons a reduction in axo-dendritic transport proteins is observed can be speculated on but are not fully explained by our proteomic analyses, which highlights a current gap in understanding. The potential mechanism, in axo-dendritic transport, would support a hypothetical role for Nab2 in localization of *Vang* mRNA that is further supported by the evidence for Nab2 regulating localization of Vang protein (**Chapter 3**). These data combined suggest that Nab2 function in neurons may be more critical than in other cell types because of the need to transport

mRNA to the distal ends of the neuron. The term for *anterograde trans-synaptic signaling* is not the only term enriched in the *Nab2* null proteomic dataset related to synaptic signaling and function and is particularly interesting in the context of recent work done in mouse models of PCP mutants (Ban et al., 2021; Feng et al., 2021; Fenstermaker et al., 2010; McKenzie et al., 2019; Onishi et al., 2020; Salinas & Zou, 2008; Schmitt et al., 2006; Shafer et al., 2011; Thakar et al., 2017a, 2017b; Zou, 2004a, 2004b, 2012). Most hypotheses for the function of Nab2 have related to morphological defects arising during neurodevelopment, largely due to the early finding of Nab2 MB morphological defects. Unfortunately, the patients identified with human *ZC3H14* mutations are isolated in rural Iran and have never undergone structural imaging scans for potential morphological defects in their brains to support or dissuade this assumption that morphological defects result from ZC3H14 loss. An approach to provide data in support or opposition of this morphological hypothesis could come from having MRI structural scans of patients with *ZC3H14* mutations. The proteomic links to synaptic signaling suggest an alternate hypothesis where the defects leading to intellectual disability in human patients, and memory defects in flies and mice, may be in part due to defects in synaptic function and not simply morphological connectivity. The presence or absence of morphological defects alone do not exclude the potential for synaptic dysfunction, but regardless of the results, having MRI scans would help focus future research. Model systems-based experiments would be needed to address questions around synaptic function.

Because Nab2 and PCP have functional links in neurodevelopment (**Chapter 3**), it is interesting that PCP mutations in *Celsr3*, the mouse ortholog of fly *fmi/stan*, lead to loss of ~50% of glutamatergic synapses, while mutations in *Vangl2* increase glutamatergic synapses in mouse hippocampus (Thakar et al., 2017a). Both the *Celsr3* and *Vangl2* mutations are accompanied by defects in hippocampus-dependent behavior, including spatial learning and memory (Thakar et al.,

2017a), and these behavioral defects are similar to the learning and memory defects in *Zc3h14* knockout mice and *Nab2* null flies (Kelly et al., 2016; Rha et al., 2017; Thakar et al., 2017a). Even though dissecting *Drosophila* neuro-connectivity is beyond the scope of this thesis, the insight into synaptic function of PCP mutants has interesting potential implications for Nab2 function. Considering *Nab2/Zc3h14* fly and mouse models have similar behavioral deficits as PCP mutants, it is possible to speculate that a similar function may underly *Nab2/Zc3h14* as has been identified in PCP mutants (i.e., alteration of glutamatergic synapse quantity). This speculative idea that glutamatergic synapses may be disrupted in *Nab2* null flies is supported by the *Nab2* null proteome changes relating to synapse function and supported by the fact that one of the main brain structures altered by Nab2 loss, the MBs, output to glutamatergic neurons (Aso, Sitaraman, et al., 2014). The 21 mushroom body output neurons that synapse along the MBs are responsible for the output of singles to neuropils outside the MBs (Aso, Sitaraman, et al., 2014) (section 1.3.b). MB output largely to glutamatergic neurons (i.e., 7 of the 21 MBONs), with the remaining MBONs outputting to GABAergic and cholinergic neurons (Aso, Sitaraman, et al., 2014). Additionally, changes in the function of these MBONs directly drive behavioral changes in the fly (Aso, Sitaraman, et al., 2014). Therefore, while speculating, it is not beyond the realm of possibility that glutamatergic synapse function is disrupted in *Nab2* null flies. Unfortunately, changes to the glutamatergic MBONs would be undetectable among the thousands of neurons stained in our Vang-eGFP/nc82 experiments and would require more detailed future experiments to address this possibility. It is unlikely that Nab2 would selectively regulate glutamatergic neurons but considering the recent links of PCP to glutamatergic synaptic formation and links of Nab2 to PCP pathway regulation, glutamatergic synapse function may be a good starting place for investigation. In aggregate, these

data suggest glutamatergic synaptic disruption may be the basis for *Nab2/Zc3h14* learning and memory deficiency and may be an area for further study in the future.

4.2.b Implications of Nab2 interaction with the PCP pathway

The overarching research question has been to understand why Nab2 function is specifically important in neurons, and the data linking Nab2 to the PCP pathway may provide insight into Nab2 mechanisms important in neurodevelopment (**Chapter 3**). During exploration of the Nab2-PCP genetic interactions, we utilized two systems to characterize neurodevelopment in the central nervous system, using mushroom body (MB) axons, and in the peripheral nervous system, using class IV dorsal dendritic arbor C (ddaC) neurons, which together allow analysis of pre- and post-synaptic compartments. This dual axo-dendritic analysis provided deeper insight into what Nab2 is doing within a neuron during its growth. Specifically, these analyses revealed that changing PCP component dosage can dominantly modify Nab2 axonal and dendritic phenotypes, as with Vang and Appl, and further identified Vang as a potential Nab2 target via a localization assay (**Chapter 3**). The findings from **Chapter 3** provide a framework for understanding Nab2 neurodevelopmental defects. Further examination of the Nab2-PCP data can yield implications of a speculative nature that were not included in-depth in the **Chapter 3** discussion.

A deeper, PCP focused analysis of the *Nab2* null brain proteome revealed PCP gene ontology terms including *planar cell polarity*, *regulation of planar cell polarity*, *protein localization in planar polarity*, and *planar cell polarity effectors*. These terms give an indication of how Nab2 and PCP component mRNAs may interact (explained below). The planar cell polarity effectors term focused on neuronal specific downstream effectors of the PCP pathway and included

Appl, Akap200, Mlp84B, and Tum. Whether the regulation of these PCP effector proteins by Nab2 is direct or indirect is difficult to determine from proteomic data alone, but could easily be due to indirect effects of Nab2 altering the PCP pathway upstream of these neuronal specific downstream effectors. In contrast, direct regulation of effector proteins like Appl could imply that Nab2 has an additional role in the function of the adult brain and is not limited to the development of it. In *Drosophila*, Appl directly functions in the development of MBs and maintains adult function of the MBs, illustrated by memory deficits resulting from Appl being knocked down in only the adult MBs (Preat & Goguel, 2016; Soldano et al., 2013). Additionally, Appl is the APP-Like ortholog of human amyloid precursor protein (APP) that functions in memory and neuro-plasticity and is implicated in development of Alzheimer's disease (Preat & Goguel, 2016). In sum, these insights into Nab2 regulation of proteins involved in maintaining adult brain function complement the proteomic findings that Nab2 may function in anterograde trans-synaptic signaling (**Chapter 2**; section 4.2.b) and together imply that there is an underappreciated role in the adult brain for Nab2 in maintaining proper function.

The planar cell polarity and regulation of planar cell polarity GO terms suggest that core components of the PCP pathway (Figure 1-6) are affected, and GO term protein localization in planar polarity suggests that the core components may be affected due to dysregulation of proteins involved in PCP core component localization. Importantly, the only core component protein modified is Vang that was decreased in abundance 5-fold. Heterozygous alleles of Vang also dominantly modify both MB and ddaC phenotypes (**Chapter 3**), and loss of Nab2 restricts Vang-eGFP protein expression to the cell bodies in the cortical surface of the brain and excludes Vang-eGFP localization from the distal ends of neurons (axons and dendrites), which make up the central complex neuropil (**Chapter 3**). These effects on Vang imply that the impact of Nab2 on the PCP

pathway may be specifically due to regulation of Vang. The variance of Vang modification of Nab2 phenotypes (i.e., suppress in MBs and enhance in ddaCs) may speak to a difference in Nab2-Vang interaction or a difference in PCP function across neuronal contexts (see below section 4.2.d).

4.2.c A model of Nab2 function in neurons

Even though the precise molecular mechanisms employed by Nab2 remain largely undefined, the findings in this dissertation provide significant insights into the neuronal function of Nab2. This work has been built upon previous studies that revealed roles for Nab2 in the nucleus regulating poly(A) tail length (Kelly et al., 2016), alternative splicing (Jalloh et al., 2020), and nucleocytoplasmic transport (Bienkowski et al., 2017; Morris & Corbett, 2018; Rha et al., 2017), in complex with other RBPs (Fmr1 and Atx2) (Bienkowski et al., 2017; Rounds et al., 2021), and in the cytoplasm in mRNA trafficking and local translation (Bienkowski et al., 2017; Corgiat & List et al., 2021). The work presented in this dissertation provides new data that has led to an updated model of Nab2 function (Figure 4-1). We hypothesize that while Nab2 functions in the nucleus (Figure 4-1 *"in the nucleus"*) performing many important post-transcriptional functions, importantly for neurons, Nab2 cycles into the cytoplasm (Figure 4-1 *"in complex"*) where it participates in the trafficking of mRNAs to the distal ends of neurons (both axons and dendrites) (Figure 4-1 *"in the cytoplasm"*). More specifically, we hypothesize that Nab2 regulates Vang mRNA in the distal ends of neurons impacting PCP pathway function (Figure 4-1 *"new insights"* and Vang mRNA in **red**). We further hypothesize that it is the regulation of this Vang mRNA, and possibly other PCP component mRNAs, that are largely responsible for the neurodevelopmental function of Nab2. This model is similar to past models for Nab2 function in the nucleus, but the

data presented in **Chapters 2 & 3** updates the model for the cytoplasmic and neurodevelopmental roles. In aggregate, this model suggests roles for Nab2/ZC3H14 in post-transcriptional regulation in both the nucleus and cytoplasm and that the importance of Nab2 in regulating varying mRNA targets differs based on space, time, and tissue type.

4.2.d Open questions and future directions

There are a handful of major open questions that have arisen from this work including: which Nab2 interactions and targets are relevant to neuronal phenotypes, which PCP components does Nab2 interact with during neurodevelopment, and is the Nab2-PCP interaction conserved in mammals?

The first of these questions, *Which Nab2 interactions and targets are relevant to neuronal phenotypes?*, directly relates to the implications from our proteomic findings (**Chapter 2**; section 4.2.a). There were many changes in the *Nab2* null proteome (346 differentially expressed proteins), and many are likely to be indirect effects of Nab2 loss. A general example of indirect regulation is that Nab2 regulates protein A, but changes in protein A in turn lead to protein expression changes in protein C and protein E as well, even though Nab2 does not directly regulate proteins C or E. Regardless of whether *Nab2* loss directly or indirectly changes the protein abundance, the changes represented in this dataset are those most relevant to the observed neurodevelopmental defects. While the most important RBP targets are typically considered to be the RNAs to which the RBPs directly bind, both the direct and indirect changes to the proteome are relevant to the observed neurodevelopment phenotypes. An example of a protein that is likely important to neurodevelopment in the *Nab2* null brain is Contactin (Cont), a GPI-anchored cell adhesion molecule that ensheathes neurons and glia and has functions including axon guidance and dendrite

self-avoidance. Cont protein is increased 1.5-fold in the *Nab2* null brain (**Chapter 2**) but is not associated with Nab2 via RIP-seq (Rounds et al., 2021). Cont is a prime example of a protein that could very well impact neurodevelopment and is altered by Nab2 loss but is not bound by Nab2 (i.e., indirectly regulated). Continuing to understand the functions of indirect targets could help further our understanding of changes in the *Nab2* null brain and potentially better understand protein changes that lead to intellectual disabilities. The focus of the ongoing research in our lab group is largely on the overlap of our RNA-seq (Jalloh et al., 2020), RIP-seq (Rounds et al., 2021), and proteomic (Corgiat & List et al., 2021) datasets. However, insight into neurologic phenotypes can also be gained from investigating the targets that do not overlap across the dataset like Cont. These non-overlapping targets are 'low-hanging fruit' for genetic interaction experiments. Investigating the overlapping targets will require more in-depth, molecular mechanism experiments (i.e., targeted RIP, localization assays, ribosome occupancy), while the non-overlapping targets can be more easily initially probed using genetic interaction tests in the fly may and may be equally important for observed phenotypes. Simple experiments would include taking the *GMR-Nab2* rough eye phenotype and testing for genetic interaction with *Cont* and alleles of similar, non-overlapping proteins. In contrast to the indirect Nab2 targets, Nab2 direct targets that are both bound by Nab2 and changed in the *Nab2* null proteome are more easily explained. These directly regulated targets are proteins that appear to be directly bound by Nab2 and changed in the proteome and should be pursued as potential targets. For example, Futsch, the fly ortholog of microtubule-associated protein-1β (Map1β), has synaptic and neuromuscular function and its mRNA both bound by Nab2 and its protein abundance is changed in our the proteomic dataset (Bienkowski et al., 2017; Corgiat & List et al., 2021; Rounds et al., 2021). These directly bound RNAs have more promise as important Nab2-targets and would be worth testing

for genetic interaction in MB and ddaC systems as shown with PCP alleles in **Chapter 3**. A third class of proteins that may be relevant to the neuronal phenotypes are those that change in the *Nab2* null pupal brain but were unidentifiable in the adult head RIP-seq, such as Vang. The relevance of Nab2 regulation of some targets may only occur during the initial stages of neurodevelopment (i.e., 0-96 hours after puparium formation) and therefore would not have been identified by the RIP-seq of *Nab2* null adult heads (Corgiat & List et al., 2021; Rounds et al., 2021).

The findings in **Chapter 3** suggest the changes to PCP pathway components are important for neurodevelopment, which brings up the second outstanding question: *Which PCP components does Nab2 interact with during neurodevelopment?* **Chapter 3** data reveals genetic interactions with multiple PCP pathway components that correlate with proteomic changes. Nab2 regulation of Vang provides a good example of Nab2-PCP interaction. *Vang* alleles modify *Nab2* null phenotypes during developmental stages, and Vang protein expression is reduced in 24 hours after puparium formation (apf) brains. Furthermore, Vang protein localization is altered in brains of 48 hours apf *Nab2* null pupae. These data suggest that Nab2 regulates Vang expression during neurodevelopment, but through what mechanism Nab2 regulates Vang expression remains unclear. Interpretation of the data, as previously discussed (**Chapter 3**; section 4.2.c), would suggest a role for Nab2 in *Vang* mRNA trafficking. The most logical test of this hypothesis would be to perform targeted RIP in pupal brains looking for Nab2 binding of *Vang* mRNA. Additionally, it would be interesting to probe whether mutant alleles of other core PCP components modify *Nab2* null MB and ddaC phenotypes and if they are directly regulated via binding or indirectly, possibly through modification of the PCP pathway as a whole. mRNAs encoding core PCP components *prickle* (*pk*), *diego* (*dgo*), *furrowed* (*fw*), and *flamingo/starry night* (f*mi/stan*) remain prime targets to investigate for genetic and physical interactions with Nab2. Prickle is particularly

interesting because Prickle protein is directly bound by Vang and is required to amplify asymmetry of the core PCP complex (Bastock et al., 2003; Cho, Pierre-Louis, Sagner, Eaton, & Axelrod, 2015). The mouse ortholog, Prickle-2, is localized in postsynaptic density and interacts with PSD-95 (Hida et al., 2011), which is particularly interesting because mouse ZC3H14 colocalizes with PSD-95 in dendritic spines of hippocampal neurons, suggesting that Nab2/ZC3H14 may deliver PCP component mRNAs to this location for translation (Bienkowski et al., 2017; Rha et al., 2017).

Another open question related to the Nab2-PCP interaction question relates to 'cell autonomy'. In neurons the PCP pathway is currently thought to function through two primary mechanisms: regulation of intracellular actin polymerization dynamics or intercellular interactions of PCP components (i.e., Fz and Vang) that guide the growth cone through the surrounding neuronal substrate (Reynaud et al., 2015). Prickle, Disheveled, and Diego all act intracellularly to promote asymmetry of the core PCP complexes but also function to activate intracellular pathways (Y. Yang & Mlodzik, 2015). In the case of regulating actin polymerization, PCP pathway components can function in different and opposing ways. For example, Disheveled promotes actin polymerization by activating and localizing Rho-family GTPases, while inturned recruits a PCP effector, Multiple wing hairs (Mwh), that inhibits actin polymerization (Y. Yang & Mlodzik, 2015). By regulating the balance of actin polymerization in the growth cone of a projecting neuron, it is possible for PCP to regulate neuronal projection and guidance (Figure 1-6). The Wnt receptor Derailed (Drl) provides an example of intercellular PCP guidance by interaction with the neuronal substrate. Drl is expressed in the dorsomedial lineage neuropil surrounding the MBs and forms a complex with Wnt5, and is thus able to direct MB axon projection via repulsion of the MB intrinsic Drl-2-2 receptor (Reynaud et al., 2015) (Figure 4-2). Our data to date indicate that Nab2 functions cell-autonomously to regulate development of MB and ddaC neurons (**Chapter 3**) (Corgiat & List

et al., 2021; Kelly et al., 2016). However, it is unclear whether the dominant modification of *Nab2* null phenotypes by PCP alleles is cell-autonomous or non-autonomous. Using Nab2 and Vang interactions as an example to explore this idea of cell autonomy further, either the cell autonomous or non-autonomous functions of the PCP pathway in neurons could explain how Nab2 and Vang interact. For example, it is possible that when Nab2 is lost, less Vang protein is produced, or Vang fails to localize properly, leading to its degradation. In a PCP cell-autonomous scenario, if Vang is needed intracellularly at the growth cone to promote localization of Inturned (Y. Yang & Mlodzik, 2015), then deregulation of Vang by loss of Nab2 could lead to disruption of actin polymerization inhibition potentially leading to the over projection of ddaC neuron arbors and fusion of MB β-lobes in *Nab2* null flies. There are two main ways the PCP interaction could be cell non-autonomous. First, in a PCP cell non-autonomous scenario, Vang could be needed in the growth cone of a neuron to guide it along the surrounding neuronal substrate, via intercellular attraction between Vang and Fz. Alternatively, it is equally possible that Vang is needed in the surrounding neuronal substrate (i.e., neurons, glia, or mesoectoderm) to steer the growth cone and the drop in Vang in *Nab2* null brains is due to an effect on a Vang-interactor needed to stabilize Vang. Because the experiments presented in **Chapter 3** used genomic alleles of PCP components to test interactions with Nab2, it is unclear whether modification of Nab2 phenotypes is due to the cell autonomous or non-autonomous mechanisms. Another 'low-hanging fruit' experiment would be to take the existing triple recombinant stock (*Nab2* null, *ppk>Gal4*, *CD8:GFP*) (generated for the experiments in this dissertation) and cross in RNAi knockdowns of PCP components specifically in the dendritic arborization neurons. If *Vang* RNAi modified *Nab2* null ddaC phenotypes similarly to heterozygous *Vang*, it would indicate that the interaction of Nab2 and Vang alter intracellular, cell autonomous functions of PCP. Alternatively, if *Vang* RNAi had no

modification of the ddaC phenotype, it would suggest that Nab2 disrupts the localization of PCP components within the neuron, disrupting the intercellular, cell non-autonomous functions of PCP in a similar way to the *derailed* example (Figure 4-2).

One minor question unrelated to PCP has also arisen in the course of these studies: *Does Nab2 have a role in regulating synaptic function in the adult brain?* The potential for Nab2 to regulate adult brain function seems quite possible from the proteomics data (**Chapter 2**; section 4.2.a) and would ideally be addressed using electrophysiological analysis of synaptic transmission (i.e., patch-clamp recordings) to quantitatively measure synaptic transmission in the *Nab2* null brain. While an electrophysiological approach would be ideal, there is minimal experience in the lab with this work, and the functionality of *Drosophila* as a model could be better utilized by selectively knocking down Nab2 in the brain and testing for behavioral deficits. A stock was generated during this work but never utilized that has a temperature sensitive *Gal80* paired with the neuronal driver (*C155*) and *Nab2* RNAi. This stock could be grown to adulthood, bypassing any consideration for *Nab2* during development, and then be temperature shifted to knockdown *Nab2* in neurons and tested for behavioral deficits, providing a simple first pass at addressing the role of *Nab2* in adult brain function.

Finally, there is a major remaining question relating to conservation of Nab2 function: I*s the Nab2-PCP interaction conserved in mammals?* There is now a large body of evidence for a Nab2-PCP interaction in *Drosophila.* Proteomics data is consistent with Nab2 regulating PCP proteins in the fly brain (**Chapters 2 & 3**), and the overlap of *Zc3h14* and *Nab2* null proteomics suggests an importance in PCP regulation in both the fly and mouse models (Corgiat & List et al., 2021; Rha et al., 2017). Genetic evidence supports these proteomic data with parallel genetic interactions between *Nab2* and PCP alleles in the fly eye (W. H. Lee et al., 2020), MBs (**Chapter**

3), and ddaC neurons (**Chapter 3**).. Additionally, Vang localization defects in the *Nab2* null brain reveal a possible molecular mechanism for Nab2-PCP regulation (**Chapter 3**). There is also similarity of PCP-like phenotypes between the *Nab2* null fly and *Zc3h14* null mouse. The *Nab2* null fly has PCP-like defects in the fly wing, specifically bristle orientation defects (W. H. Lee et al., 2020). In the *Zc3h14* null mouse, the mouse cochlea has defects in the hair cell layers of the Organ of Corti (**Chapter 3**). Of the six proteomic changes shared between whole *Nab2* null fly brain and *Zc3h14* null mouse hippocampi, three are related to the PCP pathway (core PCP component Vang, PDZ-domain protein X11Lβ, and oxioreductase Wwox) (Corgiat & List et al., 2021). With PCP-like defects and shared proteomic changes in the *Zc3h14* knockout mouse, it seems quite possible that the Nab2-PCP interactions observed in the fly could translate to the *Zc3h14* mouse. Considering that the mouse is significantly more difficult to do genetic modification tests on than the fly, the best course of action in the mouse would be to choose and pursue the one most likely PCP regulated candidate. Vang, at this moment, is that best candidate considering the data in the fly and mouse both point towards Vang as a Nab2/ZC3H14 target. Therefore, crossing a *Vangl2* mouse mutant into the *Zc3h14* knockout mouse background would be a good starting place. Once that mouse was generated, one could look at the hippocampal proteome, dendritic spine morphology, and learning and memory assays for potential modification of *Zc3h14* phenotypes.

4.3 Conclusions

In summary, the combined research presented in this dissertation utilizing a *Drosophila melanogaster* model of Nab2 has produced insights into the neuronal function of the Nab2 RBP, advancing our understanding of Nab2/ZC3H14 developmental roles and how mutations in

ZC3H14 may lead to intellectual disability in humans. The experiments performed here, using genetic, biochemical, and cell biological approaches, explore the impacts of Nab2 loss on the brain and peripheral nervous tissue. This work has uncovered a novel role for *Drosophila* Nab2 in fly dendritic development and pupal axonal development, provided a window into the Nab2-regulated brain proteome, and between Nab2 and components of the planar cell polarity pathway. Additional analyses of the identified Nab2-PCP interactions particularly determine that Nab2 is required for accumulation of the core PCP protein Vang in the axo-dendritic-enriched brain neuropil. These newly appreciated roles of Nab2 in dendritic development and as a modulator of PCP pathway activity are supported by evidence in the *Zc3h14* knockout mouse hippocampi with dendritic spine defects and in the cochlea with PCP defects (**Chapter 3**) (Rha et al., 2017). Together these results imply that Nab2 may function in the cytoplasm to support transport and translation of PCP component mRNAs in the distal ends of the neuron (Figure 4-1). However, many questions remain, notably: *Which Nab2 interactions are relevant to each neuronal phenotype?*, *Which PCP mRNAs are directly bound by or indirectly regulated by Nab2?*, and *How conserved from flies to mammals is the PCP related function of Nab2?* These questions necessitate further research that will best be conducted synergistically using both the *Drosophila* and mouse models of Nab2/ZC3H14. In aggregate, the work in this dissertation highlights a theme presented throughout, that post-transcriptional regulation implemented by RBPs allows for the highly tuned spatiotemporal control of gene expression that is necessary in neurons and that neurons are highly susceptible to the disruption caused by RBP mutation and loss. Continued study of Nab2/ZC3H14 will help elucidate the targets and mechanisms that underline RBP-regulated neurodevelopment and brain function, with the ultimate goal of understanding why Nab2/ZC3H14 function is critical in neurons and how that relates to *ZC3H14*-linked intellectual disability in humans.

4.4 Figures

Figure 4-1

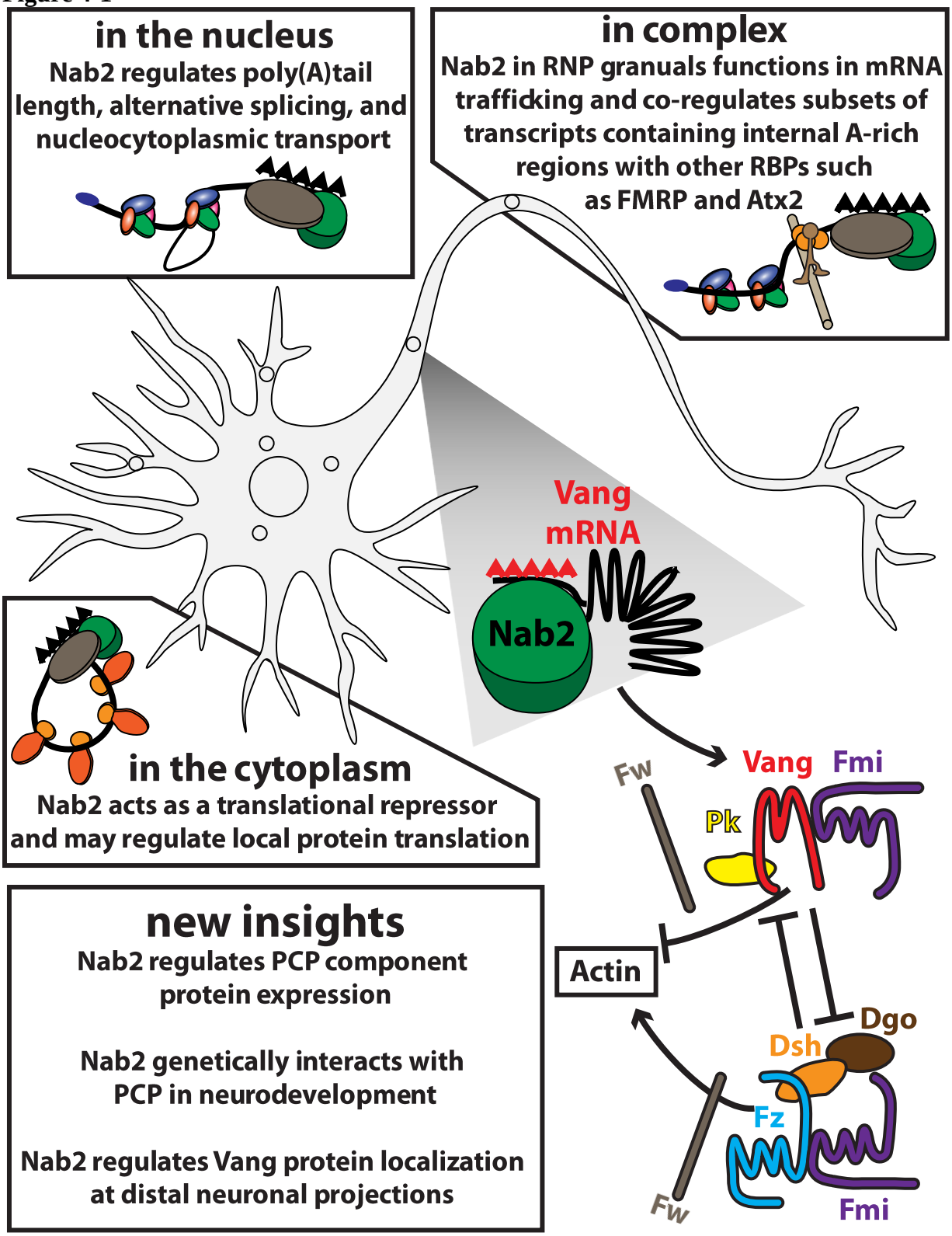

Figure 4-1: A model of Nab2 function in neurons.

Nab2 functions at multiple levels of post-transcriptional regulation and the work in this dissertation has led to an updated model of Nab2 function in neurons. *"in the nucleus"*; Nab2 limits the length of poly(A) tails (Kelly et al., 2016; Pak et al., 2011), regulates alternative splicing (Jalloh et al., 2020), nucleocytoplasmic transport (Bienkowski et al., 2017; Morris & Corbett, 2018; Rha et al., 2017). *"in complex"*; Nab2 function in complex with other RBPs to regulate RNA expression, including FMRP and Atx2 (Bienkowski et al., 2017; Rounds et al., 2021).*"in the cytoplasm"*; importantly for neurons, Nab2 cycles into the cytoplasm (where it participates in the trafficking of mRNAs to the distal ends of neurons (both axons and dendrites) (Bienkowski et al., 2017; Rha et al., 2017). *"new insights"*; highlights of new findings generated by the work in this dissertation. These findings are used to generate the hypothesis that Vang mRNA (in **red**) is regulated by Nab2, possibly through mRNA trafficking and localization, and that dysregulation of Vang that occurs with loss of Nab2 alters PCP pathway function, which may be largely explain the observed neurodevelopmental function of Nab2.

Figure 4-2

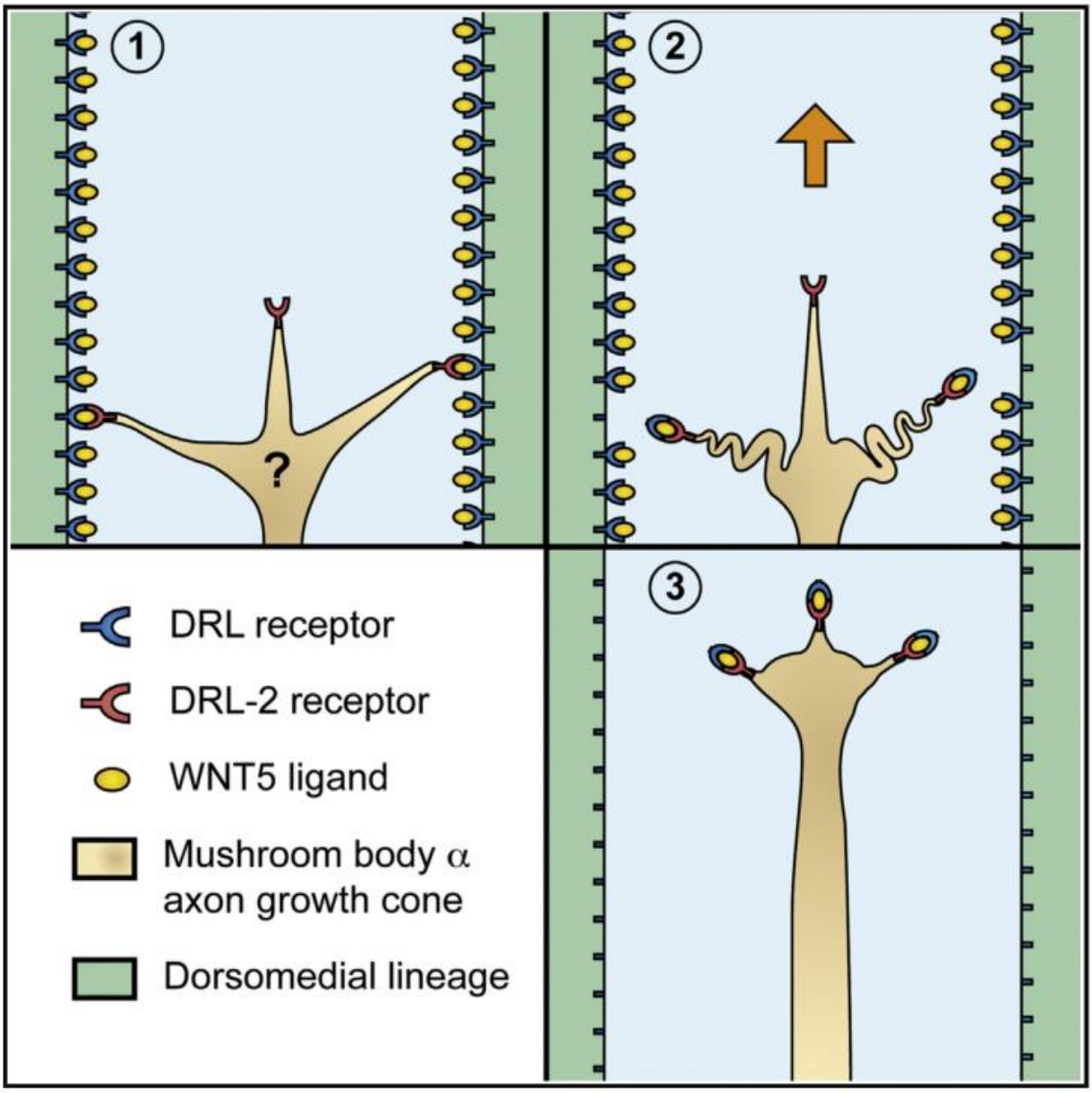
1
?
2
3
DRL receptor
DRL-2 receptor
WNT5 ligand
Mushroom body α axon growth cone
Dorsomedial lineage

Figure 4-2: Reynaud model of PCP mediated growth cone guidance.

Reynaud et al. found that even though *derailed* (*DRL)* receptor was not expressed in mushroom body axons, the receptor was responsible for guiding the axon growth by localizing WNT5 ligand on the substrate surrounding the growing neuron. DRL receptor and WNT 5 guide axons through repulsive interactions with the surrounding cells allowing the neuron to crawl over the neuronal substrate. Figure from (Reynaud et al., 2015).

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