Regulation of Neuronal Dendrite Development and Migration by the Atypical Cadherin Fat3

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Regulation of neuronal dendrite development and migration
by the atypical cadherin Fat3

A dissertation presented

by

Alexandra Krol

to

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Regulation of neuronal dendrite development and migration by the atypical cadherin Fat3

Abstract

Neuronal shape and position are critical to the formation and function of neuronal circuits. Although neurons develop axons and dendrites cell-autonomously in vitro, how extracellular cues in vivo direct neurite specification and placement remains poorly understood. The role of atypical cadherin Fat3 in amacrine cell (AC) development illustrates how the same extracellular cue can guide both dendrite formation and migration.

In the mouse retina, ACs have a bipolar morphology during their migration. Upon reaching the nascent inner plexiform layer (IPL), they elaborate one neurite into the IPL and retract the other. Loss of Fat3 leads ACs to develop an extra dendrite outside the IPL as well as errors in migration. We found Fat3 protein is concentrated at the IPL throughout AC development, suggesting Fat3 detects a directional signal. Here we investigated the signaling pathways upstream and downstream of Fat3 that mediate its role in AC development.

In Drosophila, Fat’s ligand is Dachsous. Fat and Dachsous binding is modulated by the kinase Four-jointed. Our analysis of mutant retinas determined that Fat3 and vertebrate Four-jointed genetically interacted. However knockout studies of vertebrate Dachsous homologues suggested they are not relevant Fat3 ligands in the retina. Instead, analysis of retinas missing retinal ganglion cells suggested Fat3-mediated homophilic adhesion between ACs may be important.
Sparse loss of Fat3 from ACs also led to extraneous neurites, suggesting Fat3 acts cell autonomously. *Ex vivo* live imaging revealed both migration and neurite dynamics were less directed in Fat3 mutant ACs. We hypothesized Fat3 acts to target asymmetric localization of cytoskeletal regulators to the leading neurite. To identify downstream Fat3 effectors, we performed a pulldown assay using the Fat3 intracellular domain. We identified several cytoskeletal regulators as candidate binding partners. We focused on the actin regulators Ena/VASP and demonstrated a direct interaction with the Fat3 intracellular domain. In the retina, Ena/VASP localized with Fat3 to the IPL, and loss of Fat3 changed Ena/VASP distribution. Furthermore, forcing uniform membrane recruitment of Ena/VASP in developing ACs phenocopied loss of Fat3. Together these results suggest Fat3 polarizes the activity of cytoskeletal effectors to help direct AC migration and dendrite placement.
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Chapter 1: General introduction
Neuronal morphogenesis

Mature neurons are highly polarized cells with distinct dendritic and axonal compartments and often elaborate morphologies. Each cell must also be correctly positioned and oriented relative to neighboring neurons and the overall nervous system to engage in accurate connections. To build the architecture of the nervous system requires neurons to migrate, to elaborate neurites in the correct places and to develop axo-dendritic polarity. Each of these events: neurite outgrowth, directed migration, and the acquisition of dendritic and axonal characteristics, requires cellular polarity and hence a polarized cytoskeleton. Therefore at the level of the cytoskeleton, these events are linked and share many molecular mechanisms. Of course while some aspects of cytoskeletal organization are shared, the dynamics of the cytoskeleton is also different in each developmental event. However shared themes emerge. First, the microtubule and actin cytoskeleton act in a coordinated manner. Both are critical for polarized behaviors as is the coordination between the two. Second, in the absence of an external cue the cytoskeleton can ‘self organize’ to a great extent. Third, extracellular cues act to initiate, coordinate, and maintain cytoskeletal polarization. Lastly, these developmental events are linked temporally; therefore it is important not to only consider them in isolation as each one defines the initial state of the next: Initial polarization precedes migration or the emergence of axo-dendritic polarity. Migration transitions to neurite elaboration with the dendrite forming from either the leading or trailing neurite and as a last step, dendrites and axons elaborate and acquire distinct properties.
**Polarity initiation**

To generate a polarized state requires a symmetry breaking event and the creation of initial cytoskeletal asymmetry. Many events in neuronal development, including migration and dendritogenesis, require cellular polarity. Therefore it is critical to understand the molecular and cellular mechanisms that are and are not necessary to initiate polarity.

The breaking of neuronal symmetry and the emergence of polarity have been most extensively studied *in vitro* using hippocampal cultures (Bradke and Dotti, 2000; Dotti et al., 1988). Dissociated neurons, upon plating, undergo spontaneous polarization or repolarization in distinct and defined stages. Stage 1: Neurons form multiple protrusions. Stage 2: Neurons form multiple symmetric neurites which dynamically extend and retract. Stage 3: One neurite lengthens rapidly and is specified as the axon, while the remaining neurites are specified as dendrites or retract. Stage 4: All neurites elaborate and acquire mature dendritic and axonal properties. Dissociated neurons’ ability to form axons and dendrites suggests the establishment of neuronal polarity is a cell-intrinsic event. The first symmetry breaking event seems to be axon specification, suggesting it may be the critical decision point. This is supported by the observation that when an axon is cut off, another neurite will develop into an axon in its place (Dotti and Banker, 1987).

The classic *in vitro* experiments suggest that axon formation is stochastic. However more recent studies, which have examined the process in more detail, suggest otherwise. There is some evidence that the first neurite that forms is instructive and has an increased potential to become the axon *in vitro*. The first neurite also seems to
determine the position of the second neurite, which forms at the opposite pole (Calderon de Anda et al., 2008). Moreover, the position where the first neurite forms may not be random either.

Initially the position of the centrosome and the Golgi apparatus was believed to be instructive as the centrosome is often at the site of axon formation. Among other pieces of evidence, manipulations which lead to multiple centrosomes can lead to multiple axons (de Anda et al., 2005). On the other hand, mutations in Drosophila which eliminated the centrosome did not seem to interfere significantly with neuronal morphogenesis (Basto et al., 2006). Furthermore, observations with higher temporal resolution subsequently suggested that neurite formation often precedes centrosome localization and is unrelated to centrosomal position (Gärtner et al., 2012, 2014a). Instead, experiments using drug manipulations to inhibit the actin or the microtubule cytoskeleton determined that initial neurite outgrowth requires F-actin polymerization and precedes the localization of the centrosome-Golgi complex, which additionally requires microtubules and PI3-kinase (Gärtner et al., 2012).

Neurite outgrowth is closely preceded by adhesive signaling. Plating neurites on stripes of N-cadherin and poly-l-lysine versus poly-l-lysine alone leads to neurite outgrowth on the N-cadherin-facing side of the neuron. Critically, asymmetric N-cadherin also accumulates and precedes neurite outgrowth when cells are plated on a homogeneous surface. This also seems to reflect the situation in vivo where endogenous N-cadherin is enriched at the apical surface of neurons preceding neurite outgrowth, at the apical surface (Gärtner et al., 2012, 2014a). Therefore even in vitro, the choice of which neurite grows out first does not appear to be either entirely
stochastic, or be determined by which neurite is closest to the centrosome, but instead is influenced by extracellular cues, or asymmetries present pre-dissociation or from the preceding mitotic event.

In vivo the roles of extracellular cues and the plane of mitosis are becoming increasingly clear. For instance in Drosophila sensory neurons, the formation of the first neurite, which subsequently becomes an apical dendrite, is linked with the orientation of the mitosis. The mitotic cortical furrow defines the apical pole of the cell, depositing remnants of its markers including Aurora A. This immediately precedes PIP2 enrichment, followed by the accumulation of apical determinants such as alpha and beta catenin, DE Cadherin and Par-3. Changing the orientation of the mitotic furrow, also changes the location of first neurite outgrowth (Pollarolo et al., 2011). While the link between asymmetries derived from mitotic exit and neuronal polarization is less clear in other systems, several examples of important extracellular cues have been found. For instance Sema 3a is present in a gradient in the cortex and orients neurons to promote migration (Chen et al., 2008), apical dendrite extension (Polleux et al., 2000) and suppress axon extension (Shelly et al., 2011). However what cues guide the very-most initial polarization of the cortical neurons remains to be explored.

These studies demonstrate that, to some extent, the elaborate morphology of individual neurons can be achieved cell-intrinsically through a self-organizing cytoskeleton in vitro. As is further described below, once polarity or asymmetry is initiated, cytoskeletal regulators can self-organize to reinforce this polarity. However even at the most initial stage of neuronal polarity, extrinsic cues play a critical role, even
in contexts where it appeared they may not. These initial asymmetries are then amplified to generate axons and dendrites. Similar initial polarization events must also precede the polarity of a migrating neuron with a leading and trailing neurite.

**Migration**

Between initial polarization and dendrite specification, most neurons migrate. Therefore dendrites are often formed from either the leading or the trailing neurite of a previously migrating cell. As a consequence, the cytoskeletal state of neuron at the end of migration is the initial state of most cells undergoing axo-dendritic polarization. Migrating neurons have a highly polarized morphology and underlying cytoskeleton (Cooper, 2013; Marín et al., 2006). Each step of the migratory movement-leading process extension, nuclear translocation and trailing process retraction-involves coordinated activity of microtubules and actin and polarization of the cytoskeleton.

**Modes of migration**

Several modes of migration have been characterized, but broadly speaking they can be divided into radial, i.e. directed perpendicular to ventricular surfaces, and tangential, i.e. directed parallel to ventricular surfaces.

Radial migration has been best characterized in the migration of excitatory pyramidal cells of the cerebral cortex (Nadarajah and Parnavelas, 2002). Radially migrating neurons have a bipolar shape with a largely unbranched leading process. Two modes of radial migration have been identified in the cortex. In the first mode, neurons use radial-glia as scaffolds for migration. In the second mode, radial migration is glia-
independent. Live imaging reveals these two modes of migration are characterized by different dynamics (Nadarajah et al., 2001). Neurons migrating along glia with a saltatory movement: periods of slow migration punctuated by periods of rapid forward movement. The rapid movement corresponds to a surging forward of the nucleus into the leading neurite, and the slow migration corresponds to trailing neurite retraction, and leading neurite extension. Neurons migrating in a glial-independent fashion extend their leading neurites directly into the marginal zone. This is followed by relatively smooth translocation of the nucleus and cell body towards the marginal zone (Nadarajah et al., 2001; Sekine et al., 2011).

Tangential migration has best been characterized in inhibitory neurons migrating from the medial ganglionic eminence to the cerebral cortex (Martini et al., 2009; Valiente and Marín, 2010). As neurons migrate tangentially, they extend a branch from their leading neurite, or sometimes a second process from their soma. The centrosome and other organelles are recruited to the branchpoint, upon which the nucleus progresses and concurrently or soon after, one of the branches is stabilized while the other retracts or becomes the trailing process (Bellion et al., 2005; Martini et al., 2009; Ward et al., 2005). Formation of neurites, stabilization, and retraction occurs in response to guidance cues, and contributes to guidance (Evsyukova et al., 2013).

Neurons can also alternate from one mode of locomotion to another during the course of migration. For instance cortical pyramidal neurons progress through several stages as they move towards the pial surface (Kriegstein and Noctor, 2004; Noctor et al., 2004). Upon exit from mitosis, neurons move radially a short distance from the ventricular space to the SVZ (Noctor et al., 2004). Thereupon they pause and take on a
highly dynamic multipolar shape, extending and retracting thin neurites (Tabata and Nakajima, 2003). During this stage many neurons progress slowly towards the pial surface; others however remain stationary and some move tangentially, translocating their nucleus along a stabilized lateral process. Neurons then either directly reassociate with the glial fiber and resume radial migration, or first migrate backwards towards the ventricle and make contact with it prior to resuming radial migration (Noctor et al., 2004).

**Cellular mechanisms of migration**

While all of the modes of migration have differences, all modes share the polarization of the neuron to achieve directional extension of a leading process, translocation of the nucleus and trailing process retraction.

*Leading neurite*: Leading neurite dynamics vary widely between the different modes of migration, as described above, and initial extension occurs largely independently of the rest of the neuron. Only upon leading neurite stabilization does the nucleus translocate (Ayala et al., 2007; Lambert de Rouvroit and Goffinet, 2001). The cytoskeletal dynamics that lead to the exploratory dynamics and stabilization of the leading processes are likely to be similar to the growth cones found at the tips of growing axons and dendrites. The growth cone has a highly organized and precise arrangement of actin and microtubules. At the periphery, actin bundles drive the protrusion of filopodia, while an actin meshwork forms a lamellipodium spanning in between. The actin underlying the fillipodia and lamellipodia is highly dynamic, with constant polymerization at the tips, combined with a retrograde flow of actin towards the
neurite shaft driven by the motor myosin II. Severing of actin can also be important, providing additional actin ends for growth. The plus ends of microtubules emerge from a linear arrangement of microtubules at the shaft to invade and actively explore the periphery of the growth cone (Lowery and Van Vactor, 2009). In recent years, the central role of microtubule actin coupling and coordination in neurite dynamics has become increasingly clear (Cooper, 2013). Presence of F-actin bundles inhibits microtubule entry into the periphery, while capture of microtubules by actin can also promote microtubule entry into filipodia and stabilization at the cell cortex. In radial migration, for the leading neurite to remain associated with the glia, adhesion between the leading neuronal tip and the glia is important, mediated by astrotactin and integrins in particular (Marín et al., 2010). Other modes of migration are influenced by cues and receptors identified in axon guidance such as Ephrins and Semaphorins (Evsyukova et al., 2013).

**Nucleokinesis:** Nucleokinesis, or the forward movement of the nucleus, is in many ways the defining feature of neuronal migration, since extension and retraction of processes occurs in multiple other contexts (Valiente and Marín, 2010). Nucleokinesis occurs most commonly in several steps. First, a cytoplasmic swelling forms in the leading process. Next, the centrosome and accompanying organelles including the Golgi and mitochondria move into the swelling. Lastly, the nucleus follows. Microtubules link the centrosome with the nucleus, surrounding the nucleus. Dyneins moving along the microtubule provide a pulling force that is believed to be the main force moving the nucleus forward. At the rear of the nucleus, actomyosin contraction contributes a pushing force to neuronal migration. More recently, several studies have questioned the
centrality of the centrosomal movements, as modes of migration have been observed where the nucleus leads the centrosome occasionally (Sakakibara et al., 2014). In this case, the movement of the nucleus cannot be driven by dynein pulling forces. Furthermore in the retina, the centrosomes do not lead the nucleus during migration (Zolessi et al., 2006), while in Drosophila, elimination of the centrosomes altogether has little effect on neuronal morphogenesis (Basto et al., 2006) However in cortical neurons, disrupting dynein function or actomyosin contraction, blocks the forward movement of the nucleus (Tsai et al., 2007). Therefore it is likely that different neurons and perhaps different modes of migration rely to differing extents on the different forces underlying nucleokinesis.

**Trailing neurite retraction:** Little is known about active regulation of trailing neurite retraction. At the most, it is believed to be a byproduct from the process of actomyosin contractility (Schaar and McConnell, 2005). As discussed, actomyosin contractility can contribute to the forward movement of the nucleus. Squeezing of the nucleus forward leaves behind a segment of trailing neurite, previously occupied by the neuron. Some neurons do not form a trailing neurite during migration, while others such as pyramidal neurons of the cortex extend an axon during migration.

Migration is an important step in the development of many neurons. As a highly polarized and dynamic process, migration illustrates many principles of polarity. First, the actin and microtubule cytoskeleton are dynamic, highly organized structures and tightly coordinated to allow the progression of the leading process and the movement of the nucleus. Second, similar cellular events can be achieved by different mechanism.
For instance, both actomyosin contraction or microtubule-dynein pulling can generate nuckleokinetic forces; different neuronal type depend more on one or the other. Similarly, leading process pathfinding can occur with the advance of a single neurite or through the extension of branches. Third, migrating cells can change direction rapidly, illustrating how an extracellular cue can reorient neuronal polarity. Indeed some neurons can repolarize completely, so the trailing neurite becomes the leading neurite (Martinez-Molina et al. 2011). Lastly, the same neuron can switch completely from one mode of migration and polarization to another in response to the extracellular environment, illustrating both the dynamic nature of the polarized migratory state, and the influence of extracellular cues.

**Dendrite formation**

Dendrites and axons are largely defined by the structural and functional difference of their respective cytoskeletons. While mature dendrites typically have microtubules of mixed orientation, axons have microtubules oriented predominantly with plus-ends towards the tips. This organization has consequences not only for the morphology of these compartments, but is also critical in defining dendrite and axon-specific transport and identity. In turn, selective transport of proteins and organelles further reinforces compartment identity and microtubule organization. Following initial polarization, most often derived from the preceding migratory neuronal state, microtubules stabilization and actin polymerization are coordinated to establish morphological polarity.
Microtubules

As previously discussed, in *in vitro* hippocampal cultures, the choice of one neurite to become an axon from multiple symmetric neurites seems to be a defining step. Upon one neurite being specified as the axon, all others either form dendrites or retract. This suggests that the cytoskeletal events that mark the transition of a generic neurite to an axon represent the beginning of cytoskeletal feed forward loop that results in the formation of dendrites. Prior to a single neurite extending rapidly and marking the transition from Stage 2 to Stage 3, all neurites appear the same dynamically and morphologically. However these neurites already exhibit differences in their microtubules, as evidenced by the selective movement of motor proteins along them (Jacobson et al., 2006; Petersen et al., 2014). Kinesin-1 translocates selectively to axons in mature neurites, and a constitutively active form accumulates at axonal tips, while motors that traffic vesicles marked by Rab 11b are destined for dendrites in mature neurites. In stage 2 neurites, constitutively active kinesin-1 selectively targets only one or two neurites (Jacobson et al., 2006) while Rab 11b vesicles target all other neurites (Petersen et al., 2014). During this stage, neuronal identity seems to be dynamic. The accumulation of Kinesin-1 can switch to another neurite, and Rab11b-tagged vesicle traffic switches in a corresponding manner. Importantly however, prolonged Kinesin-1 accumulation at a neurite precedes the burst of neurite outgrowth which marks it as having acquired axonal identity (Jacobson et al., 2006). The differences in molecular motor trafficking probably reflect an underlying difference in posttranslational modifications of microtubules, which are also seen in stage 2 microtubules (Hammond et al., 2010). These may reflect a difference in microtubule
stability, since taxol treatment that increases microtubule stability led to a more even
distribution of posttranslational microtubule modifications. However such a difference
may well be quite small since no difference was seen in neurite microtubule stability and
turnover using FRAP experiments (Hammond et al. 2010). Stability of microtubules
does seem to be important since localized light-activation of taxol in a single neurite is
sufficient to promote an axonal fate (Witte et al., 2008). Conversely, destabilization of
microtubules by a small amount of nocodazole leads to multiple dendrites (Witte et al.,
2008).

Actin

Changes in actin in the growth cone probably precede, or at minimum are
coordinated with changes in microtubules. Actin organization for instance can mediate
microtubule entry into the growth cone. Typically, prior to a neurite growing and
becoming the axon, the neurite also exhibits a greatly enlarged growth cone with
destabilized actin (Bradke and Dotti, 1999). Furthermore, inducing actin destabilization
in a single neurite, with drugs such as cytochalasin D leads to its becoming the axon.
Similarly destabilizing all actin by global application of the drugs or by inactivating the
Rho family of GTPases using toxin B leads to multiple axons (Bradke and Dotti, 1999).

Golgi

The Golgi apparatus seems to be differentially required for dendritic growth
versus axonal growth. The Golgi is important for secretion and trafficking of receptors,
as well as serving as a microtubule organizing center. In a screen for reduced dendritic
arbors with a maintenance of axons in *Drosophila* neurons, three genes were identified, all regulators of ER-to-Golgi transport. Their mutations led to a dispersion of the Golgi both in the soma and in dendritic Golgi-outposts. Furthermore, while the dendritic arbors were severely reduced, axon outgrowth was unaffected over the observed timecourse (Ye et al., 2007). Indeed blocking secretory trafficking from Golgi with BFA, or budding from the TGN with dominant negative protein kinase D1 all led to a decrease in dendrite outgrowth both *in vivo* and *in vitro* (Horton et al., 2005; Ye et al., 2007) while preserving axonal growth.

The positioning and distribution of the Golgi seem critical for its function in dendrite development. In cells with asymmetric dendrites, the Golgi is asymmetrically distributed. For instance in hippocampal neurites *in vitro*, the Golgi enters into the largest dendrite, while GABAergic interneurons lack a primary dendrite and have a more uniform distribution of Golgi. *In vivo*, the Golgi is also orientated into the primary dendrite in cortical pyramidal neurons (Horton et al., 2005) and in Purkinje cells of the cerebellum (Tanabe et al., 2010). Importantly, the orientation of the Golgi appears to be predictive of where the primary or apical dendrite will form. While initially Purkinje cells in the cerebellum extend and retract multiple dendritic neurite, eventually all neurites retract except for one which elaborates into a single apical dendrite. Live imaging in zebrafish embryos shows that the Golgi localized to the base of the future apical dendrite prior to morphological differences in the neurites. Furthermore, cell autonomous dominant negative expression of aPKC lead to dispersal of the Golgi, the formation of multiple Golgi and the retention of multiple primary dendrites by Purkinje cells (Tanabe et al., 2010). Similarly, overexpression of Grasp-65, which helps hold
Golgi stacks together, in hippocampal cultures leads to dispersal of the Golgi while preserving Golgi secretion. This leads to the formation of a symmetric dendritic arbor while preserving the rate of dendrite outgrowth (Horton et al., 2005).

The distribution of the Golgi outposts into dendritic arbors, particularly to branch points, is also an important consequence of microtubule differences and differential trafficking. Mutations in Drosophila of the minus end directed dynein and plus end directed kinesin motor complexes led to changes in Golgi distribution towards more proximal dendrites as well as entry into the axon (Satoh et al., 2008; Zheng et al., 2008). This leads to problems in dendrite outgrowth and branching, although the dynein mutations also lead to thicker axons. More direct interference with Golgi positioning, by expressing dominant negative lava lamp, preventing Golgi association with the dynein motor, also leads to defects in dendrite development (Ye et al., 2007). The presence of Golgi outposts in dendrites is also associated with dendrite branch extension and changes in Golgi outpost distribution lead to changes in dendrite morphology (Ye et al., 2007). Part of the role of Golgi outposts may be secretory and part may be through the acentrosomal nucleation of microtubules through associated gamma-tubulin (Ori-McKenney et al., 2012).

In summary, to generate axo-dendritic polarity, initial asymmetries lead to the coordination of actin polymerization and microtubule stabilization. Continued outgrowth is dependent on a continuation of coordinated actin and microtubule dynamics. In the case of dendrite development, the localization of the Golgi is also important. The role of the Golgi is a case in point of how initial asymmetries are amplified. Initial localization of
the Golgi seems dependent on microtubule organization. However recruitment of the Golgi and Golgi outpost leads to further microtubule nucleation.

**Outstanding questions**

*In vivo*, neuronal polarization, migration and dendritogenesis are closely linked events for which the dynamics and polarity of the underlying cytoskeleton are critical. The precise composition, distribution and activity of many cytoskeletal regulators generate the forces, dynamics and directionality of the cytoskeleton. By acting upon these cytoskeletal regulators, extracellular cues can provide a polarizing cue that orients the cell with the tissue.

We have a good understanding of how the cytoskeleton is regulated. Many cytoskeletal effectors are known that bind to and directly regulate actin and microtubule stability and structure. These include actin regulators that mediate actin polymerization, branching and severing, as well as actin motor proteins. Equally there are also many microtubule regulators that regulate stability, polymerization, catastrophe and severing, regulators at the plus and minus tips of microtubules, as well as a plethora of plus and minus end directed motor proteins. There are also increasing numbers of actin and microtubule cross linking proteins. On top of this there are regulators that recruit, promote and inhibit the activity of many of these effectors. All of these contribute to the regulation of actin and microtubule dynamics.

As described above, we also have a good idea of the microtubule and actin dynamics that occur during many steps in development. However what is less clear are the ways in which the cytoskeleton polarity is coordinated with surrounding cells and the
tissue. To address these questions it is necessary to have an *in vivo* model where the spatial nature of the extracellular cues is preserved.

**Retinal development**

The retina has proved to be a powerful system for elucidating many aspects of neuronal polarization, migration, and dendrite targeting. The retina is a highly accessible portion of the nervous system with a very clear and stereotyped architecture. In particular, the strict laminar organization highlights changes in cell morphology and connectivity. The retina consists of three nuclear layers that contain the cell bodies and two plexiform layers, which contain all the synapses (Figure 1.1, right hand side). The outer nuclear layer contains rods and cones. The rods and cones connect to bipolar cells in the inner nuclear layer; the bipolar cells connect to retinal ganglion cells in the retinal ganglion cell layer. Horizontal cells and amacrine cells reside in the inner nuclear layer, modulating connections in the outer and inner plexiform layers respectively. In the mouse, the retinal ganglion cell layer also contains a substantial number of displaced amacrine cells. Because of this precise arrangement of the retina, a change in morphology such as two dendritic arbors rather than one becomes relatively easy to detect.

The retina develops from a pseudostratified epithelium of progenitors oriented with their apical side towards the outer limiting membrane. These progenitors undergo interkinetic nuclear migration between the outer limiting membrane and the basal lamina, divide and give rise to postmitotic neurons near the outer limiting membrane. Upon exiting the mitotic cycle, neurons migrate basally towards their final positions
Figure 1.1 Amacrine cell development and architecture of the retina.

Amacrine cells (green) are born in the neuroblast layer (NBL; left) and establish specific connections with retinal ganglion cells (black) and bipolar cells (red) in the inner plexiform layer (IPL) of the mature retina (right). (GCL) Retinal ganglion cell layer; (INL) inner nuclear layer; (OLM) outer limiting membrane; (ONL) outer nuclear layer; (OPL) outer plexiform layer. Text and figure modified from Deans et al., 2011. Reprinted with permission.
(Norden et al., 2009). Retinal progenitors produce the different cell types of the retina in a defined, sequential, but overlapping time course. Retinal ganglion cells are born first, followed by cones, horizontal cells, amacrine cells, rods, Muller glia and bipolar cells (Schweers and Dyer, 2005; Voinescu et al., 2009).

One nice set of studies shows the advantage of the retina as a model system and how it can be used to begin to study the roles of extracellular cues in the context of a tissue. As mentioned previously, hippocampal neurons plated in culture form a single axon and a dendritic arbor following a multipolar stage of neurite outgrowth (Dotti et al., 1988). Similarly retinal ganglion cells when they are cultured undergo a multipolar neurite stage and eventually extend an axon (Zolessi et al., 2006). Similar to hippocampal cells, the constitutively active Kif5c, used as an axonal marker, oscillitates between the different neurites before staying in the one that then grows into an axon (Randlett et al., 2011a). However in vivo, retinal ganglion cells extend a single neurite towards the basal lamina, and Kif5c is always localized to the basal process, accumulating at the tip following contact with the basal lamina (Randlett et al., 2011a). Outgrowth and specification of the axon turn out to be dependent on contact with Laminin 1α present at the basement membrane (Randlett et al., 2011a). Furthermore, experiments with hippocampal cultures have shown that apical junctional complex-associated proteins such as par-3 and par-6 localize to the tip of the growing axon and are important for polarization in vitro (Nishimura et al., 2004; Shi et al., 2003, 2004). However in the retina, these proteins remain apically localized throughout migration and axon specification and outgrowth (Zolessi et al., 2006). These experiments show how important the tissue environment is for physiological cellular behaviors. One further
study showed that apical retraction of the trailing neurite was controlled via Slit-Robo regulation of Ncadherin-mediated adhesion of the apical neurite to the outer limiting membrane (Wong et al., 2012). This illustrates how relevant extracellular cues can begin to be understood in the context of the retina, but highlights the need to link the effect of the cues to molecular changes in the cytoskeleton.

Amacrine cells of the retina present a good model to study how oriented dendrite outgrowth occurs. Amacrine cells have many dendritic morphologies (MacNeil et al., 1999), which likely correspond to different functions. However they share a strict orientation of their dendritic arbor into the inner plexiform layer (IPL). Therefore changes in dendrite orientation are easy to assess. Furthermore, most amacrine cells lack a classic axon, so that polarization of amacrine cells can be considered separate from axon specification.

Amacrine cells exit the mitotic cycle between E9-P4, peaking at E15-E17 (Hinds and Hinds, 1983; Voinescu et al., 2009). Following differentiation, amacrine cells migrate towards the nascent IPL containing processes from retinal ganglion cells, which are born first (E9-E16) and have already migrated to the retinal ganglion cell layer (Figure 1.1). Birthdating studies show some correlation between birth order of amacrine cells and the position of their cell bodies, with the first born being closest to the RGC layer (Voinescu et al., 2009). Upon encountering the IPL, amacrine cells stop, elaborate their leading processes to form connections with the retinal ganglion cells and each other, and retract their trailing processes (Figure 1.1, Godinho et al., 2005; Hinds and Hinds, 1978, 1983). The exact sequence or migrating amacrine cell morphologies in the mouse is unclear. Live imaging in zebrafish, suggests migrating amacrine cells appear
to have a multipolar morphology with exuberant extension and retractions of neurites (Godinho et al., 2005). However early EM studies suggest a bipolar shape in migration followed by elaboration into the IPL and retraction of the trailing process although some multipolar cells are seen close to the IPL (Hinds and Hinds, 1983). Golgi studies in the chicken retina suggest that some amacrine cells have a bipolar migratory morphology while others have a multipolar migratory morphology (Prada et al., 1987). One large caveat to the early studies is that cell identity and the sequence of events had to be inferred, for obvious reasons. Clearly however, an important decision for amacrine cells is which neurites to retract and which to elaborate into the IPL.

**Fat3 in amacrine cell development**

Previously the lab uncovered a role for the atypical cadherin Fat3 in amacrine cell development. During amacrine cell development, Fat3 is expressed by amacrine cells and retinal ganglion cells. In the absence of Fat3, amacrine cells no longer reliably retracted their trailing neurite. Instead, Fat3 mutant amacrine cells near the IPL tended to have longer trailing neurites (Figure 1.2), and 23% of calretinin positive amacrine cells retain a trailing neurite. Consequently, Fat3KO amacrine cells go on to develop two dendritic arbors: a correct arbor projecting to the IPL and an ectopic arbor projecting away from the IPL (Figure 1.3). All subtypes of amacrine cells examined (Figure 1.3 A-D and Deans et al., 2011) with the exception of cholinergic amacrine cells, develop an extra neurite in the absence of Fat3, although some cells of each subtype are unaffected. The ectopic neurites recruit contacts from neighboring neurons leading to the formation of an ectopic layer of neurites and synapses (Figure 1.3 G,H). While most
Figure 1.2 Fat3KO amacrine cells near the IPL have longer trailing neurites.

A) A montage of representative Ptf1a-cre;Z/EG labeled cells in P1 Fat3KO mice. Neurite length was quantified for cells at positions (a) and (b) in wildtype and Fat3KO retinas. (C) Migrating cells in the middle of the neuroblast layer (a) have trailing processes of equal lengths in wildtype and mutants. In contrast, Fat3KO cells near the IPL (b) have longer trailing processes than wildtype cells in the same location (*** P<0.0001 by unpaired two-tailed t-test). Mean is represented by a straight line. Scale bars: 10µm. Text and figure modified from Deans et al., 2011. Reprinted with permission.
Figure 1.3 Amacrine cells develop a second dendritic arbor and extra plexiform layers in Fat3KO retina.

(A,B) At P11, calretinin labels a subset of amacrine cells and retinal ganglion cells that form three bands in the WT IPL (A). This persists in the Fat3KO retina (B) but labeled dendrites are also present in a new layer in the INL. We refer to this layer as the outer misplaced plexiform layer (OMPL) due to its position and composition of ectopic dendrites. (B,C) Dab1-positive AII cells form a single layer at the boundary between the INL and the IPL. In Fat3KOs (H), AIIIs are divided into two groups by the OMPL; an example of an AII trapped in the middle of the INL is marked (*). In Fat3KOs, AII cells also extend dendrite branches to the OPL, and a smaller number of dendrites project into GCL (brackets). (I,J) The morphology of rod bipolar cells is also altered in the Fat3KO retina, with endings frequently mislocalized in the IMPL (brackets). (G,H) VGAT immunolabeling of AC dendrites and synaptic vesicles in the wildtype and Fat3KO OPL and IPL at P11. Staining also highlights the IMPL (arrow) and OMPL (arrowhead) in Fat3KOs. Retinas were counterstained with DAPI (blue). Scale bars: 50 µm (A–B, G–H); 20 µm (C–F).

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Figure 1.3, Continued

A. Calretinin

B. OMPL

C. Dab1

D. OMPL

E. PKCalpha

F. OMPL

G. VGAT, DAPI

H. wildtype Fat3KO
contacts appear to be with other amacrine cells, bipolar cell axons are also recruited (Figure 1.3 E-F). Amacrine cells with cell bodies in the inner nuclear layer form an ectopic plexiform layer in the middle of the inner nuclear layer, while displaced amacrine cells with cell bodies in the retinal ganglion cell layer form an ectopic plexiform layer underneath the retinal ganglion cell layer. However there is also a second phenotype; loss of Fat3 leads to an increase in the number of amacrine cells migrating into the retinal ganglion cell layer. Lastly, the functions of Fat3 in amacrine cell migration and morphology appear to be separable. Amacrine cell specific loss of Fat3 leads to amacrine cells with extra neurites. However amacrine cells no longer migrate into the retinal ganglion cell layer. The synaptic layer below the retinal ganglion cells is also lost in an amacrine cell-specific conditional knockout, suggesting it is derived from amacrine cells that migrate too far in the full knockout.

The Fat3KO phenotype highlights an important event in amacrine cell development: the transition from a migratory morphology to correct neurite elaboration. In general, little is know about mechanisms leading to trailing process retraction. In particular, the phenotype raises the question of how Fat3 signaling leads to retraction of the trailing neurite.

Fat family

Fat3 belongs to the Fat family of atypical cadherins. These are characterized by an extremely long extracellular domain consisting of 3+4 cadherin domains followed by Laminin and EGF domains as well as a transmembrane domain, and unique intracellular domain. There are two Fat members in Drosophila, Fat and Fat-like and
four in Vertebrates, Fat 1-4. Fat 4 is most closely related to *Drosophila* Fat and Fat1-3 are most closely related to *Drosophila* Fat-like (Figure 1.4) (Tanoue and Takeichi, 2005).

Primarily from work in *Drosophila*, the roles of Fat are fairly well understood both *in vivo* and at a cellular level (Sopko and McNeill, 2009). In contrast, a coherent role for the Fat-like family of Fats has still to emerge (Sadeqzadeh et al., 2014a). While several phenotypes have been identified, it is still too early to say whether there is a common theme. Similarly while many intracellular domain binding partners for Fat1 have been identified, a clear picture of Fat-like signaling has not emerged.

**Phenotypes**

*Drosophila* Fat is the founding member of the Fat family and as such has been most extensively studied. *Drosophila* Fat has key roles both in setting up planar cell polarity of tissues and in controlling cell growth. It seems that some but not all of these roles are conserved for vertebrate Fat4. The generation of Fat4 null mice has revealed that Fat4 also plays a role in planar cell polarity but so far does not support a role in regulation of growth such as for *Drosophila* Fat (Saburi et al., 2008). Novel roles for Fat4 may also exist in the nervous system. Knockdown studies have shown a role for Fat4 in apical membrane architecture of neural progenitors of the cerebral cortex (Ishiuchi et al., 2009).

Although a coherent picture of Fat-like function has not yet emerged, it seems overall that Fat-like’s and Fat’s functions are distinct. The discovery of *Drosophila* Fat-like is relatively recent therefore only a couple of studies have investigated its function.
Figure 1.4 Schematic representation of the Fat, Fat-like and Dachsous family of atypical cadherins and their interactions.

*Drosophila* has one Fat, one Fat-like and one Dachsous cadherin. Mammals have one Fat cadherin, Fat4, and three Fat-like cadherins, Fat 1-3, and two Dachsous cadherins, Dachsous1 and 2. Binding in trans, as illustrated, has been demonstrated between Fat and Dachsous in *Drosophila* and Fat4 and Dachsous1 in mammals. This binding in trans is modulated by phosphorylation of the extracellular domains by the golgi kinase Fourjointed in *Drosophila*. Fjx1 is the mammalian Fourjointed homologue and interacts genetically with Fat4, therefore its phosphorylation of the extracellular domains of Fat4 and Dachsous1 is likely conserved.
*Drosophila* Fat-like mutants show failures in tubular organ formation (Castillejo-López et al., 2004) and egg chamber formation (Viktorinová et al., 2009, 2011). At a cellular level, failure in egg chamber formation is linked with the failures of actin-polarity of the surrounding follicle epithelium (Viktorinová et al., 2009). In vertebrates, the role of Fat1 has been explored the most and knockout mice exhibit a variety of phenotypes including kidney failure, exencephaly, anophthalmia, (Ciani et al., 2003) and problems in muscle morphogenesis (Caruso et al., 2013). At a cellular level, kidney failure is associated with failures in renal glomerular slit-juncture formation (Ciani et al., 2003), while muscle morphogenesis failures are associated with a decrease in polarization of muscle cells during migration (Caruso et al., 2013). As described above, loss of Fat3 in mouse leads to failures in dendrite development (Deans et al., 2011). In zebrafish, Fat3 has also been linked with polarized intercalation during chondritogenesis, as well as chondrocyte cell fate determination (Le Pabic et al., 2014).

Altogether, it is still unclear what role or roles Fat-likes have. In *Drosophila* Fat-like and Fat phenotypes seem distinct. However in vertebrates the situation may be more complicated. Genetic studies with mouse mutant for Fat4 and Fat1 or Fat3 show both synergistic and antagonistic phenotypes depending on the tissue (Saburi et al., 2012). Therefore perhaps at least a portion of Fat-like signaling may overlap or be related to Fat signaling.

**Upstream signaling**

Signaling upstream of Fat has been extensively studied in *Drosophila*, but is much less understood in vertebrates. Fat signaling is regulated by the closely related...
atypical cadherin Dachsous, which has 27 cadherin domains. Loss of either Fat or Dachous leads to similar defects in both planar cell polarity and growth control (Sopko and McNeill, 2009). Fat and Dachsous interact via adhesion of their extracellular domains, which can mediate cell aggregation in vitro (Matakatsu and Blair, 2006). This interaction is modulated by phosphorylation of both extracellular domains by the golgi-kinase Four-jointed. Phosphorylation by Four-jointed inhibits binding of Dachsous to Fat and promotes binding of Fat to Dachsous (Brittle et al., 2010; Simon et al., 2010) (Figure 1.4). As both Four-jointed and Dachsous are expressed in a gradient in tissues such as the wing, the phosphorylation promotes the asymmetric and graded activity of the uniformly expressed Fat across tissues (Sopko and McNeill, 2009). In vertebrates this signaling cassette seems to be at least somewhat conserved. Fat4 and and homologue of Four-jointed, Fjx1 interact in the planar polarized morphogenesis of the inner ear and kidney (Saburi et al., 2008). Furthermore Dachsous1 has been shown to mediate cellular adhesion with Fat4 (Ishiuchi et al., 2009). Dachsous1 mutants phenocopy Fat4 in planar cell polarity of the inner ear and kidney (Mao et al., 2011). Fat4 and Dachsous1 also are co-expressed in the brain in neural progenitors, although knockdown of Dachsous1 does not phenocopy Fat4 in this system (Ishiuchi et al., 2009). By contrast, signaling upstream of Fat-like is still an open question. In zebrafish, Fat3 and Dachsous2 both contribute to chondrocyte polarity (Le Pabic et al., 2014). However mutation of Dachsous unlike that of Fat-like does not lead to an egg chamber phenotype in Drosophila (Viktorinová et al., 2009). Furthermore, Fat2 expression in heterologous cells can mediate adhesive aggregation (Nakayama et al., 2002).
Therefore some evidence suggests Fat-like cadherins can bind homophilically, whereas other evidence suggests a Dachsous cadherin acts upstream of both Fat-likes and Fats.

**Downstream signaling**

Fat-likes and Fat share little to no identity in their intracellular domains. Therefore signaling pathways downstream of Fat-likes are more likely to be the most relevant to signaling downstream of Fat3. More generally however, it has been found that the two main functions of Fat in *Drosophila* – planar cell polarity and growth control, seem to be separable at the level of the intracellular domain (Matakatsu and Blair, 2012; Pan et al., 2013). Distinct regions of the Fat intracellular domain are sufficient to rescue either growth control or planar cell polarity, although different groups associated different regions for these two functions. Furthermore, while the intracellular domain of Fat4 was able to rescue some aspects of planar cell polarity in *Drosophila*, it was not able to rescue growth control, consistent with a lack of growth control phenotypes in the Fat4 knockout mouse model (Pan et al., 2013). These findings suggest that Fat and probably Fat-like domains are modular in function and the intriguing possibility that the functions have changed, even between closely related molecules, with evolutionary time.

Of the Fat-like family, downstream signaling pathways have largely only been identified for Fat1. Fat1 has been found to bind a significant number of proteins through specific interactions with its intracellular domain (Sadeqzadeh et al., 2014a). Of most interest to the Fat3 phenotype in amacrine cells, *in vitro* studies suggest Fat1 has functions in cell migration and cell polarity. Knockdown of Fat1 in cell culture leads to defects in cell migration, and impaired repolarization of cells in an *in vitro* wound-healing
assay (Moeller et al., 2004; Tanoue and Takeichi, 2004). It also leads to changes in actin organization in the cells. These changes are associated with, although not definitively linked to, the binding of Fat1 to Ena/VASP, a major family of actin regulators (Moeller et al., 2004; Tanoue and Takeichi, 2004). Ena/VASP binds to both monomeric and filamentous actin as well as to profilin-actin complexes and promotes the formation of long unbranched filaments (Krause et al., 2003).

Perhaps pointing to a second, separate function of Fat1, the intracellular domain of Fat1 can be cleaved, and can translocate to the cell nucleus through a cryptic nuclear localization signal that is revealed upon cleavage (Hou et al., 2006). Fat1 has also been shown to share some colocalization in cell-culture with Atrophin and beta-catenin and to bind them in pulldown assays, although a specific binding site was not identified for either partner (Hou and Sibinga, 2009; Hou et al., 2006). Atrophin is a transcriptional co-repressor. Of note, it is one of the few identified direct binding partners of Fat and acts together with Fat and Fat4 to direct planar polarity (Fanto et al., 2003; Saburi et al., 2008). Atrophin also binds to and genetically interacts with Fat3 in zebrafish in the morphogenesis of chondrocytes (Le Pabic et al., 2014). However in contrast to Fat1, analysis of the Fat3 intracellular domain using SeqNLS (Lin and Hu, 2013) fails to predict a nuclear localization sequence. Nonetheless, perhaps Atrophin signaling might represent an intersection between Fat and Fat-likes.

Additional binding partners that have been identified for Fat1 point to additional, very different functions. As just one example, Fat1 has recently been shown to bind caspase 8, thereby acting in the death-receptor apoptotic pathway in cells (Kranz and Boutros, 2014). Therefore Fat1 has so far been found to bind a large range of effectors,
however it is poorly understood how these binding partners contribute to the function of Fat1 specifically, or Fat-likes in general *in vivo*.

**Summary**

Neurons can polarize cell autonomously, but a symmetry-breaking event is always required, which commonly comes from an extracellular cue. Many events in neuronal development require polarized cellular activity. Furthermore, cell polarity must also be coordinated with the tissue and surrounding neurons. It is also important to consider steps of neuronal development as a continuum, such as migration and dendrite outgrowth. Here we used the Fat3KO phenotype as a molecular entry point to understand the molecular and cellular mechanisms of the transition from migration to dendrite development for amacrine cells of the retina. As Fat3 signaling is poorly understood, we also sought to understand signaling upstream and downstream of Fat3 in the context of amacrine cell development.
Chapter 2: Signaling upstream of Fat3 in the retina

Alexandra Krol, Michael R. Deans, C.O. Copley, Andrew F. Tucker, Lisa V. Goodrich

A. Krol purified the Fat3 antibody, performed all experiments and analysis except for Figure 2.4. For Figure 2.4 M. R. Deans and his technician C.O. Copley identified the Fat3+/−, Fjx1 −/− phenotype and performed immunostainings and confocal imaging. A.Krol generated the mice and provided tissue. A. Tucker generated in situ. Linda Hu helped with the affinity purification of the Fat3 antibody. The Dachsous 2−/− allele was generated by Francoise Helmbacher and kindly shared prior to publication. Helen McNeill provided Dachsous 2−/− and Dachsous 1 overexpressor retinal tissue. Ken Irvine provided Dachsous 1−/− retinal tissue. Part of this work is previously published and reprinted with permission from Neuron.
Introduction

A critical aspect of nervous system organization is the high specificity of neuronal connections. One mechanism used by neurons to establish specific connections is the expression of an adhesive code. The specificity of adhesive interactions is used in many aspects of neuronal development including, but not limited to, pathfinding decisions in neurite outgrowth and migration as well as synaptic target selection (Hirano and Takeichi, 2012). The restriction of neurites into lamina is also a common theme in the nervous system and can promote the formation of connections with the right synaptic partners.

Previously the lab uncovered a role for the atypical cadherin Fat3 in the development of amacrine cells of the retina (Deans et al., 2011). Amacrine cells project a dendritic arbor that is strictly restricted to the inner plexiform layer (IPL), a layer of connections between retinal ganglion cells, bipolar cells and amacrine cells. However in Fat3KOs, amacrine cells form a second, incorrectly positioned, dendritic arbor (Deans et al., 2011). During amacrine cell development, Fat3 is expressed by amacrine cells and retinal ganglion cells. Amacrine cells are born near the outer limiting membrane of the retina. As amacrine cells migrate towards the IPL, amacrine cells extend a leading and a trailing neurite. Amacrine cells retract the trailing neurite upon the completion of migration. In Fat3KO and wildtype retinas, migrating amacrine cells tend to have the same trailing neurite length. However once amacrine cells reach the IPL, amacrine cells in the Fat3KO tend to have longer trailing neurites compared to wildtype. This suggests the amacrine cells in the Fat3KO fail to reliably retract their trailing neurites at
the end of migration and instead the trailing neurite forms an extra, misplaced dendritic arbor.

Until amacrine cells have contacted the IPL, wildtype and Fat3KO amacrine cells have trailing neurites of the same length (Deans et al., 2011). This implies that the Fat3 signaling that leads to trailing neurite retraction occurs following contact with the IPL. In support of this idea, Fat3 protein is localized to the IPL during retinal development (Nagae et al., 2007). As mentioned, Fat3 is expressed in retinal ganglion cells and amacrine cells. Therefore Fat3 could be acting as either a cell-surface receptor in amacrine cells to detect a cue in the IPL, or Fat3 could be acting as the cue in the IPL being detected by amacrine cells. In favor of Fat3 acting as a receptor in amacrine cells, amacrine cells develop extra neurites in an amacrine cell specific knockout of Fat3. These findings raise the question of what acts upstream of Fat3 to help restrict amacrine cell dendrites to the IPL.

Fat3 belongs to the Fat-like family of atypical cadherins. Very little is known about signaling upstream of any of the Fat-likes, however signaling upstream of the closely related atypical cadherins Fat has been studied extensively in Drosophila. Fat signaling is regulated through adhesion to another closely related atypical cadherin called Dachsous. Mutations of Dachsous largely phenocopy mutations of Fat, leading to disregulation of tissue growth and the loss of planar polarized tissue organization (Sopko and McNeill, 2009). Fat and Dachsous bind heterophilically and are able to mediate aggregation of heterologous cells (Matakatsu and Blair, 2004). This adhesive interaction is also important for their localization; Fat and Dachous stabilize each other at cell contacts, so that loss of Dachsous leads to reduction of Fat from adjacent cell
contacts (Matakatu and Blair, 2004). In vertebrates there are two homologues of *Drosophila* Dachsous, Dachsous 1 and Dachsous 2. Dachsous 1 has been shown to act upstream of Fat4. *In vivo*, single mouse mutations of Dachsous 1 and Fat4 largely phenocopy each other, leading for example to similar disruptions in kidney development (Mao et al., 2011; Saburi et al., 2008). In support of Dachsous1 and Fat4 acting in the same pathway, double mutants show little enhancement of skeletal disruptions compared to either single mutant. At a cellular level, the depletion of one decreases the localization of the other in adjacent cells both *in vivo* and *in vitro* (Ishiuchi et al., 2009). Furthermore, *in vitro*, Fat4 and Dachsous1 mediate the aggregation of transfected cells and have been shown to bind to each other in trans. Therefore the adhesive interaction in *Drosophila* between Fat and Dachsous appears to be conserved in vertebrates between Fat4 and Dachsous 1.

The golgi kinase Fourjointed also acts upstream of Fat signaling, modulating adhesion between Fat and Dachsous by phosphorylating the extracellular domains of both cadherins. Phosphorylation of Fat promotes the binding of Fat to Dachsous and phosphorylation of Dachsous inhibits the binding of Dachsous to Fat. In some tissues in *Drosophila* Fourjointed and Dachsous are expressed in opposing gradients to set up a gradient of Fat activity (Brittle et al., 2010; Simon et al., 2010). In vertebrates, the Fourjointed homologue Fjx1 also interacts with Fat4 genetically, suggesting the function of Fjx1 is conserved (Saburi et al., 2008).

Altogether, evidence points to members of the Fat family mediating adhesion via heterophilic interactions with members of the Dachsous family. There is also a recent report from zebrafish that finds Fat3 and Dachsous 2 interact genetically to mediate
chondrocyte polarity and differentiation (Le Pabic et al., 2014). Therefore the top two candidates for being a ligand for Fat3 are the two Dachsous family members, Dachsous 1 and Dachsous 2. If adhesion with Dachsous is conserved between Fat and Fat-likes, Fjx1 would be predicted to modulate such an interaction. Lastly the localization of Fat cadherins as well as many other cadherins is stabilized at cell contacts through adhesive interaction with their ligand. Therefore, we predict absence of a ligand for Fat3 would affect Fat3’s localization.

In this chapter we test the components of the upstream signaling pathway thus far identified for Fat cadherins: Dachsous and Fourjointed. We also ask whether the ligand for Fat3 could come from retinal ganglion cells, the only other cell type that contributes to the IPL early in amacrine cell development. The story that emerges is complicated. It appears that neither Dachsous 1 nor Dachsous 2 nor retinal ganglion cells are necessary for Fat3 signaling or localization to in the IPL. However there is a genetic interaction with Fjx1 indicating that some components of ‘classic” signaling upstream of Fat may be relevant.

Results

Fat3 is concentrated in the IPL from the earliest stages of amacrine cell development.

Prior to contact with the IPL, amacrine cells in wildtype and Fat3KO retina do not exhibit differences in trailing neurite length. However following contact, amacrine cells in the Fat3KO tend to have longer trailing neurites and go on to form an extra dendritic arbor (Deans et al., 2011). These observations suggest that Fat3 acts following
amacrine cell contact with the IPL. Previously Fat3 protein had been reported to localize to the IPL during retinal development (Nagae et al., 2007). However the spatial and temporal pattern of Fat3 localization relative to amacrine cell development and contact with the IPL was unknown. To determine the localization of Fat3 protein, we generated an antibody for Fat3. Specificity of the antibody was tested by western blot on wildtype and Fat3KO lysates as well as on Fat3KO tissue (data not shown). Amacrine cells were labeled using Ptf1a Cre, which is specific for amacrine cells and horizontal cells (Fujitani et al., 2006), in combination with the genetic reporter Z/EG, which recombines at low frequency and so generates sparse labeling of amacrine cells. From early IPL formation (E17.5, Figure 2.1A), Fat3 protein was concentrated at the IPL with a more diffuse signal in the retinal ganglion cell layer. Migrating amacrine cells did not show detectable Fat3 protein (Figure 2.1A). Later, at P0 when many amacrine cells would be found near IPL in the process of retracting trailing neurites, Fat3 was still only detected in the IPL and not in trailing neurites (Figure 2.1B). Fat3 protein continued to be concentrated at the IPL throughout amacrine cell development (Figure 2.1C). At P11, Fat3 localizes to at least five, and probably all, sublaminae of the IPL (Figure 2.1D). Therefore, Fat3 protein is always concentrated at the leading neurite tip and eventually the dendrite of amacrine cells, although it is possible that a more diffuse localization is present in migrating amacrine cells that is below the level of detection.

Retinal ganglion cells are not require for Fat3 signaling in amacrine cells

Having found that Fat3 is localized to the IPL from early in development, we next asked what cells could provide a ligand for Fat3 signaling. At the beginning of amacrine
Figure 2.1 Fat3 is enriched in amacrine cell dendrites from the earliest stages of IPL development.

A–D) Fat3 protein (red) is present in the IPL at E17.5 and increases as more ACs are added (labeled green; GFP reporter from Ptf1a-cre; Z/EG) until the mature pattern emerges at P11. In contrast, migrating cells do not exhibit enhanced Fat3 immunolabeling (arrowhead, A, A’). Note that double labeling of GFP and Fat3 requires a chicken anti-GFP antibody that introduces some background in the GCL at P0. Scale bar 20 µm. Figure modified from Deans et al., 2011. Reprinted with permission.
cell development the only cells that contribute to the IPL are retinal ganglion cells and other amacrine cells. An attractive hypothesis is that retinal ganglion cells could provide an initial cue for amacrine cells to project into the future IPL. To determine whether the ligand for Fat3 signaling is in retinal ganglion cells, we removed all retinal ganglion cells from the retina using a Math5 knockout model. Math5 is a transcription factor required for retinal ganglion cell differentiation (Brown et al., 2001). In the Math5 knockout, almost no retinal ganglion cell neurons develop and instead precursors form extra amacrine cells (Feng et al., 2010). If retinal ganglion cells were necessary for Fat3 signaling, we would predict loss of retinal ganglion cells would lead to extra amacrine cell neurites. To test this, amacrine cell lamination was examined in Math5 mutants. In the absence of retinal ganglion cells, amacrine cells still restricted their neurites to the IPL as revealed by VGAT immunostaining (Figure 2.2A). Furthermore, Fat3 protein was also still localized to the IPL (Figure 2.2B). Therefore a signal from retinal ganglion cells appears unnecessary for Fat3 localization and Fat3 signaling may continue to restrict amacrine cell dendrites to the plexiform layer. To test whether Fat3 signaling is still important in the absence of retinal ganglion cells, a doubleknockout of Fat3 and Math5 was generated (Figure 2.2C). In the absence of retinal ganglion cells, removing Fat3 recapitulates the Fat3KO phenotype leading to the formation of extra plexiform layers in the inner nuclear layer and below the retinal ganglion cell layer. These results indicate that only amacrine cells are necessary for localizing Fat3 and signaling upstream of Fat3.
Figure 2.2 Retinal ganglion cells are not required for Fat3 signaling in amacrine cell dendrite formation.

A) Math5KO retinas do not form ectopic plexiform layers as revealed both by DAPI staining (gray) and VGAT labeling (green). B) Fat3 immunolabeling (red) persists in Math5KO, confirming that Fat3 protein is localized to amacrine cell dendrites and does not require a signal from RGCs to maintain this localization. C) In Fat3;Math5 DKO s, VGAT-positive OMPL (outer misplaced plexiform layer) and IMPL (inner misplaced plexiform layer) formation is prominent, similar to Fat3KOs, confirming that Fat3 activity is responsible for the development of normal amacrine cell morphologies in the Math5KO. All specimens were collected at P11. Scale bar 50 µm. Figure and text modified from Deans et al., 2011. Reprinted with permission.
**Analysis of Fat and Fat-like cadherin expression in the developing retina**

To identify which Fat and Dachsous cadherins were expressed in the retina during amacrine cell development, reverse transcription PCR was performed on retina tissue for all the Fat-Ds family cadherins (Figure 2.3). Dachsous 1 and Dachsous 2, Fat4 and Fat1 were also present during retinal development. Therefore Dachsous 1 and Dachsous 2 are present and could serve as a ligands for Fat3. Since Fat4 is also present, Dachsous 1 could also be acting together with Fat4 in the retina.

![Figure 2.3 RT PCR for Fat and Dachsous members.](image)

RT PCR demonstrates presence of additional related cadherins Fat1 and Fat4 as well as Dachsous 1 (Ds1) and Dachsous 2 (Ds2) in the retina. RT PCR on E17.5 retina and brain, and P5 retina and kidney demonstrate that all Dachsous-Fats are detected in the brain and kidney (Ds1 882 bp, Ds2 397 bp, fat1 729bp, Fat2 827bp, Fat3 200bp).

*Fat3 genetically interacts with Fjx1 to regulate plexiform layer formation.*

Fourjointed is a kinase that modulates through phosphorylation interactions between Fat and Dachsous; together Fat, Dachsous and Fourjointed act as a signaling cassette to pattern many different tissues(Brittle et al., 2010; Ishikawa et al., 2008). To test whether the vertebrate homologue for Fourjointed, Fjx1 may also act upstream of
Fat3, we asked whether Fjx1 is expressed during amacrine cell development. *In situ* hybridization for Fjx1 at P3 and P11 revealed that Fjx1 has a dynamic expression pattern (Figure 2.4 A,B) with overlap with Fat3 expression (Deans et al., 2011). At P3, Fjx1 is expressed most intensely in the retinal ganglion cell layer, where there are amacrine cells and retinal ganglion cells and at the top of the developing inner nuclear layer, which probably corresponds to bipolar cells and maybe some amacrine cells (Figure 2.4 A). At P11, expression is restricted to the retinal ganglion cell layer (Figure 2.4 B).

Therefore Fjx1 is present at a time and place to interact with Fat3 or its ligand. Next, genetic interactions between Fat3 and Fjx1 were investigated. No phenotype was detected in Fjx1KO retinas, as visualized by VGAT staining (Figure 2.4 D). This fits with a function for Fjx1 in modulating Fat-Dachsous interactions, since Fj phosphorylation is not needed for adhesion between Fat and Dachsous to occur. Neither was enhancement of the Fat3 phenotype seen in a double mutant of Fat3 and Fjx1 (data not shown). Again, this fits with the upstream role for Fjx1, which predicts Fjx1 should have no effect in the absence of Fat3. However, a phenotype emerges when one copy of Fat3 is removed in a Fjx1KO background. A thin extra layer of connections is formed underneath the retinal ganglion cell layer, as visualized by VGAT staining (Figure 2.4 E). This extra layer only forms in the periphery of the ventral retina, as identified by the presence of blue cones. In the dorsal retina, no extra layer is formed (Figure 2.4 F).

These results suggest that Fat3 and Fjx1 act in the same pathway and that Fat-like and Fat upstream signaling is conserved.
Figure 2.4 Fat3 genetically interacts with Fjx1 to regulate plexiform layer formation.

A,B) In situ hybridization for Fjx1 at P3 (A) and P11 (B) shows a dynamic pattern of Fjx1 expression in presumptive bipolar cells (asterisk) and the GCL at P3, and prominent expression in the GCL at P11. C–F) VGAT-labeling (green) at P11 reveals formation of an extra plexiform layer in Fat3+/−;Fjx1−/− retinas (E, arrows). This ectopic layer is not present in Fat3+/−;Fjx1+/− (C) or Fjx1−/− (D) control retinas. Extra layer in Fat3+/−;Fjx1−/− mice is restricted to the periphery of the ventral retina, a region that is enriched for SOP expressing blue cones (blue). No IMPL develops in dorsal regions (F). Scale bars: 100 µm (A, B); 50 µm (C–F). Figure and text modified from Deans et al., 2011. Reprinted with permission.
**Analysis of mutants**

The genetic interaction between Fat3 and Fjx1 suggests that Fat3 might share the same ligand as Fats. There are two vertebrate Dachsouses. If either Dachsous 1 or Dachsous 2 serves as a ligand for Fat3, then loss of Dachsous would be predicted to phenocopy loss of Fat3. Phenotypic analysis was performed on P11 Dachsous 1 KO or Dachsous 2 KO retinas and heterozygous littermate controls (Figure 2.5). No changes in the overall organization of the retina or individual neuronal morphology were detected. DAPI staining of the nuclei (Figure 2.5 A,D,G), and VGAT staining of plexiform layers (Figure 2.5 C,F,I) did not point to either extra synaptic layers or an increase in retinal ganglion cell layer numbers. Neither were changes detected in cell morphologies of calretinin positive amacrine cells (Figure 2.5 B,E,H), a population of amacrine cells strongly affected in the Fat3 mutant.

Dachsous and Fat3 were predicted to interact through adhesive interactions, therefore loss or overexpression of a ligand was predicted to change Fat3 localization. Although there was no obvious phenotype in Dachsous mutants, Fat3 localization could be a more sensitive measure of ligand absence. Fat3 localization was examined by immunostaining (Figure 2.6). However no change in Fat3 localization was observed in Dachsous1 mutants (Figure 2.6A,B). It was not possible to examine Fat3 localization in Dachsous 2 mutant retinas since fixation conditions of obtained tissue was not compatible with the Fat3 antibody as evidenced by failure of successful immunostaining on control retinas provided. Since Dachsous 1 and Dachsous 2 share a great deal of homology, including in their extracellular domain, overexpression of Dachsous1 could reveal a role of either Dachsous1 or Dachsous2. Unfortunately animals overexpressing
**Figure 2.5 Retinas of neither Dachsous mutant show a Fat3-like phenotype.**

Immunostaining of P11 retinas. A-C) Wildtype. (D-F) Dachsous 2 null. G-I) Dachsous 1 null. A,D,G) DAPI immunostaining for nuclei show three unbroken nuclear layers in all genotypes. B,E,H) Calretinin immunostaining labels a subset of amacrine cells with extra neurites in Fat3 null retinas, however no extra neurites are seen. C,F,I) VGAT labels synaptic layers, however no extra layers are seen.
Figure 2.6 Fat3 protein is correctly localized to the IPL in Dachsous 1 mutant and overexpressor tissue.

Fat3 immunostaining in A) Wildtype and B) Dachsous 1 mutant tissue at P11 concentrated at inner plexiform layer. Animals overexpressing Dachsous 1 survive only to P0. At P0, Fat3 staining is more diffuse at the inner plexiform layer, but looks similar in C) Wildtype and D) Dachsous 1 overexpressor.
Dachsous 1 died postnatally. P0 retinas were examined and therefore it was not possible to evaluate changes in cell morphology or retinal organization. Fat3 localization appeared unchanged (Figure 2.6 C,D), although it is difficult to assess at P0.

Discussion

Fat3 acts in amacrine cells to restrict their dendritic arbors to the IPL and Fat3 signaling appears to occur following amacrine cell contact with the IPL. However what signal Fat3 might detect in the IPL and from what cell is unknown. Here we developed an antibody to more closely associate Fat3 localization with amacrine cell development. In line with a possible role associated with the IPL, Fat3 was found always concentrated at the IPL, in amacrine cell dendrites and leading neurites, and was not detected in cell bodies, even of migrating amacrine cells, although it is always possible that signal was below the level of detection. Furthermore, neither Fat3 function in amacrine cells nor localization was dependent on a signal from retinal ganglion cells.

Although little is known about signaling upstream of Fat-like cadherins, signaling upstream of Drosophila Fat is well established. The closely related atypical cadherin Dachsous interacts through adhesive interactions with Fat. This adhesive interaction is also modulated by phosphorylation by the golgi kinase Fjx1 (Sopko and McNeill, 2009). Therefore we tested whether, Fjx1, Dachsous 1 or Dachsous 2 played roles upstream of Fat3.

A genetic interaction with Fjx1 was found, consistent with Fjx1 acting upstream of Fat3. However our analysis of mutant retinas suggests that neither Dachsous1 nor
Dachsous2 is a binding partner for Fat3. It remains possible that both Dachsous 1 and 2 are binding partners and one can compensate for the loss of the other. Therefore it will be necessary to analyze double knockout retinas to determine whether they phenocopy the Fat3 knockout and firmly exclude Dachsous as a ligand for Fat-likes. However in support of Dachsous not being upstream of Fat-likes, mutation of the single Drosophila Dachsous does not phenocopy oocyte developmental defects following mutation of Fat-like (Viktorinová et al., 2009).

If not Dachsous 1 or Dachsous 2, what might the ligand be for Fat3? The only adhesive interaction or upstream genetic interaction that is well characterized in the Fat/Ds family of cadherins is between Drosophila Fat/ Vertebrate Fat4 and Drosophila Dachsous/ Vertebrate Dachsous 1. However, perhaps a closer examination of the cadherin phylogeny is needed.

Many attempts have been made to organize the cadherins into evolutionary and functional groups. Since the number of extracellular cadherin repeats varies greatly, from 2-34, determining which cadherin repeats to align raises difficulty. More recently however several different studies have localize the binding specificity of several cadherin interactions to the initial cadherin domains (Brasch et al., 2012). This has prompted the classification of cadherins based upon the first few extracellular domains. Furthermore, during the great expansion of cadherin molecules in evolution, adhesive and signaling properties may have been selected for independently. Therefore, consideration of alignments based upon extracellular conservation, while it may not reproduce the evolutionary relationships, may be the better predictor of adhesive interactions (Hulpiau and van Roy, 2009, 2010; Sotomayor et al., 2014).
Several different alignments including ones based on the first three extracellular domains suggest that Fat, Dachsous, and Fat-like families are evolutionarily distinct and of ancient origins. Indeed, a closer examination of the phylogeny reveals that Fat4, Ds1 and Ds2 are more closely related to each other in their extracellular domains than to any of the Fat-like molecules or to any other cadherin. There is therefore perhaps less evidence that the Fat-likes bind to Dachsous than grouping Fat-likes with Fats suggests. This analysis suggests several alternatives to Fat-likes binding to Dachsous:

**Fat-likes interact with Fat-likes**

Conservation suggests that Fat and Dachsous extracellular domains are similar. Therefore the Fat-Ds interaction may be closer to a "homophilic" interaction than a heterophilic one. This might therefore suggest that Fat-likes are also involved in either homophilic interactions or adhesion with other Fat-like members. Therefore in the retina, Fat3 may be its own ligand or bind to Fat1, the other Fat-like member detected by RT-PCR. In support of a homophilic interaction, Fat2 can mediate homophilic aggregation of heterologous cells (Nakayama et al., 2002). However experiments in *Drosophila* argue against a homophilic interaction determining Fat-like localization. Analysis of Fat-like in oocyte follicular cells suggests that the localization of Fat-like does not alter upon loss of Fat-like from adjacent cells (Viktorinová et al., 2009).

**Fat-likes interact with Fats**

Alternatively Fat3 might bind to Fat4. Perhaps the critical binding sites mediating adhesion specificity in the Fat-like extracellular domains are more similar to Dachsous 1 and 2 than to Fat4, since sequence analysis indicates that Fat-likes are similarly different overall to Dachsous 1, 2 and Fat4. Unexpectedly, Fat4 and Fat3 share critical
sequence motifs with protocadherin 15 and protocadherin 23, respectively, that mediate binding between the two protocadherins (Sotomayor et al., 2012, 2014).

**Fat3 and Fjx1**

If Dachsous 1 and 2 are not ligands for Fat3, then what is the mechanism by which Fat3 and Fjx1 interact genetically? The nature of the genetic interaction suggests that Fjx1 acts upon either Fat3 or Fat3’s ligand. However, while the extracellular domain of Fat1 is phosphorylated, it is not phosphorylated by Fjx1 (Sadeqzadeh et al., 2014b). Given that the extracellular domains of Fat1 and Fat3 are similar, this may also suggest that Fjx1 also does not phosphorylate the extracellular domain of Fat3. Perhaps instead the genetic interaction indicates an interaction between Fat3 and Fat4, which, given its homology to *Drosophila* Fat, is likely to be phosphorylated by Fjx1.

In conclusion, upon analysis of Dachsous mutants, and a re-examination of the Fat, Fat-like and Dachsous phylogeny, three top new candidate for Fat3 emerge: Fat1, Fat4 and Fat3 itself. It will be difficult to test the hypothesis that Fat3 acts homophilically in the retina as its extremely large size makes altering its expression pattern in the retina difficult. In the future, to test the other two candidates, Six3 Cre (Furuta et al., 2000) will be used to create retina specific deletions of Fat1 and Fat4 since knockouts of Fat1 or Fat4 are perinatally lethal (Ciani et al., 2003; Saburi et al., 2008). Since almost all Fat, Dachsous, and Fat-like family members are present in the retina, the retina presents a good model system in which to dissect Fat and Fat-like interactions further in the future.
Materials and Methods

Some methods have been reproduced from published text with the permission of Neuron.

Mice
Mice: Fjx1 (A. Vortkamp, U. Duisburg-Essen); Ptf1a-cre (C. Wright, Vanderbilt U.); Math5<sup>Cre</sup> (L.Gan, U. Rochester), Fat3 (M. Deans), Z/EG (JAX).

Mouse Breeding and Experimental Intercross Strategies: For experimentation, fat3KOs were generated by intercross matings of heterozygotes and PCR genotyped. For lineage tracing experiments, Fat3+/−; Z/EG males were crossed to Ptf1a-cre +/- ;Fat3+/− females. Fat3CKOs were generated by breeding Fat3 Floxed/Floxed males to Ptf1a-cre +/- ;Fat3+/− females. Ptf1a-cre +/- ;Fat3 Floxed/- animals were analyzed as conditional knock-outs (Fat3CKO) with Ptf1a-cre +/- ;Fat3Floxed/+ littermates as controls. Math5 mutant mice were generated by intercrossing Math5Cre heterozygotes and Math5;Fat3 double knock-outs were obtained by intercrossing Math5 Cre/+;Fat3+/− animals. Fat3 and Fjx1 interaction studies were completed by intercrossing Fat3+/− ;Fjx1+-/− males with Fat3+/− ;Fjx1+/− female mice and littermates were used as experimental and controls.

Antibodies
Polyclonal antibodies against the C-terminal 245 amino acids of Fat3 were prepared using a His-tagged antigen injected into mouse and rabbit (Primm Biotech, Cambridge, MA). Subsequent standard affinity purification was done on rabbit antisera using a GST-C-terminal Fat3 fusion protein produced in E. coli. All other antibodies are commercially
available as listed. Calretinin (Chemicon AB1550), chicken anti-GFP (Aves GFP-1020), goat anti-PKCalpha (Santa Cruz SC208), mouse anti-PKCalpha (SIGMA PS704), SOP (Santa Cruz SC14363), VGAT (Synaptic Systems 131 003), AlexaFluor-conjugated secondaries (Invitrogen), and DyLight-conjugated secondaries (Jackson Immunoresearch).

**In Situ Hybridizations (ISH)**

In situ hybridizations were completed for fjx1 using a probe corresponding to nucleotides 931–1563 of NM_010218.

For ISH retinas were dissected and fixed as described for immunofluorescent labeling. Cryosections were dried in a 50°C oven and then postfixed using ice cold 4%PFA for 10 minutes, treated 1µg/ml Proteinase K (Sigma P-6556) in room temperature (RT) PBS for 10 minutes and then fixed again in ice cold 4%PFA. Acetylation was completed using 0.1M triethanolamine/0.25% acetic anhydride for 15 minutes at RT. Between each of these steps sections were washed 2X5' with PBST (PBS with 0.1% Triton X-100) at RT, shaking gently. ISH buffer consisted of 10mM Tris, 600mM NaCl, 1mM EDTA, 0.25% SDS, 1X Denhardt's, 50% formamide 300µg/ml Yeast tRNA (Sigma #R6750). Slides were pretreated in hybridization buffer for 1-4 hours at 60°C, then hybridized with 1-2µg/ml DIG-labeled anti-sense RNA probes in hybridization buffer supplemented with 25% Dextran Sulfate. Slides and probes were hybridized overnight at 60°C. Following hybridization slides were washed with 5xSSC at 65°C for 10 minutes, 50% formamide/1X SSC at 65°C for 30 minutes, and TNE (10mM Tris pH 7.5, 500mM NaCl,
1mM EDTA) at 37°C for 10 minutes. Non-specific background was eliminated by
treatment with 20µg/ml RNAse A (Sigma R-5503) prepared in TNE at 37°C for 30
minutes followed by washes in 37°C TNE, 65°C 2X SSC, and twice in 65°C 0.2X SSC
for 20 minutes each. Following washes slides were transferred to MABT (100mM Maleic
acid, 150mM NaCl, Tris to pH 7.5) and blocked for immunohistochemical detection of
DIG with 20% heat-inactivated sheep serum/MABT for 1 hour. Slides were incubated
overnight in alkaline phosphatase conjugated sheep antiDIG antibodies (Roche) diluted
1:2000 in MABT/10% sheep serum. Slides were later washed 3X in MABT, followed by
alkaline phosphatase buffer at room temperature for 20 minutes each. Histochemical
detection was completed using BM Purple (Roche) and incubation at RT in the dark for
4-48 hours

**RT PCR**

Tissue for RNA was collected in Trizol (Invitrogen) and homogenized. RNA was purified
using chloroform precipitation, treated with DNAsel Roche (Cat. No. 04 716 728 001),
then phenol chloroform extracted. cDNA was produced using SuperScriptIII Reverse
Transcriptase (Invitrogen: Cat. No 18080-093)

PCR was performed with the following primers:

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SK0056 CAGCGCCTTGGCCACCTGAA  Fat2_RT reverse primer
product length = 827

Sk005 gcagaagatgtggacagcaaa  old Fat3_RT forward primer  product length = 256
Sk006 tggcaggttaacactggaa  old Fat3_RT reverse primer  product length = 256

SK0051 CTCGCAACCCCTGCCAGCAT  Fat4_RT forward primer
SK0052 GTTAGGCCCCGACCAGCCGA  Fat4_RT reverse primer  product length = 704

**Immunofluorescence**

Retinas were collected at P11, enucleated and fixed in 4% PFA in Sorenson’s buffer at 4C for 2 hours, sucrose protected in 30% sucrose O/N. Tissue was embedded in Neg-50 by freezing on metal cooled with liquid nitrogen. 14um cryosections were blocked and permeabilized for 30 min at room temperature using 10% goat or donkey serum and 0.1% TritonX-100 in PBS. Sections were incubated 1-2 overnights at 4°C in block solution without triton with primary antibody. Then sections were incubated at room temperature for 1 -2 hours, with appropriate secondaries at 1:1000. Sections were counterstained with DAPI (1:10,000) and mounted using 50% glycerol/50% sorenson’s solution.

Images were collected on Nikon E600 and E800 fluorescent microscopes or Olympus Fluoview and Zeiss LSM510 confocal microscopes.
Chapter 3: Polarized neuronal behaviors depend on Fat3 and the asymmetric regulation of the cytoskeleton

Alexandra Krol, Steven J. Henle and Lisa V. Goodrich.

A. Krol designed, performed and analyzed all experiments with the exception of the following. All live imaging experiments were designed, performed and analyzed by S. Henle. For the mitochondria localization experiment, A. Krol designed the experiment together with S.J. Henle and cloned the constructs. S. J. Henle performed the experiment and analyzed the data.
Introduction

Mature neurons have a highly polarized morphology that typically includes multiple dendrites specialized to receive information and one axon to transmit information. Correct polarization into these compartments is critical for neuronal function and directional signaling. However neuronal polarity is not just important for setting up axo-dendrite polarity. Instead, many steps of neuronal development, such as migration, require a polarized state: for one side of the cell to have different properties than the other. To create a polarized state requires a symmetry breaking event and typically the propagation of the asymmetry across the cell.

The generation of polarized morphologies and behaviors also requires a polarized cytoskeleton, and correspondingly asymmetric activity of cytoskeletal regulators. To some extent the creation of a polarized cytoskeleton can be cell autonomous. For instance neurons in culture appear to acquire axo-dendritic polarity without external cues (Bradke and Dotti, 2000). This suggests the ability of cytoskeleton and its regulators to amplify and propagate initial asymmetries across a cell (Stiess and Bradke, 2010). However extracellular cues can be important symmetry breaking events that initiate polarity and set up the axis of polarity, particularly in the context of the tissue (Barnes and Polleux, 2009; Gärtner et al., 2014b). Extracellular cues may also be important to maintain neuronal polarity during migration and dendritogenesis.

Dendrite elaboration is usually preceded by neuronal migration and the initial dendrite often forms from either the leading or trailing neurite of the migrating neuron. Therefore the polarity of the cytoskeleton and the distribution of its regulators at the end
of migration is the initial state of polarity at the beginning of dendrite formation. As a consequence, mutations that disrupt polarity of the cytoskeleton have the potential to disrupt both migration and dendrite morphology.

Previously, we found that a single-pass transmembrane protein, Fat3 is important in both dendrite development and migration (Deans et al., 2011). Fat3 is expressed both by amacrine cells and retinal ganglion cells of the retina. Amacrine cells project dendritic arbors only into the inner plexiform layer (IPL), a layer of neuropil between amacrine cells, bipolar cells and retinal ganglion cells. In a Fat3 knockout, amacrine cells no longer form a single dendritic arbor reliably. Instead, many amacrine cells retain their trailing neurites after migration, and this leads to the formation of a second arbor of neurites. In addition, some amacrine cells migrate aberrantly from the inner nuclear layer past the IPL into the retinal ganglion cell layer.

During development, Fat3 protein is concentrated in the IPL. However, in the absence of Fat3, the extra neurite is formed opposite to where Fat3 protein is normally localized (Deans et al., 2011). This raises the question of how a receptor at the leading neurite of a neuron, can lead to changes in overall morphology. One possibility is that Fat3 defines overall amacrine cell polarity. However the molecular and cellular mechanisms of Fat3 are unknown.

Fat3 belongs to the Fat family of atypical cadherins. Fats have extremely long extracellular domains with 34 cadherin repeats, as well as laminin and EGF domains. Fat intracellular domains (ICDs) share no homology to classic cadherins. In vertebrates, there are four Fat homologues (Fat1-4); Fat4 shares the most homology with Drosophila Fat, whereas Fat1-3 shares greater sequence similarity to Drosophila Fat-like (Tanoue
and Takeichi, 2005). As the founding member of the Fat family, Drosophila Fat has been extensively studied and has roles in a variety of cellular processes, including planar cell polarity and cell growth (Sopko and McNeill, 2009). In contrast Fat-like has been identified more recently and is less well understood (Castillejo-López et al., 2004).

The Fat3ICD shares extensive homology with Fat1ICD suggesting Fat1 and Fat3 downstream signaling might have similarities. In vitro, Fat1 expression can affect actin organization, at least in part by directly binding and localizing Ena/VASP proteins through two EVH1 binding sites (Moeller et al., 2004; Tanoue and Takeichi, 2004). However a role for Ena/VASP signaling in the function of Fat1 in vivo has not been determined. Fat3ICD shares sequence similarity for the two EVH1 binding sites that are capable of recruiting Ena/VASP. Therefore, Fat3 may act through Ena/VASP to guide actin dynamics in amacrine cells. Ena/VASP are good candidates as they are key regulators of actin dynamics underlying the initiation and extension of neuronal processes as well as migration (Dent et al., 2007; Kwiatkowski et al., 2007; Lebrand et al., 2004).

Here we show that Fat3 acts in amacrine cells cell-autonomously to promote directed migration and asymmetric neurite dynamics. We propose that in both cases, asymmetric Fat3 acts to polarize amacrine cells. In an effort to understand Fat3 signaling, we sought to identify downstream effectors that could mediate the effects of Fat3. We found evidence that asymmetric localization of Ena/VASP could polarize amacrine cells in a similar manner to Fat3.
Results

Amacrine cells project their dendritic arbors into the inner plexiform layer (IPL). As for other neurons, confining amacrine cell projections to a specific area serves to restrict the synaptic connections made to a specific subset of neurons. Fat3 contributes to restricting amacrine cell dendrites to the IPL; in the absence of Fat3, an extra neurite extends from the cell body, away from the IPL and into the inner nuclear layer. However Fat3 protein is concentrated in the IPL. This raises the question of how a receptor at one end of a neuron leads to changes in overall morphology and suggests Fat3 may affect cellular polarity.

*Fat3 acts cell autonomously to restrict amacrine cell neurites to the IPL*

Amacrine cells are born over many days of development, and Fat3 is expressed and concentrated to the IPL throughout. Although amacrine cell-specific removal of Fat3 leads to extra amacrine cell neurites, exactly when Fat3 acts is unclear. One possibility is Fat3 is required to initiate IPL formation by early born amacrine cells. Loss of Fat3 would then affect only early born amacrine cells; late born amacrine cells could rely on additional cues from the IPL. A second possibility is Fat3 establishes the structural and molecular composition of the IPL throughout amacrine cell development. Loss of Fat3 in early-born amacrine cells would then disrupt the IPL and have a secondary effect on late-born amacrine cell morphology. Alternatively, Fat3 could play a role in amacrine cells cell-autonomously and at throughout amacrine cell development.
First, to test whether late born amacrine cells require Fat3, the morphology of late-born amacrine cells was assessed in an amacrine cell specific knockout of Fat3. A Fat3 conditional allele (Fat3 Fl) was crossed to Ptf1a-Cre, expressed by all amacrine cells, in order to remove Fat3 from nearly all amacrine cells (Fat3cKO) as we have previously shown (Deans et al., 2011). To label amacrine cells born after P0, in vivo retinal electroporations were performed with plasmid expressing myristoylated-Venus (memVenus) in a Cre-dependent fashion. Labeling was restricted to late-born amacrine cells by performing electroporations at P0, thereby limiting plasmid uptake was to cells born after P0. Comparison of control retinas (Figure 3.1A) and Fat3cKO retinas (Figure 3.1B) reveals amacrine cells with non-IPL facing neurites in the FatcKO retinas. Therefore late-born amacrine cells are affected by loss of Fat3 from all amacrine cells (Figure 3.1A, B).

Next, we asked whether late-born amacrine cells require Fat3 if the IPL develops with Fat3 activity intact. To do this, Fat3 was removed from a sparse population of late-born amacrine cells by electroporating Cre into Fat3 Fl/- retinas. Again, electroporation at P0 ensured that retinal ganglion cells and >85% of amacrine cells (Voinescu et al., 2009) were inaccessible to Cre. Therefore development of early-born amacrine cells and the IPL proceeded normally prior to P0. As before, Cre-dependent memVenus was co-electroporated to visualize the morphology of electroporated cells. To identify amacrine cells for analysis in a morphology-independent way, a reporter specific for amacrine cells at P11 was also co-electroporated (Figure 3.1C,D). Cells were analyzed using 3D reconstructions in Amira software and any non-IPL facing neurites of cells labeled with the amacrine cell reporter were measured (Figure 3.1C’,D’). Analysis of
Figure 3.1 Removal of Fat3 from late born amacrine cells suggests a cell autonomous function.

A,B) Late born amacrine cells developed extra dendritic arbors in an amacrine specific knockout of Fat3 (arrow). Late born amacrine cells were labeled by electroporation at P0 with Cre-dependent expression of mem-Venus. Morphology of control A) and Fat3cKO amacrines was visualized at P11. C-E) Removal of Fat3 only from late born amacrine cell led to extra neurites in those cells. In C) control Fat3 Fl/+ , or D) Fat3 Fl/- retinas, the floxed allele of Fat3 was excised following Cre electroporation at P0. Morphology of amacrine cells was visualized as in A and amacrine cells were identified with Tomato expression driven by a promoter specific for amacrine cells at P11. C”, D”) Amira reconstructions of amacrine cells. C’, D’) Examples of morphologies of individual cells. E) Cumulative histogram of all lengths of all non-IPL facing neurites measured in 3D reconstructions of Cre electroporation. F) Percentage of cells with extra neurites greater than 10 um. P= 0.0001 Mann-Whitney U-test. N=4 retinas per condition, 4 images/retina.
Figure 3.1, Continued
Cre-electroporated cells revealed that significant numbers of electroporated amacrine cells developed an extra neurite pointing into the INL (28% +/- 5%) compared to the control (7% +/- 4%) (Figure 3.1 C’- F). These results suggest the effect of Fat3 loss on amacrine cells is not secondary to defects in IPL formation. Instead, the simplest explanation is Fat3 acts cell autonomously to restrict neurites to the IPL.

*Fat3 promotes directed amacrine cell migration*

These results suggest that Fat3 acts at some point during the course of an individual amacrine cells’ development. To determine the earliest effect of Fat3 on amacrine cell development, we turned to time-lapse imaging in an ex-vivo retinal slice culture. Having established late born amacrine cells are affected by Fat3 loss (Figure 3.1), analysis was restricted to late born amacrine cells. Late-born amacrine cells were labeled as before, except ex vivo, using electroporation of a Cre-dependent tdTomato fluorescent reporter plasmid into Ptf1a Cre expressing P0 retinas. Unexpectedly, live imaging revealed that amacrine cell leading neurites contacted the IPL while cell bodies were still close to the outer limiting membrane and the majority of the distance to the IPL remained to be traveled (Figure 3.2A). Since Fat3 is concentrated at the IPL, this suggested that Fat3 might affect amacrine cell migration towards the IPL. In contrast, an earlier analysis of fixed tissue was only able to detect a defect in trailing neurite retraction following completion of amacrine cell migration (Deans et al., 2011). Therefore, individual cell movements were tracked during migration, and wildtype and Fat3cKO amacrine cell dynamics were compared.
Figure 3.2  Amacrine cell migration is less directional with loss of Fat3.

A-B) Representative time-lapse images of developing wildtype (A) and Fat3 amacrine cKO (B) in an organotypic slice culture. The blue dot indicates the center of the cell body used for tracking. Scale bar, 20 µm.  C-E) Analysis of amacrine cell migration during development. Graph depict average migration rate directed toward the IPL (C), average movement of cell bodies (D), and percentage of cell movements that were in the opposite direction of the previous movement (E) for WT and Fat3 cKO mutant amacrine cells in the middle third of the retina. n=75 cells for each condition from 3 WT and 5 Fat3 cKO animals. p<.001. F-G) Representative images of a labeled cohort of WT(F) and Fat3 cKO (G) amacrine cells electroporated at P0, and then cultured for 48 hours in a retinal slice culture. Scale bar, 20 µm.  H) Quantification of the location of amacrine cell bodies labeled by electroporation at P0 and cultured for 48 hours. * indicate p<.05, Mann-Whitney U-test. I-J) Representative images of P1 WT(F) and Fat3 cKO (G) amacrine cells labeled genetically with Ptf1a Cre and tdTomato reporter. (Bright cells far away form the IPL are horizontal cells, also labeled by Ptf1a Cre).  K) Quantification of the amacrine cell position in I and J. * indicates p<.05, Mann-Whitney U-test.
Figure 3.2, Continued
Comparison of cell body migration of wildtype (Figure 3.2A) and Fat3cKO amacrine cells (Figure 3.2B) revealed multiple differences. Fat3cKO amacrine cells migrated slower towards the IPL (Figure 3.2C); however this was not due to a decrease in cell body motility (Figure 3.2D). To the contrary, Fat3cKO amacrine cells were more motile than wildtype amacrine cells. This suggested that Fat3cKO cells were migrating away from the IPL more than wildtype cells (Figure 3.2B). Further quantification showed that Fat3cKO amacrine cells were twice as likely to change direction of migration as wildtype cells (Figure 3.2E), leading to vacillation by the cell bodies. To assess the cumulative effect on migration, cell body position was quantified following an equal time in culture (Figure 3.2F-H). As expected, Fat3cKO cell bodies were overall farther away from the IPL. This delay in migration was corroborated in vivo. Amacrine cells were labeled genetically with Ptf1a Cre and a genetic tdTomato reporter and analyzed at P1, an age corresponding to the age of analyzed ex vivo cultures (Figure 3.2I-J). As mentioned before, most amacrine cells are born prior to P0. Therefore by P1, most amacrine cells had already completed migration. Nonetheless, more amacrine cells were still migrating in mutant compared to control retinas, as evidenced by the presence of labeled cells in the middle third of the retina (Figure 3.2I-K, blue dots indicate cells in the middle third) and confirmed by quantification.

**Fat3 affects trailing neurite dynamics**

Previous results suggested that loss of Fat3 led to failure in trailing neurite retraction. Therefore, time-lapse imaging was used to analyze the dynamics of trailing neurites. Trailing neurite tip distance to cell body was tracked during migration to assess
changes in trailing neurite length independently of cell body position (Figure 3.3A,B). As expected, loss of Fat3 led to slower retraction of trailing neurites (Figure 3.3C). Additionally, the Fat3cKO amacrine cell trailing neurites were more dynamic, exhibiting more frequent changes in length than wildtype amacrine cell neurites (Figure 3.3D). Trailing neurites also frequently branched, a rare event in wildtype cells (Figure 3.3E-F). Wildtype neurite branching was nearly always restricted to the leading neurite, while loss of Fat3 led to frequent episodes of ectopic branching; sometimes branches even emerged aberrantly from the cell body (Figure 3.3E). To quantify the amount of branching, a metric was developed which reflected both the number of branches formed and their duration. Quantification revealed Fat3 cKO neurons have a higher branching index (see Methods) than control neurons (Figure 3.3F).

Overall, loss of Fat3 led to loss of directed cell body migration and more motile trailing neurites, as well as an increase in symmetric neurite dynamics. These dynamics are a reflection of the state of the underlying cytoskeleton, and suggest that loss of Fat3 leads to a more symmetric cytoskeleton. Given Fat3’s asymmetric distribution, this suggested that on a molecular level, Fat3 might help polarize cytoskeletal activity.

*Fat3 can alter the distribution of Ena/VASP through a conserved binding site*

Fat3 is a single pass transmembrane receptor with an extremely large extracellular domain and an unstructured intracellular domain of 381 amino acids. Therefore, one way that Fat3 could act is by binding cytoskeletal regulators through its intracellular domain, promoting a change in their distribution. While there are no known molecular binding partners of Fat3, it is most closely related to the atypical cadherin
Figure 3.3 Trailing neurite dynamics are altered in Fat3 mutants.

A-B) Representative time-lapse images of developing wildtype (A) and Fat3 amacrine cKO (B) in an organotypic slice culture highlighting trailing process retraction. The blue dot indicates the tip of the trailing process used for tracking. Scale bar, 20 µm.  C-D) Analysis of amacrine cell migration during development. Graph depicts average trailing process retraction rate (C) and average overall tip movement (D) for WT and Fat3 cKO mutant amacrine cells. n=50 cells for each condition from 3 WT and 5 CKO animals. p<.001, Mann-Whitney U-test.  E) Representative series of time-lapse images Fat3 cKO mutant amacrine cells displaying trailing process branching. F) Quantification of branching frequency in WT and Fat3 cKO amacrine cells. Branching index = cumulative # of branches at each timepoint / # of timepoints. n = 3 WT and 5 Fat3 CKO. * indicates p < 0.001, Mann-Whitney U-test.
Figure 3.3, Continued

A

B

C

D

E

F
Fat1, which binds to Ena/VASP family members through its intracellular domain (Moeller et al., 2004; Tanoue and Takeichi, 2004). The Ena/VASP family are a major family of actin regulators that are critical in both neurite elongation and neuronal migration (Dent et al., 2007; Goh et al., 2002; Kwiatkowski et al., 2007). Ena/VASP proteins act to promote formation of filamentous actin and are regulated by recruitment to the plasma membrane (Bear and Gertler, 2009; Krause et al., 2003). Fat1 can cause Ena/VASP-dependent changes in the actin cytoskeleton in vitro. However, whether Fat1 and Ena/VASP share roles in vivo is unknown.

Alignment of the Fat1 and Fat3 intracellular domains revealed conservation at the two Fat1 Ena/VASP EVH1 binding sites. Further analysis of the Fat3 domain revealed a third candidate Ena/VASP EVH1 binding site (Figure 3.4A, 4B). If Fat3 acts to change the distribution of cytoskeletal regulators in amacrine cells, the intracellular domain of Fat3 (Fat3 ICD) would be predicted to change the distribution of Ena/VASP in a cellular context. To test this, the Fat3ICD was targeted to the mitochondria outer leaflet, a cellular location where Ena/VASP is normally not found. Fat3ICD presence at the mitochondria strongly recruited endogenous VASP, thereby altering its overall distribution (Figure 3.4C, 4D). To localize the site of the interaction, a series of Fat3ICD truncations and deletions removing the predicted Ena/VASP binding sites were targeted to the mitochondria. Deletion of all three predicted sites got rid of the interaction, while restoring just the conserved first two sites restored the interaction. Further examination of the two conserved sites revealed the first site did not affect VASP recruitment, despite the first site in Fat1 having higher affinity for Ena/VASP than the second site (Moeller et al., 2004). Instead, deleting the second Fat3 ICD site removed the
Figure 3.4  Fat3 can relocalize Ena/VASP family members through conserved binding sites

A and B) Schematic depicting three putative Ena/VASP binding sites in the Fat3 ICD. The predicted most critical residues for Ena/VASP binding are highlighted in red (A) and their relative locations are schematized in B. C-D) Representative images of VASP localization in the presence of mitochondrial-targeted Venus (C) or full length Fat3 ICD (D) E) Summary of VASP recruitment to the mitochondria using the mitochondrial recruitment assay with a series of Fat3 ICD constructs lacking the putative VASP binding domains. Boxed numbers correspond to amino acid of full length protein. F) Fat3ICD fused to GST was expressed in bacteria and used as bait in a pulldown from brain lysate. The critical amino acids in the first two putative binding sites (red in A) were mutated to alanines (grey stars). Pulldown was assessed by western blot.
Figure 3.4, Continued

A

Fat1: SDPVFFPPED--FPAPILPLPPK--F
Fat3: SEYFFPEKEFFLRSQALLPPALTDF--QTLPFPPLPG

B

Venus

Fat3 ICD

C

mitVenus

D

mitVenus, Fat3 ICD

C'

VASP

D'

E

VASP Recruitment

F

Input, brain lysate

supernatant

beads
interaction (Figure 3.4E). To more finely dissect which site was responsible for the Fat3-Ena/VASP interaction, the amino acid critical for Ena/VASP binding was mutated to an alanine in each site. Fat3ICDs with point mutations were fused to GST, purified from bacteria, and used as bait in a pulldown from neonatal mouse brain lysate. Fat3ICD pulled down both VASP and EVL (and Mena, not shown) from brain lysate. A point mutation in just the second site abolished the interaction, while a point mutation in the first had no effect (Figure 3.4F). These results indicate that Fat3ICD and Ena/VASP interact both in a cellular and biochemical context. The interaction is also likely to be direct since a point mutation in a predicted Ena/VASP binding site removes the interaction.

\textit{Fat3 and Ena/VASP localize to the IPL throughout amacrine cell development}

Next Ena/VASP expression was examined in the retina. Western blot of retina lysates confirmed that all three Ena/VASP family members are present throughout amacrine cell development (Figure 3.5A). Moreover, co-immunostaining for Mena and Fat3 in the retina at P0 and P6 demonstrated both are concentrated in the IPL at this time (Figure 3.5B-C). Therefore Ena/VASP is expressed in the right time and place to act with Fat3 in amacrine cell development (Figure 3.5). Next Ena/VASP distribution was examined in Fat3 mutant retinas. At P6, when the extra plexiform layer can first be seen, all three family members were mislocalized into the middle of the developing inner nuclear layer, where the extra plexiform layer was forming (Figure 3.6).
Figure 3.5 Fat3 and Ena/VASP localize to the IPL during amacrine cell development

A) All three Ena/VASP family members, Mena, VASP and EVL were expressed in the retina during amacrine cell development as visualized by western blot of retina lysates from indicated timepoints. B,C) Mena (B,C) and Fat3 (B’,C’) both localize to the inner plexiform layer at early in development at P0 (B) and at P6 (C), at the end of amacrine cell production.
Figure 3.6 Loss of Fat3 leads to change in Ena/VASP localization

A-B) Mena, (C-D) VASP, (E-F) and EVL localization at P6 in the wildtype (A,C,E) and mutant (B,D,E) retinas. Intensity plots corresponding to pixel intensity in indicated white rectangle are plotted to the right. Mutant retinas have an increased concentration of all three family members near the middle of the developing inner nuclear layer (B,D,F, arrows).
**Uniform Ena/VASP localization phenocopies loss of Fat3**

Altogether, the localization of Fat3 and Ena/VASP in the retina, the binding of Ena/VASP by Fat3 and the effect of Fat3 on amacrine cell directed behavior, suggests the following model. In this model, Fat3 acts cell autonomously to promote the recruitment of Ena/VASP to the leading neurite and therefore out of the trailing neurite. The resulting asymmetric distribution of Ena/VASP leads to migration towards the IPL and retraction of the trailing neurite (Figure 3.7A). Loss of Fat3 leads to a reduction of Ena/VASP recruitment to the leading neurite and hence an increase of Ena/VASP into the trailing neurite. As a result, trailing neurites are retained (Figure 3.7A’). This model predicts that uniform recruitment of Ena/VASP is sufficient to lead to trailing neurite retention. To test this prediction, we sought to change the distribution of endogenous Ena/VASP without changing Ena/VASP expression levels in amacrine cells. To that end, electroporation was used to introduce expression of a peptide previously shown to strongly and specifically bind all three Ena/VASP members (Ball et al., 2000; Niebuhr et al., 1997; Zettl and Way, 2002). To recruit Ena/VASP to the membrane uniformly, the peptide was targeted to the membrane using a myristoylation signal and fused to Venus to both visualize the distribution of the peptide and the morphology of the transfected cell. Analogous constructs have been shown to distribute Ena/VASP uniformly to membranes *in vitro* (Bear et al., 2000, 2002; Lebrand et al., 2004). This construct was expressed specifically in amacrine cells, using electroporation into Ptf1a Cre positive retinas at P0. Myristoylated Venus was used as a control. Analysis of electroporated amacrine cell morphologies at P11 demonstrated that qualitatively, an increase in uniform Ena/VASP signaling led to extra neurite retention similar to loss of Fat3 (Figure
Figure 3.7 Redistribution of Ena/VASP is sufficient to redirect amacrine cell neurites.

A) Model of proposed action of Fat3: Fat3 localizes to the IPL, biasing the distribution of any bound effectors into the IPL. A’) In the absence of Fat3, these effectors have a more uniform distribution, leading to the retention of extra neurites. B-D) Direct redistribution of endogenous Ena/VASP family members in amacrine cells leads to extra neurites, supporting the model in (A’). B-C) Either Venus as a control (B) or Venus fused to a strong recruitment peptide for all Ena/VASP family members (C) was uniformly targeted to the membrane of amacrine cells. Expression of constructs was restricted to amacrine cells using Cre-dependent expression combined with a Ptf1aCre transgenic line. Constructs were introduced at P0 using electroporation. Morphology was accessed at P11. C’) An example amacrine cell with an extra neurite facing away from the IPL. Arrows point to extra neurites. D) Quantification of the percentage of cells with extra neurites greater than 10 um in (B) and (C). Mann Whitney U Test, P = 0.001. Morphology was evaluated in 3D reconstructions and any non-IPL facing neurites were measured. N= 4 retina/condition, 4 images/retina.
3.7B and 7C). Quantification confirmed that significantly more electroporated amacrine cells had an extra neurite (13% +/- 2%) compared to the control (3% +/- 1%) (Figure 3.7D). Therefore, uniformly targeting Ena/VASP to the membrane was sufficient to lead to the retention of extra neurites and recapitulates the Fat3KO phenotype.

Discussion

Many steps of neuronal development require a neuron to be polarized. This includes asymmetric division, migration of neuronal cell bodies, as well as the growth, retraction and specification of neurites. For a cell to be polarized can mean one of two things. In the first case, polarity refers to a cell being asymmetric, so one side of the cell is different and acts differently from the other. For example, mature neurons have axons and dendrites, and these have very different morphological and functional properties. In the second case, polarity is propagated across the cell, so that events on one side of the cell affect the other. For instance when axo-dendritic polarity is being established, the specification of one neurite as an axon leads to the specification of the rest as dendrites (Cáceres et al., 2012).

As these examples illustrate, the creation of cellular asymmetry requires an initial polarizing event that propagates across a cell. Once a neuron is polarized, one end of the cell can act independently of the other. For instance axon guidance can occur independently of either the cell body or dendrites. However in other cases even once a neuron is polarized, propagation of polarity across the cell continues to be important.
For instance, in many modes of migration leading tip progression is linked to cell body movement.

The organization of the cytoskeleton establishes and maintains cellular polarity. In turn the polarization of the cytoskeleton is generated by the asymmetric distribution of cytoskeletal regulators or their activity. Together, the cytoskeleton and its regulators can reinforce and propagate an initial asymmetry (Stiess and Bradke, 2010). In vivo extracellular cues can generate the initial polarizing event and continue to reinforce the polarity (Arimura and Kaibuchi, 2007). Moreover, the cytoskeleton is anything but a passive structure. Not only does the cytoskeletal underlie the polarity of the cell, but it also generates the forces that lead to both migration and neurite outgrowth. Therefore when cytoskeletal polarization is disrupted, it leads to the disruption both of morphological asymmetry and as well as movement.

Here we present evidence that Fat3 does not just regulate the behavior of an already polarized amacrine cell. Instead, Fat3 acts to polarize and reinforce the polarity of amacrine cells during migration and trailing neurite retraction. Firstly, although Fat3 is concentrated at the leading tip of the amacrine cell, loss of Fat3 affects both the cell body and the trailing tip. Secondly, live imaging reveals that the cell dynamics of both migration and neurite retraction become less directed in the absence of Fat3: cell bodies can migrate in the wrong direction and neurites reextend. Therefore Fat3 activity affects polarity all along the cell. The simplest model suggests that Fat3 acts to polarize the cytoskeleton across the entire neuron and this leads to defects in both migration and trialing neurite dynamics. It is also possible that Fat3 generates two different polarity
signals, one of which affects trailing process retraction and the other cell body migration. Currently however it is not possible to distinguish between two possibilities.

In support of Fat3 acting to polarize the cytoskeleton to mediate effects on migration and these events needing a polarized cytoskeleton, we found that Ena/VASP is also asymmetrically distributed in the retina and is concentrated in neurites in the IPL. Ena/VASP regulates actin dynamics through direct binding to actin and profilin-actin complexes to promote the formation of long unbranched filaments (Krause et al., 2003). A triple knockout of Mena, VASP and EVL in mice leads to a loss of neurite formation in vitro and in vivo. Additional defects include neurite outgrowth, guidance and neuronal migration (Chang et al., 2006; Dent et al., 2007; Goh et al., 2002; Lebrand et al., 2004).

Ena/VASP is regulated by recruitment to the plasma membrane. Our model of Fat3 activity suggests that recruitment to one side of the cell can prevent Ena/VASP from being recruited on the other side, perhaps by depletion from the trailing neurite into the leading neurite. In support of this model, Ena/VASP distribution is changed in Fat3 mutant retinas. Fat3 may be directly responsible for this as Fat3 bound to Ena/VASP in vitro. However it is likely that Ena/VASP is only a small part of Fat3 signaling; Fat3 has a large intracellular domain and so is likely to have additional effectors.

These findings suggest a common role for Fat-likes may be the generation of actin polarity. Here we find that manipulating the organization of the actin cytoskeleton through Ena/VASP mimics the Fat3 phenotype. Similarly, loss of Drosophila Fatlike disrupts planar polarized organization of actin stress fibrils in follicle cells during oogenesis (Viktorinová et al., 2009). Fat1 too has been shown to affect actin organization in vitro (Moeller et al., 2004; Tanoue and Takeichi, 2004), although a role
for actin manipulation has yet to be been demonstrated in vivo. However while Fat3 leads to cell-autonomous changes in cellular polarity, Drosophila Fat-like’s effect on actin organization in follicle cells is non-cell autonomous (Viktorinová et al., 2009, 2011). The varied localization in tissue additionally speaks to differences in function: Fat3 is localized very asymmetrically in the amacrine cells, while Drosophila Fat-like is at all the plasma membrane of follicular cells, concentrated basally (Viktorinová et al., 2009). Fat1 localizes at adherens-like structures at slit junctions in the kidney (Yaoita et al., 2005). Studies also point to functions of Fat-likes distinct from actin polarity. For instance Fat3 genetically and physically interacts with Atrophin, a transcriptional corepressor, to mediate zebrafish chondrocyte differentiation (Le Pabic, 2014). Interactions with Atrophin have also been found with Fat1 (Hou and Sibinga, 2009). Furthermore the roles of Fat1, 3 and Fat4 in vertebrate morphogenesis point to complicated interactions, some synergistic, some antagonistic (Saburi et al., 2012). Altogether this suggests that a shared function of Fat-likes is the polarization of actin, although they are likely to have multiple functions that vary depending on the tissue.

Amacrine cell development presents an opportunity for looking at the generation of polarity in situ. Mature amacrine morphology exhibits strict polarity with neurites restricted to one side of the cell body, projecting into the IPL. This asymmetry is generated from an outwardly relatively symmetric bipolar shape during migration, although clearly neurites are asymmetric in behavior. There must also be an initial polarity that allows amacrine cells to extend a neurite towards the IPL. However the Fat3 phenotype demonstrates that initial polarity is insufficient to ensure polarized migration and trailing neurite retraction. Instead polarity must be reinforced by receptors
such as Fat3. Many cells that lack Fat3 and exhibit changes in trailing neurite dynamics still eventually retract. It is likely that multiple externally derived cues act in concert to promote polarized dynamics during amacrine cell development thereby leading to migration, neurite outgrowth, and eventually the mature morphological polarity of the neuron.

**Materials and Methods**

**Animals**

Lines used: Fat3 Fl and Fat3-/- mice (Deans et. al., 2011), Ptf1a Cre (C. Wright, Vanderbilt U.), Rosa26 Tomato, A14. Mice were kept on a mixed background.

Fat3cKOs were generated by breeding Fat fl/fl mice with Ptf1a-cre +/-;Fat3+/-, with or without tomato reporter. Controls were from the same cross, but with one Fat3 wildtype allele. Fat3 Fl/- and Fat3 Fl/+ controls for Cre electroporation experiments were generated by breeding Fat3 +/- mice to Fat3 Fl/Fl mice. Electroporations for testing the effects of uniform Ena/VASP recruitment were performed in Ptf1a Cre expressing animals generated by breeding Ptf1a Cre males crossed to wildtype CD1 females.

**Antibodies used**

Mouse pan-Mena, rabbit Mena 2197, VASP 2010, EVL 1404 from the Gertler lab. Fat3 (Deans et al., 2011). Anti-Dsred clonetech. Mouse anti Actin.

** Constructs used**
CAG-FSF VenusCaax, CAG-FSF VenusFPPPCaax, Cag-FSF Tomato, CAG-FSFVenus, CAG- FSF NeonGM130, Cag-mitVenus, Ndrg4-Cre, Ndrg4-Tomato and Ndrg4-TomatoNLS. CMV-MitVenus, CMV-mitVenusFat3ICD, CMV-mitVenusFat3ICDdeletions. pGex 6P-1-Fat3ICD, pGex 6P-1-Fat3ICDmutations. All constructs were verified by sequencing.

Vector backbone for Cre dependent expression derived from Cag loxP stop loxP GFP a kind gift from C. Cepko, same as Addgene plasmid #13769. Inserts amplified by PCR for expression of Venus- FPPPx4-Caax (ActA Ena/VASP binding site) a kind gift from F. Gertler, modified to express Venus instead of GFP. Caax box sequence used: CMSCKCVLS. Other inserts used included Venus-caax, Tomato, mitVenus, NeonGM130. Ndrg4 Cre was a gift from C. Cepko, backbone same as Addgene plasmid #13766, and was also modified to express Tomato and NLS-Tomato.

Vectors for expression of proteins targeted to the mitochondrial were created using isothermal assembly. pCMV backbone derived from pEYFP-Mem, Clonetech, N terminal mitochondrial targeting tag MVGRNSAIAAGVCGALFIGYCIYFDRKRRGDPN from Tom20 protein (Kanaji et al., 2000) introduced with primer used to PCR-amplify Venus or Venus with Fat3ICD.

Deletions of ICD were also created with isothermal assembly with sequences as indicated in figure. Full length Fat3 cytoplasmic domain (aa 4175 - 4555, all amino-acid positions are referenced to UniProt Q8BNA6). Truncation 1: 4175-4398. Deletion 1,2,3: 4175-4398,4426-4455,4469-4555. Deletion 1,2: 4175-4398,4426-4555. Truncation 2: 4175-4427. Deletion 1: 4175-4398, 4412-4555. Deletion 2: 4175-4411,4426-4555.
GST fusions used the pGEX6P-1 (GE Lifesciences) vector with PCR of Fat3ICD inserted into FseI/Ascl sites. The truncation construct was made used blunt jointing of AVRII site in Fat3ICD with Ascl site in vector, creating truncation of Fat3ICD at amino acid 4394, followed by 11 amino acids and a stop codon derived from the vector. The constructs with point mutations were created by GenScript.

In vivo retinal electroporations and fixed tissue imaging

Retinal electroporations were performed as described by Matsudo and Cepko. In brief, 5ug/ul of DNA was injected into the subretinal space of P0 mouse retinas, followed by 5 pulses of 80V delivered across the head. Mice were allowed to develop to P11 whereupon eyecups were collected and fixed in 4%PFA at 4C overnight, then processed as for immunostaining. Retinas were sectioned at 40um and imaged blind to condition with an Olympus Fluoview 1000 Confocal microscope, 60x Lens, 1.4 N.A., pinhole C.A. 90 um, zstep .4um, resolution in x, y of .103um x .103 um per pixel. Four separate stacks through the entire thickness of a section were taken of 4 retinas per condition for a total of 16 images per condition. Quantification of extra neurites was performed in Amira software following isosurface reconstruction of 3D morphologies. Analysis was limited to cells with cell bodies fully present in the stack. For the Cre electroporation experiments, analysis was limited to cells expressing Ndrg4 Tomato; all neurites not extending into the IPL were measured in 3D.

Analysis of migration in fixed tissue was as follows. Retinas were collected at P1, fixed and embedded as described for immunostaining, sectioned at 20um and immunostained with rabbit anti Dsred (clonetech) and 568 secondary and
counterstained with DAPI. A single slice was collected, 40x objective, 1.3 N.A. pinhole C.A. 90um, resolution in x, y of .198um x .198 um per pixel. 4 images were collected per retina within 500 um of the optic nerve. 4 retinas per condition were imaged for a total of 16 images per condition. Cell position in the y axis as well as the upper and lower limits of the nuclear layer were measured in ImageJ. Percentage of distance to the IPL traveled was calculated in Excel using these measurements.

Ex vivo retinal electroporation and live imaging

Ptf1a-cre positive P0 mouse retinas were dissected and electroporated as previously described (Matsuda and Cepko, 2004). Retinas were electroporated with .7ug/uL lox-stop-lox tdTomato and .3 ug/uL mNeonGreen-golgi. Retinas were then embedded in 5% low melting point argarose and sectioned at 200 uM on a vibratome. Sections were placed on organotypic culture substrates (millicell, Millipore) and cultured at 37 C for 24-72 hours as has been described previously (Surzenko et al., 2013). Amacrine cell development was visualized by chronic long term (16-24 hour) imaging on a Zeiss 710 NLO multiphoton microscope with an environmental chamber that regulates temperature CO2 and humidity. The laser was tuned to 950 nm to excite both fluorophores, a 40X 1.1 NA objective was used, and fluorescence emission was captured simultaneously by non-descanned detectors. Images were collected at 2 μm intervals throughout a 130-150 um volume near the optic nerve. Images were analyzed using the mTrackJ plugin in ImageJ (NIH), Excel, and Prism.

Retina immunostaining and imaging
Retinas were collected at P0 or P6 and fixed in 4% PFA at 4°C for 2 hours. Following sucrose protection in 30% sucrose O/N, tissue was embedded in Neg-50 and frozen on metal cooled with liquid nitrogen. 14μm cryosections were blocked and permeabilized for 30 min at room temperature using 10% goat or donkey serum and 0.1% TritonX-100 in PBS. Sections were incubated 1-2 overnights at 4°C in block solution without Triton X-100 with primary antibody. Then sections were incubated at room temperature for 1 - 2 hours, with appropriate secondaries at 1:1000. Sections were counterstained with DAPI (1:10,000) and mounted using 50% glycerol/50% sorenson's solution.

For Fat3 and Mena colocalization, z-projections were made of a stack of 4 images taken with 60x Lens, 1.42 N.A., pinhole C.A. 90 um, zstep .37um, resolution in x, y of .207um x .207 um per pixel. For visualizing Mena, VASP, EVL localization in Fat3 wildtype and mutant retinas, single images were taken with 40x objective, 1.3 N.A. pinhole C.A. 190um, resolution in x, y of .397um x .397 um per pixel

**Mitochondrial recruitment assay**

Cos-7 cells were transfected with Lipofectamine 2000 (Invitrogen) with equal concentrations of the Fat3 ICD constructs targeted to the mitochondria. After 24 hours cells were fixed with 4% PFA, and stained with 1:750 rabbit anti Vasp (Gertler). Cells were then imaged using an Olympus FV1000 microscope with a 1.42 NA, 60X objective. The colocalization was then quantified by determining the Pearson's coefficient of individual cells using the coloc2 plugin in ImageJ (NIH).

**Pull Down**
GST fusion proteins were grown in Rosetta E.Coli (Millipore) at room temperature, induced with IPTG at OD600 = 0.5-1 and harvested after 4 hours after induction. Bacterial pellets were lysed in PBS with 1% TrionX-100, 1mM EDTA, 0.1mg/ml lysozyme supplemented with 1 mM Pefabloc SC PLUS protease inhibitor (Roche, Rochester, NY), 1mM DTT. Fusion proteins in bacterial lysate were bound to glutathione sepharose beads (GE Life Sciences) in batch, 4 hours at 4C. Beads were washed 2x in PBS, 0.5% TritonX-100 supplemented with 1mMDTT and 1x in PBS, 1mM DTT. Beads were snap frozen in PBS, 25%glycerol, 1mMDTT and stored at -80C.

To make brain lysate, P1 brain was dissected and homogenized on ice in a dounce with 750ul lysis buffer added per 100mg tissue. Lysis continued on ice for 30 minutes. Homogenate was spun to clear, 10, 000 x g for 30 min. 900ul of lysate added to 50ul of beads bound to fusion protein and rotated at 4C overnight. Beads were spun 600 g, 3 minutes, unbound brain lysate supernatant was collected and beads were washed 5x with 400ul w/DTT, 10 minutes in between spins. 50ul beads was combined with 45ul lysis buffer and 25ul 4x NuPage sample buffer (Invitrogen). Following pull down, brain lysate input, supernatant and beads were analyzed by standard western blot analysis.

**Westerns**

For Ena/Mena/VASP western blots, retinas and brain tissue were stored in -80 C prior to lysis. Following thawing, samples were lysed in 50mM Tris ph7.5, 200mM NaCl, 10% glycerol, 1% NP40, 1mM EDTA, 1 mM EGTA, supplemented with 1 mM Pefabloc SC PLUS protease inhibitor (Roche, Rochester, NY), 1mM Na3OV4, 1mM DTT. Samples
were quantified using a BCA Protein Assay Kit (Pierce) then normalize to the same protein concentration. Protein was run on a Tris–Glycine polyacrylamide gel, transferred onto Immobilon-P Membrane (Millipore, Meck, Billerica, MA) in 25mM Tris, 192 mM glycine, 10% Methanol and 0.05% SDS followed by standard western blotting. Blots were reprobed for actin following stripping (thermoscientific product 2105).
Chapter 4: Signaling downstream of Fat3

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A. Krol designed, performed and analyzed all experiments with the exception of the following. Fat3Trunc and Fat3delEV mice were designed and created together with S. J. Henle. GST-Fat3ICD fusion constructs were designed together with S. J. Henle and cloned by S.J. Henle. Blastocyst injections were performed by Partners Healthcare Transgenic Core. Mass Spectrometry analysis was performed by Boston Children’s Hospital IDDRC proteomics’ core.
Introduction

Amacrine cells of the retina precisely project a single dendritic arbor into the inner plexiform layer (IPL). Migrating amacrine cells have a bipolar shape with the leading neurite extending into the IPL and a trailing neurite that is retracted at the end of migration. Fat3 protein is concentrated at the IPL and cell-autonomous loss of Fat3 causes some amacrine cells to retain the trailing neurite. While many of the mutant amacrine cells resolve their morphology, live imaging reveals that the majority of amacrine cells exhibit less directed movement of both the cell body and the trailing neurite (Chapter 3). Amacrine cell bodies often reverse their direction of travel, and amacrine cell trailing neurites exhibit more branching than controls, similar to leading neurites. The change in mutant mature amacrine cell morphology and the changes in cellular dynamics during development imply a change in the organization and state of the underlying cytoskeleton. Loss of Fat3 seems to promote a less polarized amacrine cell state, suggesting a more symmetric distribution of cytoskeletal regulators.

The highly asymmetric localization of Fat3 protein in wildtype retinas further suggests that Fat3 promotes the asymmetric localization or activity of cytoskeletal regulators and raises the question of what effectors Fat3 binds. There is some evidence that other Fat-like cadherins organize actin. In Drosophila, loss of Fat-like disrupts the planar-polarized organization of actin in egg chamber follicle cells (Viktorinová et al., 2009). Similarly, loss of vertebrate Fat1 can change the organization of actin in cells in vitro (Moeller et al., 2004; Tanoue and Takeichi, 2004). Fat1 has also been found to bind an assortment of proteins in pulldown assays, including a few cytoskeletal
regulators (Sadeqzadeh et al., 2014a). However none of these effectors have been connected with a function for Fat1 in vivo. We showed that Fat3, like Fat1, binds Ena/VASP in a pulldown assay, and can change Ena/VASP localization in vitro. Furthermore, uniform recruitment of endogenous Ena/VASP to amacrine cell membranes in vivo phenocopied loss of Fat3 and led to amacrine cell neurite retention. Together, these results suggest that a change in Ena/VASP distribution is one way in which Fat3 polarizes the amacrine cell cytoskeleton (Chapter 3). However it remains unclear whether Ena/VASP is necessary for the function of Fat3 in vivo. Furthermore, the Fat3 intracellular domain (ICD) is 381 amino acids long, and is predicted to bind many additional proteins by the online ELM (Eukaryotic Linear Motif) predictor (Dinkel et al., 2013). In particular, the ICD includes a C terminal PDZ binding domain. Therefore we set out to further examine Fat3 downstream signaling.

To dissect Fat3 signaling, we created mouse models with specific deletions within the part of the endogenous Fat3 gene encoding the Fat3ICD. Three mouse lines were created: one with an engineered deletion of the ENA/VASP Fat3ICD binding site determined in Chapter 3, and two with premature stop codons leading to the deletion of the terminal third of the ICD. Analysis of these mouse mutants demonstrated that neither the Ena/VASP binding site, nor the terminal third of the ICD, which includes the Ena/VASP binding site and the terminal PDZ binding site, is necessary for Fat3 signaling in amacrine cells. Further analysis of the Fat3 ICD suggested the ICD binding site indeed interacts with additional regulators of both the actin and the microtubule cytoskeleton. Some of these interactions remain in the truncated Fat3 protein created in the mouse mutants. In the retina, all cytoskeletal effectors examined were concentrated
at the IPL and were mislocalized in the Fat3 mutant retina. This included effectors that seemed to lack specific binding to the Fat3ICD. Furthermore a general rearrangement of actin and microtubules was observed. Therefore it is likely that loss of Fat3 is permissive for the initiation of a cascade of signaling that leads to rearrangement of the cytoskeleton and the creation of extra plexiform layers.

Results

Deletion of Fat3 ICD binding sites in vivo has no effect on retinal morphology

Uniform recruitment of Ena/VASP to the membrane is sufficient to lead to extra amacrine cell neurites (Chapter 3). Furthermore, Ena/VASP localized to the future extra plexiform layer in Fat3 mutants. To specifically test whether Fat3 recruitment of Ena/VASP is necessary for Fat3 signaling, CRISPR-Cas9 genome editing (Cong et al., 2013; Yang et al., 2013) was used to create mutant mice that produce versions of Fat3 that are no longer able to bind to Ena/VASP. In brief, oocyte injections were performed with RNA encoding the Cas9 enzyme as well as two guide strands targeting Cas9 nuclease to two sites in the fat3 locus, one just C terminal, and one just N terminal of the Ena/VASP binding site (Figure 4.1A). To create an in-frame and engineered deletion of the Ena/VASP binding site, a template repair oligonucleotide was also injected. Three mutant alleles were recovered. The Fat3 delEV allele carried an in-frame deletion of the Ena/VASP site, Fat3 delEV (Figure 4.1A,C). In addition two alleles leading to a truncation of the Fat3 protein (Fat3Trunc) were created where a Cas9-mediated cut at the first site led to premature stop codons and frame shifts N terminal to the Ena/VASP
**Figure 4.1 Design and genotyping of mutations in Fat3 intracellular domain.**

A) Schematic of position in the Fat3 intracellular domain (ICD) relative to the Ena/VASP binding site (red) where CRISPR guide RNAs were targeted and the resulting mutations. Fat3delEV consisted of a deletion of the binding site. Fat3Trunc consisted of a premature stop upstream of the binding site. PCR primers illustrate approximate position of primers used in genotyping. B) DNA sequencing of cloned genomic DNA corresponding to the wildtype and the two Fat3Trunc alleles around the CRISPR cut site locus. Fat3Trunc –CT consisted of a deletion of wildtype nucleotides CT (highlighted yellow). Fat3Trunc –GaCCT consisted of an insertion of G-CCT (highlighted orange). The wildtype allele is cut by restriction enzyme AVRII. This site is lost in the Fat3Trunc alleles. C-D) PCR genotyping for newly created alleles. Primers illustrated in (A) C) Reaction for Fat3delEV alleles. Lower band corresponds to Fat3delEV allele. D) Reaction for Fat3Trunc alleles. PCR is followed by restriction with AVRII. Wildtype alleles produce two bands, FatTrunc alleles produce a single band.
Figure 4.1, Continued

A

CRISPR

Fat3 ICD

Fat3 ICD

Fat3delEV

Fat3Trunc

PCR Primers

B

Fat3 WT

Fat3WT: CCTAGG

Fat3Trunc -CT

Fat3Trunc -GaCCT

C

Fat3delEV

Fat3Trunc

PCR

PCR + AVR II cut
binding site (Figure 4.1A,B,D). Sequencing demonstrated changes in the genomic sequence of the three mouse lines (Figure 4.1B and not shown) as did PCR genotyping (Figure 4.1C-D).

Immunostaining with the Fat3 antibody demonstrated that Fat3 protein was still correctly localized in the IPL of all three mouse lines at P11 (Figure 4.2). The Fat3 antibody is a polyclonal antibody targeted against the N terminal 245 amino acids of the Fat3 ICD (Figure 4.2 A) and further purified using recombinant full Fat3 ICD as described in Chapter 1. While the plexiform layers in the mouse retina have some autofluorescence, Fat3 signal in these three mutant retinas (Figure 4.2C-E) was comparable to that in wildtype retina and clearly greater than the signal in the Fat3 null retina (Figure 4.2F). These immunostaining results suggested the truncated Fat3 proteins were expressed and localized correctly and that at least some of the epitope recognized by the antibody is in the N terminal 245-157 amino acids of the ICD.

If any of the regions deleted were required for Fat3 signaling, we would predict the Fat3KO phenotype to be recapitulated. Therefore new mutant alleles were examined for change in lamination and amacrine cell morphology (Figure 4.3). Neither Fat3Trunc nor Fat3delEV (Figure 4.3 F-O) showed any changes in the organization of the retina similar to the changes seen in the Fat3 null retina (Figure 4.3 P-T). There was no obvious change in retinal ganglion cell layer thickness, as visualized by DAPI staining (Figure 4.3 A,F,K,P), suggesting no increase in the number of amacrine cells in that layer. Nor were any new plexiform layers seen, as visualized by DAPI staining and by VGAT staining (Figure 4.3 D,I,N,S). Since it is likely that a critical number of affected amacrine cells is needed before extra plexiform layers are formed, amacrine cell
**Figure 4.2 Fat3 protein in new Fat3 mutant alleles localizes to IPL.**

A) Illustration of Fat3 peptide originally used to generate the polyclonal antibody (from Deans et al., 2011). B-D) Fat3 immunostain of P11 retinal tissue shows Fat3 staining in IPL in Fat3 WT(B), Fat3delEV(C) and both Fat3 Trunc allele (D,E) and background autofluorescence in Fat3 null tissue (F). Tissue was immunostained in parallel and images were taken at the same settings, then brightness was enhanced equally in ImageJ.
Figure 4.3  Neither Fat3delEV nor Fat3Trunc retinas show Fat3KO-like phenotype.

Immunostaining of P11 retina tissue. A-E) Wildtype. F-J) Fat3delEV. K-O) Fat3Trunc. and P-T) Fat3null. Arrows point to the extra layer of connections in the inner nuclear layer. (A,F,K,P) DAPI staining labels the nuclei. (B,G,L,Q) Calretinin staining labels amacrine cells in the inner nuclear layer and amacrine cells and retinal ganglion cells in the retinal ganglion cell layer. (C,H,M,R) PKCal immunostaining labels rod bipolar cells. (D,I,N,S) VGAT staining and (E,J,O,T) SV2 staining labels synaptic layers. All staining demonstrates mutant retinas resemble wildtype retinas and not Fat3null retinas.
morphology was also visualized using calretinin staining (Figure 4.3 B,G,L,Q), as at least 23% of this population are affected in the Fat3 mutant. However no extra neurites were observed. Lastly, bipolar cells were visualized using PKCalpha immunostaining (Figure 4.3 C,H,M,R), since amacrine cell neurites can attract bipolar cell terminals as seen in the Fat3 mutant retinas. However no such aberrant terminals were seen in the Fat3 delEV or Fat3 Trunc retinas.

**Fat3 ICD binds to many regulators of the cytoskeleton**

One possibility for why loss of the terminal third of the ICD has no effect on Fat3 activity is that the N terminal two thirds of the Fat3ICD acts redundantly with the rest of the ICD and compensates for the loss. Alternatively the terminal third of the ICD might be irrelevant for Fat3 signaling in amacrine cells. To determine additional candidate binding partners of Fat3, we performed a pulldown assay using the Fat3ICD, followed by analysis of the binding partners using mass spectrometry. Fat3ICD fused to GST was expressed in *E. coli* and purified on Glutathione sepharose beads. As a control, GST alone was also expressed and purified on beads. To determine which associated proteins might be recruited by Ena/VASP, Fat3ICD with a point mutation that removed Ena/VASP binding (Fat3ICD-ptMutEV, Chapter 3) was also purified. Fat3ICD beads and control beads were then incubated with P0 brain lysate, and washed to decrease binding of unspecific proteins (Figure 4.4 A). Beads bound to purified Fat3ICD and binding partners were run using SDS PAGE and visualized using a Coommasie stain. Both GST beads and FatICD beads as well as control beads bound to purified Fat3ICD (and any unspecific *E.coli* proteins) were run (Figure 4.4 B). Several prominent bands in
Figure 4.4 Experimental outline for mass spectrometry experiment.

A) Diagram of steps in pulldown experiment. Fat3ICD was expressed in bacteria fused to GST and purified onto glutathione beads. The resulting beads were incubated with brain lysate and washed to remove unspecific interactions. B) Coomasie stained SDS PAGE gel of proteins associated with beads after pulldown experiment. First lane is purified full length GST Fat3ICD, some breakdown products of this protein and perhaps some bacterial proteins. Other lanes additionally show proteins from brain lysate associated with full length ICD, full length ICD with a point mutation removing Ena/VASP binding, or GST alone. Two other lanes shown were not further analyzed. Strongest stained bands correspond to protein expressed by and purified from bacteria. Orange lines indicate the bands cut out to be processed for mass spectrometry analysis.
the Fat3ICD bead lane compared to the other two lanes indicate binding partners of Fat3ICD. The Coomassie-stained gel was then cut into pieces, as indicated, and each piece was trypsin digested and analyzed by mass spectrometry.

Analysis by mass spectrometry revealed Fat3 ICD bound many more proteins than the GST control beads (Appendix 1). In particular, Fat3ICD bound to many regulators of the cytoskeleton and proteins associated with the cytoskeleton (Table 1). Interestingly this included regulators of both the actin and the microtubule cytoskeleton. As expected, the Fat3ICD but not the Fat3ICD_ptMutEV bound to a member of the Ena/VASP family, EVL. Loss of the Ena/VASP binding site also resulted in loss of binding of several other cytoskeletal regulators including APC and Drebrin, suggesting they might act in a complex with Ena/VASP. Several other interactions merit a few more words. First, Homer was also identified as a binding partner of Fat1 (Schreiner et al., 2006) and was predicted to bind based on sequence homology. Moreover, Kinesin light chain 1 and 2 as well as Kif5c were also identified as binding partners of Fat1 (Schreiner et al., 2006). Together, along with Kif5a and Kif5b, these proteins make up Kinesin 1, which is targeted specifically to axons (Huang and Banker 2012, Jacobson 2006). Secondly, casein kinase I delta and epsilon were identified, which are the vertebrate homologues of discs overgrown. Discs overgrown binds and phosphorylates the intracellular domain of Drosophila Fat (Feng and Irvine, 2009; Sopko et al., 2009). This interaction was also found to be conserved between casein kinase I delta/epsilon and Fat4 (Sopko et al., 2009).

Clearly these results must be interpreted with caution, especially since some of the interactions were on the threshold of detection. In many cases, only a single peptide
belonging to a protein was identified by mass spectrometry. Therefore significant experimental variability would be expected. For instance some interactions were identified in the pulldown with Fat3ICD_ptMutEV and not with Fat3ICD. Nonetheless, candidates identified provide an excellent starting point to further investigate signaling downstream of Fat3.

Table 4.1: Mass Spectrometry results. Cytoskeletal effectors identified from the pulldown described in Figure 4.4. Binding to proteins in grey was lost in Fat3ICD_ptMutEV pulldown.

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Identification of Fat3ICD interaction sites for mass spectrometry-identified candidates

To further assess which of the candidate binding partners identified by mass spectrometry bind specifically to Fat3ICD, additional pulldown assays from brain lysate were performed (Figure 4.5). Analysis of the Fat3 ICD using GlobPlot (Linding et al., 2003) and IUPRED (Dosztányi et al., 2005) indicates that it is largely unstructured. Such intrinsically disordered regions are predicted to interact with binding partners via short linear motifs (Dinkel et al., 2014). Therefore, short stretches of the ICD should retain binding properties. To assess binding specificity, GST fusions to six fragments tiling the Fat3 ICD were generated (Figure 4.5A). Proteins which bind specifically should bind to relatively few fragments unlike those that associate more promiscuously. To gain insight on our Fat3Trunc mouse model, two more GST fusions were created to determine which binding sites remain in the Fat3Trunc mouse (Long) and which were removed (Rest). Lastly, it is possible the ICD of Fat3 is not necessary for Fat3 function in the retina. Instead, perhaps Fat3 performs a largely adhesive function. To test this possibility in the future, we are in the process of generating a mouse containing only the membrane proximal region of the Fat3ICD. To test any intracellular binding partners that might remain in this mouse, a short fragment corresponding to this region (Short) was also generated (Figure 4.5A).

Pulldown assays from brain lysate were performed using fragments of the Fat3ICD as bait, as described before in Figure 4.4A. Full length Fat3ICD was used as a positive control and GST alone as a negative. As a positive control, VASP was found to bind as before to fragments that contained the binding sites identified in Chapter 3 and
Figure 4.5 Pulldown assay demonstrates binding specificity for some but not other candidate Fat3 effectors identified by mass spectrometry.

Pulldown assays were carried out as illustrated in Figure 4 and followed by western blot to narrow down binding sites of candidate Fat3 effectors. A) To scale illustrations of GST-fusions used in pulldown. Position of Ena/VASP binding sites (second site the strongest binding) illustrated as red stars and predicted WIRS, WAVE binding sites in yellow. N terminal fusions were created to peptides tiling the Fat3 ICD (1-6) as well as to a peptide corresponding to the portion of the Fat3ICD that remained in the FatTRUNC mice (Long) and remove in those mice (Rest). The Short peptide corresponds to a membrane proximal region that will remain in a mouse currently being generated that is intended to be a truncation of the entire Fat3 intracellular domain. GST-fusion proteins were purified on beads as in Figure 4 and incubated with brain lysate. B) Western blot for binding partners of the peptides illustrated in (A). VASP binds as predicted. WAVE2 binds to two separate sites. CLASP2 and CLASP1 show similar binding patterns, a little N terminal of the VASP binding and perhaps a low level of unspecific binding. PSD95 binds to the predicted C-terminal PDZ binding site, although it is unclear where the binding to the Long peptide comes from. Raptor binding relatively specific. Drebrin binding completely unspecific including to GST alone. This experiment suggests that binding of both actin effector WAVE2 and microtubule effectors CLASP would remain in the Fat3Trunc mice. Note: Full length binding to WAVE is partially occluded by GST-FAT3ICD protein fusion that is same size.
Figure 4.5, Continued

A. Fat3 ICD

B. Proteins detected:
- VASP
- WAVE2
- CLASP2
- CLASP 1
- PSD 95
- Raptor
- Drebrin
indicated in Figure 4.5A. Although these experiments are ongoing, binding by the WAVE Complex, CLASPS, PSD95, Raptor, and Drebrin have thus far been examined.

WAVE2 is a component of the WAVE complex, to which Cyfip1 and 2, identified by mass spectrometry, also belong. The WAVE complex is another major regulator of actin signaling (Stradal et al., 2004). It binds to several different types of binding sites. Most recently a new binding site, particularly prevalent in protocadherins and other receptors was identified, called a WIRS (Wave Interacting Receptor Sequence) (Chen et al., 2014). The WAVE complex is recruited to this short motif through the interface created by the Cyfip and Abi subunits of the WAVE complex (Chen et al., 2014). Chen et al predicted Fat3 to contain two of these motifs, one in fragment 1 & 2 and the second in fragment 3. As predicted, WAVE bound to the first fragment, however it did not bind to the predicted second site. Furthermore we found a second interaction site in fragment 6 & 7, where examination of the sequence revealed a third WIRS consensus motif. Therefore, at least one major regulator of the actin cytoskeleton is predicted to associate with the Fat3Trunc allele.

CLASP1 and CLASP2 are regulators of the microtubule cytoskeleton, and have been associated with organization of the Golgi (Miller et al., 2009). Clasp 1 and 2 appeared to have very similar binding patterns, as expected for closely related proteins, further suggesting that the binding is specific. It seems likely that Fat3 has at least two separate binding sites for CLASPs, since CLASPs bound well to both the Fat3Long and Rest fragments which only overlap by a few amino acids and to both fragment 3 and 5 which do not overlap. However, further experiments will be needed to narrow down interaction sites.
PSD95 is a major scaffolding protein, usually of postsynaptic densities. PSD95 is predicted to bind to the C-terminal Fat3 ICD binding site. This site is contained in fragments 7, and in the Rest fragment. PDZ binds as predicted to these. Fat3 also bound to the LONG fragment, and very slightly to fragment 1, which is surprising, particularly given that PSD95 did not bind a previous iteration of the LONG fragment used in Chapter 3 (data not shown).

Raptor is a regulatory component of the mTOR1 complex, which is part of a large signaling network particularly important for the regulation of cellular homeostasis (Lipton and Sahin, 2014). Raptor binds most strongly to fragments 1, 3, 4, 5.

Drebrin has been proposed to mediate actin-microtubule coupling as it interacts both with actin and microtubules (Geraldo et al., 2008). From the mass spectrometry results, it appeared to bind robustly to Fat3ICD, with the recovery of many peptides. Furthermore, binding was lost with loss of VASP binding. However from this second analysis, it appears that it bound fairly unspecifically, including to GST alone, suggesting it is not a specific binding partner of Fat3.

Overall, these preliminary pulldown results begin to clarify which interactions are likely to be real and which are not, as well as begin to narrow down the site of interaction. To determine whether the proteins interact directly or as part of a complex will require additional experiments however, particularly when specific binding motifs are unknown. In particular the WAVE complex and the CLASPs emerge as interesting targets to study in the future.
The formation and composition of the extra plexiform layer mirrors the inner plexiform layer

Previously Ena/VASP was found to localize to the IPL during amacrine cell development. Furthermore, Ena/VASP was mislocalized to the nascent extra plexiform layer forming at P6 in Fat3 mutants, suggesting that loss of Fat3 is permissive for Ena/VASP mislocalization to amacrine cell trailing neurites. To test whether other candidates were similarly localized and mislocalized, the distribution of several candidate effectors was examined in P6 retinas (Figure 4.6). All candidates examined localized to the inner plexiform layer as well as to the future extra plexiform layer (Figure 4.6, arrows). This included proteins that bound specifically to Fat3ICD such as the CLASPs (Figure 4.6 A-D) and those which bound unspecifically, such as Drebrin (Figure 4.6 G-H). Clearly no direct link to Fat3 signaling can be determined from localization to the extra plexiform layer. However, overall, the results indicated a general similarity in the development of the normal IPL and the extra plexiform layer. The results also suggested a general rearrangement of the actin and microtubule cytoskeleton. Indeed labeling of filamentous actin by phalloidin and neuronally enriched beta-III-tubulin by Tuj revealed both were present in similar patterns in the inner and extra plexiform layers (Figure 4.7). Labeling also revealed an intriguing pattern of actin and microtubule stabilization. Phalloidin and Tuj intensity seemed to be complementary, with areas of heightened Phalloidin intensity containing lower Tuj intensity and vice versa (Figure 4.7 A'-F' and A''-F''). This also appears to be the case for the extra plexiform layer, suggesting a hierarchical build-up of actin and microtubule cytoskeleton in developing plexiform layers.
Figure 4.6 Localization of cytoskeletal effectors in developing retina.

Many cytoskeletal effectors localize both to the IPL and the new plexiform layer in Ptf1aCre Fl/− retinas at P6. Immunostaining of A,C,E,G,I,K) P6 Control and B,D,F,H,J,L) P6, Ptf1a Cre, Fat3 Fl/− retinas. Arrows point to forming extra plexiform layer. Immunostaining for CLASP2 (A-B), CLASP1 (C-D), PSD95 (E-F), Drebrin (G-H), Doublecortin (I-J) and Phosphorylated Doublecortin (K-L). Both effectors shown to bind specifically and unspecifically to Fat3ICD (Figure 4.5) as well as effectors of both microtubule and actin cytoskeleton localize to developing inner plexiform layer and extra plexiform layer.
Figure 4.6, Continued
Figure 4.7  Actin and microtubules concentrate to the developing IPL and extra plexiform layer, but occupy complementary domains.

Immunostaining of P6 retinas. A-C) Control and D-F)Ptf1a Cre, Fat3 Fl/−, arrows point to forming extra plexiform layer. A,D) Phalloidin staining labels filamentous actin. B, E) Tuj staining labels largely neuron specific beta-III-tubulin. Pseudocolored to show staining intensity more clearly, warm colors indicate higher intensity (A′,A″, B′,B″,D′,D″,E′,E″). C-F) Merge of Phalloidin and Tuj staining shows little overlap. A″, B″, C′ D″, E″, F′ ) Enlargements of corresponding boxed areas indicated in corresponding images. Where actin signal is high, microtubule signal is low and vice versa.
Discussion

Loss of Fat3 leads to the stabilization of neurites in amacrine cells that should not be maintained. We have shown that the stabilization of actin, through recruitment of Ena/VASP is also sufficient to lead to extra amacrine cell neurites. However, although Fat3 binds Ena/VASP and is able to change its localization in vitro, what proportion of Fat3 signaling is through Ena/VASP? As the fat3 coding region is very large, it is technically challenging to dissect Fat3 signaling through classical rescue experiments. Instead, a mutant mouse was created, removing the Ena/VASP binding site in the endogenous Fat3 locus. However Fat3’s ability to direct amacrine cell dendrites appeared unimpaired. Moreover, two further Fat3 alleles were created with a premature stop just N-terminal of the Ena/VASP binding site, thereby eliminating translation of over a third the Fat3 ICD. This too did not appear to affect Fat3 signaling in amacrine cells. These in vivo experiments illustrate the importance of testing the relevance of signaling pathways in vivo in the native cellular context. These results also raise the question why over a third of the Fat3 ICD appears to be unnecessary and what may be compensating for it.

Compensation for loss of C terminal of Fat3 ICD

The first possibility is that the ICD of Fat3 does not play a role in signaling. Instead, Fat3 could play a primarily adhesive role through its cadherin extracellular domain. For instance classic cadherins are still able to assemble adherens junction-like structures in the absence of intracellular signaling (Brasch et al., 2012). Experiments
with *Drosophila* Fat also indicate that some activity remains in Fat which lacks the ICD (Matakatsu and Blair, 2006; Pan et al., 2013), although it is believed these effects are cell non-autonomous. To investigate the possibility that *in vivo*, Fat3 adhesion is sufficient, a truncation mouse is being created which lacks all but the membrane proximal region of the intracellular domain.

A second possibility is that Fat1 signaling can compensate for Fat3 signaling in the presence of the transmembrane domain of Fat3 and a portion of the intracellular domain. A similar interaction in cis- between the transmembrane and intracellular domain of *Drosophila* Fat and the full length Fat has been reported (Sopko et al., 2009). Therefore it is possible that Fat3 can recruit Fat1 via cis-interactions in and near the transmembrane domain. In this case, Fat1 ICD signaling could compensate for Fat3 ICD truncation. To test this possibility, double mutants of the Fat3Trunc mice and Fat1 are being generated. Since systemic Fat1 loss can lead to anopthalmia (Ciani et al., 2003), Fat1 is being removed conditionally using the early retinal neuroepithelially-expressed Six3 Cre (Furuta et al., 2000).

A third possibility is that the portion of the ICD that is lost in Fat3Trunc mice is not necessary for Fat3 signaling in amacrine cells, but is important for some other aspect of Fat3 function yet to be discovered. Lastly, it is possible that the remainder of the ICD acts redundantly with the portion that is deleted and so can compensate. The ICD is large and is largely unstructured. Such unstructured domains have been shown to interact with downstream signaling partners via short linear motifs. These motifs can be highly conserved between species but since these motifs are short and have only a few critical residues they are also highly evolvable, so that disordered regions can gain and
lose motifs throughout evolution (Dinkel et al., 2014). Only a few amino acid changes are sufficient to create new motifs that can be selected for if they promote the function of the protein, such as recruiting cytoskeletal effectors. In a long disordered region, this could produce an end product with multiple sites acting in concert, and while each site contributes to Fat3 signaling, sites could also be redundant. This could also lead to the conservation of function over sequence during evolution. For instance this might explain why both Fat3 and Fat1 bind Ena/VASP, but not through the same site (Chapter 2). This might also explain why *Drosophila* Fat-like acts upon actin organization in oocyte development (Viktorinová et al., 2009), but lacks a predicted Ena/VASP binding site and shares only one short stretch of sequence similarity with Fat1 and Fat3.

An interesting, speculative example of conservation of function over sequence during evolution is binding of the WAVE complex. As mentioned above, a recent motif was identified that mediates recruitment of the WAVE complex and was identified bioinformatically in many human receptors including Fat3 and Fat1 intracellular domains (Chen et al., 2014). A search for this motif using ScanProsite (de Castro et al., 2006) reveals that there are two such motifs in Fat1, three in Fat3 and two in Drosophila Fat-like. Sequence alignment of the intracellular domain reveals that the two WIRS motifs in Fat1 align with two of the motifs in Fat3, although there is also a third. Strikingly, the two motifs in *Drosophila* Fat-like are nowhere near the motifs in Fat1 and Fat3. Despite this, some hints suggest WAVE might play a role in *Drosophila* Fat-like signaling; mutation of the WAVE complex to remove binding to WIRS binding sites in *Drosophila* lead to a Fat-like oogenesis phenotype (Chen 2014).
In support of multiple redundant effectors binding to the Fat3ICD, pulldowns from brain lysate identified many candidate binding partners that are regulators of the cytoskeleton. It is also likely that only a subset of binding partners were detected because binding often depends on phosphorylation events. Although Fat3ICD has many potential phosphorylation sites, Fat3ICD was purified from bacteria and so was unphosphorylated. Subsequent validation of these binding partners identified indicated that some of these interactions were probably unspecific. However others did seem to be specific and associated with the portion of Fat3ICD which remained in the Fat3Trunc mouse created, indicating that they compensate for the region truncated or that the truncate region is not important for Fat3 signaling in amacrine cells.

Coordination of microtubules and actin

Of note, Fat3ICD was found to associate with regulators of both the actin and microtubule cytoskeleton. This raises the possibility that Fat3 acts to coordinate the two. Over the last decade it has become increasingly clear that while actin and microtubules can play different roles, they also act in concert and can engage in direct physical interactions (Gundersen et al., 2004; Schaefer et al., 2008). This coordination of actin and microtubule activity is necessary for multiple processes, including neurite outgrowth and migration. Furthermore, coupling between microtubules and actin is an important point of regulation. While a handful of regulators of the interaction have been identified, such as Drebrin, Map1B and APC, we are only beginning to understand how these two systems are interconnected and regulated and in particular how extracellular cues can influence the interaction.
Of the Fat3ICD candidate binding partners confirmed by Western blot, CLASP 1 and 2 represent a mechanism by which Fat3 could coordinate effects on microtubules and actin. CLASP1 and 2 belong to a group of proteins called +TIPs. +TIPs interact with the dynamic plus ends of microtubules, usually through binding to EB1-3 (end-binding) proteins which bind directly to microtubule plus ends and are emerging as important regulators of multiple aspects of microtubule function (Akhmanova and Steinmetz, 2010). CLASPs have been shown to mediate microtubule dynamics both at the Golgi and at the cell cortex. At the cell cortex, CLASPs promote contact between microtubule tips and the actin rich cell cortex (Mimori-Kiyosue, 2003). Recently, CLASPs were also shown to promote microtubule recruitment and tethering in focal adhesions, which connect the extracellular matrix to the actin cytoskeleton (Stehbens et al., 2014). Thus far, the only mechanism identified to recruit CLASP to membranes is by binding to LL5β, a phosphatidylinositol-3,4,5-triphosphate binding protein (Lansbergen et al., 2006). However it is possible that CLASPs bind to cortical actin near the membrane since binding between CLASPs and actin has been identified (Tsvetkov et al., 2007). In neurons, CLASPs associate with microtubule ends that extend into actin rich filipodia and lameliipodia (Lee et al., 2004). CLASPs have also been found to act in Drosophila neuronal midline crossing, downstream of Slit/Robo signaling through Abl kinase (Lee et al., 2004). If Fat3 really does bind to CLASPs, it would be the first receptor identified to do so, and could promote the capture of microtubules by cortical actin at the tip of the leading process. Drosophila Fat has been shown to affect microtubule organization, however the mechanism is unknown (Harumoto et al., 2010; Matis et al., 2014).
Microtubules and actin in the retina

The localization in the retina of effectors of both the actin and microtubule cytoskeleton at the developing inner plexiform layer and novel plexiform layer in Fat3 mutants emphasizes the intimate coordination of actin and microtubules. The new plexiform layer appears to recruit all the same cytoskeletal effectors as the IPL and share a pattern of actin and microtubule staining. Together, these results suggest the two plexiform layers are forming in very similar ways. Clearly it is unlikely that every protein found in the extra plexiform layer is one that was directly associated with Fat3. Instead it probably reflects the hierarchical association and stabilization of the actin and microtubule cytoskeleton through coordination of hundreds of regulatory proteins.

Materials and Methods

Mice

Lines used: Fat3 Fl and Fat3-/- mice were generated previously (Deans et al., 2011), Ptf1a Cre (C. Wright, Vanderbilt U.) Mice were kept on a mixed background.

Fat3CKOs were generated by breeding Fat Fl/Fl mice with Ptf1a-cre +/- ;Fat3+/-.

Controls were from the same cross, but with one Fat3 wildtype allele.

Antibodies used

Rabbit Mena 2197 gift from the Gertler lab. Phospho Doublecortin (anti phospho-T331, S334, JNK kinase phosphorylation sites (Gdalyahu et al., 2004), antibody # 1952.
Antibody # 1774 recognizes anti phospho-T321, (S327) and produces same pattern of staining, data not shown. Both antibodies were kind gifts of Orly Reiner).

Rabbit anti Fat3 (Deans et al., 2011). Rabbit Raptor rabbit 24ca2, Rabbit Wave2 (d2c8 Cell Signaling), Rat Clasp1 (kt66, AbSea), Rat Clasp2 (kt69, AbSea), Mouse Psd95, Mouse Drebrin (m2f6, Enzo), Goat Doublecortin (8066, Santa Cruz), Tuj, Calretinin (Chemicon AB1550), mouse anti-PKCalpha (SIGMA PS704), VGAT (Synaptic Systems 131 003), AlexaFluor-conjugated secondaries (Invitrogen), Phalloidin Alexa 488 (Invitrogen A12379).

Constructs used

pGex 6P-1-Fat3ICD, pGex 6P-1-Fat3ICDmutations and truncations. All constructs were verified by sequencing.

GST fusions used the pGEX6P-1 (GE Lifesciences) vector with isothermal assembly of Fat3ICD sequences as indicated in the figure. Full length Fat3 cytoplasmic domain (aa 4175 - 4555, all amino-acid positions are referenced to UniProt Q8BNA6).

Long: 4175-4398. Rest: 4398-4555, Short: 4175 - 4195, Frag 1-7 were 95 amino acids long each, tiling the ICD as illustrated.

Generation Fat3Trunc and Fat3delEV mice

Crispr 1 target sequence: GAGAGTGACTACTACCTAGG

Crispr 2 target sequence: GGACTTCCCTGACCAGTATG
Guide strands were cloned using isothermal cloning into the pX330 vector. Crispr guide sequences were validated by transfection of 3T3 cells and screening with the Surveyor Assay Kit (Transgenomic kit) using standard protocols.

For the RNA for injection, the Cas9 mRNA was purchased from Trilink, the Ambion MEGA shortscript T7 kit was used to make the guide RNAs. Repair oligo was purchased from IDT:
GAGGGGCAGCACACCAGGGGTAGCACACGAGCTGGAGAGGTGACTACTACCTAG
GTGGATATGATGATCAGTATGAGGCTGCCTCCTCCCTCCAGCCCACCTCACTCAG
AGCACCATGAGCCCCA
Note that two silent mutations (red) were introduced to create a new restriction enzyme site for BclI.
Partners Healthcare Transgenic Core injected the RNA at the following concentrations into: 100 ng/μL Cas9, 50 ng/μl each guide RNA, and 100 ng/μl of repair oligo

PCR primers for genotyping Fat3Trunc (followed by digest with AVRII):
SK0215 ATGAGTCTGCACCAACCAGG
SK0216 GGAATTCGCAATCCCAACA

PCR primers for genotyping Fat3 delEV:
SK0211 CTCTGATTGGATGCCTGGAG
SK0212 TCTGTGCCCTGGTTCATGTT

Retina immunostaining and imaging
Retinas were collected at P6 or P11, fixed in 4%PFA at 4C for 2 hours. Following sucrose protection in 30% sucrose O/N, tissue was embedded in Neg-50 and frozen on metal cooled with liquid nitrogen. 14um cryosections were blocked and permeabilized for 30 min at room temperature using 10% goat or donkey serum and 0.1%TritonX-100 in PBS. Sections were incubated 1-2 overnights at 4°C in block solution without Triton X-100 with primary antibody. Then sections were incubated at room temperature for 1 - 2 hours, with appropriate secondaries at 1:1000. Sections were counterstained with DAPI (1:10,000) and mounted using 50% glycerol/50% sorenson's solution. For Tuj and phalloidin stains, z-projections were made of a stack of 4 images taken with 60x Lens, 1.42 N.A. For cytoskeletal effectors, a single thick optical section was taken.

**Pulldowns**

GST fusion proteins were grown in Rosetta E.Coli (Millipore) at room temperature, induced with IPTG at OD600 = 0.5-1 and harvested after 4 hours after induction. Bacterial pellets were lysed in PBS with 1% trionx 100, 1mM EDTA, 0.1mg/ml lysozyme supplemented with 1 mM Pefabloc SC PLUS protease inhibitor (Roche, Rochester, NY), 1mM DTT. Fusion proteins in bacterial lysate were bound to glutathione sepharose beads (GE Life Sciences) in batch, approximately 50ul of beads/construct. 4 hours at 4C. Beads were washed 2x in PBS, 0.5% tritonX100 supplemented with 1mMDTT and 1x in PBS, 1mM DTT. Beads were snap frozen in PBS, 25%glycerol, 1mM DTT and stored at -80C. (For validation pulldown, beads were used immediately).

To make brain lysate, P1 brain was dissected and homogenized on ice with dounce with 750ul lysis buffer (For validation pulldown, 700ul lysis buffer was added)
added per 100mg tissue. Lysis continued on ice for 30 minutes. Homogenate was spun to clear, 10,000 x g for 30 min. 900ul of lysate (890 ul for validation pulldown) added to 50ul of beads bound to fusion protein and rotated at 4C overnight. Beads were spun 600 g, 3 minutes, unbound brain lysate supernatant was collected and beads were washed 5x with 400ul w/DTT, 10 minutes in between spins. 50ul beads was combined with 45ul lysis buffer and 25ul 4x NuPage sample buffer (Invitrogen) (for validation pulldown. 250ul 1x NuPage sample buffer (Invitrogen) diluted in lysis buffer). Following pull down, brain lysate input, supernatant and beads were analyzed by standard western blot analysis. For mass spectrometry analysis, samples were processed by the Children’s Hospital IDDRC proteomics core. Beads were run out on a Tris –Glycine polyacrylamide gel, bands cut, trypsin digested and analyzed by mass spectrometry.

**Westerns**

Protein was run on a Tris –Glycine polyacrylamide gel, transferred onto Immobilon-P Membrane (Millipore, Meck, Billerica, MA) in 25mM Tris, 192 mM glycine, 10% Methanol and 0.05% SDS followed by standard western blotting.
Chapter 5: Concluding remarks and future directions
The patterning of any tissue into a functional three-dimensional structure is a challenge. Not only must cells be arranged correctly locally, but organizational cues must be propagated globally to arrange the tissue. Building the architecture of the nervous system raises additional challenges. In vertebrates, neurons almost always migrate following terminal division, sometimes significant distances, to arrive at their final position. Individual neurons then acquire highly polarized and highly diverse morphologies. Lastly, connections must be formed with only the right neurons. Each of these steps is vital for nervous system function. Furthermore, not only must all of these steps occur correctly for individual neurons, but it must occur in a coordinated fashion so neurons are arranged correctly relative to one another. To address these challenges, neurons use and sometimes “reuse” a wide variety of signaling pathways. The atypical cadherin Fat3 is one example of a molecule that helps coordinate neuronal polarity during migration and neurite retraction with the tissue architecture. This work explores the molecular and cellular mechanisms of Fat3 signaling in amacrine cell development. Here I discuss some of the questions raised by the work as regards retinal development and Fat3 signaling.

Fat3 is related to the atypical cadherin Fat. One of the functions of Fat is to promote planar cell polarity (PCP). In epithelial structures, where PCP has been the most studied, PCP coordinates the alignment of cells in a plane, so they all ‘point’ in the same direction, like the fur on a mouse’s back (Goodrich and Strutt, 2011). Therefore classic PCP signaling addresses the challenge of propagating polarization across cells in a tissue. In classic PCP, a gradient of Fat activity is set up by Fat’s ligand, Dachsous and the golgi kinase Fourjointed, which phosphorylates Fat and Dachsous extracellular
domains (Sopko and McNeill, 2009). Through activity of Fat, cells are aligned in the gradient. It is clear that in some contexts vertebrate homologues Fat4 and Dachsous1 are being used analogously to set up PCP in tissues (Mao et al., 2011; Saburi et al., 2008). However in the nervous system Fat4 and Dachsous1 have also been found to be important in seemingly quite different roles including coordinating migration of facial branchiomotor neurons (Zakaria et al., 2014) as well as mediating subapical membrane apposition in the cortex (Ishiuchi et al., 2009). These phenotypes demonstrate how signaling pathways can serve related but distinct functions in the nervous system.

The role of the closely related Fat-like family of cadherins is poorly understood both in invertebrates and vertebrates. It is clear in Drosophila that Fat-like does not mediate classic PCP. However Fat-like does regulate planar polarity of actin filaments orientation in follicle cells of Drosophila oocytes (Viktorinová et al., 2009). In vertebrates, a clear role for Fat-likes has not emerged. Recently in zebrafish, Fat3 has been reported to mediate chondrocyte differentiation and polarity in zebrafish (Le Pabic et al., 2014). On the other hand, Fat1 mouse mutants have a range of defects including in renal development, neural tube closure and anophthalmia (Ciani et al., 2003). At a cellular level, Fat1 loss also leads to loss of muscle precursor cell polarity during migration and subsequent shape abnormalities (Caruso et al., 2013). Altogether these phenotypes do not point to common mechanism or role for Fat-likes in vivo.

Previously we found that loss of atypical cadherin Fat3 led to a reorganization of the mouse retina with the formation of an extra layer of connections and synapses in the middle of the inner nuclear layer (Deans et al., 2011). In the absence of Fat3, amacrine cells no longer reliably restrict their dendritic arbors into the inner plexiform layer (IPL).
Instead, some amacrine cells extend a second neurite away from IPL and these extra neurites go on to form aberrant connections with each other. This extra neurite appears to arise from the trailing neurite present during amacrine cell migration. Therefore, regulation of the presence or absence of a single trailing process appears to be sufficient to coordinate changes in tissue architecture.

*Fat3 cellular and molecular mechanisms*

This work set out to understand the cellular and molecular mechanisms of Fat3 activity *in vivo*. In the retina, Fat3 acts to restrict amacrine cells’ dendritic arbors to the IPL. From the earliest time point in amacrine cell development, we found that Fat3 protein is concentrated at the IPL (Chapter 2). During trailing neurite retraction, Fat3 acts cell autonomously in amacrine cells (Chapter 3), most likely in response to a cue from other amacrine cells. Although early in development, the IPL contains neurites from both retinal ganglion cells and amacrine cells, we found that Fat3 signaling from retinal ganglion cells was not necessary for amacrine cell morphogenesis (Chapter 2). Furthermore, Fat3 signaling is not limited to directing trailing process retraction (Chapter 3). Live imaging and careful analysis of migrating amacrine cell dynamics revealed multiple changes in cell dynamics were observed, both prior to and during trailing process retraction. Loss of Fat3 led to loss of directed migration and trailing neurite retraction. Cell bodies occasionally migrated in the wrong direction and the trailing neurite underwent periods of re-extension. Consequently, migration and trailing neurite retraction were slowed. Moreover, neurite branching was no longer restricted to the leading process. Instead neurites branched aberrantly from both the trailing neurite and...
the cell body. Altogether the cell-autonomous role of Fat3, its concentration at the leading neurite, and the decrease of directional cellular movement following loss of Fat3 suggests that Fat3 acts to polarize the cytoskeleton.

In support of Fat3 acting to polarize the cytoskeleton, Fat3 bound and could relocalize Ena/VASP \textit{in vitro}. Furthermore, uniform recruitment of endogenous Ena/VASP to amacrine cell membranes mimicked loss of Fat3 (Chapter 3). However further dissection of the Fat3 pathway \textit{in vivo} suggested that Ena/VASP recruitment was not necessary for Fat3 signaling. Instead Fat3 signaling is likely to be much more complicated. For instance many additional candidate binding partners of Fat3 were identified, including regulators of both the actin and microtubule cytoskeleton (Chapter 4).

\textit{Model of the role of Fat3 in retinal development}

Altogether our work suggests the following model of Fat3’s role in amacrine cell development. Following terminal division, amacrine cells extend a leading neurite into the IPL. Fat3 is concentrated in the IPL and acts cell autonomously to promote the recruitment of cytoskeletal regulators of both the actin and microtubule cytoskeleton into the leading neurite. By promoting an asymmetric distribution of cytoskeletal regulators, Fat3 leads to differential cytoskeletal dynamics in the leading and trailing process, thereby promoting both directed migration and processive trailing neurite retraction. In the absence of Fat3, cytoskeletal regulators are more evenly distributed throughout the cell, leading to less directed cellular dynamics. This small change in polarized cytoskeletal dynamics is sufficient to lead to the retention of extra neurites in a cell-
autonomous manner (Figure 5.1). When a sufficient number of amacrine cells retain trailing neurites, the trailing neurites make connections with one another, likely leading to further stabilization. These connections form an extra layer that develops a cytoskeletal composition and complement of cytoskeletal regulators very similar to the IPL, eventually leading to the formation of synapses.

Figure 5.1 Model of Fat3 function in amacrine cell development.
A) In wildtype amacrine cell development, Fat3 and cytoskeletal effectors are concentrated in the IPL. Amacrine cells are polarized leading to smooth migration and trailing neurite retraction, creating the mature asymmetric amacrine cell morphology. B) In Fat3 mutant amacrine cell development, cytoskeletal effectors may be more uniformly distributed. Amacrine cells lose polarity, leading to loss of directed migration and trailing neurite retraction and ultimately some mature amacrine cells retain an extra neurite.
Further functions of Fat3 in the retina

Several lines of questions remain about the role of Fat3 in retinal development that are beyond the scope of the model.

Upstream of Fat3 in the retina

Fat3 acts cell autonomously (Chapter 3) on amacrine cell morphology, suggesting Fat3 acts as a receptor. However it remains an open question what the ligand of Fat3 may be. This study made some progress towards this question, suggesting that retinal ganglion cells are not necessary for Fat3 signaling in amacrine cell morphogenesis (Chapter 2). Therefore the ligand is predicted to be present in other amacrine cells since early in development only retinal ganglion cells and other amacrine cells contribute to the IPL. Both with and without retinal ganglion cells, Fat3 protein localized to the IPL. We hypothesize that Fat3 is stabilized in the IPL through adhesive interactions with its ligand. For instance classic cadherins have been shown to mediate adhesion and clustering on one side of a membrane solely through their extracellular domains in liposomes (Brasch et al., 2012). However directed trafficking is also known to play a major role in correctly targeting receptors (Solecki, 2012). In support of the importance of adhesive interactions, Fat and Dachsous are also able to localize each other to cell-cell contacts when expressed in heterologous cells without an intracellular domain (Matakatsu and Blair, 2006). From our Fat3Trunc mouse mutants we also know at least the terminal third of the intracellular domain is not required for Fat3 localization to the IPL (Chapter 3). It is also important to note that while our model suggests that Fat3 localization is necessary for Fat3 signaling, this remains to be confirmed. For
instance, in *Drosophila* the uniformly localized Fat intracellular domain is able to rescue much of Fat function in the absence of an extracellular domain (Matakatsu and Blair, 2006). In the future, the retina will continue to be a good system in which to identify a Fat3 ligand and to test the importance of Fat3 localization for Fat3 signaling.

**Fat3 in retinal ganglion cells**

Although only the role of Fat3 in amacrine cells was examined in this work, Fat3 is also expressed in retinal ganglion cells. Additional, the Fat3KO has a second phenotype in the retina that was little touched upon. In the Fat3KO, as well as having a defect in trailing neurite retraction, amacrine cells exhibit aberrant migration into the retinal ganglion cell layer (Deans et al., 2011). However when Fat3 is conditionally removed only from amacrine cells using Ptf1a Cre, amacrine cells no longer migrate too far, although they still fail to retract trailing neurites (Deans et al., 2011) and exhibit defects in migration dynamics (Chapter 3). There are two possibilities why in the amacrine cell conditional knockout of Fat3 amacrine cells do not have the same migration defect as in the full knockout. One possibility is the Ptf1a Cre is inefficient or comes on a little too late and some Fat3 protein remains in amacrine cells in the conditional knockout. This Fat3 is sufficient to rescue the migration into the retinal ganglion cell layer, but not the trailing neurite retraction. The second possibility is that Fat3 has a cell non-autonomous role in retinal ganglion cells. Therefore, it is possible Fat3 can also act as a ligand to signal to a subset of amacrine cells to stay in the inner nuclear layer.
**Fat3 in amacrine cell leading neurites.**

A prediction of the current model is that Fat3 acts to promote leading neurite cytoskeletal dynamics. However defects in the leading neurite have not yet been observed. Experiments to examine the early dynamics of the leading neurite are ongoing, but require sparser labeling with the *ex vivo* electroporation than has been achieved thus far. While it is possible that Fat3 also affects targeting of the IPL-facing neurite, no gross laminar deficits were seen in mutants. Furthermore, Fat3 protein is distributed throughout the IPL and affects many amacrine cell subtypes so Fat3 unlikely to mediate specific laminar targeting.

It is also interesting to speculate on later roles for Fat3 in the dendrite and whether Fat3 acts to promote synapse development. Clearly Fat3 is not necessary for synapse formation since the extra plexiform layers in the Fat3 mutant develop synapses. However Fat3 protein persists long beyond the end of amacrine cell migration and neurite restriction to the IPL. Furthermore, Fat3 bound synaptic proteins such as PSD95 and Homer (Chapter 4 and data not shown), suggesting a possible later role in synapse formation.

**Amacrine cell migration**

Amacrine cell migration, as characterized by our live imaging (Chapter 3), seems distinct from other modes of migration described. Although amacrine cells are interneurons, the unbranched leading neurite dynamics and migration appear more similar, albeit with differences, to cortical projection neuronal radial migration. Two main
radial modes of projection neuron migration have been described: glial guided and glial-independent (also called somal translocation). During glial guided migration, migrating neurons associate through adhesive interactions with a radial glial progenitor spanning the cortex. Migrating neurons exhibit alternative movements between leading neurite extension and cell body migration resulting in a saltatory motion. During glial-independent migration the leading neurite is anchored in the marginal zone and the cell body motion is fast and smooth. In the cortex, early born projection neurons are seen to undergo solely glia-independent migration. Later in development, most neurons use glial-guided migration; once the leading neurite contacts the out limiting membrane, neurons transition to glia-independent migration (Marín et al., 2006; Nadarajah and Parnavelas, 2002; Nadarajah et al., 2001).

Preliminary observations suggested amacrine cells extend a leading neurite quickly into the IPL without engaging in extensive exploratory behavior. What directs initial leading neurite extension is unknown. Perhaps analogous to cortical glial guided migration, amacrine cells' leading neurite uses a neural progenitor cell, which spans the retina, as a track. However in glial guided migration leading neurite extension and cell body progression are linked, leading to saltatory movement (Schaar and McConnell, 2005). In contrast, amacrine cells appear to extend their leading neurite without pause while the cell body stays close to the apical surface of the retina. Therefore glia-guided leading neurite extension seems unlikely. Perhaps leading process extension is more similar to that of earlier born cortical neurons that do not undergo a period of glial guided migration. It has been suggested that those early born neurons inherit the leading neurite during the terminal division from the progenitor (Miyata et al., 2001),
although alternative mechanisms might also exist. Perhaps, Sema 3a is a relevant cue for leading neurite outgrowth, given the gradient of Sema3a in the cortex and its role in polarization, migration, apical dendrite outgrowth (Chen et al., 2008; Polleux et al., 2000; Shelly et al., 2011). Tangentially migrating interneurons also respond to a range of axon guidance molecules and neurotrophins (Evsyukova et al., 2013; Polleux et al., 2002; Steinecke et al., 2014). In the future, it will be interesting to determine the mechanisms directing extension and pathfinding of this leading neurite.

Mechanisms anchoring the leading neurite in the IPL once it is arrives are also unclear. The molecular mechanisms of anchoring of the leading neurite in glial-independent migration have recently been elucidated, and include reelin-triggered adhesive signaling through NCadherin (Franco et al., 2011), Nectins (Gil-Sanz et al., 2013) and Integrins (Sekine et al., 2012). Analogously, Reelin is localized to the IPL, raising the possibility of similar mechanisms leading to anchoring. However, reeler mutants are not reported to have migration defects in the retina (Rice et al., 2001). Overall it seems likely that the amacrine cell leading neurite is anchored through adhesion in the IPL. Although it would be interesting if Fat3 plays a role, no loss of anchoring was observed in the mutant and it is likely that many adhesive cues in the IPL contribute to anchoring.

Most of amacrine cell migration occurs with the leading neurite anchored in the IPL. In the cortex, migrating neuron cell body movements switch from saltatory and slow to smooth and fast following anchoring of the leading neurite (Nadarajah et al., 2001; Sekine et al., 2011). This suggests that anchoring of the leading neurite leads to faster, smoother movement of the nucleus. However preliminary observations suggest that
amacrine cell body migration is relatively saltatory and slow, despite a similarly anchored leading neurite. What could lead to the difference in nuclear dynamics? The cytoskeletal mechanisms of somal translocation have not been explicitly studied, but the previous mode of glial-guided migration is reliant on dynein-microtubule pulling forces coupling the centrosome and the nucleus. Downregulation of the microtubule minus end directed motor dynein, or its regulator Lis-1, uncouples the centrosome and the nucleus and stops nucleokinesis (Tsai et al., 2007). If dynein and centrosome coupling continue to be important in somal translocation, this suggests a possible mechanistic difference. Migrating neurons in the retina, including amacrine cells, have their centrosome in the apical, trailing neurite (Hinds and Hinds, 1983; Randlett et al., 2011b). Therefore similar dynein-centrosome forces are unlikely to be at work. Indeed, interkinetic nuclear migration of neural precursors in the retina, although a different process, was shown to be entirely dependent on actomyosin and not on microtubules (Norden et al., 2009). Therefore perhaps in amacrine cells, actomyosin forces, which act at the base of the nucleus in cortical cells, might act to generate a saltatory movement. Actomyosin forces have also been shown to be important in the migration of interneurons from the medial ganglionic eminence, and can produce a periodic motion. Indeed, when microtubules are destabilized in these interneurons, nucleokinesis occurs more rapidly (Martini and Valdeolmillos, 2010).

Of note, amacrine cell migration as observed in this study (Chapter 3) is very different to that previously described in zebrafish retinas (Godinho et al., 2005). There, migration appeared multipolar with numerous neurites exhibiting exploratory behavior. One possible explanation of this difference is the population of amacrine cells being
studied. In particular, it is possible that earlier born amacrine cells have different
migration and neurite dynamics. Early and late born amacrine cells might also respond
to different cues. Future experiments will also address the cellular dynamics of earlier
born amacrine cells. Another possibility is that mechanisms of amacrine cell migration
may have changed over evolution.

Retinal lamination

The restriction of neurites into laminae is a common theme in the nervous
system. Retinal lamination is a particularly clear example and therefore is a good
system to understand how such organization is established. Indeed several other
adhesion molecules have been found to be important in retinal lamination. In the chick
retina, homophilic interactions between immunoglobulin superfamily adhesion
molecules Sidekicks, Contactins and Dscams mediate specific synaptic layer targeting
in the inner plexiform layer. Loss or gain of function of these molecules is sufficient to
change the synaptic layer targeted by axons or dendrites of expressing neurons
(Yamagata and Sanes, 2008, 2012; Yamagata et al., 2002). In the mouse retina, similar
experiments demonstrate Cadherin 8 and Cadherin 9 mediate layer specific targeting of
direction selective bipolar cells (Duan et al., 2014), albeit through heterophilic not
homophillic interactions. These adhesive interactions in the inner plexiform layer appear
to be important for mediating terminal target selection. In contrast, Fat3 acts to polarize
the entire cell and leads to failure to retract a trailing neurite. Furthermore, as discussed
above, Fat3 is unlikely to lead to laminar targeting defects.
Instead Fat3 acts more similarly to the Sema 5A/5B - Plexin A1/A3 signaling pathway (Matsuoka et al., 2011). Plexin A1 and A3 are expressed by amacrine cells and localize to the IPL. Plexin A1 and A3 mediate repulsion from Sema5A and 5B, which are expressed in the nucleoblast layer throughout amacrine cell development. Together, they act to restrict amacrine cell dendrites and, directly or indirectly, other cell neurites to the IPL. Loss of either the Semas or the Plexins leads to a phenotype very similar to loss of Fat3: amacrine cells form extra neurites and an extra layer of connections.

How similar are the roles of Fat3 and Sema5A,B/PlexinA1,3 in amacrine cell development? At the gross level, the outcome seems very comparable. However, it is unclear how similar the mechanisms are that lead to extra neurite formation and retention. In particular, loss of Sema5A,B/PlexinA1,3 might lead to neurite re-extension, rather than prevent retraction. Sema5A,B/PlexinA1,3 signaling might also affect just the neurites, rather than the whole cell including its migration. In the future it would be interesting to determine how the Fat3 and Sema5A,B/PlexinA1,3 pathways interact in amacrine cell morphogenesis.

The most striking similarity following loss of either pathway is the formation of an extra layer of connections and synapses. While many mutations lead to neurite overgrowth, they do not lead to the formation of orderly new layers of connections. Why then do these mutations in the retina lead to the formation of new layers? One possibility is the architecture of the retina. In Fat3 mutants, this extra layer forms just below Mueller cell bodies, so perhaps Mueller cell bodies act as a barrier to restrict the extra neurite from extending too far and promote neurite-neurite association to a
localized area. Another possibility is amacrine cells have an intrinsic ability to drive layer formation. This is suggested by the finding that amacrine cells neurites stratify prior to retinal ganglion cells (Godinho et al., 2005), nor are retinal ganglion cells (Kay et al., 2004) or bipolar cells necessary for stratification (Green et al., 2003; Tomita et al., 2000). However stratification autonomy does not seem to be a unique property of amacrine cells. Indeed bipolar cells are able to organize into a synaptic layer despite loss of all synaptic partners (Randlett et al., 2013).

From immunostains of the cytoskeleton and cytoskeletal regulators in the Fat3 mutant (Figure 4.6, 4.7), it appears that the development of the extra plexiform layer is strikingly similar to that of the normal IPL. Although possible, the initial development of these extra neurites is unlikely to proceed from exactly the same signaling pathways following loss of Fat3 or PlexinA1/A3 signaling. Indeed, development of plexiform layers following loss of different cell types is also likely to initiate following different molecular cues. The formation of the synaptic layers from diverse cues indicates several things. First, neurites in vivo, whether destined to become dendrites or not, are able to acquire mature dendritic properties such as synapse formation. Furthermore, these neurites are able to organize into a plexiform layer. This emphasizes that it is as necessary to direct where a neurite will not form as it is to direct where it will form. Secondly, there are many different ways to initiate retention of extra neurites. Most likely, once a neurite has crosses a cytoskeletal state “threshold” the microtubule and actin regulators act in a reinforcing feedforward loop to continue this progression. This has been suggested in vitro by the induction of axon formation by the stabilization of microtubules by spatially restricted taxol treatments (Witte et al., 2008) or by destabilizing actin with cytochalasin
Indeed, we found that increasing recruitment of a single family of actin regulators Ena/VASP was sufficient to retain an extra neurite (Chapter 3).

**Fat-like signaling**

While much of this study has been focused on the role Fat3 plays in amacrine cells, it also offers important steps forward towards understanding of Fat-like signaling. Relatively little is known about Fat-like signaling; in particular its role *in vivo* is poorly understood.

**Upstream**

Very little is known about signaling upstream of any of the Fat-like cadherins. Therefore based upon known ligands of Fat, we predicted either Dachsous 1 or 2 as a ligand for Fat3. However our mutant analysis suggested that neither was necessary (Chapter 2), although it still remains possibly they act redundantly since it was not possible to analyze double mutants. Dachsous not being a ligand for Fat-likes is supported by the finding in *Drosophila* that Dachsous mutants do not phenocopy loss of Fat-like in oocyte development (Viktorinová et al., 2009). Subsequent examination of the phylogenetic relationships of the Fats, Fat-likes and Dachsous suggests that Fat is more similar to Dachsous than to Fat-like in the extracellular cadherin domains, which are proposed to mediate their adhesion (Chapter 2, Hulpiau and van Roy, 2010; Sotomayor et al., 2014). Therefore Fat3 appears equally likely to bind homophilicaly, or to another Fat-like, Fat, or Dachsous.

**Downstream**
The Fat3KO phenotype, both the change in mature amacrine cell morphology and the change in cellular dynamics of migration and trailing neurite retraction, suggests that Fat3 acts to polarize the cytoskeleton in amacrine cells. Our finding that Fat3 acts cell autonomously suggests that Fat3 acts as a receptor to mediate its activity on the cytoskeleton. In particular we present evidence that Fat3 mediates effects on the actin cytoskeleton. For example, uniform membrane recruitment of Ena/VASP, most likely through its well-known effects on actin, leads to a Fat3-like retention of the trailing neurite (Chapter 3). Fat1 has also been shown to recruit Ena/VASP and cause changes in cell actin organization in vitro (Moeller et al., 2004; Schreiner et al., 2006; Tanoue and Takeichi, 2004). In addition, in vivo, Fat1 loss leads to changes in myoblast cell shape (Caruso et al., 2013), although whether the effect is cell-autonomous is unknown. Altogether, these results give support to polarization of actin being a common mechanism of vertebrate Fat-like cadherins. Thus far, Fat-like in Drosophila has not been shown to act as a receptor, but does lead to cell non-autonomous effects on actin fibril polarization (Viktorinová et al., 2009).

Further in vitro and in vivo experiments examining signaling downstream of Fat3 suggest the mechanism for Fat3 mediated effects on the cytoskeleton is likely to be complicated. In particular, the lack of phenotype following deletion of the terminal third of the intracellular domain raises the question of whether the intracellular domain is required at all. Mass spectrometry analysis of Fat3 binding partners from a pulldown from brain lysate suggested Fat3 bound many effectors of the actin and microtubule cytoskeleton. Some of these effectors were validated and bind regions remaining following truncation of the intracellular domain. In particular, binding of the WAVE
complex suggested the truncated Fat3 could retain actin-signaling activity. It also points to a possible mechanistic difference between Fat1 and Fat3. Although Fat1 has the same predicted WAVE binding sites, Fat1 was found not to bind WAVE in vitro (Chen et al., 2014; Tanoue and Takeichi, 2004). The finding that Fat3 interacts with several regulators of the microtubule cytoskeleton suggests that Fat3 could coordinate the actin and the microtubule cytoskeleton. Of note, *Drosophila* Fat has been shown to provide directional information for PCP signaling through effects on microtubule organization (Harumoto et al., 2010; Matis et al., 2014), however a molecular mechanism has not been described. Our finding that Fat3 might bind to +TIP microtubule regulators such as CLASP2 suggests Fat3 could act to anchor microtubules into the leading process and/or promote microtubule entry. +TIPs, including CLASP2, are thought to mediate microtubule-actin interactions at the cell cortex (Akhmanova and Steinmetz, 2010). However although CLASP2 can be targeted to the membrane downstream of receptor signaling via the phosphatidylinositol-3,4,5-triphosphate binding protein LL5beta (Hotta et al., 2010; Lansbergen et al., 2006; Stehbens et al., 2014), an interaction between CLASP2 and a receptor has not yet been observed. Furthermore, the function of CLASPs downstream of receptors in the nervous system is only beginning to be understood (Lee et al., 2004).

In the future, more work will be needed to further dissect the nature of the interactions identified. As well as validating interactions further, it will be important to determine which proteins interact with Fat3 as part of a complex, and which bind directly. Perhaps the Fat3 intracellular domain acts as a scaffold for cytoskeletal effectors, or perhaps the interactions Fat3 mediates are transient. Most intrinsically
disordered regions, such as the Fat3 intracellular domain, mediate interactions via short linear motifs (Dinkel et al., 2014). These interactions tend to be weak compared to domain-domain binding, indicating Fat3 may mediate relatively transient interactions. Such transient interactions could act to ‘concentrate’ binding partners at the recruitment site, such as in the neurite in the IPL. Alternatively the interactions could be more stable and perhaps link the cytoskeleton directly to the membrane.

Concluding remarks

This work has established a foundation for understanding the mechanisms and cellular functions of the Fat3 signaling pathway specifically, and the little-understood Fat-like cadherins more generally. Fat3 signaling in the retina offers a molecular entry point to continue to dissect Fat-like signaling as well as potentially to study the coordination of the actin and microtubule cytoskeletal in neuronal development in vivo. Much work remains to be done on signaling downstream of Fat3 and upstream signaling remains an open question. The retina, with the presence of most Fat,Fat-like, and Dachsous members presents a good opportunity to work out both individual family members’ functions in the nervous system as well as potential interactions between the different family members.

This work also establishes amacrine cell morphogenesis as a system in which to study the transition from migration to directed neurite outgrowth. In the future, amacrine cells in the retina may also provide a good system for visualizing the whole path of development: from polarization to migration to orientated dendrite arborization and
synaptogenesis. The clear architecture of the retina provides a good window into how extracellular and intracellular cues lead to polarization and to the exact connectivity that is critical for nervous system function
References


microtubule plus ends to the cell cortex through a complex with LL5beta. Dev. Cell 11, 21–32.


Appendix 1

These are the full mass spectrometry results from Chapter 4.

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Protein types highly prevalent in the CRAPome, a mass spectrometry resource of unspecific interactions commonly found in experiments. (Mellacheruvu et al., 2013)

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Ribosomal proteins, very unlikely to be real, as known background of many mass spectrometry experiments.

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