Drosophila Fezf Coordinates Laminar-Specific Connectivity Through Cell-Intrinsic and Cell-Extrinsic Mechanisms

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Accessibility
Drosophila Fezf coordinates laminar-specific connectivity through cell-intrinsic and cell-extrinsic mechanisms

Abstract

Laminar arrangement of neural connections is a fundamental feature of neural circuit organization. Identifying mechanisms that coordinate neural connections within correct layers is thus vital for understanding how neural circuits are assembled. In the medulla of the Drosophila visual system neurons form connections within ten parallel layers. The M3 layer receives input from two neuron types that sequentially innervate M3 during development. Here we show that M3-specific innervation by both neurons is coordinated by Drosophila Fezf (dFezf), a conserved transcription factor that is selectively expressed by the earlier targeting input neuron. In this cell, dFezf instructs layer specificity and activates the expression of a secreted molecule (Netrin) that regulates the layer specificity of the other input neuron. We propose that employment of transcriptional modules that cell-intrinsically target neurons to specific layers, and cell-extrinsically recruit other neurons is a general mechanism for building layered networks of neural connections.
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#gradschoolpapi #papisfritas

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Invictus

by William Ernest Henley

Out of the night that covers me,
Black as the pit from pole to pole,
I thank whatever gods may be
For my unconquerable soul.

In the fell clutch of circumstance
I have not winced nor cried aloud.
Under the bludgeonings of chance
My head is bloody, but unbowed.

Beyond this place of wrath and tears
Looms but the Horror of the shade,
And yet the menace of the years
Finds and shall find me unafraid.

It matters not how strait the gate,
How charged with punishments the scroll,
I am the master of my fate,
I am the captain of my soul.
siempre pa’lante
Dedication

This dissertation is dedicated to Marilyn Correa.


Thank you for your love and your compassion. Thank you for your wings.
I. Introduction
Layers are a common structural motif throughout the nervous system

An organism’s ability to properly sense and respond to the environment is dependent on precise connectivity between neurons. During development, neurites (i.e. axons and dendrites) must identify their proper synaptic partners by navigating through a complex extracellular environment that is often packed with projections from many other neurons. Proper development of the nervous system is dependent on mechanisms that enable neurons to achieve proper synaptic specificity by recognizing appropriate synaptic partners, and neglecting others. This selection process lays the foundation for proper function of the nervous system and is thus critical to ensure organism survival. Although proper neuronal wiring is of critical importance, we have a limited understanding of the mechanisms that mediate this process.

Proper connectivity is thought to be facilitated by grouping correct synaptic partners into discrete domains, which are often organized into parallel laminae. Laminar organization is presumed to be important for several reasons. Segregation of proper synaptic partners into the same lamina, and away from non-partners, is thought to increase the likelihood that proper connections are made (Baier, 2013). Layered connectivity is also thought to be an economical method of wiring. As neural activity is energetically costly for the neuron, limiting the length of the neurites is advantageous (Wang and Clandinin, 2016). Lastly, layer specific connectivity is the structural basis for parallel processing of neural information. In developing an accurate percept of the environment, the nervous system first splits sensory information into basic features, such as motion and color. Signals encoding unique aspects of the environment travel via initially parallel neural pathways, eventually converging to form a complex perception (Azeredo da Silveira and Roska 2011; Clark and Demb, 2016; Nassi and Callaway, 2009; Roska and
Werblin 2001). While laminar connectivity underlies proper brain function, the basic principles that direct development of laminar organization are not well understood.

Layer specific connectivity is a prominent and consistent structural motif throughout the nervous system, reflecting its importance to proper brain function. For example, in the mammalian cortex, the somas of specific neuron types, and synapses between distinct synaptic partners, are organized into six layers (Layers I-VI) (Custo Greig and Woolworth et al., 2013). Connections between particular neuron types are formed in a layer specific manner. Layer V pyramidal neurons, for instance, receive synaptic inputs from pyramidal neurons whose axons project from Layers II/III (Harwell et al. 2012). Another laminar region of the vertebrate nervous system is the spinal cord, which is comprised of 10 lamina (Lamina I-X) that process distinct types of neural information. For example, Lamina I receives input from nociceptive primary afferent neurons (Rexed, 1952; Rexed, 1954; Todd, 2010). As a final example, multiple regions of the vertebrate and insect visual systems, such as the inner plexiform layer (IPL) of the vertebrate retina and medulla neuropil of the fly optic lobe (see below), are comprised of layer specific circuits (Sanes and Zipursky, 2010). Thus, layers are a characteristic feature of the nervous system.

Developmental studies in the vertebrate and insect visual systems have offered great insight into the mechanisms that direct layer specific connectivity. The characteristics of both systems were first observed and compared by Santiago Ramon y Cajal, who found that when the somas of fly neurons are hypothetically relocated without adjusting neurite positions, the wiring diagrams of both systems appeared strikingly similar (Cajal and Sanchez, 1915). Since Cajal’s initial observations, we have confirmed that the fundamental structure of the vertebrate and insect visual systems are indeed comparable. In both, the neuropil regions are devoid of cell
bodies, and composed of only neurites. As such, neuropil development is a function of interactions between axons and dendrites. Circuits in both are arranged in two perpendicularly oriented pathways: columnar units that each represent a single point in the visual field, and parallel layers within which distinct types of information are processed. The accessibility, compact organization, and stereotypic structure of the visual system are all reasons why it has been established as an advantageous model to study layer specific connectivity. More specifically, studies in the IPL of the vertebrate retina and the medulla neuropil of the fly optic lobe have played a central role in shedding light on the developmental mechanisms that govern this process (Sanes and Zipursky, 2010).

The IPL of the vertebrate retina is comprised of five sublaminae (S1-S5), which are innervated by three general classes of neurons. Bipolar cells (BCs) are interneurons that relay sensory information from photoreceptors to the IPL, where this input is processed between BC terminals and dendrites from amacrine cells (ACs) and retinal ganglion cells (RGCs). ACs are interneurons whose neurites are confined to the IPL, while RGCs are the major output projection neurons from the retina to the brain. Discrete neural information is split among BC subtypes, which send this information to different sublaminae in the IPL. ACs and RGCs are also composed of distinct subtypes whose dendrite positions throughout IPL sublaminae determine the aspect of the visual scene to which they are tuned. This organization results in parallel channels of information processing in the IPL (Duan et al., 2014).

A classic example of the parallel flow of visual information in the IPL is the manner in which neural activity encoding the onset of light (ON) and the offset of light (OFF) are processed. In this example, neural information concerning ON stimuli is processed in the proximal half of the IPL, while the distal half contains neural activity corresponding to OFF
stimuli (Famiglietti and Kolb, 1976; Nelson, et al., 1978). ACs and RGCs are considered ON and/or OFF cells depending on which sublaminae they stratify, and thus, the stimuli to which they are responsive. While more recent studies have suggested that a circuit model in which ON and OFF sections of the IPL are mutually exclusive is an oversimplification (Hoshi et al. 2009), it remains an iconic example of parallel processing in the vertebrate IPL.

As in the vertebrate visual system, laminar structures are prominent in the fly eye. In the Drosophila melanogaster optic lobe, the medulla is a laminar neuropil composed of 10 layers (M1-M10), which are innervated by more than 100 different neuron types (Fischbach and Dittrich, 1989; Nern et al., 2015). The medulla receives direct visual input from photoreceptors R7 and R8, as well as indirect input from R1-R6 via five types of lamina neurons (L1-L5) (Fischbach and Dittrich, 1989; Nern et al., 2015). Each input neuron is represented within columnar units creatively referred to as columns, each of which processes information from a specific point in the visual field, and thus all together represent a retinotopic map (Maisak et al., 2013). Within the medulla, information is processed between input neurons and various classes of downstream neurons, such as transmedullary neurons (Tm), the cellular analog of RGCs in the vertebrate retina (Sanes and Zipursky, 2010) that relay visual information to downstream neuropil, and medulla intrinsic neurons (Mi), whose neurites are confined within the medulla (Fischbach and Dittrich, 1989). Neurite positions are largely indicative of synapse locations, although some neurites do not stratify and form connections throughout the length of the axon (Takemura et al., 2013, 2008, 2015). The diversity of neurite arborization patterns throughout the medulla is extraordinary. Some neuron types arborize in one specific layer, while others may innervate multiple. Furthermore, certain neurons are confined to single columns, while others may span multiple columns, enabling them to pool visual information from different points in
space (Fischbach and Dittrich, 1989). The medulla is thus a complex network of intricately shaped neuron types, whose beauty still captivates me as much as it did at the beginning of my graduate studies.

Parallel flow of information through distinct layers is also used in the medulla to process the visual scene. For example, color vision is encoded by R7 and R8 photoreceptor cells, which are responsive to UV light and blue-green light, respectively, and whose axons terminate in different layers where they form most of their synapses (Schnaitmann et al., 2018; Takemura et al., 2013, 2008, 2015). Another well-studied example is the ON and OFF pathways, which encode information regarding light increments and decrements, respectively. In this system, L1 and L2 lamina neurons are respectively components of the ON and OFF pathways, and they deliver such information to non-overlapping medulla layers (Arenz, et al., 2017; Bausenwein et al., 1992; Behnia et al., 2014; Takemura, et al., 2013). Layer specific connectivity in the medulla is thus the structural basis for how information is processed in the fly eye.

Developmental strategies for layer assembly

A wealth of studies since the 1940’s strongly indicates that layer specific connectivity, and synaptic connectivity in general, involve interactions between cell surface molecules and the extracellular environment (Yoge and Shen, 2014). In the chemoaffinity hypothesis, Roger W. Sperry posits that synaptic targets find each other via molecular identification tags on the cell surface (Sperry, 1943, 1944, 1963). Since Sperry’s landmark experiments, the field has identified several gene families that encode cell surface proteins involved in important neurodevelopment processes, such as axon guidance (Stoeckli, 2018) and tiling (Sanes and Zipursky, 2010). These
families include Cadherins, Semaphorins, Plexins, and others (de Wit and Ghosh, 2016). While there has been major headway made in identifying the molecular cues involved in circuit formation, we have not yet developed a comprehensive understanding of how these factors are employed by the nervous system to achieve proper connectivity.

There are several methods by which the nervous system establishes layer specific connectivity. One method involves the repurposing of classic axon guidance mechanisms to direct laminar targeting. In the chemoaffinity hypothesis, Sperry hypothesized that gradients of guidance molecules may direct neurites to their proper position. Indeed, later studies in the chick tectum would confirm that RGC projections are topographically positioned based on interactions between Eph kinases present in molecular gradients in the tectum, and their ligands, ephrins (Drescher et al., 1995; Cheng et al., 1995; McLaughlin and O’Leary, 2005). Work from Baier and colleagues have shown that molecular gradients may also direct RGC axon layer specificity in the zebrafish tectum. These studies suggest that Slit, a secreted guidance molecule (Rothberg et al., 1988), is present in the tectum as a molecular gradient, which positions RGC in different lamina via interactions with the Slit receptor Robo2 on the RGC axons (Xiao and Baier, 2007; Xiao et al., 2005; Xiao et al., 2011). In the fly medulla, the classic guidance molecule Netrin (Harris et al., 1996; Mitchell et al., 1996), is concentrated in the M3 layer, where it stabilizes R8 photoreceptor growth cones via interaction with the Netrin receptor Frazzled (Akin and Zipursky, 2016; Pecot et al., 2014; Timofeev et al., 2012) (Figure 1). Thus, classical cell surface interactions are adopted by the developing nervous system to construct laminar circuitry.

A longstanding and intriguing hypothesis has been that a cell surface molecular code directs layer specific connectivity between synaptic partners. Fundamental studies have provided evidence that layer specificity is at least partially governed by cell surface recognition molecules.
Figure 1. The classic guidance molecule Netrin is repurposed in the fly visual system to locally stabilize layer specific targeting. (A) Commissural axons in the vertebrate neural tube (red) achieve long range axon guidance to cross the midline in a Netrin (purple)-dependent manner. (B) In the fly medulla, Netrin (pink) is secreted from L3 growth cones (green) and concentrated in the M3 layer, where it stabilizes the incoming R8 axons (blue). (A) is adapted from Hand and Kolodkin, 2017.

between synaptic pairs. Sanes and colleagues have shown that in the chick IPL, the cell-type specific expression of homophilic transmembrane molecules regulates sublaminae connectivity (Yamagata et al. 2002; Yamagata and Sanes, 2008, 2012). In the chick retina, subfamilies of
immunoglobulin-containing superfamilies (IgSFs) are expressed in largely non-overlapping groups of neurons that innervate the IPL (i.e. BCs, ACs and, RGCs). The proteins encoded by IgSF genes are present in discrete layers throughout the IPL. Moreover, distinct neuron types that target the same layer have been shown to express the same transmembrane molecule. For example, Sidekick-1 (Sdk-1), a homophilic molecule (Goodman et al., 2016; Tang et al., 2018), has been shown to be necessary and sufficient for targeting of a specific sublamina. Similar evidence has been found for other IgSF groups such as Dscams and Contactins (Yamagata et al. 2002; Yamagata and Sanes, 2008, 2012).

Recently, interactions between members of IgSFs have been proposed to underlie layer specific connectivity in the fly optic lobe. Zipursky and colleagues found that during development, lamina neurons (L1-L5), express unique combinations of IgSF genes from several gene families. For example, defective proboscis retraction (dpr) genes (Nakamura et al., 2002), of which there are 17, exhibit unique expression patterns among L1-L5 neurons (Tan et al., 2015). Moreover, the cognate interacting molecules for dprs, dpr-interacting proteins (DIPs), were found to be expressed by corresponding synaptic partners (Cosmanescu et al., 2018; Tan et al., 2015). The expression patterns of dprs and DIPs raise the intriguing possibility that they may coordinate connectivity between synaptic partners. A recent study by Zipursky and colleagues has shown that switching the combination of genes expressed could alter layer specificity (Xu et al., 2018). Whether this a general function for dprs remains to be seen. Still, it appears that synaptic partner matching in both the vertebrate IPL and fly medulla is at least partially regulated by interactions between cognate cell surface molecules in synaptic pairs.

Studies from several labs suggest a model in which layers emerge from step-wise cell type-specific interactions, which first begins with neurite restriction to broad domains. In general,
these studies suggest that initial broad domains are established by the complementary expression of cell surface molecules that position neurites. Work from Kolodkin and colleagues imply that broad domain targeting is a prerequisite for layer specificity in the IPL of the mouse retina. The transmembrane proteins Semaphorin-6A (Sema-6A) and Plexin-A4 (Plex-A4), two known repulsive interacting partners, are present in a non-overlapping manner in the OFF and ON regions, respectively. Normally, tyrosine-hydroxylase positive neurons (TH) and M1-type melanopsin intrinsically photosensitive retinal ganglion cells (M1-RGCs), two synaptic partners, connect in distal most sublamina in the OFF region. In Plex-A4, Sema-6A double mutant mice, TH neurons and M1-RGCs improperly target the ON region. As the complementary expression of Sema-6A and Plex-A4 is observed during development, these data suggest that TH neurons and M1-RGCs are first restricted to the OFF region prior to innervating a sublamina (Matsuoka et al, 2011). A follow up study showed that starburst amacrine cells (SACs) also depend on this mechanism for proper layer specificity (Sun et al., 2013). While this study does not directly show neurite targeting of these cells throughout development, other studies indicate that other cell types establish diffuse arbors that developmentally coincide with the first observed complementary expression of Sema-6A and Plex-A4 (Duan et al., 2014; Lui and Sanes 2017; Peng et al., 2017). Additionally, a previous study shows that a similar mechanism regulates layer specific, subcellular targeting of mossy fibers in the hippocampus (Suto et al., 2007), suggesting that this model of layer assembly may be used in laminar structures in other parts of the nervous system.

Studies in the fly medulla indicate that an analogous design of neurite restriction to direct layer specificity exists in the fly eye (Figure 2). The fly medulla is comprised of three regions, the outer medulla (M1-M6) and inner medulla (M8-M10), which are separated by the serpentine
Figure 2. Broad domain restriction precedes layer specific innervation. (A) In the *Drosophila melanogaster* visual system, L3 neurons (green) are restricted to a broad neuropil domain during an early stage of targeting. Sema-1a on L3 growth interacts with PlexA on medulla tangential neurites (MeT; light blue), a repulsive interaction that prevents them from targeting deeper. Additionally, CadN-mediated adhesion between growth cones redundantly promotes restriction to this region. (B) L3 growth cones then undergo a morphological change, which includes retraction and lateral extension (yellow chevron symbols with red outline) within the developing M3 layer. (C) The process illustrated in (B) results in layer specific innervation of the M3 layer by the L3 growth cone.

layer (M7) (Fischbach and Dittrich, 1989). During development, lamina neurons are restricted to the outer medulla by repulsive interactions between the transmembrane Sema-1A in the growth cone and Plex-A receptors in the serpentine layer. N-Cadherin (CadN)-mediated homophilic attraction between lamina neuron growth cones acts redundantly to Sema-1a/Plex-A repulsion to promote restriction to the outer medulla. When both CadN and Sema1a are knocked out, lamina neurons extend beyond the outer medulla, which ultimately disrupts laminar target specificity.
Each lamina neuron type then establishes layer specific innervation throughout the outer medulla layers (Nern et al., 2008; Pecot et al., 2013). Thus, in the fly eye, layer specific targeting is preceded by neurite restriction to broad, primitive domains.

A likely scenario is that the developing nervous system employs each of these models to establish complex wiring patterns. Moreover, it is unclear how the nervous system coordinates different design strategies for layer assembly. To date, we do not have a precise understanding of the principles and molecular cues that underlie the precedent broad domain restriction and final layer emergence. As broad domain targeting precedes layer specific innervation and connectivity, we believe that this is a critical stage in the stepwise development of laminar circuits. Achieving a comprehensive understanding of the strategies that direct layer assembly will require knowledge of the molecular mechanisms that govern broad domain restriction and coordinate between different methods of layer assembly.

The fly eye: a fruitful model to study layer assembly

This mechanistic investigation necessitates a model system that possesses several important characteristics. At the top of this list is a compact, stereotyped system that is particularly accessible and enables one to confidently assess the consequences of their controlled genetic perturbations. A second critical feature of an ideal system would be genetic tractability that allows exquisite genetic access to specific neuron types throughout development. Finally, as we would like to ultimately examine the functional consequences of our manipulations, a model system that is amenable to physiological and behavioral assays is also necessary.
The fruit fly *Drosophila melanogaster* is an advantageous model that fulfills all of the criteria listed above. Visual system neurons in the fly, in particular those that innervate the medulla neuropil, exhibit incredibly stereotyped morphology, layer specificity, and connectivity. Greater than one hundred different neuron types establish distinct laminar patterns throughout the 10 layers of the medulla. These laminar patterns are largely indicative of synapse location of, although some neurites do not stratify and form connections throughout the length of the axon (Takemura et al., 2013, 2008, 2015). The input neurons of the medulla (R7, R8, and L1-L5) are also genetically accessible, enabling the investigation of cell-autonomous gene function and synapse development (Chen et al., 2014; Lee and Luo, 1999; Millard and Pecot, 2018). The plethora of genetic tools available in the fly has also allowed neuroscientists to measure neural activity and observe fly behavior, leading to the elucidation of the neural components that regulate information processing such as motion detection in the fly visual system (Arenz et al., 2017). Thus, the fly eye is an ideal model to investigate the molecular mechanisms that govern layer specific connectivity.

The *Drosophila melanogaster* visual system is composed of the retina and several neuropil regions that are devoid of cell bodies, containing only processes from neurons and glia (Chotard and Salecker, 2007). The retina is a compound structure consisting of an array of modular units known as ommatidia. Each unit detects visual information via photoreceptors, or R cells (R1-R8). The lamina, the first neuropil, receives input from R1-R6 photoreceptors, which synapse onto select lamina neurons (L1-L3). Retinotopy is established in the lamina, where columnar units known as cartridges each process a distinct point in visual space (Maisak et al., 2013). Photoreceptors R7 and R8, as well as all five lamina neuron types (L1-L5) project through the lamina and terminate within specific layers in the medulla neuropil, wherein
retinotopy is conserved in parallel channels called columns (Maisak et al., 2013). Orientated orthogonally to the columns are the 10 layers that constitute the medulla (M1-M10). The neuropil is split into two segments, the outer medulla (M1-M6) and inner medulla (M8-M10), which are separated by medulla tangential neurites traversing through the serpentine layer (M7). Within the medulla, visual information is processed between input neurons and various neuron classes that also innervate the neuropil. One example is the medulla intrinsic (Mi) neurons, interneurons whose neurites are confined to the medulla. Another class, transmedullary medulla (Tm) neurons, relay visual information to the lobula and lobula plate, two downstream neuropil that are together referred to as the lobula complex. Neural activity processed within the fly optic lobe is ultimately transmitted to higher order central brain regions (Fischbach and Dittrich, 1989).

Within the medulla, information is first processed in the outer region, which receives direct input from neurons R7, R8 and L1-L5. These input neurons all establish unique laminar positions throughout the M1-M6 layers. R7 and R8 cells terminate within the M6 and M3 layers, respectively, maintaining a tubular morphology instead of arborizing within their terminal layers, though each neuron does form most of their connections at those positions (Takemura et al., 2013, 2008, 2015). The L1-L5 lamina neurons arborize throughout layers M1-M5 in a manner that is largely non-overlapping. The terminal positions of these cell types thus encompass all layers within the outer medulla (Fischbach and Dittrich, 1989).

We choose to study the development of these input neurons to gain insight into the developmental rules that underpin layer specific connectivity. The morphology, layer specificity, and connectivity of these cells are incredibly stereotyped and well-characterized (Fischbach and Dittrich, 1989; Nern et al., 2008; Pecot et al., 2013; Takemura et al., 2013, 2008, 2015). As
mentioned above, there are a vast array of genetic tools available to study these cell types. In particular, we can use the clonal technique MARCM (mosaic analysis with a repressible cell marker) to sparsely target and label individual lineages, enabling the assessment of cell-autonomous gene function (Lee and Luo, 1999). Physiological assays to study the functional properties of these neurons are also well-established (Fisher et al., 2015). For these reasons, we anticipate that examining the mechanisms that direct layer specific connectivity of these input neurons will lead to general principles that underlie layer assembly.

**Outer medulla layer assembly occurs in a step-wise manner**

Although R7, R8 and L1-L5 all exhibit distinct morphologies and layer specificities in the adult eye, examining their growth cones during development reveals that these neurons first establish primordial positions in a somewhat overlapping manner. At 24 hours after pupal formation (h APF) (i.e. early pupa), the developing outer medulla is partitioned into two primitive, broad domains, the distal and proximal outer medulla. R7 growth cones are positioned in the proximal most edge of this region, while R8 growth cones are temporarily positioned at the distal most edge, the M0 (Ting et al., 2005). L1-L5 growth cones target the region between R7 and R8 growth cones. L1, L3, and L5 target the proximal domain, while L2 and L4 target the distal domain. Thus, these neurons initially target in an overlapping manner, which is not the case for their final laminar patterns (Figure 3).

Several studies by Zipursky and colleagues have shown that lamina neuron growth cones are in part restricted to the outer medulla via redundant cell surface-mediated mechanisms (Nern et al., 2008; Pecot et al., 2013). The transmembrane proteins Sema-1A and CadN act cell-
Figure 3. Lamina neuron layer specificity is preceded by an overlapping growth cone targeting pattern. (A) During early pupal development, the outer medulla is partitioned in two regions, the distal and proximal domains. L1-L5 growth cones are positioned in one of these two domains. (B) The primordial targeting pattern exhibited in (A) is followed by a series of morphological changes that results in the layer specific arborization patterns observed in the adult.

autonomously to restrict L1, L3 and L5 neurons to the outer medulla. When Sema-1a and CadN are both knocked out, L1, L3, and L5 target beyond the outer medulla, which disrupts their segregation into their respective layers later in development (see below). Data from this study suggest that Sema-1a on the growth cones encounters Plex-A in the developing serpentine layer, producing a repulsive interaction. In addition, CadN-mediated homophilic interactions between growth cones attract them to the outer medulla (Pecot et al., 2013). L2 and L4 neurons, on the other hand, are restricted to the distal domain via an unknown mechanism.

As development progresses, the medulla expands, likely due to innervation from other neurites, and the input neuron growth cones segregate into their developing layers. In some
cases, growth cones merely remain in their original positions, which will become their final layers. For example, the proximal most region that R7 targets will ultimately become the M6 layer. On the other hand, other types undergo morphological changes that result in arborization within discrete layers. In the case of the L3 neuron, the growth cone retracts from the proximal domain, while simultaneously expanding laterally, ultimately segregating into the developing M3 layer. By 48 h APF (i.e. midpupa), the layers are discernible, based on the terminal positions of input terminals (e.g. R7 and L3) (Nern et al., 2008; Pecot et al., 2013).

The emergence of discernible layers by midpupal development is followed by the delayed innervation of other neurons into those layers. During midpupal development, R8 growth cones begin to extend from the M0 layer towards the developing M3. Within the M3 layer, the R8 terminal is stabilized by interactions between Netrin in the neuropil, and the Netrin receptor Frazzled on the growth cone. Interestingly, L3 growth cones, which segregate into the M3 layer before R8, are the source of Netrin (Pecot et al., 2014; Timofeev et al., 2012). Layer assembly in the outer medulla is thus a dynamic, step-wise process involving cooperative interactions between distinct cell types.

Several key questions must be answered if we are to develop a solid understanding of the principles underlying layer specific connectivity (Figure 4). It is currently unclear which mechanisms determine the decision between stopping at the distal outer medulla or innervating the proximal region, which, according to the current evidence, is critical for laminar targeting. Secondly, we do not know how timely, interdependent layer innervation is coordinated between distinct growth cones. For example, R8 targeting succeeds the segregation of L3 growth cone into the M3 layer in a manner that is dependent on Netrin, an L3-derived cue (see above). How
are these steps coordinated by the developing nervous system? Finally, we must still determine if layer specificity is requisite for synaptic specificity.

In this dissertation, we examine the molecular mechanism that directs L3 neurons to the proximal outer medulla, and ultimately the M3 layer. We find that the conserved transcription factor, dFezf, cell-autonomously controls L3 innervation of the proximal domain, which is critical for L3 layer position and connectivity. In RNA-sequencing experiments, we show that cell surface genes are enriched in the list of differentially expressed genes in dFezf mutant L3 neurons. Interestingly, many of the cell surface genes affected are members of the same IgSF gene families, suggesting that dFezf targets a battery of related genes to direct L3 targeting and connectivity. We also illustrate that dFezf coordinates L3 and R8 targeting of the same layer. dFezf activates Netrin in L3 neurons, which is secreted into the M3 layer, where it stabilizes R8 terminals that target the layer directly. Thus, dFezf acts as a molecular link between different steps of layer assembly. Considering the conservation of Fez family of transcription factors across species (Eckler and Chen, 2014), and the structural and mechanistic similarities between layered neuropil in the fly and vertebrate visual systems (Sanes and Zipursky, 2010), we anticipate that this strategy is a fundamental principle of layer assembly.
Figure 4. Open questions in layer assembly of the outer medulla. (A) While the mechanisms that restrict lamina neuron growth cones to the outer medulla during early pupal development are partially understood, the mechanisms that direct growth cones to the distal or proximal domains remain enigmatic. (B) The secretion of Netrin into the M3 layer by the L3 neuron is a critical step in coordinated layer specificity between the L3 and R8. The upstream regulator of Netrin expression in the L3 is unclear. (C) Once within the proper layer, neurons must choose their appropriate synaptic partners. The molecular logic that underlies synaptic pairing within the layer is not well understood.
References


II. Drosophila Fezf coordinates laminar-specific connectivity through cell-intrinsic and cell-extrinsic mechanisms

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Introduction

Precise neural connectivity underlies the structural organization and function of the nervous system. In the nervous systems of diverse organisms, neural connections are arranged into layered networks, wherein each layer is defined by a unique cellular composition and the axonal arborizations of different input neurons are restricted to specific layers. This organizational strategy is thought to optimize the precision of synaptic connectivity and information processing. Layered connections define many regions of the vertebrate nervous system, including the cerebral cortex (mammals), the spinal cord and retina, and connections in the insect optic lobe are also organized in a layer specific manner. Elucidating how neurons organize into layered networks is crucial for understanding how the precision of neural connectivity is achieved, and may provide key insights into neural function.

Previous studies in both vertebrates and invertebrates have identified molecules that are necessary for layer specificity in particular neuron types (reviewed in) (Baier, 2013 Huberman; et al., 2010; Sanes and Yamagata, 1999; Sanes and Zipursky, 2010). However, the molecular mechanisms governing the assembly of specific layers remain poorly characterized. For example, how different neurons innervate the same layer, and whether they do so through shared or different mechanisms is not known. To address this, we study layer formation in the Drosophila melanogaster visual system, wherein the cell types that innervate specific layers are well-described and genetically accessible.

In the fly optic lobe (Figure 5A), visual input converges on the medulla, wherein it is processed within parallel layers. The medulla receives input directly from R7 and R8 photoreceptors, which are UV and blue/green sensitive, respectively, and indirectly from broadly
Figure 5. The Drosophila visual system and lamina monopolar neurons. (A) Anatomy of the Drosophila visual system (Adapted from Fischbach and Dittrich, 1989). The optic lobe
comprises four consecutive neuropil regions called the lamina, medulla, lobula and lobula plate. (B) Cartoon of lamina neuron axons in adult flies. The nearly mutually exclusive axonal arborizations of lamina neurons help define layers M1-M5. (C) Cartoon of lamina neuron growth cones in early pupal development. Prior to innervating discrete layers, lamina growth cones terminate in broad distal or proximal domains within the outer medulla.

tuned photoreceptors (R1-R6) through second order lamina monopolar neurons L1-L5 (lamina neurons). Photoreceptor and lamina neuron axons innervate discrete medulla layers and synapse with medulla neurons that process information and transmit signals to higher centers. Information from specific regions of the visual field is processed in modular columnar units, organized perpendicular to the layers. Lamina and photoreceptor axons carrying input from the same point in space converge on targets in the same column. Input from neighboring points in space is processed in neighboring columns, establishing a retinotopic map. The medulla comprises ten layers (M1-M10) organized into outer (M1-M6) and inner (M8-M10) regions that are divided by tangential processes that form the serpentine layer (i.e. M7) (Fischbach and Dittrich, 1989) (Figure 5A). The cell bodies of medulla neurons are excluded from the layers, and thus layered connections develop within a dense meshwork of cellular processes. Laminar-specific connections within the inner plexiform layer (IPL) of the vertebrate retina develop in a similar manner. Medulla layers are defined in adult flies by the morphologies of the axon terminals and dendritic branches of particular cell types, which in general overlap completely or not at all (Figure 5B). The positions of these processes are largely indicative of the location of synapses, although some neurites do not stratify and form synapses en passant (Takemura et al.,
2013, 2008, 2015). In total, ~40,000 neurons that fall into more than sixty cell types form connections within one or more layers (Fischbach and Dittrich, 1989).

Studies of lamina neuron and photoreceptor axon development indicate that medulla layers emerge dynamically from broad domains. The morphologies of L1-L5 axons define layers M1-M5 in the outer medulla of adult animals (Fischbach and Dittrich, 1989) (Figure 5B). However, in early pupal development the outer medulla is a fraction of its adult size, and lamina growth cones terminate in two broad domains (Figure 5C) (Nern et al., 2008; Pecot et al., 2013). L2 and L4 growth cones terminate in a distal domain of the outer medulla, while L1, L3 and L5 growth cones terminate in a proximal domain. The mechanisms underlying specificity for the distal or proximal domain are not known. Nevertheless, these findings indicate that lamina neurons innervate broad domains prior to segregating into discrete layers. In the mouse IPL, it has been proposed that neurons initially innervate broad domains defined by the complementary expression of repulsive cell surface molecules (Matsuoka et al., 2011). Taken together, these studies suggest the precedence of broad domain innervation prior to layer innervation may represent a conserved developmental strategy for constructing layer specific circuits.

Characterization of the targeting of L3 axons and the axons of R8 photoreceptors to the M3 layer has provided insight into how discrete layers emerge from broad domains. L3 and R8 axons innervate M3 sequentially during development. R8 neurons are born first and project axons that terminate near the distal surface of the medulla (known as M0) (Ting et al., 2005). L3 axons within the same column project past R8 axons and terminate within a broad domain in the proximal outer medulla (discussed above) (Nern et al., 2008; Pecot et al., 2013). Subsequently, L3 growth cones segregate into the developing M3 layer through a stereotyped structural rearrangement that is regulated by mechanisms that are not well understood (Pecot et al., 2013).
Within M3, L3 growth cones secrete Netrin, which acts locally to regulate the attachment of R8 growth cones within the layer after they extend from M0 (Akin and Zipursky, 2016; Pecot et al., 2014; Timofeev et al., 2012). Thus, the M3 layer develops in part through the stepwise innervation of L3 and R8 axons, and R8 layer specificity depends on a signal from L3 growth cones.

Taken together, these developmental studies point to a model, wherein outer medulla layers emerge in a stepwise manner from broad domains through a precise sequence of interactions between specific cell types. To gain insight into the molecular mechanisms that control stepwise layer assembly we concentrate on assembly of the M3 layer, and in this study focus on L3 neurons. Previously, we found that Drosophila Fez f (dFezf)/Earmuff, the Drosophila ortholog of the Fezf zinc finger transcription factors in vertebrates (Fezf1/2) (Hashimoto et al., 2000a, 2000b; Matsuo-Takasaki et al., 2000; Pfeiffer et al., 2008; Weng et al., 2010), is selectively expressed in L3 neurons in the lamina (Tan et al., 2015). Thus, we reasoned that dFezf might regulate the expression of genes that control cell-specific aspects of L3 development, including broad domain specificity, layer specificity, and synaptic specificity within the target layer. Interestingly, our genetic analyses of dFezf function in L3 neurons revealed a critical role for dFezf in regulating the M3-specific innervation of both L3 and R8 axons. DFezf functions cell-autonomously to promote the targeting of L3 growth cones to the proximal domain of the outer medulla, which is essential for L3 layer specificity. We find that members of the dpr cell surface gene family (Nakamura et al., 2002) are prominent direct or indirect dFezf targets in L3 neurons. We propose that dFezf regulates growth cone targeting by controlling the expression of dpr proteins that mediate interactions with target cells in the medulla. In addition, we show that dFezf non-cell-autonomously regulates R8 layer specificity.
through the activation of Netrin expression in L3 neurons. When dFezf function is lost in L3 neurons, L3 and R8 axons innervate inappropriate layers while the dendrites of a common synaptic target (Tm9) innervate the M3 layer normally. As a result, synaptic connectivity with the target cell is disrupted. We conclude that dFezf represents a transcriptional module that constructs M3 layer circuitry by controlling the stepwise layer innervation of specific cell types through cell-intrinsic and cell-extrinsic mechanisms. We propose that the use of such modules to coordinate neural connections within correct layers is a widespread strategy for building laminar-specific circuits.

Results

DFezf is selectively expressed in L3 neurons throughout development and in adult flies

As a first step towards characterizing the function of dFezf in L3 neurons we examined its expression during development. Previously, we showed that dFezf is expressed in L3 neurons at 40 hr after pupal formation (h APF) (Tan et al., 2015), when L3 growth cones are in the process of segregating into the developing M3 layer (Pecot et al., 2013). To assess if dFezf is also expressed in L3 neurons at other stages of development and determine whether expression remains restricted to L3 neurons, we assessed dFezf immunostaining in the lamina using confocal microscopy. We discovered that dFezf is expressed in L3 neurons from early pupal stages (at least as early as 12 hr APF) through to adulthood, but is not expressed in late third instar larvae when lamina neurons begin to differentiate (Figure 6A–D). DFezf expression was not detected in lamina neuron precursor cells or any other cells besides L3 at any stage of development in the lamina. The timing and selectivity of dFezf expression is consistent with dFezf playing an important role in regulating L3 development.
Figure 6. DFezf is cell autonomously required for L3 layer specificity and axon terminal morphology. (A–D) Confocal images showing dFezf protein expression in the lamina from the late 3rd instar larval stage through to adulthood (newly eclosed flies). DFezf expression (green)
Figure 6 (continued) was observed through immunohistochemistry using a specific antibody. Elav immunostaining (blue) labels all lamina neurons (LNs). At least five brains were examined per time point. Scale bar = 5 microns and applies to all images. (A) DFezf (green) is not expressed in the lamina during the 3rd Instar larval stage. Lamina neurons (blue) differentiate in close proximity to R1-R6 axons (white, mAB24B10) forming columnar-like cartridges oriented orthogonally to the lamina plexus (thick white band) comprising R1-R6 terminals. (B–D) DFezf (green) is expressed exclusively in L3 neurons in the lamina during pupal development and in newly eclosed adults. L3 neurons (magenta) express myr::GFP (anti-GFP) driven by 9–9 GAL4 (Nern et al., 2008). Asterisks in (B) indicate dFezf-expressing lamina neurons (green + blue) that are most likely L3 neurons that have yet to turn on GAL4 expression (youngest L3 neurons). (E–N) MARCM + STaR experiments in adult flies. See Materials and methods section for a detailed description. Scale bars when shown are five microns with the exception of the scale bar shown in (E), which is twenty microns. L3 clones (green) expressed myr::tandem Tomato (tdTomato) and were visualized using a DsRed antibody. (E) Broad view (confocal image) of the morphologies of wild type L3 clones (green) in the lamina and medulla, which are indicated by R1-R8 axons (magenta, mAB24B10). Boxed regions indicate examples of the regions shown in confocal images in F–I, M and N. (F–I) Confocal images of terminals from wild type or dFezf1 L3 neurons (green), within the outer medulla (M1-M6), defined by R7 and R8 axons (magenta, mAB24B10). (J) Adult MARCM quantification. N is the total number of neurons counted per genotype. 10 brains were analyzed for each genotype. (K, L) Confocal images show L3 clones (magenta) within the outer medulla and, within L3 terminals, Brp (green) expression and localization. Brp expressed from its native promoter within a bacterial artificial chromosome (BAC) was selectively tagged with smFPV5 in L3 MARCM clones, and visualized using an anti-
V5 antibody. N-Cadherin (CadN) immunostaining (blue) serves as a neuropil marker. All dFezf1 L3 neurons displayed Brp puncta in their terminals. At least five brains were analyzed for each genotype. (M and N) Morphologies of dendrites from wild type or dFezf1 L3 neurons in the lamina. The lamina is indicated by R1-R6 axons (magenta, mAB24B10). (O–Q) MARCM experiments analyzed at 24 or 48 hr APF. DFezf1 L3 clones (green) express myr::GFP (9–9 GAL4) and are visualized using a GFP antibody. Co-labeling of L3 somas and transcription factors specific for other lamina neuron subtypes (using specific antibodies) show that the mutant neurons do not express these proteins. (P and Q) R1-R6 axons (blue, mAB24B10) demarcate lamina cartridges and the lamina plexus.
DFezf is cell autonomously required for L3 layer specificity

To investigate the function of dFezf in L3 neurons we used mosaic analysis with a repressible cell marker (MARCM) (Lee and Luo, 1999) in conjunction with synaptic tagging with recombination (STaR) (Chen et al., 2014). This allowed us to generate single, fluorescently labeled wild type or dFezf mutant L3 neurons while simultaneously visualizing the active zone protein Bruchpilot (Brp), expressed from its native promoter (see Materials and methods for detailed description). The dFezf null allele used in these experiments (dFezf1) contains a single A to T nucleotide change leading to the substitution of a leucine for a conserved histidine in the third zinc-finger domain (Weng et al., 2010). We found that L3 neurons homozygous for dFezf1 displayed defects in axon terminal morphology and layer specificity in the medulla. Wild type L3 neurons elaborate stratified axon terminals within the M3 layer (100%) (Figure 6E, F, J). The large majority (84%) of the axons of dFezf1 L3 neurons terminated inappropriately within layers distal to M3 (i.e. M1-M2) (Figure 6G, J) and had abnormally shaped terminals (compare Figure 6F and G). The remaining 16% of the axons either terminated in the M3 layer (Figure 6H, J) or in more proximal layers (e.g. M4-M6) (Figure 6I, J), and these also had terminal-like swellings in M1-M2 (Figure 6H, I). Presynaptic Brp puncta were present in mis-targeting L3 terminals (Figure 6K, L), suggesting that mutant neurons formed synaptic connections within incorrect layers. However, whether these represent active synapses, and if so whether they are established with appropriate partners, inappropriate partners, or both is unclear. In the lamina, L3 neurons display a unique dendritic projection pattern in which all dendrites project to one side of the neurite (Figure 6M). Dendritic processes were largely normal in dFezf null neurons (compare Figure 6M and N). In addition, the mutant L3 neurons did not express transcription factors specific to other lamina neurons (Figure 6O–Q) and still expressed L3-specific markers early in
development (see Figure 7), showing that disrupting dFezf did not cause an obvious change in cell fate. However, some L3-specific genes are downregulated in dFezf L3 neurons, and genes expressed in other lamina neurons become upregulated (see below). Taken together, these findings demonstrate that dFezf is cell autonomously required for proper axon terminal morphology and layer specificity, yet dispensable for dendrite formation.
Figure 7. DFezf is required for the targeting of L3 growth cones to the proximal domain of the outer medulla. (A–R) MARCM experiments analyzed at 24 or 48 hr APF. L3 clones expressed myr::GFP driven by 9–9 GAL4 and were visualized in confocal images using a GFP
**Figure 7 (continued)** antibody. In confocal images, mAB24B10 (magenta) was used to label photoreceptor axons, which demarcate the lamina and medulla. Within the medulla, mAB24B10 labels R8 and R7 growth cones, which define the boundaries of the outer medulla. In some images R7 growth cones may appear faint. This reflects normal variations in antibody staining between brains of slightly different ages (e.g. ±2 hr). No defects in photoreceptor axon targeting were observed in these experiments. (A) Broad view of the morphologies of wild type L3 clones (green) within the lamina and medulla (indicated by R1-R8 axons, magenta [mAB24B10]) at 24 hr APF. The solid boxed region indicates an example of confocal images shown in B-D. The dashed boxed region shows an example of a newly born L3 clone in the most lateral part of the medulla array similar to N and O. M-L indicates medial and lateral. Scale bar = 20 microns. (B–D, G–J, M–Q) Confocal images show a zoomed in view of L3 growth cones within the outer medulla. At these stages of development, the boundaries of the outer medulla are defined by R7 and R8 growth cones (magenta, mAB24B10). Scale bars = 5 microns. (E, K, L, R) Quantification of MARCM experiments. N is the total number of neurons per genotype that were counted. (E) WT (seven brains), dFezf1 (12 brains), CadN, Sema-1a, dFezf1 (two brains). (all) means that all L3 clones were counted while (yng) indicates that the L3 clones present within the most lateral five medulla columns containing R7 growth cones were counted (i.e. youngest L3 clones). Prox. OM = proximal domain of the outer medulla, Distal OM = distal domain of the outer medulla. (F) Broad view of the morphologies of wild type L3 clones in the lamina and medulla (indicated by R1-R8 axons, magenta [mAB24B10]) at 48 hr APF. The solid boxed region is an example of regions shown in confocal images in G-J. Scale bar = 20 microns. (I and J) Arrowheads indicate abnormal swellings within the developing M1-M2 layers. (K) Wild type (six brains), dFezf1 (six brains). (L) Prox. OM = proximal domain of the outer medulla, Distal
OM = distal domain of the outer medulla. (P and Q) Arrowheads indicate the depths within the medulla at which L3 growth cones terminate. The serpentine layer (indicated in Q) lies directly beneath the outer medulla. (R) CadN, Sema-1a (two brains), CadN, Sema-1a, dFezf1 (two brains).
DFezf is necessary for targeting to the proximal domain of the outer medulla.

To determine how disrupting dFezf affects L3 axon targeting during development, we compared the axonal morphologies of wild type and dFezf1 neurons during pupal development using MARCM. In early pupal development (~24 hr APF), the axons of wild type L3 neurons terminate in the proximal domain of the outer medulla directly above R7 growth cones (Figure 7A, B, E). The axons of dFezf1 neurons incorrectly terminated within the distal domain of the outer medulla (Figure 7C–E). Under these conditions L3 growth cones terminated just underneath the growth cones of R8 photoreceptors (Figure 7C), and occasionally terminated slightly more distally so that they completely overlapped with R8 growth cones (Figure 7D). By mid-pupal development (~48 hr APF), the growth cones of wild type L3 neurons have segregated into the developing M3 layer (Figure 7F, G, K). However, we found that all of the growth cones of dFezf1 L3 neurons displayed abnormal morphologies, defects in layer specificity, or both. Most of the abnormal growth cones (67%) exclusively innervated the developing M1 and M2 layers (Figure 7H, K). The remaining 33% of the growth cones terminated in the developing M3 layer (Figure 7I, K) or in more proximal layers (e.g. M4-M6) (Figure 7J, K). However, each of these growth cones also displayed abnormal swellings in distal layers (i.e. the developing M1 and M2 layers) (Figure 7I, J, arrowheads). These defects in morphology and layer innervation are consistent with the abnormalities observed in adult flies under these conditions. We observed similar deficits in L3 growth cone targeting in MARCM experiments when we used a different dFezf mutant allele (dFezf2) that eliminates the entire dFezf open reading frame (Weng et al., 2010) (Supplementary figure 1). Moreover, defects in growth cone targeting during pupal development, as well as abnormal layer innervation in adult flies caused by dFezf1 or dFezf2 were completely rescued by a single copy of a bacterial artificial
chromosome (BAC) containing the dFezf locus (Figure 7L, M, and Supplementary figure 2).

Together, these findings demonstrate that dFezf is cell-autonomously required for the targeting of L3 growth cones to the proximal domain of the outer medulla, which may be necessary for the subsequent segregation of the growth cones into the developing M3 layer.

We reasoned that the growth cones of dFezf null L3 neurons could directly terminate within the distal domain of the outer medulla, or could initially target correctly to the proximal domain and then retract. Direct mis-targeting would be consistent with a change in axon target specificity, while retraction would suggest a defect in stabilizing growth cone position. To distinguish between these possibilities, we took two approaches. First, in MARCM experiments we focused on the L3 clones that had most recently innervated the medulla (i.e. the youngest clones, see dashed box in Figure 7A). Unlike the youngest wild type clones, the large majority of the nascent dFezf1 clones (82%) terminated within the distal domain of the outer medulla similar to their older counter parts (Figure 7E, N, O), consistent with most of these neurons directly innervating this region. Second, we investigated growth cone targeting when dFezf was disrupted in combination with CadN and Sema-1a. CadN and Sema-1a function synergistically to restrict L3 growth cones to the outer medulla during pupal development, preventing innervation of the serpentine layer and inner medulla (Pecot et al., 2013). We hypothesized that if the growth cones of dFezf1 L3 neurons initially target correctly and then retract, then in the absence of CadN and Sema-1a function the growth cones would inappropriately innervate the serpentine layer and inner medulla, as in CadN and Sema-1a double mutant neurons. Conversely, if the growth cones of dFezf1 L3 neurons directly mistarget to the distal outer medulla, this should prevent mistargeting beyond the outer medulla caused by the loss of CadN and Sema-1a function, and the triple KO phenotype should resemble the loss of dFezf function only. MARCM experiments
revealed the latter to be correct. L3 neurons lacking the function of dFezf, CadN and Sema-1a predominantly innervated the distal domain of the outer medulla in early pupal development (Figure 7E, P), similar to L3 neurons lacking dFezf function only (Figure 7C–E), although the triple KO neurons more frequently terminated within the R8 growth cone layer. Importantly, the triple KO neurons did not mistarget beyond the outer medulla like CadN/Sema-1a double mutant L3 neurons (Figure 7Q, R). Combined with our analyses of newly born dFezf: L3 neurons, these data suggest that L3 neurons lacking dFezf function directly innervate the distal domain of the outer medulla, which is consistent with a switch in broad domain specificity.

*DFezf acts instructively to regulate growth cone targeting to the proximal domain of the outer medulla*

DFezf could function permissively or in an instructive manner to regulate the targeting of L3 growth cones to the proximal domain of the outer medulla. To discriminate between these possibilities, we performed mis-expression experiments in other lamina neurons. If dFezf function is instructive, then mis-expressing it in L2 or L4 neurons, which normally target to the distal domain of the outer medulla (Figure 8C), should cause their growth cones to terminate in the proximal domain. By contrast, as L1 and L5 growth cones normally terminate in the proximal domain (Figure 8C), dFezf mis-expression in these neurons should not affect growth cone targeting. For technical reasons we decided to focus on L2 and L5 neurons. In L5 neurons, we mis-expressed cDNA encoding dFezf-HA using 6–60-GAL4, which has been shown to selectively drive expression in L5 neurons in the lamina (Nern et al., 2008). In these experiments all L5 neurons and a small number of medulla and lobula neurons expressed dFezf-HA, as determined through HA immunostaining (Figure 8A, and Supplementary figure 3A), and a subset of L5 neurons were specifically labeled by myr::GFP (Figure 8A). We found that the
expression of dFezf-HA had no effect on L5 growth cone position in early pupal development. The growth cones of dFezf-expressing L5 neurons innervated the proximal domain of the outer medulla in a manner indistinguishable from wild type neurons (Figure 8B–D). In adult flies, L5 terminals are confined to the M5 layer, and L5 neurons also arborize within distal layers M1 and M2 through branching that occurs off the main neurite during mid-late pupal development (Figure 8E). DFezf expression did not affect the layer position or morphology of L5 terminals, but caused defects in the distal arborizations of the vast majority of L5 neurons (Figure 8F–H). 82% of L5 neurons mis-expressing dFezf displayed reduced distal arborizations that failed to extend into the M2 layer (Figure 8F, H), and 15% of neurons extended distal branches into neighboring columns (Figure 8G, H). These findings, along with the fact that dFezf null L3 neurons inappropriately arborize in distal medulla layers, suggests that dFezf function may antagonize innervation of the distal outer medulla.
Figure 8. DFezf plays an instructive role in regulating growth cone targeting. (A–H) L5 mis-expression experiments. DFezf-HA was expressed in L5 neurons using 6–60 GAL4, and sparse labeling of L5 neurons (myr::GFP, a-GFP) was achieved using the FLP-Out method (see Materials and methods for detailed description). (A) DFezf-HA (magenta) is expressed in all L5 neurons, a subset of which are labeled with myr::GFP (green) using FLP-Out. L5 neurons are easily identified based on position, as their somas are located directly distal to the lamina plexus defined by R1-R6 axons (blue, mAB24B10), which appears as a thick band. (B and C) Confocal images of L5 growth cones (green) within the outer medulla at 24 hr APF. R7 and R8 growth cones (blue, mAB24B10) define the boundaries of the outer medulla. (D) Quantification of L5 mis-expression experiments at 24 hr APF. N = the number of cells counted. (E–G) Confocal images of L5 axons (green) within the outer medulla in adult flies. Outer medulla layers are identified using R8 and R7 axons (mAB24B10, blue) as a reference. (H) Quantification of L5 mis-expression experiments in adult flies. N = the number of cells counted. (I–O) Misexpression
Figure 8 (continued) of dFezf-HA in L2 neurons in MARCM experiments. DFezf-HA was expressed in lamina neuron clones (green, myr::GFP, α-GFP) by 11–164-GAL4 (Nern et al., 2008). L2 neurons (magenta) were labeled with myr::tdTomato (α-DsRed) driven by 16H03-LexA. L2 MARCM clones are double positive for myr::GFP and myr::tdTomato and appear white. (I–I’’) DFezf-HA (blue, a-HA) is expressed in L2 MARCM clones (white) that are double positive for myr::GFP and myr::tdTomato. (J and K) Confocal images showing L2 growth cone targeting within the outer medulla at 24 hr APF. R7 and R8 growth cones (blue, mAB24B10) define the boundaries of the outer medulla. Scale bar = 5 microns. (L) Quantification of L2 mis-expression experiments at 24 hr APF. N = the number of cells counted. (M and N) Confocal images showing L2 axon targeting within the outer medulla in adult flies. R7 and R8 axons (blue, mAB24B10) define outer medulla layers. Scale bar = 5 microns. (O) Quantification of L2 mis-expression experiments in adult flies. N = the number of cells counted.
To assess the effects of mis-expressing \textit{dFezf} in L2 neurons, we expressed cDNA encoding \textit{dFezf-HA} in lamina neurons using a pan-lamina neuron GAL4 driver (11–164-GAL4) in MARCM experiments, and labeled L2 neurons using an L2-specific LexA driver (16H03-LexA). The expression of \textit{dFezf-HA} was assessed through HA immunostaining (Figure 8I–I’’). In these experiments, GAL80 prevented GAL4 expression (and therefore \textit{dFezf-HA} expression) in lamina neurons that did not undergo mitotic recombination (non-clones), which represented the large majority of lamina neurons. In lamina neuron clones GAL4 induced the expression of \textit{dFezf-HA} and GFP, and the GFP positive clones that also expressed the L2 marker were confirmed as L2 clones. Only isolated L2 clones within each column were considered, eliminating the possibility of non-autonomous effects from \textit{dFezf} mis-expression in other lamina neurons in the same column. Using these criteria, we found that, in early pupal development, the growth cones of all L2 neurons mis-expressing \textit{dFezf-HA} inappropriately terminated within the proximal domain of the outer medulla (Figure 8J–L), and in adult flies the large majority of these neurons (88%) incorrectly innervated the M3 layer (Figure 8M–O). Similar results were found in MARCM experiments wherein a sparser distribution of lamina neuron clones was generated, and only \textit{dFezf-HA} expressing L2 neurons that were isolated in the home column and not next to lamina neuron clones in neighboring columns were considered (Supplementary figure 3B–D). In all cases \textit{dFezf-HA} expressing L2 neurons still displayed terminal-like swellings within the M2 layer, indicating a partial rather than complete change in layer specificity. These experiments demonstrate that mis-expressing \textit{dFezf} in L2 neurons is sufficient to induce innervation of the proximal domain of the outer medulla, and cause ectopic innervation of the L3 target layer M3. Collectively, our mis-expression studies in L2 and L5 neurons support that \textit{dFezf} acts instructively to direct growth cone targeting. We hypothesize that \textit{dFezf} achieves this by
antagonizing innervation of the distal outer medulla and promoting innervation of the proximal outer medulla (see discussion).

**DFezf regulates a complex program of cell surface gene expression**

To gain insight into the mechanism by which dFezf regulates L3 growth cone targeting, we sought to identify genes that are differentially expressed between wild type and dFezf null L3 neurons, as these would represent candidate direct or indirect dFezf targets. To accomplish this, we isolated fluorescently labeled wild type or dFezf null L3 MARCM clones by FACS at 40 hr APF (Figure 9A, B), prepared cDNA libraries, and sequenced the libraries using an Illumina platform. Five replicates were performed for wild type and mutant genotypes, and to minimize technical variability all ten cDNA libraries were prepared and sequenced at the same time. Differential expression analysis identified 455 differentially expressed (DE) genes (padj <0.05) (Figure 9C), and changes in the expression of several of these were verified at the protein level (Supplementary figure 4). Using previously curated databases (Kurusu et al., 2008; Tan et al., 2015), we found that 110 of the 455 DE genes are cell surface and secreted genes (Supplementary figure 5), consistent with a role for dFezf in regulating cell-cell communication. Large fractions of DE genes were up or downregulated (190 and 265 genes, respectively) (see Figure 9C), indicating that dFezf function serves to both activate and repress gene expression, either directly or through intermediate regulators. This also suggested that defects in growth cone targeting when dFezf is disrupted could result from the downregulation of particular genes, the upregulation of genes, or both.
Figure 9. DFezf regulates a complex program of cell surface gene expression. (A and B) MARCM was performed to generate and specifically label single wild type or dFezf1 L3 neurons at 40 hr APF. L3 clones were labeled with a nuclear reporter (H2A-GFP) and a transmembrane reporter (myr::tdTomato) by 9–9-GAL4. (A) A representative confocal image shows that L3 neurons are the only cell type labeled by both GFP (green) and tdTOM (magenta). Photoreceptor neurons (R1-R8) (blue, mAB24B10) are labeled as a reference for the lamina and medulla. Scale bar = 20 microns. (B) A representative FACS plot shows that L3 neurons expressing H2A-GFP and myr::tdTomato separated as a tight cluster from background cells (100K events are shown). (C) A heat map (row scaled) of differentially expressed genes that includes data from all
Figure 9 (continued) samples. Each row represents the normalized expression of a differentially expressed gene, and each column represents a sample. Presenting the data in this manner shows that samples clearly separate based on genotype, and that large fractions of differentially expressed genes are either up or downregulated. (D) Table showing dpr genes that are differentially expressed between wild type and dFezf null L3 neurons. The genes are listed in order of significance (padj). The top three most significant DE genes overall, dprs 17, 13, and 1 are shown in red, and the rest of the genes are shown in green. TPM (transcripts per million mapped reads) values are averages across all samples for a given genotype.
Since CadN and Sema-1a play a crucial role in L3 growth cone targeting (Nern et al., 2008; Pecot et al., 2013), we hypothesized that dFezf might regulate their expression as part of a gene program controlling growth cone position. However, CadN and Sema-1a mRNA levels in wild type and dFezf mutant L3 neurons were not significantly different (Supplementary figure 6A,B), demonstrating that dFezf is not necessary for their expression at 40 hr APF. In line with this, CadN and Sema-1a double mutant L3 neurons display defects in dendrite morphology (Supplementary figure 6C), but dendrites were qualitatively normal in the absence of dFezf function (Supplementary figure 6C), indicating that disrupting dFezf does not affect CadN and Sema-1a function. Together, these data strongly support that dFezf acts in parallel to CadN and Sema-1a to regulate L3 growth cone targeting within the outer medulla (see discussion).

Many genes are differentially expressed between wild type and dFezf null L3 neurons, and are thus candidates for regulating growth cone targeting. However, ranking the DE genes based on significance revealed several intriguing candidates. For example, using these criteria, the top three genes are dprs 17, 13, and 1, respectively, which are members of the dpr cell surface family (Nakamura et al., 2002). The mRNA levels of all three genes change more than 1000-fold when dFezf is disrupted in L3 neurons (Figure 8D). Dpr 17 is exclusively expressed in L3 neurons in the lamina and dpr1 is expressed in L3 and L4 neurons (Tan et al., 2015). Both are downregulated in the absence of dFezf function, and for dpr17 we confirmed this at the protein level (Supplementary figure 4A–B’). Dpr13 is highly expressed in L2 neurons and moderately expressed in L4 neurons (Tan et al., 2015), and is upregulated in dFezf null L3 neurons. In addition to these, eight other dpr genes are differentially expressed between wild type and dFezf null L3 neurons (Figure 8D), suggesting that dpr genes are prominent direct or indirect dFezf targets.
Dpr proteins are immunoglobulin (Ig) domain containing cell surface molecules (21 dprs) that bind heterophilically to dpr interacting proteins (DIPs) (9 DIPs) (Carrillo et al., 2015; Özkan et al., 2013), which are also cell surface molecules and members of the Ig superfamily (IgSF).

Dpr and DIP proteins are expressed in a matching manner between lamina neurons (including L3) and their synaptic targets in the medulla, and specific dpr-DIP interactions, or combinations of them, have been proposed to encode synaptic specificity (Tan et al., 2015). Thus, one possibility is that dFezf regulates L3 growth cone position by controlling the expression of specific dpr genes, whose products mediate interactions with appropriate DIP-expressing target cells in the medulla. In the absence of dFezf function, an abnormal complement of dprs is expressed (L3 dprs are downregulated and dprs expressed by other lamina neurons are upregulated), and this may promote interactions with incorrect target neurons, altering growth cone position and laminar specificity (see discussion).

In summary, a large number of genes change in their levels of expression when dFezf is disrupted in L3 neurons, and similar numbers of these are up or downregulated. Nearly a quarter of the DE genes are cell surface and secreted molecules, suggesting that dFezf regulates communication between L3 and other cells. Dpr genes are strong candidates for acting downstream of dFezf to regulate growth cone targeting. However, as many candidates exist, additional studies are necessary to identify the genes relevant for this process (see discussion).

*DFezf is necessary and sufficient for netrin expression*

L3-derived Netrin is necessary for the M3-specific innervation of R8 photoreceptor axons (Pecot et al., 2014; Timofeev et al., 2012). Our RNA-seq analyses revealed that the mRNA levels of Netrin-A and B (NetA/B) were drastically reduced in dFezf null L3 neurons compared to wild type neurons at 40 hr APF (Figure supplementary FA, B). This suggested that, in addition to cell
autonomously regulating L3 targeting, dFezf non-autonomously regulates R8 layer specificity via activation of Netrin expression in L3 neurons. To test this, we first assessed Netrin protein expression in L3 neurons during development. Previous gene expression and genetic studies implied that L3 neurons express Netrin protein (Pecot et al., 2014; Tan et al., 2015; Timofeev et al., 2012), but this had not been shown directly. To address this, we used a NetB-GFP protein trap, wherein endogenous NetB is tagged with GFP. We focused on NetB protein because NetB is expressed at much higher levels than NetA in L3 neurons (Supplementary figure 6A,B) (Tan et al., 2015), and the expression of NetB in L3 neurons is sufficient to support proper R8 layer specificity in the background of a NetA/B deletion (Timofeev et al., 2012). At 24 hr APF we observed NetB expression in the perinuclear region of the oldest lamina neurons (most anterior) (Figure 10A–D’). Using markers specific for different lamina neuron subclasses we discovered that NetB is expressed by L3, L4 and L5 neurons, but not L1 or L2 neurons. We assessed NetB expression in L3 by labeling L3 somas with FLAG-tagged dFezf (dFezf-FLAG) expressed from its native promoter within a BAC (Supplementary figure 7). At 48 hr APF NetB expression remained specific to L3, L4 and L5 neurons in the lamina, although it was most strongly expressed in L3 neurons at this stage (Figure 10E–H). Thus, while NetB immunoreactivity is first detected within the M3 layer at ~40 hr APF (Timofeev et al., 2012), which coincides with the segregation of L3 growth cones into the developing M3 layer, L3 neurons express NetB much earlier in development. In addition, we found that NetB is also expressed by L4 and L5 neurons during pupal development, and thus may regulate circuit assembly within multiple developing layers.
**Lamina cortex (lamina neuron cell bodies)**

**Figure 10. Cellular expression of NetB in the lamina during pupal development. (A–H)**

**NetB expression in the lamina at 24 or 48 hr APF.** Endogenous NetB was visualized using a NetB-GFP protein trap (α-GFP). All confocal images are representative examples, and at least five brains were examined per genotype. All scale bars = 5 microns. (A–D’) In the lamina, lamina neurons are born in a wave (Anterior = oldest, Posterior = youngest). The yellow dashed lines in (A–C) indicate the anterior-posterior region of the lamina where NetB expression is first observed. In D and D’ anterior-posterior is indicated by A–P. (A) At 24 hr APF, NetB (green) was expressed in the anterior side of the developing lamina (to the left of the yellow dashed line) containing the oldest lamina neurons. NetB was expressed in L3 neurons (magenta, dFezf-FLAG). The youngest L3 neurons on the posterior side of the lamina that had not turned on NetB...
Figure 10 (continued) expression are indicated by asterisks. (B) At 24 hr APF, NetB (green) was detected in L4 and L5 neurons (magenta, α-bsh) in the oldest region of the lamina (to the left of the yellow dashed line). NetB (green) was not detected in L1 neurons (blue, anti-svp, asterisks in the anterior region). (C) Consistent with findings shown in B, at 24 hr APF NetB (green) was expressed in the oldest L4 neurons (magenta) in the anterior region of the lamina (left of the dashed yellow line). L4 neurons were labeled using an L4-specific driver (31C06-LexA, LexAop-myr::tdTomato). At this stage 31C06-LexA drives expression in a small population of L4 neurons in the anterior region of the lamina. R1-R6 axons (blue, mAB24B10) provided a reference for the lamina. The image shown here is taken from a pupa that is slightly older than in A and B, as NetB is expressed in nearly all the lamina neurons in the field of view. (D and D’) NetB (green) was not expressed in L2 neurons (magenta) at 24 hr APF. L2 neurons were visualized using a specific driver (16H03-LexA, LexAop-myr::tdTomato) that drives expression in the most anterior (oldest) L2 neurons at this stage. R1-R6 axons (blue, mAB24B10) serve as a reference for the lamina. Asterisks show the positions of L2 somas. The image shown here is taken from a pupa that is slightly older than in A and B, as NetB is expressed in all the lamina neurons in the field of view. (E) NetB (green) is expressed in all L3 neurons (magenta, dFezf-FLAG) at 48 hr APF. (F) All L4 (magenta, 31C06-LexA, LexAop-myr::tdTomato) and L5 (blue, α-Pdm3) neurons express NetB at 48 hr APF. NetB expression in L4 and L5 neurons is weaker than in L3 neurons (green only). (G) NetB (green) is not expressed in L1 neurons (magenta, α-Svp) at 48 hr APF. (H) NetB (green) is not expressed in L2 neurons (magenta, α-Bab1) at 48 hr APF.
To test whether dFezf is required for NetB protein expression, we examined NetB expression during pupal development when dFezf function was disrupted in L3 neurons. Disrupting dFezf in single L3 neurons in MARCM experiments drastically reduced NetB immunostaining in L3 cell bodies at 48 hr APF (Figure 11A–B’). To assess whether dFezf is necessary for NetB expression in the M3 layer, wherein it is thought to be secreted from L3 growth cones, we performed RNAi experiments to disrupt dFezf in the vast majority of L3 neurons. The expression of dFezf RNAi in the lamina eliminated detectable dFezf immunoreactivity in L3 neurons (Figure 11C, D), and considerably reduced NetB immunostaining in L3 somas and the developing M3 layer at 48 hr APF (Figure 11E, F). Together, these experiments demonstrate that dFezf is cell autonomously required for NetB protein expression in L3 neurons.

To determine if dFezf is sufficient to induce NetB expression, we mis-expressed dFezf-HA in lamina neurons using a pan-lamina GAL4 driver and assessed NetB expression in L2 neurons (Figure 11G–J). L2 neurons mis-expressing dFezf still expressed Bab1 (Figure 11J), a transcription factor shown to be selectively expressed in L2 neurons in the lamina (Tan et al., 2015), indicating that dFezf expression did not cause an obvious change in cell fate. We found that, while no wild type L2 neurons expressed NetB at 48 hr APF (Figure 11I), 99% of dFezf-HA expressing L2 neurons ectopically expressed NetB (Figure 11J). Thus, in the context of L2 neurons dFezf is sufficient to induce NetB expression. Collectively, our RNA-seq and protein expression experiments support that dFezf, either directly or through intermediate regulators, activates Netrin expression in L3 neurons.
Figure 11. DFezf non-autonomously regulates R8 layer specificity through activation of Netrin expression in L3 neurons. (A–B’) NetB expression (green, α-NetB) in the somas of wild type or dFezf1 L3 neurons was assessed at 48 hr APF in the lamina in MARCM experiments using confocal microscopy. L3 clones (magenta) expressed myr::GFP (9–9-GAL4) and were visualized using a GFP antibody. (A–A’) Asterisks indicate the positions of wild type L3 clones (magenta). The images are representative of NetB expression (green) assessed in five brains. (A) R1-R6 axons (blue, mAB24B10) were used as a reference for the lamina. Scale bar = 5 microns. (B–B’) Asterisks indicate the positions of dFezf1 L3 clones (magenta). The images are representative of NetB expression (green) assessed in six brains. (B) R1-R6 axons (blue, mAB24B10) were used as a reference for the lamina. Scale bar = 5 microns. (C and D) DFezfRNAi was expressed in lamina neurons and their precursor cells using 9B08-GAL4. DFezf expression (green) in L3 neurons was assessed through immunostaining (α-dFezf). R1-R6 axons
Figure 11 (continued) (magenta, mAB24B10) provided a reference for the lamina. (C) Representative confocal image of dFezf expression in control flies, which include flies containing dFezfRNAi only or 9B08-GAL4 (seven brains total). Scale bar = 5 microns. (D) Shows a representative confocal image of dFezf expression in knockdown flies (eight brains examined). (E and F) NetB (green, α-Net-B) expression in the lamina and within the M3 layer was assessed in control brains and when dFezfRNAi was expressed in lamina neurons and their precursors using 9B08-GAL4. R1-R8 axons (magenta, mAB24B10) provided a reference for the lamina and outer medulla. Arrowheads indicate NetB expression in medulla neurons. (E) Shows a representative confocal image of NetB expression (green) in control flies, which contain dFezfRNAi only or 9B08-GAL4 (four brains total). Net-B was prominently observed in L3 cell bodies in the lamina, within the M3 layer in the medulla and in medulla neuron cell bodies (arrowheads) on a consistent basis. Scale bar = 20 microns. (F) A representative confocal image from flies containing dFezfRNAi and 9B08-GAL4 (six brains). NetB (green) was consistently reduced in L3 cell bodies in the lamina and the M3 layer, but not in medulla neuron cell bodies (arrowheads). (G–J) DFezf-HA was expressed broadly in lamina neurons by 11–164-GAL4. (G and H) Confocal images of DFezf-HA expression (green, α-HA) in lamina neurons (~24 hr APF). The lamina was identified by visualizing R1-R6 axons (magenta, mAB24B10). (G) Representative confocal image from flies containing DFezf-HA or 11-164-GAL4 only (at least five brains). No HA labeling was detected in the lamina of these flies. (H) Shows a representative confocal image from flies containing DFezf-HA and 11-164-GAL4 (at least five brains). Prominent HA staining (green) was always observed in the lamina of these flies at 24 hr APF. (I and J) Confocal images of NetB expression (green) (NetB-GFP, α-GFP) in the lamina at 48 hr APF. L2 neurons (magenta) were labeled using α-bab1, and R1-R6 axons
Figure 11 (continued) (blue, mAB24B10) provided a reference for the lamina. (I) Representative confocal image from flies containing DFezf-HA or 11-164-GAL4 (four brains). No L2 neurons (magenta) expressed Net-B (green) (n = 308). NetB-expressing cells in the image are L3 neurons. Scale bar = 5 microns. (J) Representative confocal image from flies containing DFezf-HA and 11-164-GAL4 (four brains). 99% of L2 neurons (magenta) expressed NetB (green) (n = 310). (K–M) DFezfRNAi was expressed in lamina neurons and their precursors using 9B08-GAL4 and R8 axon targeting was assessed within the outer medulla. (K and L) Confocal images showing R8 axon targeting within the outer medulla in adult flies. R8 axons (green) were visualized using an R8-specific marker (Rh6-GFP, α-GFP). L3 axons (magenta) are labeled by 22E09-LexA, LexAop-myr::tdTomato. DFezf knockdown inhibits 22E09-LexA activity, and serves as a positive control for the efficacy of dFezfRNAi. R7 and R8 axons (blue, mAB24B10) provide a reference for outer medulla layers. (K) A representative image from flies containing DFezfRNAi or 9B08-GAL4 only (six brains total). Scale bar = 5 microns. (L) A representative image from flies containing DFezfRNAi and 9B08-GAL4 (four brains). Arrowheads indicate the depths at which R8 axons terminate. (M) Quantification of R8 axon targeting. N = the number of R8 axons counted.
**DFezf in L3 neurons is non-autonomously required for R8 layer specificity**

To directly determine if dFezf function in L3 neurons is required for R8 layer specificity, as is predicted by its role in regulating Netrin expression, we disrupted dFezf via RNAi and assessed R8 axon targeting using an R8-specific marker. Normally, R8 axons terminate in the M3 layer (Figure 11K, M). When dFezf was disrupted 45% of R8 axons terminated within inappropriate layers (Figure 11L, M). 29% of the axons terminated in layers proximal to M3 (i.e. M4-M6) and 16% terminated in distal layers (i.e. M0-M2). The penetrance of mis-targeting in our experiments is similar to that reported by Timofeev and colleagues in a Netrin null background (53%) (Timofeev et al., 2012). However, they reported that only 2% of R8 axons mis-targeted to more proximal layers (versus 29% in our experiments). Differences in R8 mis-targeting between ours and previous experiments could reflect non-specific effects of disrupting dFezf in L3 neurons (e.g. due to mis-targeting L3 axons), or indicate that L3 neurons provide a dFezf-dependent cue in addition to Netrin that is important for the targeting of R8 axons (see discussion). Nonetheless, our experiments show that dFezf in L3 neurons is required for R8 layer specificity. Together with previous research, these findings along with our analyses of Netrin gene and protein (NetB) expression indicate that dFezf regulates R8 layer specificity, to a large extent, through activation of Netrin expression in L3 neurons.

**Disrupting dFezf in L3 neurons does not affect the layer innervation of Tm9 neurons**

Within the M3 layer L3 axons synapse onto the dendrites of transmedullary nine neurons (Tm9) (Gao et al., 2008; Takemura et al., 2013; Takemura et al., 2015), and synapses between R8 and Tm9 have also been reported (Gao et al., 2008). Tm9 neurons are a subtype of projection neurons that transmit information from the medulla to the lobula neuropil. As disrupting dFezf in L3 neurons caused defects in L3 and R8 layer innervation but did not impair the formation of
presynaptic sites in L3 neurons (Figure 6K, L), we assessed whether connectivity with Tm9 neurons is perturbed under these conditions. To test this, we used cell-specific drivers to assess overlap between L3 axons and Tm9 dendrites within the same medulla columns during development, under conditions when L3 neurons were wild type or mutant for dFezf in MARCM experiments. During early pupal development (~24 hr APF) in wild type columns, L3 growth cones and Tm9 dendrites targeted to similar depths within the outer medulla, but appeared to be restricted to opposite sides of the column (Figure 12A,B–B”). In contrast, by mid-pupal development (~48 hr APF) L3 growth cones and Tm9 dendrites overlapped significantly within the developing M3 layer (Figure 12C, D–D”). In adult flies L3 terminals were flattened and overlapped with the proximal portion of Tm9 dendrites within the M3 layer (Figure 12E, F–F”).

When dFezf was disrupted in L3 neurons, the positions of L3 growth cones and Tm9 dendrites within the same columns were altered in early pupal development (Figure 12G–G”). Frequently, the growth cones and dendrites were positioned at different depths within the outer medulla. At mid-pupal development, L3 growth cones and Tm9 dendrites did not overlap within the column, with most of the growth cones innervating developing layers in the distal medulla (M1-M2) while Tm9 dendrites innervated the developing M3 layer normally (Figure 12H–H”). In adult flies, we found that compared to wild type L3 clones, dFezfΔ clones were weakly labeled by an L3-specific GAL4 driver (9–9-GAL4) (Figure 12I, I”). Despite the weak labeling, we observed that L3 axon terminals and Tm9 dendrites innervated different depths within the neuropil, and the morphologies of Tm9 dendrites in columns containing wild type or dFezfΔ L3 neurons were indistinguishable (Figure 12I–I”). The decreased labeling observed in mutant clones is likely due to a reduction in 9–9-GAL4 activity during pupal development rather than
Figure 12. Disrupting dFezf in L3 neurons does not affect the layer innervation of Tm9 neurons. (A–I”) Confocal images from MARCM experiments analyzed at 24 hr APF, 48 hr APF or in newly eclosed adults. The images shown are representative of neurons assessed from at least five brains per genotype per time-point. L3 clones (green) expressed myr::GFP (9–9 GAL4) and were visualized using a GFP antibody, wild type Tm9 neurons (magenta) expressed myr::tdTomato (24C08-LexA) and were visualized with a DsRed antibody. R1-R8 axons (blue, mAB24B10) define the lamina and outer medulla. (A, C, E) Broad views showing the entire lamina and medulla and the morphologies of wild type L3 (green) and Tm9 neurons (magenta). The solid boxed regions show examples of the regions of the outer medulla shown in B-B’, D-D’, F-F’, G-G’, H-H’, and I-I’. (B, D, F, G, H, I) Dashed lines indicate the estimated boundaries
of a medulla column using R7 and R8 axons (blue) as a reference. (F and F”, I and I’) Asterisks mark the positions of medulla columns containing L3 clones.

cell death, as dFezf1 L3 neurons in adult flies were strongly labeled with a pan-neuronal driver (i.e. the brp locus) (see Figure 6G–I, L, N). Thus, dFezf may be required to maintain 9–9-GAL4 expression in L3 neurons.

To summarize, in these experiments we did not observe overlap between the axons of dFezf null L3 neurons and Tm9 dendrites during development or in adult flies, strongly pointing to disrupted connectivity between these neurons under these conditions. In addition, it is also likely that dFezf is partially required for R8-Tm9 connectivity, as a significant number of R8 axons terminate in layers distal to M3 in the absence of dFezf function (Figure 11L, M), and R8 axons and Tm9 dendrites are unlikely to overlap in these columns.

Discussion

Laminar organization of synaptic connections is a principal feature of nervous system structure. Thus, determining how cells arrange their connections into layered networks is critical for understanding how the nervous system is assembled. Molecules that are necessary for layer specificity in particular neuron types have been identified in different systems. However, molecular strategies that organize the assembly of specific layers have remained elusive. Here, we illuminate a transcriptional mechanism that coordinates different cell types to the same layer in the Drosophila visual system (summarized in Figure 13). Based on our findings and previous studies, we propose that specific layers are built in a stepwise manner through use of
transcriptional modules that cell-intrinsically instruct targeting to specific layers, and cell-extrinsically recruit other circuit components. Moreover, based on research in the mammalian nervous system we propose that this represents an evolutionarily conserved strategy for building laminar-specific neural circuits.

**DFezf cell-intrinsically instructs axon target specificity**

Early in pupal development, lamina neuron axons project into the outer medulla and terminate in two broad domains (Figure 13C), which later give rise to refined layers (Figure 13B). These broad domains represent a primitive form of layer organization, and thus determining how they are established is likely to be central to understanding how specific layers are constructed. The targets of lamina neuron axons within broad domains are unknown, and could include synaptic partners, intermediate targets, guidepost cells, and molecules embedded in the extracellular matrix. Our findings demonstrate that dFezf regulates broad domain specificity in L3 neurons, and indicate that this developmental step is crucial for L3 laminar specificity, synaptic connectivity, and for the proper assembly of the M3 layer.

We show that dFezf functions in parallel to CadN and Sema-1a in L3 neurons to regulate targeting to the proximal domain of the outer medulla. We envision three models by which proper growth cone position could be achieved through the combined actions of these molecules. (1) In the first model, precise growth cone position is achieved by preventing innervation of inappropriate regions. Here, dFezf prevents growth cone termination within the distal domain of the outer medulla, potentially by repressing the expression of cell surface molecules that interact with targets in this region. And CadN and Sema-1a prevent the growth cones from extending beyond the proximal domain, into the serpentine layer and inner medulla. Thus, proper growth
cone targeting is achieved through mechanisms that prevent both superficial and deeper innervation. (2) Alternatively, growth cone position may be controlled though a direct targeting
**Figure 13. DFezf coordinates the formation of laminar-specific connections.** (A) Early in medulla development, dFezf promotes the targeting of L3 growth cones to the proximal versus distal domain of the outer medulla. DFezf may regulate this step by controlling a program of dpr gene expression. (B) L3 growth cones segregate into the developing M3 layer and secrete Netrin, which regulates the attachment of R8 growth cones within the layer. DFezf also regulates this step by activating the expression of Netrin in L3 neurons. (C) Within the M3 layer, L3 and R8 axons synapse onto Tm9 dendrites. When dFezf function is lost in L3 neurons, L3 and R8 axons innervate inappropriate layers while Tm9 dendrites innervate the M3 layer normally. As a result, connectivity with Tm9 neurons is disrupted.

mechanism. In this model, dFezf could promote targeting to the proximal domain of the outer medulla by activating the expression of cell surface genes that interact with targets in this region. In this context, CadN and Sema-1a would then regulate growth cone consolidation within the
proximal domain of the outer medulla. (3) And finally, dFezf function could serve to both prevent termination in the distal domain of the outer medulla, and direct targeting to the proximal domain (combination of (1) and (2)).

In the absence of dFezf function, cell surface genes that are normally expressed by L3 neurons are downregulated, and cell surface genes expressed by other lamina neurons become upregulated. Thus, we favor the third model involving both prevention of superficial termination and directed growth cone targeting. We hypothesize that dFezf function serves to (1) activate the expression of cell surface genes that promote interactions with targets in the proximal domain of the outer medulla, and (2) repress the expression of cell surface genes that mediate interactions with targets in the distal domain. DFezf could accomplish this by directly binding the loci of cell surface genes to control their expression, by acting through intermediate factors, or a combination of both. Dpr genes are prominent dFezf targets, and one interesting possibility is that dFezf regulates growth cone targeting by controlling a program of dpr gene expression. Dprs that are activated by dFezf function in L3 neurons may mediate interactions with DIP-expressing synaptic targets in the proximal domain of the outer medulla. In contrast, dprs that become upregulated in dFezf mutant L3 neurons may mediate inappropriate interactions with DIP-expressing targets in the distal domain of the outer medulla.

Future studies will be dedicated to determining which dFezf target genes regulate L3 growth cone targeting, and the mechanisms by which dFezf regulates their expression. Since, in addition to dprs, many cell surface genes change in their levels of expression in the absence of dFezf function, RNA-seq analyses performed at earlier stages of development and in dFezf misexpression experiments (e.g. in L2 neurons) may help narrow down the list of relevant target genes. In addition, to circumvent issues of functional redundancy it will be important to
undertake combinatorial genetic approaches targeting similar genes (e.g. the dprs) in loss- and gain-of-function experiments. That a number of transcriptional regulators are differentially expressed between wild type and dFezf mutant L3 neurons (Supplementary figure 5) suggests that dFezf, at least in part, acts through intermediate regulators to control gene expression. Identification of direct dFezf targets will provide a way of testing this and addressing how dFezf controls gene expression in L3 neurons.

*DFezf regulates layer innervation through a cell-extrinsic mechanism*

Our genetic investigation of dFezf function in L3 neurons revealed a cell-extrinsic role for dFezf in regulating R8 layer specificity through the activation of NetA/B. Whether these genes are direct or indirect dFezf targets remains unclear. Preliminary bioinformatics analyses did not identify dFezf binding motifs within the NetA/B loci. However, additional experiments are necessary to rigorously assess this, and determine how NetA/B expression is controlled in L3 neurons.

Many different neurons have been shown to form synapses within the M3 layer (Takemura et al., 2013; Takemura et al., 2015), and whether L3-derived Netrin is necessary for the M3-specific innervation of neurons other than R8 remains an open question. The Netrin receptor Frazzled is broadly expressed in the medulla (Timofeev et al., 2012), and Netrin released from L3 growth cones is well-suited to serve as an M3-specific signal to other cell types, due to its concentration within the layer. Genetic studies disrupting dFezf function and assessing the morphologies of neurons that innervate M3 would provide a way of testing whether L3-derived Netrin broadly organizes M3 circuitry, or specifically controls R8 layer innervation.
In the absence of dFezf function in L3 we observed R8 axons inappropriately innervating distal layers (i.e. M0-M2), as expected in the absence of Netrin function (Akin and Zipursky, 2016; Timofeev et al., 2012), and a significant fraction of R8 axons terminating in proximal outer medulla layers (M4-M6), which was unexpected. In vivo time lapse imaging studies showed that the axons of Frazzled (Netrin receptor) null R8 neurons targeted to M3 normally and then retracted, indicating that Netrin/Frazzled signaling is required for growth cone stabilization within the target layer, but is dispensable for recognition of the target layer (Akin and Zipursky, 2016). Thus, additional molecules regulate R8 layer specificity. One interesting possibility is that in addition to Netrin, dFezf regulates the expression of a gene(s) important for R8 axons to ‘recognize’ the M3 layer, and in the absence of this cue a subset of R8 axons overshoot M3 and innervate proximal layers. Consistent with this idea, the genetic ablation of L3 neurons caused a significant percentage of R8 axons to terminate in proximal outer medulla layers (Pecot et al., 2014). In addition to Net-A/B, dozens of cell surface or secreted genes are downregulated in L3 neurons at 40 hr APF when dFezf is disrupted. Thus, there are a number of candidates that could act downstream of dFezf in parallel to Netrin to regulate R8 layer specificity. Disrupting these genes in L3 neurons and visualizing R8 axons would provide a way of identifying additional L3-derived cues necessary for R8 layer innervation.

A transcriptional mechanism for controlling the stepwise formation of layer specific circuits

Previous studies indicate that medulla layers are not pre-established regions that serve as a template for circuit formation, but rather that layers are built over time from broad domains as the neurites of different cell types are added in a precise order. Thus, central to elucidating the molecular and cellular logic underlying assembly of the medulla network is identifying the molecular and cellular components involved in the sequence of interactions giving rise to
specific layers, and discovering commonalities or connections between interactions and how different layers are assembled. Insight into the cellular mechanisms giving rise to the M3 layer circuitry was provided by the discovery that R8 innervation of M3 relies on a signal from L3 axons (Netrin) (Pecot et al., 2014; Timofeev et al., 2012), which innervate M3 at an earlier stage despite being born later in development (Selleck and Steller, 1991). These studies and the fact that L3 and R8 neurons contribute input to common pathways suggest that the cellular mechanisms governing circuit formation reflect functional relationships between neurons, rather than birth order.

Less progress has been made in identifying the molecular logic underlying the stepwise assembly of layer specific circuits. This is likely due in part, to the fact that how neurons innervate particular layers is cell type-specific, rather than layer specific. For example, while R8 neurons depend on Netrin for layer specificity, Netrin is dispensable for the layer innervation of L3 and Tm9 neurons (unpublished results). Thus, identifying commonalities in the mechanisms used by neurons to innervate layers has been challenging. However, here we show that L3 and R8 layer innervation is linked through dFezf, which controls a program of gene expression that regulates the layer innervation of both neurons. Thus, while L3 and R8 utilize different molecules to achieve layer specificity, the expression of key molecules required by each neuron is controlled by the same transcription factor.

Using the same transcriptional pathway or module to control genes that function cell-intrinsically and cell-extrinsically to regulate layer innervation represents a simple mechanism for controlling the series of interactions that lead to circuit formation. Employing specific transcriptional modules in specific neurons would ensure that those neurons innervate the correct target layer, and also express the genes necessary for the targeting of other circuit components.
We hypothesize that this strategy is widely employed to regulate the assembly of circuitry within the medulla. For example, cell-specific transcription factors analogous to dFezf in L3 have been identified in each of the other lamina neuron subtypes (Tan et al., 2015), and the positions of lamina neuron arborizations in the medulla are well suited to produce signals that regulate the precise layer innervation of other cell types (Figure 13B). Indeed, CadN-based interactions between L2 terminals and L5 axons are necessary for L5 branching within the M2 layer (Nern et al., 2008). Thus, we hypothesize that the function of cell-specific transcriptional modules in lamina neurons coordinates the assembly of discrete outer medulla layers.

We also speculate that the function of dFezf in L3 neurons is conserved in laminated regions of the mammalian nervous system. For instance, in the inner plexiform layer of the mouse retina, which is analogous to the medulla in structure and function (Sanes and Zipursky, 2010), Fezf1 is expressed in a subset of bipolar cells that innervate specific sublaminae (i.e. layers) (Shekhar et al., 2016). Bipolar cells are analogous to lamina neurons, and we hypothesize that Fezf1 in specific types of bipolar cells functions analogously to dFezf in L3 to coordinate laminar-specific connectivity.

In the cerebral cortex of mice, Fezf2 cell-intrinsically controls the identity of subcortically projecting pyramidal neurons that predominantly reside in layer V (Chen et al., 2005a; Chen et al., 2005b; Molyneaux et al., 2005), and pyramidal neuron identity was shown to be important for the laminar positioning of specific classes of GABAergic neurons (Lodato et al., 2011). While it remains unclear as to whether Fezf2 in pyramidal cells regulates the expression of molecules that act non-autonomously to recruit specific inhibitory neurons (analogous to Netrin), one interesting possibility is that Fezf2 in the cortex organizes laminar-specific circuitry through cell-intrinsic and cell-extrinsic mechanisms similar to dFezf in the medulla. In
conclusion, we expect our findings regarding the function of dFezf in the Drosophila visual system reflect an evolutionarily shared strategy for assembling laminar-specific neural circuits.

Methodology

Description of replicates

In general, the replicates for each experiment are indicated by the number of cells and brains examined (denoted in the figures and figure legends). There are essentially three types of replicates we consider. Within each optic lobe, each column is replicated hundreds of times, different lobes of the same brain are replicates, and the lobes of different brains are also replicates. In all experiments, the phenotypes reported are consistently reproducible for each type of replicate considered (i.e. within and between different animals)

Fly strains

Flies were raised on standard cornmeal-agar based medium. Male and female flies were used at the following development stages: 3rd instar, 12 hr after pupal formation (h APF), 24 hr APF, 48 hr APF, 72 hr APF and newly eclosed adults (within 5 hr of eclosion).

The following strains were obtained from Bloomington Stock Center (Indiana University):

10xUAS-IVS-myr::GFP [attP2], tubP-GAL80, FRT40A, R27G05-FLPG5.PEST [attP40], 20XUAS-RSR.PEST [attP2], 10XUAS-IVS-myr::GFP [su(Hw)attP8], GMR16H03-lexA [attP40], 10xUAS-FRT-stop-FRT-myr::GFP [su(Hw)attP5], UAS-Dcr-2, GMR24C08-lexA [attP40], GMR9B08-GAL4 [attP2], TRiP.JF02342 [attP2] (dFezf RNAi), 20xUAS-RSR.PEST [attP2], Rh6-EGFP
The following strains were obtained from Janelia Research Campus:

R27G05-FLPG5.PEST [attp18], 22E09-LexA, 39D12-LexA

The following stocks were generated in the Pecot Laboratory for this study:

18B02-dFezf, 18B02-dFezf-C1-3xFLAG

The following strains were gifts from other laboratories:

9-9-GAL4, 6-60-GAL4, 11-164-GAL4 (U. Heberlein), LexAop-myr::tdTomato [su(Hw)attP5] (S.L. Zipursky), UAS-dFezf-3xHA, dFezf₁, dFezf₂ (C.Y. Lee), CadN1-2 Δ14 (T.R. Clandinin)

Construction of transgenic animals

Generation of the BRP presynaptic marker 79C23S-RSRT-STOP-RSRT-smFPV5-2A-LexA

Restriction free cloning (van den Ent and Löwe 2006) was used to insert smFP_V5 (Viswanathan et al., 2015) (addgene: pCAG_smFPV5 plasmid# 59758) in between RSR and the 2A peptide (2A) within ‘cassette F’ (see below), which was made by Chen and colleagues for generation of the original Brp STaR marker (Chen et al., 2014). This replaced the original V5 tag and generated:

Cassette G: GS linker-RSR-STOP-RSRT-smFP_V5-2A-LexAVP16

[Cassette F: GS linker-RSR-STOP-RSRT-V5-2A-LexAVP16]

The modification of the BAC CH321-79C23S (Chen et al., 2014) was performed using the recombinaseering protocol described previously (Sharan et al., 2009).
Generation of 18B02-dFezf and 18B02-dFezf-C1-3xFLAG

A bacterial artificial chromosome (BAC) (CH321-18B02) containing the dFezf locus was ordered from BACPAC Resources, and inserted into the VK33 genomic site, resulting in the 18B02-dFezf strain.

The modification of the BAC was performed using the recombineering protocol described previously (Sharan et al., 2009, Nature Protocols).

The wild type 18B02 BAC was transformed into the SW102 E. coli strain (Warming et al., 2005). RpsL-Kan cassette with homology arms flanking the first stop codon of the dFezf locus was amplified using the following primers:

Forward: ACCATCAGCAGCAGCAAAAGACTCTCGGAGACCTTCATAGCCAAGGTGTAA
GCTTCAGCGTCCGCAAGCACTCAG

Reverse: GAGGTCGACCCCGTGGAGCTGTTCAAGCTTTCCGCATAGTATCGCTGTCAG
GGGTGGGCGAAGAACTCCAGCATGA

The PCR product was transformed into the 18B02-harboring SW102 cells, and the RpsL-Kan cassette was inserted before the first stop codon by homologous recombination.

1 kb of DNA fragment centered by the first stop codon of the dFezf locus was PCR amplified from the genomic DNA using the following primers:

Forward: CTCACCTTCCACATGCACAC

Reverse: GCGTGTCTACACGGAACTCA
This fragment was then cloned into pGEM-T vector (Promega, Madison, USA). Using the resulting construct as template, site-directed mutagenesis was performed by PCR using the following primers so that the 3xFLAG sequence was inserted before the first stop codon of the dFezf locus on the plasmid:

Forward: GAGACCTTCATAGCCAAGGTGTTTGACTACAAAGACCATGACGGTGATTATAAAGATCATGACATCGATTACAAGGATGACGATGACAAGTGACAGCGATACTATGC GGAAAGC

Reverse: CGAGAGTCTTTTGCTGCTGCTGATG

Using the resulting construct as the template, PCR was performed using the following primers to amplify the DNA fragment having 3xFLAG inserted before the first stop codon of the dFezf locus and flanked by ~500 bp of homology arms:

Forward: CATCCGCAAAGCCAGCAACA

Reverse: GAGGTGGCCATCGAGGATAT

The PCR product was then transformed into the SW102 cells that harbored modified 18B02 with RpsL-Kan cassette inserted before the first stop codon of the dFezf locus. The RpsL-Kan cassette was then replaced by 3xFLAG by recombination, resulting in a BAC that has 3xFLAG inserted right before the first stop codon of the dFezf locus. The 3xFLAG tagged BAC was inserted into the VK33 genomic site, resulting in the 18B02-dFezf-C1-3xFLAG strain.
**Immunohistochemistry**

Fly brains were dissected in Schneider’s medium and fixed in 4% paraformaldehyde in phosphate buffered lysine for 25 min. After fixation, brains were quickly washed with phosphate buffer saline (PBS) with 0.5% Triton-X-100 (PBT) and incubated in PBT for at least 2 hr at room temperature. Next, brains were incubated in blocking buffer (10% NGS, 0.5% Triton-X-100 in PBS) overnight at 4°C. Brains were then incubated in primary antibody (diluted in blocking buffer) at 4°C for at least two nights. Following primary antibody incubation, brains were washed with PBT three times, 1 hr per wash. Next, brains were incubated in secondary antibody (diluted in blocking buffer) at 4°C for at least two nights. Following secondary antibody incubation, brains were washed with PBT two times, followed by one wash in PBS, 1 hr per wash. Finally, brains were mounted in SlowFade Gold antifade reagent (Thermo Fisher Scientific, Waltham, MA).

Confocal imaging was accomplished using either a Leica SP8 laser scanning confocal microscope or an Olympus FV1200 Laser Scanning Microscope. Fiji (Schindelin et al., 2012; Schneider et al., 2012) was used to create z stack images.

The primary antibodies used were as follows: anti-GFP (chicken, 1:1000, ab13970) and anti-HA (mouse, 1:1000, ab1424) were purchased from Abcam. Anti-V5 (mouse, 1:200, MCA2892GA) was purchased from Bio-Rad. Anti-HA (rabbit, 1:1000, 3724S) was purchased from Cell Signaling Technologies. Anti-DsRed (rabbit, 1:200, 632496) was purchased from Clontech Laboratories, Inc. Anti-chaoptin (mouse, 1:20, 24B10), anti-elav (rat, 1:200, 7E8A10), anti-CadN (rat, 1:20, DN-Ex 8) and anti-seven-up (mouse, 1:10, 2D3) were purchased from Developmental Studies Hybridoma Bank. Anti-FLAG (mouse, 1:1000, F1804) was purchased...
from Sigma-Aldrich. Anti-dFezf (rabbit, 1:100) was a gift from C.Y. Lee. Anti-bsh (guinea pig, 1:200) was a gift from S.L. Zipursky. Anti-pdm3 (guinea pig, 1:500) was a gift from J.R. Carlson. Anti-bab1 (rabbit, 1:200) was a gift from S.B. Carroll. Anti-NetB (guinea pig, 1:50) was a gift from B. Altenhein.

The secondary antibodies used were as follows: Goat anti-chicken IgG (H + L) Alexa Fluor 488 (A-11039), Goat anti-rabbit IgG (H + L) Alexa Fluor 568 (A-11011), Goat anti-mouse IgG (H + L) Alexa Fluor 647 (A-21236), Goat anti-mouse IgG1 Alexa Fluor 568 (A-21124), Goat anti-guinea pig IgG (H + L) Alexa Fluor 568 (A-11075), Goat anti-Rat IgG (H + L) Alexa Fluor 647 (A-21247) were all purchased from Thermo Fischer Scientific and were all used at a dilution of 1:500.

**MARCM and MARCM + STaR experiments**

In most MARCM experiments, FLP recombinase was expressed in lamina neuron precursor cells using R27G05-FLP.G5.PEST (attP40) to induce mitotic recombination, generating single lamina neuron clones, which were visualized using cell-specific GAL4 drivers. To generate sparse lamina neuron clones in L2 mis-expression experiments (Supplementary figure 3B-D), R27G05-FLPG5.PEST (attp18) was used to induce mitotic recombination.

In experiments utilizing STaR and MARCM FLP recombinase in lamina neuron precursor cells induced mitotic recombination generating a subset of L3 neurons that were homozygous for the control chromosome (FRT40) or dFezfi. In L3 clones R recombinase was expressed (9–9 GAL4) and induced recombination within the Brp locus in a bacterial artificial chromosome (79C23S) resulting in the incorporation of smFP_V5 at the C-terminus. In addition, this resulted in the co-translation of LexA, which activated the expression of LexAop-myr::tdTomato to visualize the
morphology of L3 clones. Together, this allowed visualization of L3 morphology and endogenous pre-synaptic sites with single cell resolution in wild type conditions or when dFezf was disrupted, in an otherwise normal fly. smFP_V5 contains ten V5 epitopes built into a superfolding GFP backbone (Viswanathan et al., 2015). We find that it is considerably more sensitive than the original V5 tag in the STaR Brp constructs.

**FLP-out experiments**

To generate sparsely labeled L5 neurons, we used an interruption cassette (UAS-FRT-STOP-FRT-myr::GFP) to drive stochastic expression of myr::GFP with an L5 GAL4 driver (6-60-GAL4). Stochastic expression was accomplished through inefficient expression of FLP recombinase in lamina neurons (R27G05-FLP::PEST [attp18]). For reasons that are unknown, this FLP source results in stochastic recombination allowing for visualization of single neurons.

**RNA-seq experiments**

For each genotype (control, dFezf) 120 crosses were set, with 15 females and seven males in each cross. After three days, each cross was flipped every day for a week. From those vials we picked out Tb-negative white pre-pupae that formed within a one-hour window and placed the pupae in separate vials. GFP-positive pupae were scored under the fluorescence microscope and removed from the vials. The rest of the pupae were kept at 25°C. After 40 hr, we dissected the staged pupae within an hour in cold Complete Schneider’s Medium (CSM: 50 mL of CSM consists of 5 mL of heat inactivated fetal bovin serum, 0.1 mL Insulin, 1 mL PenStrep, 5 mL L-Glutamine, 0.4 mL L-Glutathione and 37.85 mL Schneider’s medium, filter sterilized). About 50–100 brains were dissected for each independent replicate experiment. After dissection of the brains, the optic lobes were collected and pooled together in 1x Rinaldini’s solution (100 mL
Rinaldini’s solution consists of 800 mg NaCl, 20 mg KCl, 5 mg NaH₂PO₄, 100 mg NaHCO₃ and 100 mg Glucose in H₂O, filter sterilized) for subsequent dissociation. We used a modified dissociation protocol based on one described previously (Harzer et al., 2013). The pooled lobes were washed once with 500 uL 1x Rinaldini’s solution and then incubated at 37°C for 15 min in dissociation solution that consists of 300 uL papain (100 U/mL) and 4.1 uL Liberase at a final concentration of 0.18 Wu/mL. After the enzyme digestion, the lobes were washed twice with 500 uL of 1x Rinaldini’s solution and then twice with 500 uL of CSM. The lobes were then disrupted in 200 uL of CSM by pipetting up and down until the solution became homogeneous. The cell suspension was then filtered through a 30 um mesh 5 mL FACS tube, and diluted with another 500 uL of CSM. The FACS sorting was done on the BD FACS Aria with a nozzle size of 70 um. GFP and DsRed double positive cells were sorted directly into 300 uL of RLT buffer (Qiagen, RNeasy kit) containing 1:100 beta-mercaptoethanol. Total RNA was purified with Qiagen RNeasy kit and eluted in 15 uL of water. After all the samples from five replicates (each genotype) were available, we concentrated the RNA into 5 uL by speed vac. 1.5 uL of the concentrated RNA was used to make cDNA library by Smart-seq2 as previously described (Picelli et al., 2014). The quality control of the libraries was done on the Bioanalyzer by Tapestation. The pooled libraries were then loaded onto a single flow cell and sequenced by NextSeq High Output. Between five and fourteen million mapped reads were obtained for each sample.
Quantification and statistical analyses

General

The quantification for immunohistochemistry experiments can be found either within the figure or in the figure legend. In all cases, N refers to the number of cells scored.

Analysis of RNA-seq data

The quality of the sequencing files was initially assessed with the FastQC tool. They were then trimmed to remove contaminant sequences (like polyA tails), adapters and low-quality sequences with cutadapt. These trimmed reads were aligned to the drosophila genome (Flybase release dmel_r6.15_FB2017_02) using STAR aligner. Alignments were checked using a combination of FastQC, Qualimap, MultiQC and custom tools. Counts of reads aligning to known genes were generated by featureCounts. In parallel, Transcripts Per Million (TPM) measurements per isoform were generated using Salmon. Differential expression at the gene level was called with DESeq2. We used the counts per gene estimated from the Salmon by tximport as input to DESeq2 as quantitating at the isoform level has been shown to produce more accurate results at the gene level. As described in the DESeq2 documentation, the differential expression is assessed using a Wald Test and p-values are adjusted for multiple testing using Benjamini-Hochberg FDR correction.
References


III. Conclusion
Conclusion

In this study, we discovered a transcriptional strategy underlying stepwise layer assembly. DFezf, an evolutionary conserved transcription factor (TF), intrinsically regulates the broad domain restriction, and layer specificity of one neuron type (L3), and extrinsically directs another type (R8) to the same layer. We believe that this stratagem may be a fundamental principle of layer specific targeting. Moreover, given the evolutionary conservation within the Fez family of TFs (Eckler and Chen, 2014), as well as the structural similarities shared between the layered neuropil of insect and vertebrate species (Sanes and Zipursky, 2010), we anticipate that this scheme may be widely applicable.

This study has provided a key mechanistic insight into the development of L3 neuron layer specific connectivity. During development, L3 growth cones are first restricted to a broad domain before achieving layer specificity. First, L3 growth cones are restricted to the outer medulla via the redundant functions of CadN and Sema-1a (Nern et al., 2008; Pecot et al., 2013). Within the outer medulla the growth cone targets the proximal domain, a primitive layer. Prior to our study, the mechanism that directs the L3 beyond the distal domain and into the proximal domain was not known. We have shown that dFezf cell-autonomously directs L3 to the proximal domain, which is critical for its final layer specificity and connectivity within the M3 layer. According to our data, dFezf functions in parallel to CadN and Sema-1a. Thus, L3 growth cone targeting is a result of the three independent mechanisms: CadN- and Sema-1a-mediated restriction to the outer medulla, and dFezf-mediated positioning in the proximal domain.

We also showed a pivotal role for dFezf in extrinsically regulating R8 cell layer specificity. Previous studies established that L3-derived Netrin-A and -B (Net), are secreted into the M3 layer, where they stabilize the R8 terminal. In the absence of Net, R8 terminals terminate
superficially (Akin and Zipursky, 2016; Pecot et al., 2014; Timofeev et al., 2012). Prior to our study, regulation of Net expression in L3 neurons was unknown. Here, we show that knocking out dFezf in L3 neurons eliminates Net expression and results in superficial R8 targeting. Unexpectedly, a subset of R8 neurons in this experiment aberrantly terminates beyond the M3 layer. This result is consistent with previous studies that showed a similar phenotype when L3 is eliminated (Pecot et al., 2014), and suggests that at least one other dFezf-dependent factor enables the R8 to recognize the M3 layer, though the possibility that this phenotype is caused by the absence or displacement of the L3 terminal has not been ruled out. These data indicate that the L3 neuron plays an important role in directing the R8 layer specificity through a dFezf-dependent Net signal.

Our work provides a molecular link between two different strategies of layer specific targeting. A single transcriptional program connects two modes of connectivity, via cell-intrinsic and cell-extrinsic methods. This transcriptional stratagem simplifies the development of complex wiring found in layered neuropil like the medulla.

**A general strategy for layer assembly**

Fundamental morphological characterization of the neuron types in the fly optic lobe demarcated medulla layers based on the arborization patterns of cells such as L1-L5 neurons (Fischbach and Dittrich, 1989). More recent studies have shown that lamina neuron growth cones segregate into their respective layers by midpupal development, when layers are first apparent. The preceding layer innervation by lamina neurons when compared to other neuron types like the R8 suggest that they could play an instructive role in directing layer specific
targeting of other neurons. Indeed, this is what has been observed between the L3 and R8 cells (Akin and Zipursky, 2016; Pecot et al., 2014; Timofeev et al., 2012). An intriguing possibility is that this is a common strategy used by lamina neurons in assembling laminar circuits, as well as other cells in the fly visual system.

We envision a scenario where each lamina neuron establishes the molecular identity of their respective layers by either secreting a molecule, or presenting a molecule at the cell surface. The L3 growth cone secretes Netrin, which concentrates in the M3 layer, where it acts to stabilize R8 growth cones (Akin and Zipursky, 2016; Pecot et al., 2014; Timofeev et al., 2012). Each lamina neuron has been shown to express secreted factors and cell surface genes during this stage of development (Tan et al., 2015). For example, the L2 neuron, which specifically innervates the M2 layer, exclusively expresses *slit* (Rothberg et al., 1988). We posit that the L2 growth cones secrete Slit into the M2 layer, where it rejects or permits the innervation of the layer. This strategy could explain instances where two different neuron types innervate the same layer via different mechanisms.

The cell-type specific expression of secreted factors and cell surface molecules is likely the result of cell-type specific transcription factors. It is clear that each lamina neuron expresses unique transcription factors during development, which may confer their unique expression profiles (Tan et al., 2015). Indeed, our work has identified dFezf as the regulator for Netrin expression in the L3 neuron. The TF *Ush (U-shaped)* (Cubadda et al., 1997) is specifically expressed in L2 neurons. As such, we hypothesize that within the L2 Ush drives expression of *slit*, which the cell uses to establish the layer’s molecular profile, as dFezf does with Netrin in the L3. We anticipate that within each lamina neuron exist a cell-type specific transcriptional program that activates the expression of a factor that will ultimately represent the molecular
identity of the layer. As other neuron types that innervate the medulla have also been found to express cell type-specific TFs, we anticipate that this strategy may be conserved throughout the fly visual system (Hasegawa et al., 2013).

Interestingly, pyramidal neurons that express FEZF2, the mammalian homolog of dFezf, direct the laminar targeting and connectivity of other neuron types in the mammalian cortex. Layer V pyramidal neurons have been shown to direct Layer V connectivity via the secretion of Sonic hedgehog (Shh). Circuit formation between Layer II/III neurons onto pyramidal neurons in Layer V requires shh expression in Layer V, and the expression of Shh receptor Boc (Brother of CDO) in Layer II/III neurons. Connectivity between Layer II/III and Layer V neurons was disrupted in shh and boc loss-of-function experiments (Harwell et al., 2012). One possibility is that shh is a FEZF2 downstream target, in which case FEZF2 would be an extrinsic regulator of Layer II/III neuron connectivity in Layer V, mimicking the relationship between dFezf and Net in the L3.

Regulating growth cone targeting through cell surface gene expression

Prior to our study, it was unclear how lamina neuron growth cones choose between the distal and proximal regions of the outer medulla early in development. We have shown that the TF dfFezf directs L3 growth cones to the distal region. As growth cone targeting is mediated by molecular interactions at the cell surface (Huberman et al., 2010), we favor a model wherein dFezf directs L3 targeting via regulation of cell surface gene expression. More generally, we anticipate that TFs in other lamina neurons also direct their growth cones via cell surface gene regulation.
There are three possible methods through which dFezf could be directing L3 growth cone targeting through cell surface gene expression. One possibility is that dFezf acts as a transcriptional repressor, shutting down cell surface genes that promote targeting of the distal domain. In the second scenario, dFezf acts as a transcriptional activator, turning on cell surface genes that promote targeting of the proximal domain. Lastly, a third scenario would be a combination of the first two, with dFezf acting as both an activator and a repressor.

Our RNA-seq studies in wild type and dFezf mutant L3 neurons revealed that dFezf functions as both an activator and repressor, either directly or through intermediate regulators. We have shown that when dFezf is knocked out in L3 neurons, certain cell surface genes are upregulated, while others are downregulated. Intriguingly, we found L3-specific cell surface molecules among the list of downregulated genes, and cell surface genes normally expressed in other lamina neurons were also enriched in the list of upregulated genes. We predict a model wherein dFezf activates L3-specific cell surface genes, while repressing cell surface genes expressed in other lamina neurons, to ensure that L3 targets the proper broad domain.

A striking observation from our RNA-seq experiments was that distinct families of cell surface genes are differentially expressed when dFezf is knocked out. One prime example is the defective proboscis retraction (dpr) immunoglobulin superfamily (IgSF) of cell surface genes (Nakamura et al., 2002). Previous studies have shown that each lamina neuron type expresses a unique combination of dprs during development. Moreover, dpr interaction proteins (DIPs), the cognate ligands for dprs (Carillo et al., 2015), are expressed by corresponding synaptic partners in the target region (Cosmanescu et al., 2018; Courgeon and Desplan, 2019; Menon et al., 2019; Tan et al., 2015; Xu et al., 2018). As such, a dpr code has been proposed as a possible molecular
mechanism for lamina neuron layer specificity and connectivity (Tan et al., 2015). Our data suggest that dprs may be involved in early growth cone positioning.

The potential for a dpr-mediated model of layer assembly and connectivity becomes more intriguing when our observations in dFezf mutant L3 neurons are compared to the dpr code present in other lamina neuron types. For example, dpr13 is specifically expressed in L2 neurons, which normally target the distal domain in a manner strikingly similar to the L3 dFezf mutant phenotype. Dpr13 is activated in dFezf mutant L3 neurons, consistent with dpr13 possibly promoting distal domain targeting. Furthermore, dFezf misexpression in L2 neurons is sufficient to misdirect their growth cones to the proximal domain, and eventually the M3 layer. Future gain- and lost-of-function experiments in L2 and L3 neurons will examine if dpr13 is the distinguishing molecular factor that underlies the decision between distal and proximal domain targeting. More generally, examining the function of the dpr family in L1-L5 will determine if a dpr code underlies early growth cone positioning of each neuron.

The mechanisms that regulate segregation into layers after lamina neuron growth cones have targeted primitive outer medulla domains remains poorly understood. The ongoing expression of dFezf suggests that the TF is also involved in other steps in L3 development. We hypothesize that dFezf also regulates later steps of L3 layer targeting through cell surface gene expression. More generally, we anticipate that each cell specific TF is responsible for layer segregation of its respective neuron. Dprs have recently been shown to be involved in layer specific targeting in the fly medulla (Xu et al., 2018; Menon et al., 2019; Courgeon and Desplan, 2019). We posit that interactions between dprs on lamina neuron growth cones and DIPs in the developing target region direct layer emergence in the outer medulla.
While segregation of synaptic partners into layers is thought to increase the likelihood of connectivity (Baier, 2013), it is not sufficient to induce connectivity between two neurons. For example, the L3 and R8 neurons make intimate contact within the same layer but they do not form connections (Takemura et al., 2013, 2008, 2015). Thus, there must be additional recognition mechanisms that enable partners to select on another within a confined space. We envision that, in this context, synaptic pairs connect via interactions between cell surface molecules. IgSF molecules such as dprs and DIPs have been hypothesized to direct such connectivity in the fly medulla (Tan et al., 2015). Moreover, IgSF molecules have been shown to be involved in directing layer specific wiring in other systems (Yamagata et al. 2002; Yamagata and Sanes, 2008, 2012; see introduction). We hypothesize that combinations of cell surface molecules like dprs are the molecular basis for synaptic specificity within the same layer. The connections between lamina neurons and their postsynaptic partners have been well-established (Takemura et al., 2013, 2008, 2015). As such, we can test our hypothesis by manipulating the combinations of IgSF molecules expressed in a specific cell, and examine how the connectome changes.

A critical consideration is the possibility that dFezf may not target cell surface genes directly, and instead may do so via intermediate regulators (i.e. other TFs). Several TFs are also differentially expressed when dFezf is knocked out. Importantly, as is the case for differentially expressed cell surface genes, we find upregulated TFs in dFezf mutant L3 neurons are normally expressed in other lamina neurons. For example, the TF ush (Cubadda et al., 1997) is specifically expressed in wild type L2 neurons (Tan et al., 2015), and is activated in L3 neurons when dFezf is knocked out. In one possible model, dFezf represses ush in L3 neurons, which would otherwise promote targeting to the distal domain via regulation of a/an unknown cell surface
gene(s). In the same model, this ush-mediated mechanism would normally function in L2 neurons. Future rescue experiments in which both dFezf and an upregulated TF like ush is knocked out will test this possibility.

Understanding which genetic loci are directly targeted by dFezf could lend insight into how dFezf is regulating L3 development. Through collaboration with the Weirauch laboratory, preliminary bioinformatic analyses have identified dFezf binding sites in several differentially expressed genes, including cell surface genes and TFs, suggesting that they may be directly regulated by dFezf. Future ChIP-seq experiments will reveal chromosomal loci that are bound by dFezf during development, thus revealing which differentially expressed genes are direct dFezf targets.

**Fez family function in other systems**

DFezf is the Drosophila homolog of a highly conserved transcription factor family in vertebrates consisting of two genes, Fezf1 and Fezf2. There are two particular functional domains that exhibit high percentages of homology when compared across species. The engrailed homology 1 (Eh1) domain, a short < 10 amino acid sequence located near the N-terminus, is a conserved region that is well-known for its interaction with Groucho, a co-repressor (Copley, 2005; Smith and Jaynes, 1996; Tolkunova et al., 1998). The Fez family of TFs also possess six C2H2 zinc finger DNA-binding domains, which confer the DNA sequence specificity of the protein (Pavletich and Pabo, 1991; 1992), though they also mediate interactions with RNA and other proteins (Gamsjaeger et al., 2007).
Recent studies in the mouse retina have implicated Fezf1 and Fezf2 in development of bipolar cells (BCs) and retinal ganglion cells (RGCs). Using single cell sequencing, Sanes and colleagues discovered that BC1A and BC1B, two subtypes of OFF bipolar cells, express Fezf2 and Fezf1, respectively (Shekhar et al., 2016). This study discovered BC1B, a previously unknown BC subtype with a strikingly similar morphology to the L3 neuron. Whether Fezf1/2 cell-autonomously direct their respective cell types to the OFF layer remains to be seen, though the cell-specific expression of Fezf1/2 in these cells suggests they may play cell-specific roles. Additionally, the Watanabe laboratory showed that Fezf2 knockouts resulted in a selective reduction of a subset of Chx-10-positive OFF bipolar cells, presumably BC1A (Suzuki-Kerr et al., 2018), consistent with the possibility that Fezf2 is involved in BC1A development. At this point, it is unclear if Fezf1/2 dictate cell-fate of BC1A and BC1B, or if they direct connectivity post-mitotically like dFezf in the L3 neuron. One possibility is that Fezf1/2 playing a similar role as the TF Sat1b in the mouse retina, which has been shown to direct ON layer stratification through Contactin-5 expression (Peng et al., 2017).

The post-mitotic role for dFezf in L3 development is surprising given its known function in other areas of the fly brain, and the function of its vertebrate homolog in the mammalian cortex. In the fly brain, dFezf has been shown function as a transcriptional repressor to prevent the dedifferentiation of intermediate neural progenitor cells (INPs) back into Type II neuroblasts, promoting neuronal generation (Weng et al., 2010; Janssens et al., 2014). Thus, dFezf function in the fly eye differs compared to its function in the central brain. In the eye, dFezf is expressed after the terminal differentiation of the lamina precursor cell, and functions post-mitotically to direct L3 axon targeting, whereas in the central brain, dFezf is active in progenitor cells to promote neuron generation.
Extensive studies in the mammalian neocortex have found that Fezf2 regulates the generation and development of deep layer cortical neurons. In the neocortex, a layered structure comprising six laminae (V1-V6), projection neurons are categorized in part based on the areas to which they project. Corticofugal projection neurons project axons out of the cortex to the thalamus (corticothalamic projection neurons [CThPNs]) or sub-cerebral areas (sub-cerebral projection neurons [SCPNs]). In contrast, callosal projection neurons (CPNs) project axons to other areas within the neocortex. The somas of each neuron type are located in specific cortical layers (e.g. SCPNs are highly concentrated in layer V) (Greig and Woodworth et al., 2013). Fezf2 is highly expressed in layer V neurons, and at lower levels in layer VI neurons, as well as radial glia cells (RGCs), the progenitor cells that give rise to all cells in the neocortex (Inoue et al., 2004; Shim et al., 2012; Eckler et al., 2014). Layer V neurons in Fezf2 null mice fail to target sub-cerebral areas and exhibit several characteristics of CThPNs and CPNs (Molyneaux et al., 2005; Chen et al., 2005; Chen et al., 2005; McKenna et al., 2011), including, for example, their electrophysiological properties (Chen et al., 2008). The cumulative work of several research groups suggests a model wherein Fezf2 is part of a cross-repression transcriptional network with other transcription factors such as SATB2 (CPNs) and TBR1 (CThPNs), which direct cell fate of neocortical progenitors (Molyneaux et al., 2005; Chen et al., 2008; McKenna et al., 2011; Alcama et al., 2008; Britanova et al., 2008; Bedogni et al., 2010; Han et al., 2011).

Cortical studies by Arlotta and colleagues have provided evidence that Fezf2 plays a post-mitotic role in corticospinal motor neuron (CSMN) development. Using a gain-of-function approach coupled with RNA-seq, Fezf2 was shown to be sufficient to induce the expression of CSMN genes like EPHB1. Importantly, EPHB1 null CSMN phenocopy the axon targeting defects exhibited by Fezf2 null mutants, suggesting that Fezf2 directs CSMN targeting via
EPHB1 (Lodato et al., 2014). In dFezf mutant L3 neurons, cell surface genes are the largest category of differentially expressed genes, suggesting that dFezf regulates layer specificity through activation and/or repression of cell surface molecules. Thus, while the dFezf homolog plays an earlier role in cell fate determination, it appears to play an active role in axon targeting of the differentiated cell.

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In this dissertation, we have discovered a novel transcriptional strategy underlying layer assembly. A single transcription factor can cell-intrinsically direct the layer specificity of one neuron type, while cell-extrinsically directing another to the same layer. Previously, general principles of layer assembly have been difficult to uncover because different neuron types can target the same layer using distinct strategies. The transcriptional scheme illustrated here is an elegant solution to this problem. The transcription factor dFezf serves as a molecular link between two separate and different modes of layer specific targeting. We envision that this motif is used in other by transcription factors in other lamina neurons to instruct targeting of the outer medulla. Furthermore, the structural and molecular similarities in layered neuropil across species, and the conservation of Fez family of transcription factors, suggest that this stratagem may be used in other complex systems.
References


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IV. Appendices
Supplementary figure 1. dFezf2 L3 neurons show mis-targeting phenotypes similar to those caused by the dFezf1 allele. (A–C) As in Figure 11, MARCM experiments were performed to generate dFezf2 L3 clones (green) labeled by myr::GFP driven by 9–9 GAL4. At 48 hr APF, L3 clones are visualized by confocal microscopy using a GFP antibody. Confocal images show L3 growth cones within the outer medulla which is defined by R7 and R8 axons (magenta, mAB24B10). The abnormal targeting and morphologies of the growth cones of mutant neurons resembles that observed for the growth cones of dFezf1 neurons (Figure 11H–J). All scale bars = 5 microns. (D) Quantification of MARCM experiments at 48 hr APF. N is the number of neurons that are quantified.
Supplementary figure 2. A BAC containing the dFezf locus rescues defects in growth cone targeting, layer specificity and axon terminal morphology caused by dFezf null mutations. (A–F) MARCM experiments analyzed at 48 hr APF or just after eclosion (Adult). L3 neurons are made homozygous for dFezf¹ or dFezf² in the presence of a single copy of a bacterial artificial chromosome (BAC) containing the dFezf locus (18B02). (A, C, E) Confocal images of L3 clones (myr::GFP driven by 9–9 GAL4) in the outer medulla. R7 and R8 axons (magenta, mAB24B10) define the boundaries of the outer medulla (24 and 48 hr APF) or provide a reference for outer
Supplementary figure 2 (continued) layers (adult). Scale bars = 5 microns. (B, D, F)

Quantification of MARCM experiments. N = the number of cells counted. (C) Arrowhead indicates a thin process from an L3 terminal extending toward the M6 layer. ~30% of L3 clones in this experiment display this minor abnormality. This likely reflects a partial defect in growth cone retraction from the proximal outer medulla.
Supplementary figure 3. DFezf mis-expression in L5 and L2 neurons. (A) 6–60 GAL4 drives expression of UAS-dFezf-3xHA (magenta, α-HA) in L5 neurons in the lamina (white arrowhead) and a subset of medulla (green arrowhead) and lobula neurons (yellow arrowhead). DFezf-HA is localized to cell bodies. Lamina (la), medulla (me), lobula (lo). (B–D) DFezf mis-expression in L2 neurons using a ‘weak’ FLP construct (27G05-FLP[attP18]) to generate sparse lamina neuron clones. 39D12-LexA was used to label L2 neurons (magenta, myr::tdTomato: α-DsRed), and L2 clones also expressed myr::GFP and so appear white in confocal images. (B and C) Confocal images of wild type and dFezf-HA expressing L2 axons in the outer medulla of adult flies. (D) Quantification of L2 mis-expression experiments in adult flies. L2 neurons were considered ‘isolated’ if they were the only clone within a column, and surrounded by columns without other MARCM clones. Thirteen of the sixteen L2 neurons mis-expressing dFezf-HA mis-targeted to M3. Of the thirteen mis-targeting neurons seven were ‘isolated’. These findings strongly support that dFezf cell autonomously instructs L2 mis-targeting in mis-expression experiments.
**Supplementary figure 4. Validation of mRNA changes identified through RNA-seq at the protein level.** The endogenous protein expression of a subset of DE genes identified through RNA-seq was visualized in wild type or dFezf null L3 neurons (9–9-GAL4, myr::tdTomato, α-DsRed) using GFP-protein traps (Nagarkar-Jaiswal et al., 2015), in MARCM experiments. All the experiments were performed at 40 hr or 48 hr APF to be consistent with the RNA-seq data. Each panel shows a confocal image within the region of the lamina wherein lamina neuron cell bodies reside (i.e. the lamina cortex). All scale bars = 5 microns. (A–B’) Dpr17-expressing cells (green, α-GFP). Asterisks indicate the positions of wild type (A and A’) or mutant (B and B’) L3 clones (magenta). Dpr17 protein is considerably reduced in L3 neurons lacking dFezf function. (C–D’) Nrm expression (green, α-GFP). Asterisks indicate wild type (C and C’) or mutant (D and D’) L3 clones (magenta). Nrm protein is strongly reduced in dFezf null L3 neurons. (E–F’) Beat-IIb expression (green, α-GFP). Asterisks indicate wild type (E and E’) or mutant (F and F’) L3 clones (magenta). Beat-IIb protein expression is considerably downregulated when dFezf is

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Supplementary figure 4 (continued) disrupted. (G–H’) CG34113 expression (green, α-GFP). Asterisks indicate wild type (G and G’) or mutant (H and H’) L3 clones (magenta). CG34113 protein is drastically reduced in the absence of dFezf function. (I) Table showing levels of expression for the genes assessed in A–H’ in wild type or dFezf null L3 neurons at 40 hr APF. The TPM (transcripts per million mapped reads) levels shown are an average of all samples for each genotype.
Supplementary figure 5. Biological categories of DE genes. This shows the numbers of DE genes that fall within particular biological categories based on previously curated databases (Kurusu et al., 2008; Tan et al., 2015). The proportion of genes that are up or down regulated are indicated in different colors.
Supplementary figure 6. Expression of CadN, Sema-1a and Netrin-A/B in wild type and dFezf null L3 neurons. (A) Heatmap of normalized counts for CadN, Sema-1a, Netrin-A and B (NetA/B) after applying an rlog transformation. This is a variance-stabilizing transformation, which transforms the count data to the log2 scale in a way that minimizes differences between samples for rows with small counts, and which normalizes with respect to library size. CadN and Sema-1a remain strongly expressed in the absence of dFezf function, while NetA and B are drastically reduced. (B) The average TPM (transcripts per million mapped reads) values for CadN, Sema-1a and NetA/B across all wild type and mutant samples are shown. (C) Disrupting Sema-1a and CadN in L3 neurons in MARCM experiments (as in Figure 11Q) drastically reduced L3 dendritic branches, while dendrites are not affected by the loss of dFezf function. L3 clones expressed myr::GFP driven by 9–9-GAL4 and were visualized in confocal images using a GFP antibody. R1-R6 axons (mAB24B10) were used as a reference for the lamina. Note that the wt and dFezf1 images shown here are the same as in Figure 6M and N.
Supplementary figure 7. Characterization of dFezf-FLAG (BAC). (A and B) A BAC (18B02) containing the dFezf locus was modified to incorporate sequence encoding three consecutive FLAG epitopes (3xFLAG) at the C-terminus prior to the translational stop. DFezf-FLAG (green) was detected in L3 somas by confocal microscopy using a FLAG antibody at 48 hr APF or in newly eclosed flies. All dFezf-FLAG expressing cells also expressed endogenous dFezf (magenta, α-dFezf), and all cells expressing endogenous dFezf expressed dFezf-FLAG. This confirms that dFezf-FLAG recapitulates the normal pattern of dFezf expression in the lamina.