Understanding the biochemical basis of *Drosophila* Fat function

By

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A thesis submitted in conformity with the requirements for the degree of Master of Science
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Abstract

*Drosophila* Fat is a large atypical cadherin molecule. Genetic assays show that Fat has multiple function, however, the mechanism of Fat function is poorly understood. Hence, I undertook a biochemical approach to determine the mechanistic function of Fat.

Previous data indicated that Fat might be processed; I further confirmed the precursor-product relationships between these proteins. I then looked at sub cellular localization of Fat. My preliminary data suggests that the smaller 110 kDa forms of Ft goes to the nucleus.

To characterize the interaction between Fat and Atro, only known binding partner of Fat, I conducted pull-down assays that indicate Fat has multiple binding sites for Atro. However, the interaction is weak, and different experimental conditions will be needed to characterize the interaction.

The only known downstream target of both Fat and Atro in PCP is *four-jointed*. I provided evidence that *fjlacZ1.2kb* is regulated by the Ecdysone receptor.
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LIST OF ABBREVIATIONS:

atro atrophin
ds dachsous
D-V dorsal ventral
EcR ecdysone receptor
ex expanded
fj four jointed
FRT Flp recombination target
ft fat
fz frizzled
GFP green fluorescent protein
ICD intracellular domain
hpo hippo
mats mob as tumor suppressor
mer merlin
MF morphogenetic furrow
nrx neurexin
PCP planar cell polarity
sav salvador
TMD transmembrane domain
UAS upstream activating sequence
wb western blotting
wg wingless
wt wild-type
wts warts
yki yorki
Chapter 1

Introduction
Chapter 1: Introduction

1.1 Planar cell polarity (PCP) and its diverse functions

The epidermis of adult *Drosophila melanogaster* has approximately 500,000 cells, ~5000 of which (~1%) make bristles suggesting that bristles could sprout randomly. However, even the most scattered bristles- the tergite microchaetes- are extremely precise in their pattern formation. In fact, the basic layouts of the bristles has been conserved for 50 million years (Grimaldi and Engel, 2006; Simpson et al., 1999).

Polarization of epithelial cells on their apicobasal axis is a well-studied event, which is critical for epithelial integrity and function. However, in addition to apical-basal polarity, cells in most tissues also require positional information in the plane to generate polarized structures to move or orient themselves in a direct fashion. This type of polarization of a field of cells is referred to as planar cell polarity (PCP).

A number of model systems have been used to study PCP (Fig 1). In flies, planar polarity mutants disrupt the orientation of wing hairs, creating swirls, and randomize the precisely organized ommatidia, which are composed of photoreceptors arranged in the shape of a trapezoid (Casal et al., 2006; Casal et al., 2002; Fanto et al., 2003; Simon, 2004; Strutt et al., 2002; Yang et al., 2002). Mutations in vertebrate core PCP genes disrupt inner ear hair cell organization, oriented cell divisions and tubule elongation (Curtin et al., 2003; Gong et al., 2004; Jones and Chen, 2007; Montcouquiol et al., 2003; Saburi et al., 2008). The PCP pathway has also been implicated in neural tube closure and convergent extension.
Convergent extension (CE) is the narrowing and lengthening of an embryonic field along a defined axis in vertebrates (Keller, 2002; Veeman et al., 2003; Wallingford et al., 2002).

Recently, molecules playing a role in PCP have also been found to play a role in oriented cell division, leading to cystic kidney disease (Saburi et al., 2008), and in migration of tissue in dorsal closure (Kaltschmidt et al., 2002).
Fig 1. Model systems used to study PCP and Organization of tissues:
a-b | Proximal distal orientation of hairs in the wild-type wing of *Drosophila* pointing distally(a) and in PCP mutants forming swirls(b). c,d | PCP on the paw of mouse: Hairs point distally in wild-type (c) and forms swirls in *Fz6* (*Frizzled6*, a seven-pass transmembrane receptor) mutants(d). e,f | PCP in the *D. melanogaster* eye: Ommatidia are composed of photoreceptors, which are arranged precisely in the shape of a trapezoid(e). In PCP mutants, this precise arrangement of photoreceptors is lost and the organization of the ommatidia too is lost in *Stbm* (*Strabismus*, a novel four-pass transmembrane protein) mutants (f). g,h | Individual sensory hair cells of the mammalian cochlea generate polarized bundles of actin based stereocilia shown in green. The stereocilia bundles are arranged in perfect orientation in wild-type mice (g). In PCP mutants (*Vangl2* mutants), stereocilia bundling is randomized (h). (Taken from Seifert and Mlodzik 2007).
1.2 Models used to study PCP in *Drosophila*

In *Drosophila*, planar polarity has been studied in different cell types in different model systems. One of the model systems used to study PCP is the hair on the wings, where PCP is apparent in the orderly arrangement of the actin hair that grows from the distal end of cells (Adler, 2002; Shulman et al., 1998). Adult cuticles have been one of the model systems that have been preferred by some geneticists because of its simple body plan (Casal et al., 2002; Lawrence et al., 2002). However, research in our laboratory focuses mainly on the larval and adult eyes of *Drosophila* for studying the coordinated orientation of cells (Fig 2). Apart from *Drosophila* being an organism with genetic and molecular pliability, phenotypes in the *Drosophila* eye are recognized with relative ease and allow the study of genes that would otherwise compromise viability.
(a) The fly eye is composed of 800 ommatida. Each ommatidium has clusters of photoreceptors arranged in the shape of a trapezoid. Bristles on the back of the fly and hairs on the wing point posteriorly and distally display PCP.

(b) Schematic comparison of the wild-type and PCP mutant (*frizzled, fz* or *dishevelled, dsh*) phenotype in the wing and eye, where normally formed structures have lost their organization within the plane of the epithelium (PCP defects are highlighted in red).

(c) Clonal analysis of *fz* shows both cell-autonomous and non-autonomous PCP defects. Non-autonomous defects are seen only on the distal side of a clone depicted in blue, the genotypically mutant tissue is in red. (Taken from Saburi and McNeill 2005.)
1.2.1 Morphology of the adult ommatidium in the *Drosophila* eye

The *Drosophila* compound eye emerges from the monolayer of an eye imaginal disc. The adult eye consists of 800 identical units called ommatidia. Each ommatidium consists of eight photoreceptor cells, each associated with a rhabdomere (R1-R8) (Harris et al., 1976), and are arranged in opposite orientation across the equator. Based on the position of each ommatidia in the fly eye, they are either in a dorsal or ventral orientation shown by green and red arrows, respectively, in Fig 3B. Above the bundle of eight rhabdomeres (8R) cells, each ommatidium has four cone cells that secrete the lens. Between the bundles are the pigment cells that prevent blurring of vision by absorbing the light. There are altogether 11 pigment cells per ommatidium: 2 PPCs, 6SPCs, 3TPCs (primary, secondary, tertiary pigment cells) where the SPCs and TPCs are shared between neighboring ommatidium (Fig 3C) (Kumar and Ready, 1995).

Opsins, are a group of light–sensitive 35-55kDa membrane bound G protein-coupled receptors of the retinylidene protein family (Terakita, 2005). Due to the expression of the Rh1 opsin sub-type and their large size, photoreceptors R1-R6 are highly sensitive to light in the blue wavelength. R7 and R8 are smaller photoreceptors that can express different opsins. R7 can express either Rh3 or Rh4 and R8 can express either Rh5 or Rh6 opsins. However, the expression of opsin in R7 and R8 is interdependent (Chou et al., 1996; Chou et al., 1999). The combination of opsin expression is Rh3/Rh5 or Rh4/Rh6 for R7/R8 photoreceptors, making them sensitive to UV light and blue-green light respectively (Chou et al., 1996; Chou et al., 1999; Fortini and Rubin, 1990; Harris et al., 1976). The epithelium of the eye is stratified and, if one makes sections through the
ommatidium, a trapezoid is formed by R1-R7, since R8 exists below R7 making it out of plane of the R7 photoreceptor in a planar section.

Fig 3. Morphology of the Drosophila eye
A) A scanning electron microscope image of a Drosophila compound eye. (B) A dorsal (green arrow) and a ventral (red arrow) ommatidium. The rhabdomeres (the round blue dots) of R1–R7 photoreceptors are arranged in a trapezoid. R8 is not visible in this plane of section. (C) Front view of one of the facet’s lattice is shown (right). At left is the side view of the entire conical ommatidium. Cross sections are sketched (right) at different levels. Abbreviations: 1°, 2°, 3° are primary, secondary and tertiary pigment cells respectively (Taken from Yang et al 2002).
1.3 Development of ommatidia in the *Drosophila* compound eye

The eye develops from the single layered epithelial sheet of the eye imaginal discs. Patterning of the ommatidia begins at mid third instar stage from the posterior of the eye disc and moves anteriorly (Wolff and Ready, 1991). This process takes around two days to complete and can be visualized by an indentation, the morphogenetic furrow in the disc (Wolff and Ready, 1991). Anterior to the furrow, the position of the equator is determined between *fringe (fng)* expressing ventral cells and *fng*-non expressing dorsal cells (Irvine and Vogt, 1997). As the furrow moves forward, the clusters of cells that will give rise to ommatidia begin to appear behind it. Posterior to the furrow, ommatidial assembly begins with the differentiation of R8 cells, followed by a pair wise addition of R2 and R5 cells on either side of R8 (Wolff and Ready, 1993). Photoreceptors R3 and R4 are added next, forming an ommatidial precluster. Photoreceptor R1, R6 and R7 are then added, which completes the ommatidial precluster (Wolff and Ready, 1993). The dorsal and ventral preclusters are initially indistinguishable, but the mirror image polarity of the dorsal and ventral clusters arise as soon as the precursors of the R3 and R4 take up distinct cell fates. The precursor that is located closer to the equator becomes R3 and the one that is away from the equator adopts the R4 fate (Strutt and Strutt, 1999; Yang et al., 2002). Once the R3 and R4 cell fate is decided, the dorsal and ventral clusters rotate 90° in opposite directions to establish the final mirror-image polarity pattern (Strutt and Strutt, 1999) (Fig 4A).
Fig 4. Schematic of R3/R4 cell fate specification and ommatidial rotation.

A. Schematic of precluster formation in the eye disc. As the morphogenetic furrow (mf) moves forward, ommatidial preclusters appear behind it. Ommatidial assembly begins with R8 differentiation followed by the pairwise addition of photoreceptors R2 and R5 precursors on either side of R8 and then R3/R4 precursors, which are not in contact (column 1). Following cell-cell contact between the R3 and the R4 precursors (column 2), the two cells adopt distinct fates. The one located closer to the equator adopts the R3 fate (red) and the other one becomes R4 (green). Following R3/R4 fate specification, the cluster then rotates 90° generating the final pattern (column 3 and 4). (B) A diagrammatic representation of Frizzled’s roles in R3/R4 fate determination. Higher Fz activity in the equator leads to higher Delta (Dl) activity in the equatorial cell, resulting in greater Notch (N) activation in the neighboring polar cell and R4 specification. Abbreviations: E, equatorial cell; P, polar cell; Eq, equator; Mf, Morphogenetic furrow. Refer to page 9 for detailed description (Taken from Yang et al 2002).
1.4 Function(s) of Fat during development

*Drosophila* Fat (Ft) is a cadherin molecule (Mahoney et al., 1991). Ft was first identified as its recessive mutations gives rise to viable phenotype involving changes in body shape and wing vein pattern (Mohr, 1923, 1929). The role of Ft has been extensively studied in fate determination of photoreceptors in the *Drosophila* eye and in planar polarity (Rawls et al., 2002; Yang et al., 2002). Ft plays a role in proximal distal patterning in some of the appendages (Cho and Irvine, 2004; Lawrence et al., 2002; Matakatsu and Blair, 2004; Strutt et al., 2004). Ft has also been classified as a tumor suppressor gene, since its loss of function leads to tissue overgrowth (Bryant et al., 1988, Bennett and Harvey, 2006; Mahoney et al., 1991; Mao et al., 2006; Silva et al., 2006).

1.4.1 Molecular Structure of Fat

The Ft subfamily of cadherins is conserved from flies to mammals (Tanoue and Takeichi, 2005). *Drosophila* Fat was first cloned in 1991 and is comprised of an extracellular region, a transmembrane domain and an intracellular region. In its extracellular region it contains 34 cadherin repeats, four EGF repeats and two Laminin-G domains. It has a single transmembrane domain and a largely uncharacterized cytoplasmic domain (Mahoney et al., 1991). There are two Ft cadherins in *Drosophila*: Fat and Fat-like and four in vertebrates: Fat-J, Fat1, Fat2, and Fat3 all with a predicted molecular mass of 500-600 kDa (Mahoney et al., 1991; Tanoue and Takeichi, 2005). *Drosophila* Fat plays a role in PCP, proximal distal patterning and in growth (Bryant et al., 1988; Cho and
Irvine, 2004; Fanto et al., 2003; Ma et al., 2003; Yang et al., 2002). Interestingly, the intracellular region of Ft can rescue the growth defects of ft mutants, suggesting that the intracellular domain of Ft contains information required for growth control (Matakatsu and Blair, 2006). *Drosophila* Fat-like is expressed in salivary glands and tracheal cells and is required for morphogenesis and maintenance of tubular structures (Castillejo-Lopez et al., 2004).
1.4.2 Function(s) of Fat in eye development

1.4.2.1 Role of Fat in planar cell polarity in the eye.

The key event that determines the orientation of the ommatidia is the R3/R4 fate decision (Strutt and Strutt, 1999; Tomlinson and Struhl, 1999). Extensive study suggests that the activity of the seven-pass transmembrane Wnt receptor, Frizzled (Fz), plays an important role in R3/R4 fate and that cells with higher Fz activity become R3. The ultimate determinant of the R3/R4 cell fate decision is the competition for Notch (N) between the two adjacent precursor cells. This competition is biased by the presence of higher Fz signaling in the more equatorially located R3/R4 precursor. This leads to increased Delta (Dl), a ligand for Notch, in the equatorial cell, activating N signaling in the adjacent precursor cell (Cooper and Bray, 1999; Fanto and Mlodzik, 1999; Tomlinson and Struhl, 1999; Yang et al., 2002) (Fig 4 B).

The asymmetric activity of Fz at the cell interfaces of R3/R4 cells or at the neighboring cell interfaces in wing imaginal discs depends on the recruitment of a defined complex of factors often referred to as the core PCP complex. Before the onset of Fz/PCP signaling, the entire core is localized uniformly around the apical-lateral cortex. Genetic and cell biological experiments in the wing and eye suggest that the activity of Fz on one side of the cell triggers a redistribution of symmetrically arranged membrane-localized factors. The result is the formation of two complexes that interact across cell boundaries in a polarized pattern, creating the first visible sign of planar cell polarity. One of the complexes is comprised of Frizzled (Fz), Dishevelled (Dsh), (a cytoplasmic protein containing DIX, PDZ and DEP domains), and Diego (Dgo), a cytoplasmic ankyrin repeat
protein. The other complex is comprised of Prickle (Pk), a cytoplasmic protein with LIM and PET domains, and Strabismus (Stbm), a four-pass transmembrane protein. These two complexes antagonize each other. Here, the different complex components are referred to as PCP factors. The importance of the formation of these complexes is reiterated by the fact that loss of one PCP factor affects the localization of other PCP factors and results in the loss of coordinated polarity of corresponding structures like bristles (Axelrod, 2001; Bastock et al., 2003; Das et al., 2002; Jenny et al., 2005; Strutt et al., 2002).

While these experiments demonstrated a role for these complexes in PCP, they were not able to address the events that initiated Fz asymmetric activity. However, early genetic experiments suggested a possible role for a gradient of activity across a field of cells that may serve as a directional cue for PCP. The gradient would result in a difference in Fz activity that would be recognized and amplified by adjacent cells via a Fz receptor. The obvious candidate for this role was Wingless (Wg), since it is the only known Wnt family molecule in Drosophila that triggers Fz activity (Tomlinson et al., 1997). However, extensive studies have so far failed to conclusively identify any role for Wg in PCP signaling (Casal et al., 2002; Lawrence et al., 2002).
Fig 5. Fat regulates planar cell polarity:
Sections (C, D, F) and schematic diagrams (C’, D’, F’) of wild-type, ft mutant and ft clones in the fly eyes. In the wild-type eye (C, C’) the trapezoid shapes formed by the photoreceptor rhabdomeres of the dorsal ommatidia point upwards (red arrows), the ventral ommatidia (blue arrows) point in the opposite direction; with the equator in between (yellow line). In ftfd/ftchance transheterozygous (D, D’), the organization of the ommatidia is lost and polarity randomized. (F) ft’ tissue is marked by the absence of pigment. Non-autonomous effects can be seen at the polar side of the clones where the polarity of wild-type ommatidia is reversed shown by white arrows. Red and blue arrows (F’) indicate ommatidia with dorsal and ventral polarity, respectively (Taken from Fanto et al 2002).
As mentioned earlier, Ft plays an important role in the correct specification of R3/R4 cell fate but it is not a member of the core PCP complex. In fact, ft mutants can be easily distinguished from that of the core PCP genes based on their phenotype (i.e. loss of Fat results in Dorsal - Ventral flips)(Fig 5), indicating that it directs the primary decision regarding R3/R4 cell fate. Loss of core PCP molecules result in random arrangements of ommatidia, indicating that a later event is being compromised (Rawls et al., 2002; Yang et al., 2002). More recently, a detailed examination of the relationship between Ft and Frizzled in PCP signaling in the abdomen has indicated that these pathways can act in parallel to influence PCP (Casal et al., 2006).

Clonal analysis of the phenotype of Ft clones is particularly interesting with respect to its gradient activity. Ommatidia at the equatorial margin of ft clones have normal and correct polarity, while those in the polar margin are flipped in the D-V axis. Strikingly, this reversed polarity defect of ft clones continues into wild-type tissue, indicating a non-autonomous effect. This results in an ectopic equator on the polar side of the clone. These observations support the idea of Ft playing a role in communicating a gradient of activity to Fz, however they do not rule out the possibility that there could be other players. Since Ft is a transmembrane protein expressed uniformly across the eye disc, it has been proposed that while Ft may communicate the gradient, it is not the source (Yang et al., 2002).

Clonal analysis of Dachsous and Four-jointed has suggested a role in the communication of PCP gradient across the eye (Simon, 2004). Dachsous is another large cadherin molecule and is expressed in a gradient in the eye: high at the two poles and low at the equator (Clark et al., 1995; Yang et al., 2002). It is the only known extracellular
binding partner of Ft, and genetic data suggests that it functions antagonistically upstream of Fat (Matakatsu and Blair, 2006; Rawls et al., 2002; Simon, 2004). Four-jointed is expressed in a gradient along the D-V axis, with highest expression at the equator (Simon, 2004). Fj is a type II transmembrane/ secreted protein, however secretion is not required for planar polarity function and Fj is most highly active when anchored in the Golgi (Ishikawa et al., 2008; Strutt et al., 2004). Recently four-jointed has been reported to encode a protein kinase that phosphorylates a subset of serine or threonine residues within the extracellular cadherin repeats of Ft and Ds. An acidic sequence motif (Asp-Asn-Glu or DNE) similar to the glycosyltransferase conserved sequence motif (Asp-X-Asp or DXD, where X is any amino acid) is essential for fj activity both in-vivo and in-vitro (Ishikawa et al., 2008; Wiggins and Munro, 1998).

PCP signaling downstream of Ft is poorly understood, and so far only Atrophin has been shown to interact physically with Fat. Atrophin has been linked to Ft-PCP signaling by the observation that the non-autonomous phenotype of atro is similar to ft mutant clones. Atro also plays a role in R3 fate, shown by the presence of pigment granules in R3 photoreceptor rhabdomeres located in the polar border of the clones with incorrect polarity (Fanto et al., 2003). Atro is a transcriptional co-repressor, and influences the expression of fj (Yang et al., 2002) similar to Ft. In contrast to Ft, however, Atro has not been reported to influence growth (Fanto et al., 2003).
1.4.2.2 Role of Ft in growth control:

To ensure that an organ’s appropriate size is produced during development, rates of cell proliferation, cell growth and apoptosis must be tightly controlled (Hafen and Stocker, 2003; Leevers and McNeill, 2005). Signaling via the Tor kinase, Insulin receptor, Wingless and Decapentaplegic pathways control organ size during development. Ft has been implicated in one of these, the Hippo signaling pathway (Bennett and Harvey, 2006; Cho and Irvine, 2004; Colombani et al., 2003; Silva et al., 2006; Willecke et al., 2006).

Though the role of Ft is best understood in PCP, it was first identified genetically for its proximal-distal function; hypomorphic fat mutant flies are viable and characterized by broad thorax, abdomen and wings (Mohr, 1929). Null alleles of ft result in extremely overgrown imaginal discs and pupal lethality (Fig 6B). This overgrowth is achieved by an extended third instar larvae stage, which is on average 3.2 days longer than in heterozygous siblings (Bryant et al., 1988). Interestingly, the overgrown wing discs are of hyperplastic nature, which is defined by overgrown discs that retain their monolayer structure and ability to differentiate following transplantation. ft null discs have a single layered epithelium with a relatively normal pattern of expression of Apterous, Decapentaplegic, Wingless and Engrailed (Bryant et al., 1988; Garoia et al., 2000).

The overgrowth of ft tissue is solely due to excess cells, since loss of Ft does not alter cell size nor does it alter the cell cycle profile (Garoia et al., 2005). Analysis of ft
Fig 6. Overgrowth phenotype of Ft in wing imaginal discs.
Wing discs and their respective genotypes are shown at the same magnification. 
*ft*− shown above is *ft*<sup>G-rv/ftd</sup>. (A) Wild type. (B) Overgrowth phenotype in *ft*−. (C) Rescue of *ft*− overgrowth phenotype with *act-gal4* and UAS-*ft*. (D) Rescue of *ft*− overgrowth phenotype with intracellular domain of Ft (UAS-*ftΔECD*) and *act-gal4*. (Taken from Matakatsu and Blair 2006).
clones together with wild-type clones in the same disc revealed that the size of the clones depends on the age of the larvae, and larger ft clones compared to the wild-type clones suggested ft mutant cells cycle faster than wild type without reduction in cell size (Garoia et al., 2005).

Careful characterization of ft mutant cells show that they resemble hippo (hpo) or warts (wts) mutant cells with increased cell proliferation and increased transcription of diap1 genes, Drosophila inhibitor of apoptosis protein. The Hpo/Wts pathway includes the FERM domain proteins (named for the four proteins in which this domain was originally described: F for Band 4.1, E for Ezrin, R for Radixin, M for Moesin), Expanded (Ex) and Merlin (Mer), the serine-threonine kinases, Wts and Hpo, the scaffolding protein Salvador (Sav), the Wts –activating protein, Mats and the downstream target Yorkie (Yki), which is a transcriptional enhancer (Harvey et al., 2003; Huang et al., 2005; Jia et al., 2003; Kango-Singh et al., 2002; Lai et al., 2005; Pantalacci et al., 2003; Tapon et al., 2002; Udan et al., 2003; Wu et al., 2003). Ft expanded (ex), double mutant clones have defects similar to ft or ex single mutants, suggesting that they modulate organ size by functioning in the same pathway. Overexpression studies also show that Ex or Hpo can suppress the loss-of-function phenotype of ft, placing ft upstream of ex and hpo (Bennett and Harvey, 2006).

Another striking phenotype linking Fat to Hpo signaling is the suppression of the ft phenotype by 50% yki gene dosage, which otherwise has no effect in a wild type background. Fat is required for membrane localization, stability, or both, of Ex but not Merlin (Mer), another FERM domain protein. This also suggests that the Ft-Ex cascade appears to function in parallel to Mer. Expression of an activated form of Ft stimulates the
phosphorylation of Hpo and Wts in cell culture assays (Bennett and Harvey, 2006; Silva et al., 2006; Willecke et al., 2006).

However, the evidence linking Ft to the Hippo pathway is largely based on genetic data. Hence, determining biochemical links between Ft, Ex, Mer and the Hippo kinase cascade will serve to establish a bona fide biochemical pathway linking Ft, Ex, and Mer to Hippo activation. As mentioned previously, the intracellular domain of Ft has been reported to rescue ft overgrowth phenotype as shown in Fig 6D (Matakatsu and Blair, 2006).

Ft interacts with Atro physically and genetically but Atro does not have any growth phenotype suggesting that Ft controls growth through some other mechanism.
1.5 Thesis Goals

*Drosophila* Ft is a large cadherin molecule comprised of an extracellular domain, a transmembrane domain and a cytoplasmic domain. Preliminary data exists in our laboratory suggesting that Ft is processed from a 560 kDa precursor to 110 kDa and 430 kDa proteolytic fragments and that the 110kDa fragment of Ft4 is localized in the nucleus of mouse ES cells. On the basis of these preliminary data, I undertook a biochemical approach to understand the function of Ft.

The goals of my project were

1. Investigate the precursor-product relationship between full-length Ft and its proteolytic fragments.

2. Further analyze the intracellular localization of Ft (full-length and proteolytic fragments).

3. Map the interaction region(s) between Ft intracellular domain and its only known partner, Atrophin.

4. Study the regulation of *fj*, the only known PCP target of both Fat and Atro, by the ecdysone receptor.
Chapter 2

Materials and Methods
Chapter 2: Materials and Methods

2.1 General Drosophila melanogaster techniques

2.1.1 General Drosophila culturing

Flies were grown on a yeast/cornmeal/molasses mixture in either bottles or vials at 25°C unless mentioned.

2.1.2 Staging of Larvae

To isolate third instar larval discs and brain complexes, larvae with prominent mouth hooks and developed spiracles were selected.

Stocks used:

1. w; UAS Ft-HA; hs Gal4
2. w; UAS Ft-HA/TM6
3. w; UAS FtICD/TM6
4. w; GMR Gal4 (2nd Chromosome)
5. actin Gal4 (3rd Chromosome)
6. tubulin Gal4 (3rd Chromosome)
7. UAS EcR RNAi/TM6
8. hsflp; actin >> Gal4 UAS GFP
2.1.3 Pulse Chase assay for Fat using hs Gal4;UAS Fat-HA transgenic flies

Flies were heat-shocked for 45 min at 37°C and then transferred to 18°C. Third instar larvae with prominent mouth hooks and developed spiracles were isolated. Isolated larvae were dissected for their brain complexes in PBS with protease inhibitors (Complete™, Roche, Catalog no. 1 697 498) at different time points (0 hr after heat shock until 24 hrs). The dissected sample was transferred immediately to sample buffer containing urea (50 mM Tris-HCl; pH 6.8, 2% SDS, 10% Glycerol, 1% -β Mercaptoethanol, 12.5 mM EDTA, 0.02 % Bromophenol Blue, 2M Urea). The sample was heat treated for 3 minutes in a heating block at 100°C, homogenized with a pipette and stored in the freezer (-20°C). The samples were separated by SDS-PAGE (3-8% gradient gel) then transferred to a nitrocellulose membrane. The membrane was then washed in PBS with 0.1% Triton X-100, blocked in 5% milk in PBS with 0.1% Triton X-100 and probed with an anti-HA antibody (Rabbit anti- HA, Santa Cruz, 1:1000).

5X Sample buffer (50 ml):

12.5 ml Tris-HCl, pH 8, 0.5 M

5 g sodium dodecylsulphate (SDS)

500μl β-mercaptoethanol, 100%

25 ml glycerol, 100%

30 mg bromophenol blue
2.1.4 Drosophila cell culture and transfection

Drosophila S2 cells (Invitrogen) were grown at 21-24 °C in Schneider’s Insect Medium (Sigma) supplemented with 10% foetal bovine serum (Sigma). One day before transfection, 2-6 x 10⁴ cells were plated in 500 µl of growth medium with 10% foetal bovine serum. For each transfection, 0.4 µg of DNA was diluted in 25 µl of Opti-MEM® I Reduced Serum Medium without serum and mixed gently. 5 µl of Lipofectamine™ was diluted in 20 µl of Opti-MEM® I Medium without serum and mixed gently. Lipofectamine™ was mixed gently before use.

Diluted DNA was combined with diluted Lipofectamine™ (total volume= 50 µl), mixed gently and incubated for 45 minutes at room temperature. After the incubation, 150 µl of Opti-MEM® I Medium was added to the tube containing the complexes. Growth medium was removed from the cells and replaced with 200 µL of complex-containing medium in each wells. The medium was evenly spread in the wells by rocking the plate slowly and left in the CO₂ incubator for 72 hours at 37 °C. After 72 hours of incubation, 400 µl of growth medium containing 20% serum was added without removing the transfection mixture. The cells were transferred back to the CO₂ incubator for 24 hours.
2.1.5 Immuno-histochemistry on S2 cells

22 x 22 mm cover slips were placed at the bottom of 6 well plates used for transfection. Unbound transfected cells at the bottom of the wells and on the cover slips were lost during the three 10 minutes washes with PBS. Once washed, the cells were fixed with 4% paraformaldehyde and then blocked with PBS containing 0.1% Triton-X 100 and 5% normal goat serum. S2 cells were incubated with primary antibodies at the dilutions listed in Table 2. Primary and secondary antibodies were diluted in PBS with 0.1% Triton-X 100 and 5% normal goat serum and cells were incubated in primary antibody overnight at 4°C and in secondary antibody for 2hrs at room temperature. Secondary antibodies were from Jackson Immuno Labs and diluted 1:400; the fluorophore used was fluorescein. Between antibody steps, cells were washed thrice for ten minutes each in PBS with 0.1% Triton-X 100. Following final washes samples were mounted in Vectashield (Vector Laboratories).

2.1.6 Generation of mitotic clones

The FLP-FRT recombination technique was used to make mitotic clones. Progeny were reared at 18 °C for around 70 hours. Flpase was induced by transferring the progeny to 37°C for 30 minutes and replaced to 18°C.

\[ \text{UAS EcR RNAi ; MKRS hs flp/TM6 (X) act FRT Gal4, UAS GFP ; fj 1.2kbLacZ (parent)} \]
\[ \text{UAS EcR RNAi/act >> Gal4 UAS GFP; MKRS, hs flp/fj 1.2kbLacZ (progeny)} \]
2.2 General DNA manipulating techniques

2.2.1 DNA preparation, restriction digestion and analysis

DNA preparation and purification was performed from bacterial cultures in Luria Bertani (LB) medium prepared by the Samuel Lunenfeld Research Institute, Mt. Sinai research services according to (Sambrook et al. 1989). QIAGEN Mini and Midi preparation kits were used for purification according to the manufacturer’s instructions. Restriction digestion was performed using restriction endonucleases from New England Biolabs (NEB) in the provided reaction buffers and conditions described in the NEB catalogue. Double digestions were performed as in the double digests section of the NEB catalogue.

Agarose gels of 0.8-1.5 % were used for analyzing digested DNA using electrophoresis (Sambrook et al. 1989, using ultra-pure electrophoresis grade agarose from Invitrogen).

A QIAGEN Gel extraction kit was used for purification of the digestion reactions for fragments ranging from 100bp-10kb. Smaller fragments (<50bp) were separated from digested sample using the PCR purification kit from QIAGEN. Ligation reactions using the Roche rapid ligation kit (Cat. No. 11 635 379 001) were performed at room temperature following the manufacturer’s instructions.
2.2.2 Transformation of competent bacterial strains

For transformation of ligated plasmids, Invitrogen One Shot® chemically competent Top10 cells or BL21 cells were used. Competent cells were thawed on ice for 5 minutes. 100µl of competent cells were aliquoted to a pre-chilled 10 ml falcon tube. 50ng of DNA was added to each of the 100 µl aliquots of competent cells. DNA was delivered once the tip was inserted into the pellet of the competent cells followed by a slight tap to ensure high efficiency. The tubes were incubated on ice for 30 minutes followed by heat shock for 120 seconds in a water bath at 42°C. The tubes were placed back on ice for two minutes. 250µl of SOC medium was added to each of the tubes and incubated on the shaker at 37°C for 1hr for recovery. 50µl of the culture was smeared on LB ampicillin plates and left over night at 37 °C. The plates were checked for colonies and the efficiency of transformation the next day.

2.2.3 Polymerase Chain Reaction (PCR)

PCR reactions were carried out using Takara LA Taq™ DNA Polymerase (Takara Bio Inc, Catalog No.RR002M) or PfuUltra II fusion HS DNA polymerase (Stratagene, Catalog No.600670). Reactions were performed according to the manufacturers’ instructions using plasmid DNA templates. Cycling was performed in an epgradient S mastercycler (Eppendorf). PCR products generated from Taq polymerase reactions were used directly for cloning purpose. PCR products that were generated from Pfu were incubated with Taq polymerase at 72°C for 10 minutes for post amplification addition of 3’ A-overhanging. PCR products were purified using a PCR purification kit (QIAGEN) and their respective sizes checked using 1% agarose gel.
PCR primers used:

Table 1.

<table>
<thead>
<tr>
<th>Primers</th>
<th>Sequence (5’-3’)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bait1Nco1-Fwd</td>
<td>GCGCAGCAAAACTCCAT</td>
</tr>
<tr>
<td>Bait1Spe1-Rev</td>
<td>TCCAGAGGAGTACGTGTAA</td>
</tr>
<tr>
<td>Bait1-BegNco1Fwd</td>
<td>GCGCAGCAAAACTCCAT</td>
</tr>
<tr>
<td>Bait1-B-Spe1Rev</td>
<td>CTCCTCGATGGACGCAGTTAA</td>
</tr>
<tr>
<td>Bait1-E-Nco1Fwd</td>
<td>CTCCTCGATGGACGCAGT</td>
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<tr>
<td>Bait1-M-Nco1Fwd</td>
<td>GCTCTGCATATGTCGCTG</td>
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<tr>
<td>Bait1-M-Spe1Rev</td>
<td>TCAGCACGCTCGTGCCATAA</td>
</tr>
<tr>
<td>Bait2-Nco1-Fwd</td>
<td>AAGCAGGAGAAGATTGGTAG</td>
</tr>
<tr>
<td>Bait2-Spe1-Rev</td>
<td>GGCGCAGCAAAACTCCATGTGA</td>
</tr>
<tr>
<td>Ft7-BamHI-F</td>
<td>CGCCAGCTCCATTGCTCC</td>
</tr>
<tr>
<td>Ft7-EcoR1-R</td>
<td>CAGGCGCAGCAAAACTTCCTAA</td>
</tr>
<tr>
<td>Fat7-Beg-EcoR1-R</td>
<td>CAACCGCCACCGCCGCCCCCTAA</td>
</tr>
<tr>
<td>Ft7-End-BamHI-F</td>
<td>ACCAGTGCATCCCGCACC</td>
</tr>
</tbody>
</table>

2.2.4 Sequencing

DNA sequencing was carried out at the TCAG DNA Sequencing Facility, Toronto, Ontario. For each reaction 150-200 ng of plasmid DNA and 20-33 ng (3-5 pmol) of primer for a total volume of 7.7 ul was used to sequence the extremities of the constructs. The sequence was then pair wise aligned using ApE software (A plasmid Editor) to check for the presence of the insert.
2.3 General protein manipulation techniques

2.3.1 SDS-PAGE

Polyacrylamide gel electrophoresis was performed using the BioRad protein III system. Resolving gels were 7.5-10% polyacrylamide (37.5:1 acrylamide/bis, Bio-Rad) in 375mM Tris pH8.8, 0.1% SDS. Stacking gels were 4% polyacrylamide in 125mM Tris pH6.8, 0.1 % SDS. Protein samples were mixed with 5X sample buffer and heat-treated for 3 minutes at 100 °C prior to loading. The Pageruler Molecular weight marker (Invitrogen) was used for approximate size determination. Gels were run in Tris-Glycine running buffer (25mM Tris pH8.3, 192mM Glycine, 0.1%SDS) at 150V for 1- 1.5 hours.

2.3.2 Western blotting

Polyacrylamide gels and membranes (ECL-Hybond nitrocellulose; Amersham Pharmacia) were equilibrated in transfer buffer (48mM Tris pH8.3, 192 mM Glycine, 20 % Methanol, 0.05% SDS) for 10 minutes. Transfer was performed using the Trans Blot Electrophoretic Transfer cell (BioRad) at 20mA overnight at 4°C or 115mA for 1.5 -2 hours at room temperature. Membranes were washed in double distilled water followed by Ponceau Staining for 5-10 minutes and then documented. Membranes were blocked in PBS-Tween (PBS, 0.1 % Tween 20) with 5% milk for 1 hour at room temperature. Primary antibodies were diluted in PBS-Tween, 5% Milk and incubated with the membranes for 1-2 hours at room temperature or overnight at 4 °C. Membranes were washed for 10 minutes thrice in PBS-Tween and then incubated with the appropriate HRP-conjugated secondary antibodies in PBS-Tween for 1 hour at room temperature.
Secondary antibody incubation was followed by three 10 minutes washes with PBS-Tween and treatment with the ECL plus western blotting chemiluminescent detection agent (Amersham Pharmacia) according to the manufacturer's instructions.
**Primary antibodies:**

**Table 2.**

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Source</th>
<th>Dilution</th>
<th>Comments</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mouse $\alpha$ tubulin</td>
<td>Hybridoma bank</td>
<td>1:1000</td>
<td>Monoclonal</td>
</tr>
<tr>
<td>Mouse $\alpha$ lamin</td>
<td>Hybridoma bank</td>
<td>1:1000</td>
<td>Monoclonal</td>
</tr>
<tr>
<td>Rabbit $\alpha$ Neurexin</td>
<td>Hugo Bellen</td>
<td>1:1000</td>
<td>Polyclonal</td>
</tr>
<tr>
<td>Rabbit $\alpha$ BiP</td>
<td>John Sisson</td>
<td>1:100</td>
<td>Polyclonal</td>
</tr>
<tr>
<td>Rat $\alpha$ c-term Fat</td>
<td>Helen McNeill</td>
<td>1:1000</td>
<td>Polyclonal</td>
</tr>
<tr>
<td>Rabbit $\alpha$ HA</td>
<td>Santa Cruz</td>
<td>1:1000</td>
<td>Polyclonal</td>
</tr>
<tr>
<td>Rabbit $\alpha$ c-term atrophin</td>
<td>C. Tsai</td>
<td>1:3000</td>
<td>Polyclonal</td>
</tr>
<tr>
<td>Rabbit $\alpha$ c-term atrophin (D3a)</td>
<td>Helen McNeill</td>
<td>1:200</td>
<td>Polyclonal</td>
</tr>
</tbody>
</table>

**HRP-conjugated secondary antibodies used for western blotting:**

**Table 3**

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Source</th>
<th>Dilution</th>
</tr>
</thead>
<tbody>
<tr>
<td>$\alpha$-Mouse Ig HRP Conjugate</td>
<td>Amersham Lifescience</td>
<td>1:5000</td>
</tr>
<tr>
<td>$\alpha$-Rabbit Ig HRP Conjugate</td>
<td>Amersham Lifescience</td>
<td>1:5000</td>
</tr>
</tbody>
</table>
2.3.3 *In-vitro* translation of Atrophin c-term

*In-vitro* transcription coupled to translation was performed using an Expressway™ Mini Cell-Free E. coli Expression System (Invitrogen, Catalog No. K9901-00). 1 µg of *atro c-term* was added to the reaction buffer containing enzyme mix, amino acids, ribonucleotides and salts. The tube containing the DNA and the reaction buffer was incubated at 37°C for 30 minutes shaking. After 30 minutes, 50 µl of feed buffer was added to the reaction mix to replenish components depleted or degraded during protein synthesis. The tube was again incubated at 37°C, but for 5.5 hours. *In-vitro* translated Atrophin was stored in 20µl aliquots at -80°C to avoid freezing/thawing of the samples.

**Reaction Buffer Composition (Invitrogen)**

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>E.coli extract</td>
<td>20.00µl</td>
</tr>
<tr>
<td>2.5X IVPS(<em>In-vitro</em> protein synthesis ) E.coli Rxn Buffer</td>
<td>20.00µl</td>
</tr>
<tr>
<td>50mM AA (-Met)</td>
<td>1.25µl</td>
</tr>
<tr>
<td>75mM Methionine</td>
<td>1.00µl</td>
</tr>
<tr>
<td>T7 Enzyme mix</td>
<td>1.00µl</td>
</tr>
<tr>
<td>DNA template</td>
<td>2.00µl ~ 1µg</td>
</tr>
<tr>
<td>DNAse/RNase free dH2O</td>
<td>5.75µl</td>
</tr>
</tbody>
</table>
Feed buffer

<table>
<thead>
<tr>
<th></th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>2X IVPS Feed buffer</td>
<td>25.00µl</td>
</tr>
<tr>
<td>50mM AA (-met)</td>
<td>1.25µl</td>
</tr>
<tr>
<td>75mM Methionine</td>
<td>1.00µl</td>
</tr>
<tr>
<td>DNAase/RNAase free dH20</td>
<td>22.50µl</td>
</tr>
</tbody>
</table>

2.3.4 Production and purification of His-tagged proteins

Transformation of the "atro" C-terminal fragment cloned in vector pEXP1 was done in BL21 cells. At least four colonies were picked and incubated in 10 ml of LB ampicillin overnight. 10 ml of overnight culture was then re-suspended in 200 ml of LB ampicillin and incubated for 2 hrs in 37°C. 100µL of 1.0 M IPTG was added to each of these cultures and left for induction for 3 hours at 37°C. Isopropyl β-D-1-thiogalactopyranoside (IPTG) induces the transcription of the gene coding for β-galactosidase, an enzyme that promotes lactose utilization, by binding and inhibiting the LacI repressor. 1 ml from each of the bacterial cultures was kept aside and the rest of the samples were centrifuged and the pellet was stored in the freezer at -20°C. 1 ml culture was centrifuged and the pellet was re-suspended in 50 µl of loading buffer (1X). The samples were heat-treated for 3 minutes at 100°C and 15µl of each of these samples were loaded per well of an SDS-PAGE. The gel was stained with coomassie blue for 20 minutes at room temperature and then washed with water to remove the non-specific stain to check for induction. Cultures that show induction were removed from the freezer and re-suspended in 5ml of cold lysis buffer (PBS+ Complete protease inhibitor tablet-Roche) + 25mM Imidazole final (82 µl of 1.5M
Imidazole). Each re-suspended samples was sonicated thrice for 20 seconds with 20 seconds between each sonication on ice. 50 µl of Triton X-100 was added to each of the sample and incubated for 10 min at 4°C with shaking. After the incubation, the samples were centrifuged for 20 min, high speed at 4°C. The supernatant was isolated carefully without disturbing the pellet and incubated with 200µl of Ni beads for 2 hours with shaking at 4°C. On completion of the incubation, the samples were centrifuged, most of the supernatant was removed and the rest with the beads was transferred to 1.5ml eppendorf tubes. The Ni beads were washed thrice in pull-down buffer + 10mM imidazole prior to elution with 300µl of elution buffer + 250mM of imidazole for 30 minutes at 4°C with shaking. 4µl of samples +1 µl of 5X loading buffer was heat treated for 3 minutes at 100 °C. The samples were then run on SDS-PAGE together with known concentration of BSA samples. The gel was stained using coomassie to check the concentration of the protein. The samples were stored at -80°C in 150 µl aliquots.

2.3.5 Production and purification of GST tagged protein

pGEX-4T1-Ft constructs were transformed using BL21 cells. At least four colonies were inoculated separately in 5 ml of LB containing ampicillin overnight at 37 °C. 5 ml of overnight culture was re-suspended in 50 ml of LB ampicillin at 37 °C. After 2 hrs, IPTG (1mM final concentration: added 50 µL of 1 M IPTG) was added to the culture and left for another 3 hrs at 37°C for induction. One ml of culture was taken aside before and after the induction from each culture to check for the induction. The pellets that were obtained from rest of the samples by spinning for 10 minutes at 16,000 rpm were stored in -20°C. The one ml cultures were centrifuged for two minutes at 16,000rpm and the pellet was re-
suspended in 50 µl of loading buffer (1X) and heat treated for 3 minutes 100 °C. The samples were separated by SDS-PAGE, stained with coomassie blue for 20 min and washed overnight in water to check for induction. Pellets of the samples that show induction were thawed and re-suspended in 5ml of cold lysis buffer (PBS+ Complete protease inhibitor tablet-Roche) + 25mM Imidazole (82 µl of 1.5M Imidazole), to prevent degradation of proteins. The re-suspended pellet was sonicated thrice for 20 seconds with 20 seconds between each sonication, on ice. 50 µl of Triton X-100 was added to the sample and incubated for 10 min at 4°C shaking. The samples were centrifuged for 20 min at 6000 rpm at 4°C. While the samples were in the centrifuge, glutathione beads were prepared. 266µl slurry of glutathione beads was washed three times with 500µl of PBS and centrifuged at 3000 rpm for 30 seconds. After the final wash, glutathione beads were resuspended in 200µl of PBS. The cleared lysate was incubated in 400µl of 50% GST beads over night with shaking at 4°C. Samples were centrifuged at 3000 rpm for 30 seconds and the majority of the supernatant was removed. The rest of the supernatant, together with the beads, was transferred to a 1.5ml eppendorf tube. The beads were washed thrice with 500µl of PBS with 10 min incubation at 4°C, shaking. The protein was eluted from the beads with 300µl of elution buffer (50 mM Tris-HCl pH 8, and 10mM Glutathione) for 30 min at 4°C with shaking. 4µl of eluate + 1µl of loading buffer (5X) were heat treated for 3 minutes at 100 °C. The samples were run on an SDS gel and coomassie stained to check the concentration of the protein. The samples were stored in -80°C in aliquots of 150 µl to prevent freezing/thawing of the samples.

**Lysis Buffer (50ml):**

49.7 ml of PBS
5mM DTT (200µl of DTT 1M)
1mM EDTA (100µl EDTA 0.5M)
1 tablet of complete protease inhibitors (Roche)

2.3.6 Pull-down assay

5µl of the freshly translated Atro-C terminal eluate together with 5µl of glutathione sepharose were added to 100µl of pull-down buffer (20mM Tris pH 7.2, 1mM EDTA, 1%
TritonX-100, 1mM β mercaptoethanol) and left for 30 min at room temperature on a nutator. The samples were spun at 3000rpm for 30 seconds to precipitate the beads. The supernatant was carefully removed with the help of 27¹/₄G needle syringe. 5µg of GST-Ft fusion protein constructs were added to 100µl of supernatant per condition. The samples were incubated overnight at 4°C. The samples were centrifuged at 3000 rpm for 30 seconds, followed by three washes with 500µl of pull-down buffer (20mM Tris pH 7.2, 1mM EDTA, 1% TritonX-100, 1mM β-mercaptoethanol + 100mM NaCl). After the final wash most of the washing buffer was removed with a 27¹/₄G needle syringe. 5X loading buffer and PBS was added to the washed beads. The samples were heat-treated for three minutes at 100°C. The samples were then loaded on a 10% SDS gel followed by western blot analysis and probed using antibodies to the different proteins.

2.3.7 Bradford test

1 mg/ml stock solution of BSA (Bovine serum albumin, Sigma) was used to make a series of 100µl of 0.2, 0.4, 0.6, 0.8 mg/ml concentration solutions. 50 µl of each solution was added to 2.5 ml of Bradford reagent (Sigma) in a cuvette. The solution was mixed well
and left for 5 minutes in the dark. The absorbance was taken at 595nm and a standard curve was plotted with the reading. Three serial dilutions of the samples were generated. 50µl of each of the samples were added to 2.5 ml of Bradford reagent and incubated for 5 min at dark. After the incubation, the absorbance was measured at 595nm and the reading was plotted against standards to estimate the protein concentration.

2.3.8 Fractionation assay

Third instar larvae were dissected for brain complexes/discs in PBS with protease inhibitors (Complete™, Roche, Catalog no. 1 697 498). The dissected brain complexes/discs were uniformly homogenized and then centrifuged at 500g for 2-3 minutes. The supernatant were replaced with cytoplasmic extraction reagent 1 (CER1) and vortexed vigorously to resuspend the pellet in CER1 buffer. The samples were incubated in ice for 10 minutes followed by addition of cytoplasmic extraction reagent 2 (CER2). The tube containing CER1, CER2 and samples were vortexed for 15 seconds in order to uniformly mix the samples in the extraction reagents. The samples were then centrifuged at 16000 g for 10 minutes. The supernatant/cytoplasmic extract was collected immediately in a fresh tube. The pellet was resuspended in nuclear extraction reagent (NER) after a quick wash in cold PBS. The samples were vortexed for 15 seconds every 10 minutes for 40 minutes. The samples were then centrifuged for 10 minutes at 16000 g followed by the removal of supernatant (nuclear fraction) into a fresh tube. Samples buffer was added to the nuclear and cytoplasmic extracts and boiled for 3 minutes at 100 °C. 10 µl aliquots of the nuclear and cytoplasmic extracts were made and stored in -80°C to avoid freeze/thawing of the samples. Nuclear and Cytoplasmic Extraction Reagents Kit (Pierce Biotechnology; Rockford, IL) was used for fraction assays.
Chapter 3

Results
Results:

Chapter 3:

3.1 Localization of the intracellular region of *Drosophila* Fat

The *Drosophila* protein Fat (Ft) is required for several processes during development. Ft has been reported to regulate growth (Bryant et al., 1988; Garoia et al., 2000; Mao et al., 2006; Matakatsu and Blair, 2006; Polesello et al., 2006; Silva et al., 2006) and is required for normal establishment of planar cell polarity in the eye, wing and abdomen (Casal et al., 2002; Fanto et al., 2003; Garoia et al., 2000; Matakatsu and Blair, 2004; Yang et al., 2002). Finally, Ft is required to regulate proximodistal patterning of some of the appendages (Bryant et al., 1988; Clark et al., 1995; Matakatsu and Blair, 2006). Ft has been reported to work together with Dachsous (*ds*), *four-jointed (fj)* and *frizzled (fz)* to regulate these functions (Cho and Irvine, 2004; Matakatsu and Blair, 2006; Rawls et al., 2002; Yang et al., 2002), but the mechanism of Ft action is poorly understood. Ft is a large protein, 560 kDa in size, consisting of extracellular, transmembrane and intracellular domains (Fig 1). To further characterize the molecular function of Ft, I used a biochemical approach to study the functions of the Ft domains.
Fig 1. Schematic of dFt and its deletion mutant series:

Above is a schematic of full length Ft, as well as a series of truncation mutants that were used to make transgenic flies by Dr. Sakura Saburi. These constructs were used for in-vivo and in-vitro experiments. (A) Full length Ft comprised of 34 cadherin like repeats, 5 EGF-like repeats, 2 Laminin-G repeats, a transmembrane domain, cytoplasmic domain and a HA tag at its cytoplasmic end. (B) Ft membrane tethered domain (FtICD+TM) has the transmembrane domain, cytoplasmic domain and HA tag. (C) Fat intracellular domain (FtICD) contains only the intracellular domain and HA tag. Ft intracellular domain delta (FtICDΔ) has the C-terminal end of the cytoplasmic domain and HA tag.
3.1.1 Pulse chase assay

To understand the molecular mechanism of Ft function, I decided to take a biochemical approach. I performed a pulse chase assay to confirm the product and precursor relationship of Ft and the smaller 110kDa form detected previously by Dr. Lesley Clayton, a technician in our laboratory in London (unpublished data). I gave a heat shock pulse for 45 minutes at 37 °C to the transgenic progeny (UAS-fat-HA; hs gal4) with a C-terminal HA tag and returned the flies to 18°C for recovery. I dissected the third instar larval brain complex, brain lobes together with the eye discs, at different time points. The brain complex was homogenized and suspended in sample buffer and then heat treated for 3 minutes at 100 °C. The samples were separated by SDS-PAGE and transferred to a nitrocellulose membrane. The membrane was probed with anti-HA antibody followed by incubation with the ECL Plus western blotting chemiluminescent detection agent to check the expression profile of dFat. Expression of dFatFL (full length, predicted 560kDa) (Mahoney et al., 1991) was detectable at around 2hr after heat-shock, whereas a ~110 kDa band could be seen at around 6hr after pulse (Fig2). The 560kDa band appeared more abundant at the earlier time points, followed by the ~110kDa band. The 110kDa form appeared more abundant at later time points, compared to the 560kDa form. It is difficult to determine the relative stability of the two forms based on the western blot analysis. Nevertheless, since the 560kDa form is more abundant at earlier time points and the ~110kDa form is more abundant at later time points, it suggests a precursor-product relationship. In order to confirm that the smaller form of Ft is a product
Fig 2. \( \text{Ft}^{\text{FL}} \) and the 110kDa form show a product and precursor relationship in the pulse chase assay shown above.

Brain complexes were isolated from flies at different time points, homogenized and suspended in loading dye. The samples were then heat-treated, separated by SDS-PAGE and transferred to a nitrocellulose membrane. The blot was probed with an anti-HA antibody, since Ft has a HA tag at its C-terminus. Blots were developed with ECL chemiluminescent substrate. Western blot analysis of the pulse chase assay shows that dFat\(^{\text{FL}} \) (full length) is detected from 2hrs after heat-shock, whereas the 110 kDa form is detected from 6 hrs after heat-shock. The 560kDa band appeared more abundant at the earlier time points. The 110kDa form appeared more abundant at later time points, compared to the 560kDa form, indicating a precursor-product relationship. Constitutive expression of Ft, in which the 110 kDa form is predominant, can be seen with actin driver whereas tubulin driver leads to lethality explaining the absence of Ft. The blot was probed with anti-Lamin antibody to check the quantity of protein in each lane.
of the full-length form, Dr. Clayton isolated brain complexes from wild type and several different ft mutants. Western blot analysis of the brain complex extracts was done with an anti-Ft antibody that was raised against the cytoplasmic end of Ft (Fig 3). In wild-type brain extracts, Dr. Clayton could see both the full length and the 110kDa form of Ft present, but in the case of ft mutants both these forms were missing. This, taken together with my own pulse chase data (Fig 2), supports a product-precursor relationship of Ft full length and the 110kDa band. The predicted size of the smaller form is around 110kDa, and would include the intracellular domain, transmembrane domain and a very small piece of the extracellular domain. This smaller form of Ft is similar in size to the intracellular region of ft that has information required for growth control (Matakatsu and Blair, 2006).

Since the Ft subfamily is conserved from flies to mammals (Tanoue and Takeichi, 2005), Dr. Saburi in our laboratory first tested whether similar processed forms of Ft could be detected in for Ft4, the closest homolog of Drosophila Ft. Using a peptide antibody generated against the C-terminal of Ft4 (Cys58), she detected low amounts of the full length protein in total lysates, but found string reactivity with bands at 110 kDa and 70 kDa. This indicates that, like Drosophila Ft, Ft4 can be processed to smaller products. To begin addressing the function of these processed forms, Dr. Saburi performed a cell fractionation assay in mouse ES cells to determine the localization of mouse Ft4. Nuclear and cytoplasmic extraction from mouse ES cells was followed by western blot analysis with Cys58 antibody together with specific molecular markers for nuclear, cytoplasmic and membrane proteins. Dr. Saburi showed that the ~110kDa and ~70kDa but not the full length forms of Ft4 are enriched in the nuclear extract of these cells (Fig.4). Since
Fig 3: Smaller forms of Ft missing in ft mutants:

Fat western done using an anti-Ft antibody on brain extracts from ft mutants. Ft^{FL} and Ft^{110kDa} forms are present as shown with arrowheads, in the wild-type (WT) lanes but both these forms are missing in the case of ft mutants as shown in lanes next to the wild-type lane. This genetic experiment confirmed that the smaller form is the product of the full length Ft. Note that several immunoreactive bands, indicated here by asteriks, are detected using the Ft antibody even in the ft mutants, indicating that they are likely cross-reactive gands. This experiment was performed by Dr. Clayton (unpublished data).
Fig 4. Fractionation assay in mouse ES cells show smaller forms of Ft4 in the nuclear fraction:
Western blot analysis with a Cys58 antibody (antibody raised against the C-terminal end of Ft4) shows that Ft4 110 kDa and 70 kDa forms are present specifically in the nuclear fraction of mouse ES cells. Nuclear (c-myc, Y12), cytoplasmic (β actin), ER (BiP) and plasma membrane (Presenilin1) were probed to check the efficacy of the fractionation. This experiments was done by Dr. Sakura Saburi using Nuclear and Cytoplasmic Extraction Reagents Kit (Pierce Biotechnology; Rockford, IL) (T- Total lysate, C- Cytoplasmic fraction, N- Nuclear Fraction).
Drosophila Ft and Ft4 show sequence similarity, the next obvious experiment for us was to analyze the subcellular localization pattern of Drosophila Ft. As Drosophila is an organism with molecular and genetic pliability, this would allow us to eventually determine the functional relevance of a nuclear form of Ft.

### 3.1.2 In-vitro localization of dFt full length and truncated products

Based on overall sequence similarity, Ft4 and Drosophila Ft form one family. On the other hand, three mammalian sequence relatives Ft1, Ft2, Ft3 and Drosophila Ft-like form another family (Katoh and Katoh, 2006; Tanoue and Takeichi, 2005). Because Dr. Saburi showed that intracellular region of Ft4 is localized in the nuclear fraction of mouse ES cells and that Fat4 and Drosophila Ft have sequence similarity, I examined the localization of Drosophila Fat in S2 cells. In order to look at dFat localization in S2 cells, which do not express dFt, I transfected these cells with ft-full length (Ft\(^{FL}\)), C-terminally tagged with HA, as shown in the schematic (Fig 1).

Transfected S2 cells were fixed and probed with an anti-HA antibody and secondary antibody conjugated with a fluorophore. S2 cells were then analyzed using a confocal microscope at 40X magnification where Ft is seen to localize to the membrane and the cytoplasm of transfected cells compared to untransfected cells (Fig 6).

Fractionation assays were performed on S2 cells transfected with Ft intracellular (Ft\(^{ICD}\)) and Ft membrane tethered form (Ft\(^{ICD+TMD}\)). Using a Nuclear and Cytoplasmic Extraction Reagents Kit (Pierce Biotechnology; Rockford, IL) and 1 X 10\(^6\) cells, I fractionated the S2 cells in two fractions (cytosolic and nuclear), and analyzed the
Fig 5. dFt<sup>FL</sup>-HA is localized in the cytoplasm and membrane in S2 cells:

S2 cells transfected with dFt<sup>FL</sup>-HA were stained with an anti-HA antibody and propidium iodide as shown in green and red channel above, respectively, to check for the localization of Ft. Ft<sup>FL</sup>-HA is localized in the cytoplasm and the plasma membrane as shown in the green channel in panel A (above row). This staining pattern is absent in untransfected cells stained with anti-HA antibody and PI as shown in panel B (lower row).
total extract in parallel. Western blotting was done on cell extracts from the fractionation assay with anti-HA antibody, since \( \text{Ft}^{\text{ICD}} \) and \( \text{Ft}^{\text{ICD+TM}} \) were HA tagged at the cytoplasmic end as shown in Fig 6. Interestingly, I could see that \( \text{Ft}^{\text{ICD}} \) is localized at least partially in the nuclear fraction, similar to that shown by Dr. Saburi in mouse ES cells. In the case of \( \text{Ft}^{\text{ICD+TMD}} \), the construct was localized in the whole extract, but not in the nuclear or cytosolic fractions (Fig 6). Absence of \( \text{Ft}^{\text{ICD+TMD}} \) from the nuclear fraction could be attributed to lack of planar polarity in S2 cells or the absence of proteolytic protein(s), since \( \text{Ft} \) is not normally expressed in S2 cells. In order to rule out that these factors play a role in the subcellular localization pattern of \( \text{dFt} \), I next checked for the localization of \( \text{dFt} \) and its smaller forms by fractionating extract prepared from \( \text{drosophila} \).
Fig 6: $F_{\text{ICD}}$ and not $F_{\text{ICD+TMD}}$ localizes to the nuclear fraction of S2 cells:

Fractionation was done on S2 cells transfected with $F_{\text{ICD-HA}}$ and $F_{\text{ICD+TMD-HA}}$. Samples were separated in SDS-PAGE and then transferred to nitrocellulose membrane. The membranes were probed with HA, tubulin and lamin antibodies to check for Ft localization and the efficacy of the fractionation. Blots were developed with ECL chemiluminescent substrate. Ft intracellular domain ($F_{\text{ICD}}$) is detected in the whole lysate, as well as the cytoplasmic and nuclear fractions. The membrane tethered domain ($F_{\text{ICD+TMD}}$) can only be detected in the whole lysate and not in the cytoplasmic and nuclear fractions. Lamin and Tubulin were used as controls to label the nuclear and cytoplasmic fractions, respectively.
3.2 \textit{In-vivo} localization of dFat

3.2.1 Intracellular localization of dFat in the Brain complex

The localization of the intracellular domain of dFat in the nuclear fraction of S2 cell was very intriguing. Next, I checked the subcellular localization of Ft by employing the transgenic fly system described in figure 2. I therefore induced the expression of Ft exactly as described for the pulse chase assay (heat pulse for 45 minutes at 37°C and transferred the larvae to 18°C for recovery). I dissected brain complexes from third instar larvae after eight hours of recovery in 18°C, since at this time point we could see both the full length and the truncated form (Fig 2). Western blot analysis of the fractionation assay was done with an anti-HA antibody. I could see localization of Ft\textsubscript{FL} and the 70kDa and 110kDa forms in the cytoplasmic fraction as shown in Fig 7, but only weak reactivity in the nuclear fraction, in contrast to the S2 cell experiment.

Since Ft\textsubscript{FL} was transiently expressed in both these conditions, I then used a trans-activating system in flies to constitutively express \textit{ft}. In order to do so I used the trans-activating Gal4-UAS system from yeast (Brand and Perrimon, 1993; Fischer et al., 1988). I used transgenic flies ubiquitously expressing actin-Gal4 and transgenic flies with different forms of \textit{ft} subcloned downstream of the upstream activating sequence (UAS), \textit{UAS ft}\textsuperscript{FL-HA/TM6B} and \textit{UAS ft}\textsuperscript{ICD-HA/TM6B}. Transgenic flies having Gal4 and UAS constructs were crossed and wandering third instar larvae having both Gal4 and UAS constructs, act Gal/++; \textit{UAS ft}\textsuperscript{FL-HA}/+ and act Gal/+; \textit{UAS ft}\textsuperscript{ICD-HA}/+ were isolated with
Fig 7. Fractionation assay of transiently expressed Ft\textsuperscript{FL}-HA indicates predominant cytosolic localization of all products:

Fractionation was done on brain complexes isolated from \textit{hsGal4; UAS-Ft\textsuperscript{FL}-HA} transgenic flies. Isolated fractions were separated by SDS-PAGE and transferred to nitrocellulose membrane. The membrane was probed with anti HA antibody. Blots were developed with ECL chemiluminescent substrate. Western blot analysis with anti-HA antibody shows that Ft\textsuperscript{FL} and its truncation products are localized in the cytoplasmic fraction. We can also see traces of Ft\textsuperscript{FL} and its truncation products in the nuclear fraction, which could be as a result of membrane contamination. Tubulin, lamin, and BiP were used to mark the cytoplasmic, nuclear and ER respectively to check the efficacy of the fractionation (YW: wild-type).
the help of phenotypic markers (i.e non-tubby larvae with well developed mouth hooks and spiracles for the fractionation assay). Fractionation assays were done on these brain complexes followed by Western blot analysis with an anti-HA antibody since the constructs were HA-tagged at the C-terminus. I also probed the blot with Lamin, Tubulin and Neurexin IV antibodies as nuclear, cytoplasmic and membrane markers respectively. I could see that the FtICD-HA was found in the cytoplasmic fraction, as shown in Fig 8 unlike in the nuclear fraction of the S2 cells as shown earlier (Fig 6). Fractionation assays performed in the artificial S2 system where Ft is not normally expressed show that FtICD is localized to both cytoplasmic and nuclear fractions. Fractionation assays of the more physiologically relevant brain complexes show that FtICD is localized to the cytoplasmic fraction. Since a number of factors could play a role during the development of an organism that could control the localization of protein, I decided to repeat the fractionation assay using eye discs where Ft has been extensively studied.
Fig 8. Cytoplasmic localization of Ft^ICD-HA in the brain complex:

Fractionation assay of Ft^ICD-HA was done on the brain complex. Isolated extracts were separated by SDS-PAGE and transferred to a nitrocellulose membrane and probed with HA antibody. Blots were developed with ECL chemiluminescent substrate. Ft^ICD is localized in the cytoplasmic fraction, as indicated by an arrow. No trace of Ft^ICD in the nuclear fraction was detected. Tubulin, lamin and Neurexin (NrxIV) were used to mark the cytoplasmic, nuclear and membrane fractions, respectively to check the efficacy of the fractionation. On the right is the schematic of the intracellular domain used for the fractionation. (YW:Wild-type).
3.2.2 Intracellular localization of dFt in the eye discs

Ft has been reported to play a role in growth, polarity and proximal distal patterning (Bennett and Harvey, 2006; Bryant et al., 1988; Fanto et al., 2003; Matakatsu and Blair, 2006). One of the model systems that has been extensively used for elucidating these functions of Ft is the eye disc. To examine FtICD localization in eye discs, fractionation assays were performed on eye discs isolated from wandering third instar larvae. Western blot analysis on the eye disc extracts was done with an anti-HA antibody to detect FtICD. I also probed the blot with lamin, tubulin and neurexin IV antibodies to check the efficacy of the fractionation as shown in Fig 9. FtICD is localized in the whole extract and in the cytoplasmic fraction but not in the nuclear fraction. This discrepancy between the S2 cell results and the results in the eye discs as shown in figure 9 could be for a number for reasons. One of the reasons could be that Ft is under spatial-temporal control and cannot be detected abundantly in the nucleus of the cells. It is also possible that the overexpressed protein is not localized at detectable amounts in the nucleus in eye discs.

In order to examine Ft localization in more detail, Yonit Tsatskis, a technician in our laboratory, performed immuno-histochemistry of Ft in the eye discs. She probed the discs with anti-HA antibody as shown in Figure 10. Yonit Tsatskis was able to show that FtICD-HA is localized in the nucleus of only a subset of cells (Fig10C), marked with white arrows. FtFL-HA and FtTM+ICD-HA were localized apically and in the cytoplasm, respectively (Fig10A and 10B). Since very few cells have FtICD localized in the nucleus, this may explain why it was difficult to detect Ft in the nucleus with the biochemical techniques previously described.
Fig 9. Cytoplasmic localization of FtICD in the eye discs:

Fractionation assay of FtICD was done on eye discs. Isolated fraction were separated by SDS-PAGE then transferred to a nitrocellulose membrane and probed with anti-HA, tubulin, lamin and neurexin antibodies. Blots were developed with ECL chemiluminescent substrate. FtICD-HA is abundantly localized in the cytoplasmic fraction and unlike FtICD fractionation assays in S2 cells no traces of Ft nor its truncation constructs were detected in the nuclear fraction. Tubulin, lamin and neurexin (NrxIV) were used to probe the cytoplasmic, nuclear and membrane fraction, respectively, to check the efficacy of the fractionation. On the right is the schematic of the FtICD used for the fractionation. (YW: wild-type, WL: Whole Lysate).
Fig 10. Ft<sup>ICD</sup>-HA is localized in the nucleus of a subset of cells in eye discs:

Eye discs were dissected from third instar larvae and stained with anti HA antibody. (A) Ft<sup>FL</sup>-HA is predominantly localized to the apical surface in the eye disc and partially in the cytoplasm. (B) Ft<sup>TM+ICD</sup> is localized to the apical surface and in the cytoplasm. (C) In Ft<sup>ICD</sup>-HA eye discs, very little Ft is localized to the apical surface, but Ft<sup>ICD</sup> is present in the nucleus of a subset of cells indicated by white arrows. Experiments done by Yonit Tsatskis.
3.3 **Characterization of Fat and Atrophin binding**

Since our laboratory is interested in understanding the molecular basis of Ft function, Jamie Meredith, a technician in our laboratory, carried out a yeast two-hybrid screen to look for protein binding partners of Ft. Surprisingly, *Drosophila* Atrophin (Atro), a transcriptional co-repressor was found to interact with the intracellular region of *ft*. Atrophin, also know as Grunge, is the sole *Drosophila* homolog of human Atrophins (Erkner et al., 2002; Zhang et al., 2002). Atro is a nuclear co-repressor comprised of an ELM domain, Myb domain and a Pro rich domain (Wood et al., 2000; Zhang et al., 2002). A subsequent pull-down assay confirmed that Ft and Atro physically interact. In addition, Ft and Atro display genetic interactions: the viability of *ft* heterozygous flies is compromised when one copy of *atro* is removed (Fanto et al., 2003). Ft and Atro also share a number of phenotypic similarities. That is, clonal analysis of Atro showed non-autonomous reversal of polarity of ommatidia at the polar side of the clone. Mosaic analysis showed that Atro helps in determining the R3 cell fate. Both *ft* and *atro* mutants show blisters on the wings and defects in the closure of the thorax of flies. Despite the phenotypic similarity of *ft* and *atrophin* clones these two genes do not have completely identical phenotypes. *atrophin* clones show increased numbers of inner photoreceptor cells. They do not have growth defects as shown by *ft* mutants and act specifically in a pre-furrow adhesion process (Fanto et al., 2003).

In order to study the interaction of Ft and Atro, I planned to narrow down the region of Ft that interacts with Atrophin and identify the domain that is responsible for this interaction using a pull-down assay.
3.3.1 The N-terminal portion of Ft\textsuperscript{ICD} binds to Atrophin

Atro and Ft have been reported to interact physically and genetically (Fanto et al., 2003). The intracellular region of Ft can rescue the PCP defect of Ft and it can also rescue the growth phenotype (Fanto et al., 2003; Matakatsu and Blair, 2006). Atro, which physically and genetically interacts with Ft, has a PCP defect very similar to Ft as shown by clonal analysis. An open question is whether the intracellular domain of Ft plays a role in its function and whether the function of Ft is in turn regulated by Atro directly or indirectly.

To begin to address this question, I generated constructs containing various portions of the intracellular region of Ft fused to GST. GST-Ft fusion constructs were produced in BL21-DE3 bacteria and purified using glutathione-coupled beads. The C-terminus of Atro was synthesized \textit{in-vitro} using a transcription translation kit. The GST-Ft fusion proteins and Atro were incubated in a pulldown buffer overnight to allow for binding. Western blotting with an anti-Atro antibody was done on precipitated material to check for binding.

As previously observed, the C-terminus of Ft\textsuperscript{ICD} bound to Atro. However, I also detected an interaction between the N-terminus of Ft\textsuperscript{ICD} and Atro. In fact the binding of N-terminus of Ft\textsuperscript{ICD} seems to be consistently stronger, as compared to the C-terminus of Ft\textsuperscript{ICD} (Fig.12). Next, in order to narrow down the region of these interactions, I generated smaller constructs of Ft\textsuperscript{ICD} as shown in the schematic (Fig.11). The pull-down assay was performed with these truncated GST-Ft\textsuperscript{ICD} fusion proteins and \textit{in-vitro} translated Atro.
Fig 11. Schematic of Ft-GST constructs:
Above is the schematic representation of the deletion mutants for Ft intracellular domain that were cloned into pGEX-4T1 vector for making GST-Ft\textsuperscript{ICD} constructs for the pull-down assays. The constructs are numbered for convenience; the length of the arrows is not to scale.

Nucleotide position of Ft\textsuperscript{ICD} constructs:

Table 4.

<table>
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<th>Fragment</th>
<th>Amino acid positions of GST- Ft\textsuperscript{ICD} constructs</th>
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<tr>
<td>1</td>
<td>AA: 4916-5147, REFSEQ: NP_477497</td>
</tr>
<tr>
<td>2</td>
<td>AA: 4916-5015, REFSEQ: NP_477497</td>
</tr>
<tr>
<td>3</td>
<td>AA: 5016-5147, REFSEQ: NP_477497</td>
</tr>
<tr>
<td>4</td>
<td>AA: 4614-4922, REFSEQ: NP_477497</td>
</tr>
<tr>
<td>5</td>
<td>AA: 4614-4758, REFSEQ: NP_477497</td>
</tr>
<tr>
<td>6</td>
<td>AA: 4752-4922, REFSEQ: NP_477497</td>
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Western blot analysis was done with an anti-Atro antibody on the precipitated material as shown in Fig.13. No significant differences in the binding were detected between the truncated GST- FtICD-C terminal proteins to Atro. In the case of truncated GST-FtICD-N terminal proteins, the C-terminal end of the FtICD-N terminal (fragment 6) always showed stronger binding to Atro as shown in Fig.13. Since I could see non-specific binding of Atro with GST, I increased the stringency of the pull-down assay but this affected the overall binding, suggesting that the Atro and Ft interaction is weak. Thus another approach may be needed to properly dissect the Ft-Atro interaction.
Fig 12. Pull-down assay showing FtICD-GST binds Atro:

Pull-down assay was performed using *in-vitro* translated Atrophin C-terminal and pieces of GST-FtICD. Western blot analysis with anti-Atrophin antibody shows the amount of Atro pulled down by GST alone (Lane1), GST- N-terminal FtICD (Lane2, fragment No.4 of fig.11) and C-terminal of FtICD(Lane3, fragment No.1 of fig.11). Both GST- N-terminal FtICD (Lane2) and C-terminus of FtICD (Lane3) bind Atro. The GST- N-terminal FtICD has an apparent stronger affinity for Atro compared to the C-terminus of FtICD (Lane3). A weak band is detected for Atro in the GST alone pull-down, but the intensity of this band was comparatively much weaker than that detected with Fat fragments. *In-vitro* translated Atro-C terminal (input) and the unbound Atro-C terminal are shown in Lane 4 and Lane 5 respectively. Below the western blot is the ponceau staining showing the loading of the respective lanes.
Fig 13. The C-terminal fragment of Ft<sup>ICD</sup>-N terminal has stronger affinity for Atro:

Pull-down assay was performed by incubating in-vitro translated Atrophin and pieces of GST-Ft<sup>ICD</sup> to allow binding and performing a GST pull-down. Western blot analysis using anti-Atrophin antibody shows the amount of Atro pulled down by GST alone (Lane1), GST- C-terminal Ft<sup>ICD</sup> pieces (Lanes labeled 1-3) and GST-N-terminal Ft<sup>ICD</sup> pieces (Lanes labeled 4-6). GST-N-terminal Ft<sup>ICD</sup> and C-terminal Ft<sup>ICD</sup> (Lane labeled 1 and 4 respectively) binds to Atro and it seems that the GST- N-terminal Ft<sup>ICD</sup> has stronger affinity for Atro compared to C-terminal Ft<sup>ICD</sup> (Lane labeled 4) as shown before in Fig.12. In fact, it is the C-terminal end of GST- N-terminal Ft<sup>ICD</sup> that shows the stronger interaction (Lane labeled 6) compared to the N-terminal end (Lane labeled 5). GST alone too binds to Atro (Lane 1) but this binding is much weaker compared to amount of atro pulled down with intracellular GST-Ft<sup>ICD</sup>. The overall binding is weak and was only detected 11 out of my 19 attempts but we always see stronger affinity of Atro to N-terminal of Ft<sup>ICD</sup>. Below the western blot is the ponceau staining of the western blot showing the loading of the respective lanes. Below the ponceau staining is the schematic of the GST-Ft<sup>ICD</sup> constructs.
3.4 *four-jointed (fj) LacZ* 1.2kb is up-regulated in Ecdysone receptor RNAi clones

Fat and Atropin play an important role in PCP, but not much is known about how Fat regulates PCP. The only known downstream target of Ft and Atro in PCP is *four-jointed*. The *four-jointed* locus encodes a type II transmembrane protein with two putative cleavage sites for signal peptidases in its transmembrane domain. Four-jointed has therefore been proposed to act as a secreted signaling molecule. Recently, *fj* has been reported to function as a Ser/Thr kinase that phosphorylates substrates through its glycosyltransferase related sequence motif DNE (Asp/Asn/Glu) (Ishikawa et al., 2008). Fj is expressed in a dorsoventral gradient in the developing eye disc (Strutt et al., 2004; Zeidler et al., 1999). Loss of function clones of *fj* result in strong non-autonomous defects in ommatidial polarity, where inverted wild-type ommatidia are observed at the polar end of clones, as in the case of *ft* clones. *fj* is up-regulated in *fat* and *atro* clones (Simon, 2004; Yang et al., 2002). Since, *fj* is the only known target of *atro* and *ft*, Dr. Natasha Arbuzova, a postdoctoral fellow in our laboratory, carried out an enhancer analysis of *fj* using transgenic flies. To identify the sequence-specific transcription factors that regulate PCP in conjunction with Atro, Dr. Arbuzova further refined the enhancer response element of *fjlacZ* from 22kb to 1.2kb region. She was also able to see an up-regulation of *fjlacZ*1.2kb in *ft* and *atro* clones (unpublished data), similar to that reported for *fj lacZ* in *ft* and *atro* clones (Yang et al., 2002). After the enhancer analysis Dr. Arbuzova did an *in-silico* analysis of *fjLacZ*1.2kb sequence. In her *in silico* study of the *fj* 1.2kb region, she found a
putative binding site for the Ecdysone receptor (EcR). The steroid hormone ecdysone plays an important role in *Drosophila* metamorphosis with the help of EcR genes (Bender et al., 1997; Carney et al., 1997). EcR so far has not been reported to play a role in planar cell polarity. However, Ultraspiracle receptor (USP), which modulates EcR function by forming a heterodimer (Yao et al., 1992) has been reported to play a role in morphogenetic furrow movement and formation of photoreceptors (Zelhof et al., 1997).

To determine whether EcR has any role in *fj* regulation in the *Drosophila* eye I used the MARCM (Mosaic analysis using repressible cell marker) technique by labeling homozygous mutant cells. I generated clones of EcR where EcR was down-regulated with an EcR RNAi transgene under the UAS promoter. Knock down clones of EcR were generated using FLP-FRT system together with the Gal4-UAS system. The clones expressing EcR RNAi are marked by the expression of GFP (*hs flp; act Gal4 >> UAS EcR RNAi / UAS GFP*). Interestingly, I was able to see that *fjLacZ1.2kb* is up-regulated (Fig 14. B, B’) in the clones marked by GFP positive cells (Fig 14.A, A’). This up-regulation of *fjLacZ1.2kb* in EcR clones suggests that EcR regulates *fj* expression *in-vivo.*
Fig 14. Knock down of Ecdysone receptors up-regulates *Fj LacZ1.2kb*

In all panels, clones are marked by the presence of GFP (green) and *fjLacZ1.2kb* expression is marked in Red. (A, A’) Clones in which Ecdysone receptor is knocked down. (B, B’) Expression of *fjLacZ1.2kb* (Red). (C, C’) Overlay of A on B and A’on B’ respectively. The *fjLacZ1.2kb* expression is up-regulated (B, B’) in ecdysone receptor clones marked by the presence of GFP (A, A’). A, B and C are 60X confocal images whereas A’, B’, C’ are the magnified versions of the white boxes shown in A, B, C respectively.
Chapter 4

Discussion and Future Plans
Discussion and Future Plans:

*Drosophila* Ft, a large atypical member of the cadherin superfamily, was first identified by Mohr in 1923, and has been extensively studied for its function in growth and planar polarity (Bryant et al., 1988; Mahoney et al., 1991; Matakatsu and Blair, 2004; Rawls et al., 2002; Yang et al., 2002). However, most of these studies were based on genetic assays. To determine mechanistically how Fat functions in growth and PCP, it is necessary to obtain a biochemical understanding of Fat function. Preliminary data in our laboratory suggested that in addition to the full-length Fat, a smaller 110kDa form of Ft exists. Western blot analysis with a Ft antibody done on extracts from a variety of *ft* mutants by Dr. Clayton indeed demonstrated that Ft$^{FL}$ and the smaller 110 kDa form were absent in *ft* mutants. Since, Ft is highly conserved from flies to mammals (Tanoue and Takeichi, 2005) Dr. Saburi, a postdoctoral fellow in our laboratory validated the presence of truncated fat4 forms in mouse. She then fractionated mouse embryonic stem cells and found localization of Ft4 intracellular domain in the nuclear fraction of mouse ES cells. This preliminary data from our laboratory formed the basis of my work.

I was able to confirm the precursor-product relationship of Ft full length and the smaller 110kDa form, by performing pulse chase assays. I also conducted *in-vivo* and *in-vitro* experiments to check the subcellular localization of *Drosophila* Ft. My data from S2 cell fractionation assays shows that Ft$^{ICD}$ is localized at least partially in the nucleus. On the other hand, neither the full length nor the membrane tethered Ft could be detected in the nucleus. Ft is not normally expressed in S2 cells and I had transfected Ft in S2 cells to look at its localization. It is possible S2 cells do not make the enzymes responsible for Ft cleavage. Hence, we do not see it being localized to the nucleus.
Fractionation assays from fly tissue showed that full length Ft and truncation products are not localized abundantly in the nuclear fraction. This data was consistent with the fractionation assays performed on brain complexes and the eye discs where Ft was expressed constitutively. Yonit Tsatskis’s immunostained eye discs may explain the absence of Ft$^{ICD}$ from the nuclear fraction, where Ft$^{ICD}$ can be seen in the nucleus of only a subset of cells. Ft may be below the limit of the detection of the biochemical technique used in my experiments. However, it remains to be determined whether the nuclear localization of Ft$^{ICD}$ is functionally relevant. The functional relevance of Ft$^{ICD}$ *in-vivo* could be tested by seeing if Ft$^{ICD}$ can rescue *ft* mutants phenotypes such as overgrowth, loss of PCP and reduced crossvein spacing.

Yonit Tsatskis was able to detect Ft$^{ICD}$ in the nucleus of a subset of cells. However, *in silico* analysis of Ft$^{ICD}$ does not show a conserved nuclear localization signal. In order to narrow down the region of Ft$^{ICD}$ that is necessary for its nuclear localization, Yonit Tsatskis subdivided the Ft$^{ICD}$ construct that is localized to the nucleus. She then made transgenic flies and tested the constructs for nuclear localization by immunohistochemistry. She plans to use this strategy to narrow down the region that is responsible for nuclear localization of Ft$^{ICD}$. Once Yonit Tsatskis is able to narrow down the region responsible for the localization of Ft$^{ICD}$ to the nucleus, it will be interesting to determine whether *ft* mutants for the portion of Ft responsible for nuclear targeting have similar overgrowth, and PCP phenotype as *ft* mutants.

As discussed earlier, *ft* and *atro* mutants seem to share a number of phenotypes with PCP mutants. It was surprising to see they physically interact, since Ft is a membrane protein and Atro a transcriptional co-repressor (Fanto et al., 2003). I wanted to
characterize this interaction between the intracellular region of Ft and Atro, and started by making fusion protein constructs of smaller pieces of Ft\textsuperscript{ICD} and GST. The constructs made did not disrupt the highly conserved region of Ft\textsuperscript{ICD} (Fig1). My data from the pull-down experiments using Ft\textsuperscript{ICD}-GST fusion protein and \textit{in-vitro} translated Atro show that Atro not only binds to the C-terminal of Ft\textsuperscript{ICD}, as was previously reported, but it also binds to the N-terminal portion of Ft\textsuperscript{ICD}. I also see that the binding is stronger for the N-terminal of Ft\textsuperscript{ICD}, as compared to its C-terminal, for Atro. In fact preliminary data suggests that the binding of Atro is stronger at the N-terminal of Ft\textsuperscript{ICD}, a region closer to the transmembrane region of Ft\textsuperscript{ICD}. This suggests that Atro could have more than one binding site in the intracellular region of Fat.

Alignment of intracellular regions of \textit{Drosophila} Ft and mouse and human Ft4 show that they are conserved from flies to mammals (Fig1).

The binding of Atro and Ft-GST is weak in nature since only 11 out of 19 attempts was I able to detect an interaction between Ft and atro above the levels of my negative control (11/19). To further confirm an interaction between the two proteins the conditions of the pull-down could be modified by increasing the stringency through: 1) varying the duration of washes, 2) increasing the salt concentration and also adding more detergent and 3) changing the duration of binding. I have been using an \textit{in-vitro} translated Atro and GST-Ft constructs for my assays. An \textit{in-vitro} translated Ft combined to GST-Atro constructs may alternatively be employed for the pull-down assay. Alternative approaches, such as yeast two hybrid or co-immunoprecipitation from cells could also be employed to characterize the physical interaction between Ft\textsuperscript{ICD} and Atro.
Atro and ft clones show an up regulation of fJlacZ expression (Fanto et al., 2003; Yang et al., 2002) and recently fJ has been reported to functions as a kinase that phosphorylates Ser/Thr residues in the extracellular cadherin domain of Ft, suggesting Ft could be modified by fJ as it transits via Golgi (Ishikawa et al., 2008). Dr. Natasha Arbuzova, a postdoctoral fellow in our laboratory, carried out an enhancer analysis of fJlacZ using transgenic flies to study regulation of fJ. She made deletions of enhancer response elements of fJlacZ constructs and checked for the expression profile of these constructs. Constructs that recapitulate the fJ expression pattern were tested for regulation by Atro and Fat, in Atro and Ft clones as reported for fJlacZ (Yang et al., 2002). Dr. Arbuzova was able to narrow down the enhancer response element of fJlacZ from 24kb to 1.2Kb (fJlacZ1.2kb) and was also able to replicate the up-regulation of fJlacZ phenotype in atro and ft clones in fJlacZ1.2kb (unpublished data of Dr. Arbuzova). She carried out in-silico analysis on the 1.2kb enhancer response element of fJ and was able to find putative binding sites for the Ecdysone receptor. My data show that in Ecdysone receptor knock down clones we see an up regulation of fJlacZ1.2kb. The up-regulation of fJlacZ1.2kb is robust irrespective of the position of the clone in the eye disc. This suggests EcR may play a role in the regulation of fJ.

Three EcR isoforms – EcR-A, EcR-B1 and EcR-B2 – share a common C-terminal region that contains DNA-binding and ligand-binding domains, but differ at their N-terminal domain (Bender et al., 1997; Mangelsdorf and Evans, 1995; Talbot et al., 1993). Ecdysone signaling in Drosophila is controlled by a heteromeric receptor composed of the Ecdysone Receptor (EcR) and Ultraspiracle (USP) (Riddiford et al., 2000). Recently it has been shown that clones of EcR-B1 in the follicle cells of Drosophila result in loss of
polarity and integrity of follicle cells (Romani et al., 2008). It has also been reported that 
Usp clones lead to precocious differentiation and defects in precluster formation in eye 
dics (Zelhof et al., 1997). To further characterize the function of EcR and its role in 
\( fjLacZ1.2kb \) regulation, it will be interesting to analyze the regulation of \( fjLacZ1.2kb \) with 
different forms of EcR RNAi and monitor PCP defects up on EcR knockdown. Since EcR 
forms a dimer with USP, it might be worth testing if \( fjLacZ1.2kb \) is regulated by USP. 
One could also narrow down the region of \( fjLacZ1.2kb \) that responds to EcR.

My findings show Ft is processed and its intracellular domain is localized to the 
nucleus in S2 cells. I have also presented results suggesting Fat\(^{ICD} \) has more than one 
binding site for its only known intracellular binding partner, Atrophin, and finally I show 
that \( fjLacZ1.2kb \) is down-regulated by EcR. The majority of studies in Fat cadherins have 
been based on \textit{Drosophila} genetics; however, the results I have presented together with 
future studies will help in unraveling the molecular mechanism of Fat functions.
Fig 1. Alignment of the cytoplasmic domain of *Drosophila* Fat and mouse and human Ft4:

Alignment starts from 20 amino acid after the transmembrane domain of *Drosophila* Fat and mouse and human Fat4. Amino acids that is conserved in all the species is marked by asterix. One can see that there are two highly conserved domain near the transmembrane domain and one in the middle and one close to c-terminal end (Taken from Saburi et al 2008).
References:


