Regulation Of Long-Range Planar Cell Polarity By Fat-Dachsous Signaling

by

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Planar cell polarity (PCP) is the organization of cellular characteristics within the plane of a tissue. PCP manifests both structurally, as in the directionality of insect bristles or mammalian skin hair, or dynamically, as in vertebrate neurulation, gastrulation, and oriented cell division in the kidney. Two well-conserved pathways are known to regulate PCP in invertebrates and in vertebrates: the Frizzled/PCP pathway and the Fat-Dachsous (Ft-Ds) pathway. The latter consists of the cadherins Ft and Ds, along with the Golgi kinase Four-jointed (Fj) and the transcriptional co-repressor Atrophin (Atro). Ft and Ds can bind each other, suggesting a mechanism for signal transduction. Fj phosphorylates Ft and Ds, modulating their binding affinities for each other. Atro is proposed to link Ft-Ds signaling with downstream events in the nucleus during eye development. The details of Ft-Ds binding, and the consequences of their interactions with other members of the pathway are poorly understood.

In this work, I quantitatively analyzed Ft-Ds pathway mutant clones for their effects on ommatidial polarity in the Drosophila eye. My findings suggest that the Ft-Ds pathway regulates PCP independently of asymmetric cellular accumulation of Ft or Ds. I found that Atro has a
position-specific role in regulating polarity in the eye, that Fj dampens clonal polarity signals, and that asymmetric accumulation of the atypical myosin Dachs is not essential for production and propagation of a long-range PCP signal. My observations suggest that Ft and Ds interact to modulate a secondary signal that regulates long-range polarity, that signaling by the Ds intracellular domain is dependent on Ft, and that ommatidial fate specification is genetically separable from long-range signaling.
Acknowledgments

I thank my supervisor, Helen McNeill, for mentoring me with great patience and understanding, and for showing me how good science comes from a combination of being enthusiastic, careful, realistic, and imaginative. She tried her best to cure me of my habit of writing run-on sentences, and the many examples that doubtlessly exist in this thesis are entirely my fault.

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List of Abbreviations

aa    amino acid
AJ    adherens junction
A-P   anterio-posterior
apf   after puparium formation
atro  atrophin
Bac   bacterial artificial chromosome
BSA   bovine serum albumin
ccene1/2 cyclin E1/2
d    dachs
dco   discs overgrown
dgo   diego
diap1 Drosophila inhibitor of apoptosis protein 1
dm    Drosophila melanogaster
DMEM  Dulbecco’s modified Eagle’s medium
dpp   decapentaplegic
DPX   distyrene, plasticizer, xylene
ds    dachsous
dsh   dishevelled
<table>
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<th>Description</th>
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<tr>
<td>DSHB</td>
<td>Developmental Studies Hybridoma Bank</td>
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<tr>
<td>D-V</td>
<td>dorso-ventral</td>
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<td>ECD</td>
<td>extracellular domain</td>
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<tr>
<td>EDTA</td>
<td>ethylenediaminetetraacetic acid</td>
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<td>EGF</td>
<td>epidermal growth factor</td>
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<tr>
<td>EGFR</td>
<td>epidermal growth factor receptor</td>
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<tr>
<td>elav</td>
<td>embryonic lethal abnormal vision</td>
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<tr>
<td>EMS</td>
<td>ethyl methanesulfonate</td>
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<td>FBS</td>
<td>fetal bovine serum</td>
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<td>fj</td>
<td>four-jointed</td>
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<td>fmi</td>
<td>flamingo</td>
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<td>FRT</td>
<td>FLP recombination target</td>
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<td>ft</td>
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<td>fz</td>
<td>frizzled</td>
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<td>GAF</td>
<td>GAGA factor</td>
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<td>GFP</td>
<td>green fluorescent protein</td>
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<td>hh</td>
<td>hedgehog</td>
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<td>HM</td>
<td>Hippo motif</td>
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<td>hpo</td>
<td>hippo</td>
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<td>ICD</td>
<td>intracellular domain</td>
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<td>Abbreviation</td>
<td>Definition</td>
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<tr>
<td>JAK</td>
<td>Janus kinase</td>
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<td>kDa</td>
<td>kilodalton</td>
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<td>L3</td>
<td>longitudinal vein 3</td>
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<td>lft</td>
<td>lowfat</td>
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<tr>
<td>LIM</td>
<td>Lin11, Isl-1 and Mec-3</td>
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<tr>
<td>MARCM</td>
<td>mosaic analysis with a repressible cell marker</td>
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<td>mats</td>
<td>Mob as tumor suppressor</td>
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<td>MEF</td>
<td>mouse embryonic fibroblast</td>
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<tr>
<td>MF</td>
<td>morphogenetic furrow</td>
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<td>mm</td>
<td>Mus musculus</td>
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<td>mRNA</td>
<td>messenger RNA</td>
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<td>mupp1</td>
<td>Multi-PDZ domain protein 1</td>
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<td>naip5</td>
<td>NLR family, apoptosis inhibitory protein 5</td>
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<td>NCBI</td>
<td>National Center for Biotechnology Information</td>
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<tr>
<td>OCD</td>
<td>oriented cell division</td>
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<td>PBS</td>
<td>phosphate-buffered saline</td>
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<td>PBT</td>
<td>phosphate-buffered saline with Triton X-100</td>
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<td>PCP</td>
<td>planar cell polarity</td>
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<td>P-D</td>
<td>proximo-distal</td>
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<td>PFA</td>
<td>paraformaldehyde</td>
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<td>Term</td>
<td>Description</td>
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<td><em>pk</em></td>
<td><em>prickle</em></td>
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<td>RNA</td>
<td>ribonucleic acid</td>
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<td>RNAi</td>
<td>RNA interference</td>
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<td><em>sav</em></td>
<td><em>salvador</em></td>
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<td><em>scrib</em></td>
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<td><em>ser</em></td>
<td><em>serrate</em></td>
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<td>siRNA</td>
<td>small interfering RNA</td>
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<td><em>sple</em></td>
<td><em>spiny legs</em></td>
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<td>STAT</td>
<td>signal transducer and activator of transcription</td>
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<td><em>stbm</em></td>
<td><em>strabismus</em></td>
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<td><em>tead4</em></td>
<td><em>TEA domain family member 4</em></td>
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<td>TRiP</td>
<td>Transgenic RNAi Project</td>
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<td>UAS</td>
<td>upstream activation sequence</td>
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<td><em>vang</em></td>
<td><em>van gogh</em></td>
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<td>VDRC</td>
<td>Vienna Drosophila RNAi Center</td>
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<td><em>w</em></td>
<td><em>white</em></td>
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<td><em>wg</em></td>
<td><em>wingless</em></td>
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<td><em>warts</em></td>
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<tr>
<td><em>yap1</em></td>
<td><em>yes-associated protein 1</em></td>
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<td><em>yki</em></td>
<td><em>yorkie</em></td>
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Chapter 1

Introduction

This chapter is adapted from Sharma, P. and McNeill, H. (2013). Fat and Dachsous cadherins. 
1 Introduction

1.1 Abstract

Fat (Ft) and Dachsous (Ds) are very large, well-conserved cell adhesion molecules. They bind each other and have important, highly conserved roles in planar cell polarity (PCP) and growth control. PCP is defined as the directionally coordinated development of cellular structures or behavior. Cellular and tissue growth needs to be modulated in terms of rate and final size, and the Hippo pathway regulates growth in a variety of developmental contexts. Ft and Ds are important upstream regulators of these pathways. There are two Ft proteins in *Drosophila*, Fat and Fat2, and four in vertebrates, Fat1–4 (Figure 1). There is one Ds protein in *Drosophila* and two in vertebrates, Dachsous1–2. In this chapter, I discuss the roles of Fat and Ds family members, focusing on *Drosophila* and mouse development.

1.2 Fat and Dachsous cadherins

*Drosophila* Fat (Ft) is a very large (560 kDa) type-I transmembrane protein with 34 cadherin domains, 4 EGF-like domains, and 2 laminin G domains in the extracellular region, followed by the transmembrane and intracellular regions (Mahoney *et al.*, 1991; Rock *et al.*, 2005; Tanoue and Takeichi, 2005). The presence of 34 cadherin domains is characteristic of all members of the Fat family, although an alternate method of sequence alignment predicts 35 cadherin domains (Jin *et al.*, 2012). Mature Ft exists in a stable form that is cleaved N-terminal to the transmembrane region (Feng and Irvine, 2009; Sopko *et al.*, 2009).
Drosophila Dachsous (Ds) is another large (379 kDa) type-I transmembrane protein with 27 cadherin domains in the extracellular region, followed by the transmembrane and intracellular regions (Clark et al., 1995). Ds can be cleaved between cadherin repeats at two sites in its extracellular region (Ambegaonkar et al., 2012) (Figure 1).

Drosophila Fat2 (also known as Fat-like) is a homolog of Drosophila Fat (Castillejo-Lopez et al., 2004). Its intracellular region is, however, substantially different from that of Fat, and it has one laminin G domain in the extracellular region. There are four vertebrate Fat proteins. Fat4 is orthologous to Drosophila Fat and has two laminin G domains. Fat1, Fat2 and Fat3 are closer to Drosophila Fat2 and each have one laminin G domain (Rock et al., 2005). Like Drosophila Fat, Fat1 and Fat4 are also cleaved N-terminal to the transmembrane region (Sadeqzadeh et al., 2011; Sopko et al., 2009).

There are two vertebrate Dachsous homologs, Dachsous1 and Dachsous2 (Hong et al., 2004; Nakajima et al., 2001; Rock et al., 2005), each with 27 cadherin domains. The intracellular domains of Ds and its vertebrate homologs contain a putative β-catenin binding site (Rock et al., 2005).

fat (ft) and dachsous (ds), along with four-jointed (fj), which encodes a Golgi kinase, and dachs (d), which encodes an atypical myosin, were part of a group of genes described in the early days of Drosophila genetics as regulators of growth, limb development, and tissue patterning (Mohr, 1929; Stern and Bridges, 1926). ft is a hyperplastic tumor suppressor gene: in ft mutants, epithelial structure and cell differentiation are maintained even though the epithelium is greatly overgrown (Bryant et al., 1988). ds mutants have patterning defects similar to ft mutants (Clark et al., 1995). ft and ds are important in at least two major developmental processes: regulation of tissue growth through the Hippo pathway, and directional control of cellular morphology and behavior, called planar cell polarity (PCP) (reviewed in Goodrich and Strutt, 2011; Gray et al., 2011; Grusche et al., 2010; Halder and Johnson, 2011; Hergovich, 2012; Maung and Jenny,
2011; Saburi and McNeill, 2005; Sopko and McNeill, 2009; Staley and Irvine, 2012; Thomas and Strutt, 2012; Zhao et al., 2011).

This introduction focuses on the roles of Ft and Ds in regulating PCP and, to a lesser degree, Hippo signaling. However, Ft–Ds signaling is also involved in other processes, including neural differentiation (Dearborn and Kunes, 2004; Kawamori et al., 2011; Reddy et al., 2010) and degeneration (Napoletano et al., 2011), stem cell regulation (Karpowicz et al., 2010), cell–cell interactions (Hamaratoglu et al., 2009; Ishiuchi et al., 2009), cancer (Chosdol et al., 2009; Nakaya et al., 2007; Qi et al., 2009; Settakorn et al., 2005), and neurological disorders (Blair et al., 2006).
Figure 1. Structural representations of Fat and Dachsous homologs.

**A.** Phylogenetic relationships of Fat and Dachsous homologs of mouse (mm, *Mus musculus*) and fruit fly (dm, *Drosophila melanogaster*). Full-length protein sequences were aligned using Clustal Omega, and a Maximum Likelihood tree was constructed using MEGA 5. **B.** Structural representations of *Drosophila* Ft and Ds and their mouse homologs. Domain numbers and positions are based on Tanoue and Takeichi, 2005. Though mFat1 and mFat4 are known to be cleaved in their extracellular domains, their cleavage sites are not precisely known, and so they are not indicated.
1.3 Fat–Dachsous and PCP

1.3.1 Introduction to PCP

Many tissues display directionally organized cell structures or behaviors, which are called PCP (Bayly and Axelrod, 2011; Thomas and Strutt, 2012). Ft–Ds signaling and Frizzled/PCP (Fz/PCP) signaling are the main PCP pathways (Figure 2), regulating PCP in contexts as disparate as ommatidial orientation in the *Drosophila* eye and orientation of stereocilia in the mouse cochlea (Saburi *et al.*, 2008; Yang *et al.*, 2002). PCP is also evident in dynamic processes, such as oriented cell division in *Drosophila* imaginal discs (Baena-Lopez *et al.*, 2005) and cell intercalation during zebrafish neurulation (Ciruna *et al.*, 2006). Ft and Ds cadherins act together with Fj, D, and the transcriptional co-repressor Atrophin (Atro) to regulate PCP (Thomas and Strutt, 2012). Vertebrate orthologs of *Drosophila* ft and ds, such as *fat1*, *fat4*, and *dachsous1* also regulate PCP (Mao *et al.*, 2011a; Saburi *et al.*, 2008; Saburi *et al.*, 2012).

In *Drosophila*, Ft and Ds localize to the subapical region of epithelial cells, just apical to the adherens junction (AJ), and positively regulate each other’s membrane localization (Figure 3A-D) (Ma *et al.*, 2003; Silva *et al.*, 2006; Strutt and Strutt, 2002). Data from cell culture studies and from overexpression and mutant clones suggest that Ft and Ds form trans-heterodimers (Ma *et al.*, 2003; Matakatsu and Blair, 2004; Strutt and Strutt, 2002). Ft and Ds can also localize on opposite sides of a cell, along the polarity axis (Figure 3E) (Ambegaonkar *et al.*, 2012; Brittle *et al.*, 2012). Unlike classical cadherins, there is no evidence of homodimers of either Ft or Ds forming across cells (Brasch *et al.*, 2012; Matakatsu and Blair, 2004). Fj phosphorylates Ft and Ds in specific N-terminal cadherin domains (Ishikawa *et al.*, 2008) (Figure 1). This phosphorylation changes Ft and Ds binding affinities: binding of phosphorylated Ft to Ds increases, while binding of phosphorylated Ds to Ft decreases. *ds* and *fj* are expressed in complementary gradients in epithelial tissue (Brodsky and Steller, 1996; Clark *et al.*, 1995;
Villano and Katz, 1995; Yang et al., 2002; Zeidler et al., 1999), which is hypothesized to set up a gradient of Ft–Ds interaction across the tissue (Casal et al., 2006; Simon et al., 2010). Thus, different cells, and different sides of the same cell, would have different levels of Ft–Ds signaling. This could then lead to differential activity of Ft–Ds downstream effectors. For example, Atro activity is proposed to be higher in a cell with greater Ft–Ds activity compared to its neighbor (Fanto et al., 2003), and subcellular localization of D is regulated by Ft-Ds signaling (Ambegaonkar et al., 2012; Bosveld et al., 2012; Brittle et al., 2012; Mao et al., 2006; Willecke et al., 2008).

In sections 1.3.2 to 1.3.6, I discuss regulation of PCP by the Ft-Ds pathway in Drosophila, focusing on polarity in the eye, wing, and abdomen (Figure 4). In the remaining sections of this chapter, I discuss other roles of the Ft-Ds pathway. Key members of the Ft-Ds, Fz/PCP, and Hippo pathways are listed in Table 1.
Figure 2. *Drosophila* polarity-regulating pathways.

**A.** The Ft-Ds pathway: Ft and Ds in different cells can bind each other. Atro interacts with Ft, and also regulates transcriptional events in the nucleus, though a functional link between Atro binding to Ft and translocating to the nucleus has not been established. Atro represses fj transcription, and Fj phosphorylates specific sites in the Ft and Ds extracellular domains. Ft, Ds and Atro are likely to have other, as yet unknown targets to regulate polarity. **B.** The core Fz/PCP pathway: The atypical cadherin Flamingo (Fmi) binds the multipass transmembrane proteins Frizzled (Fz) and Van Gogh (Vang). These two complexes bind across cells and mediate each others’ intracellular localization. In the cytoplasm, Fz binds Disheveled (Dsh) and Diego (Dgo), and Van Gogh binds Prickle (Pk). A variety of cytoskeletal and nuclear effectors lie downstream of Fz/PCP signaling.
Figure 3. Ft and Ds localization.

**A-D.** Adapted from Ma et al., 2003. **A.** Wing disc with *ft* clone stained with α-Ds antibody. Ds is well localized at cell boundaries in wild-type tissue (lower part of picture) but delocalized in *ft* mutant tissue (upper part of picture). Ds is depleted from surface of mutant cells at the clone boundary (red arrows) and accumulates at the border with wild-type cells (yellow dots). **B.** Wing disc with *ds* clone stained with α-Ft antibody. Ft is well localized at cell boundaries in wild-type tissue (lower part of picture) but somewhat increased and relatively delocalized in *ds* mutant tissue (upper part of picture). Ft is depleted from surface of mutant cells at the clone boundary (red arrows) and accumulates at the border with wild-type cells (yellow dots). **C-D.** Wing discs with *ds ft* clones stained with α-Ds (C) and α-Ft (D) antibody. No accumulation of either protein occurs at clone borders (wild-type cells marked with yellow dots). **E-E’.** Adapted from Brittle et al., 2012. Eye disc expressing Ds-EGFP in patches (green), and stained with α-Arm (magenta). Cells without green signal are expressing untagged Ds. Anterior is left, equator is below. Ds-EGFP asymmetrically localizes to the equatorial side of the cell, and to a lesser degree to the posterior side of the cell.
A. Wing cuticle ridges

B. Wing hair

C. Eye ommatidia

D. Abdomen hair

Figure 4. Planar polarity in Drosophila epithelia.

**A.** Wing ridges: Folds in the wing cuticle, called ridges, are oriented along the A-P axis in the anterior wing, and along the P-D axis in the posterior wing. **B.** Wing hair: Each hexagonal wing cell produces an actin-rich projection called a hair that points distally. **C.** Ommatidia: Clusters of photoreceptors called ommatidia organize in trapezoidal shapes that point in opposite directions in the dorsal and ventral halves of the eye. **D.** Abdomen hair: The abdomen is made of repeating segments, and cells in each segment produce hair that point towards the posterior.
Table 1. Key members of the Ft-Ds, Fz/PCP, and Hippo pathways.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Ft-Ds pathway</strong></td>
<td></td>
</tr>
<tr>
<td><em>fat</em></td>
<td>atypical cadherin</td>
</tr>
<tr>
<td><em>dachous</em></td>
<td>atypical cadherin</td>
</tr>
<tr>
<td><em>four-jointed</em></td>
<td>Golgi-localized kinase</td>
</tr>
<tr>
<td><em>atrophin</em></td>
<td>transcriptional co-repressor</td>
</tr>
<tr>
<td><strong>Fz/PCP pathway</strong></td>
<td></td>
</tr>
<tr>
<td><em>frizzled</em></td>
<td>7-pass transmembrane receptor</td>
</tr>
<tr>
<td><em>van gogh</em></td>
<td>4-pass transmembrane protein</td>
</tr>
<tr>
<td><em>flamingo</em></td>
<td>7-pass atypical cadherin</td>
</tr>
<tr>
<td><em>disheveled</em></td>
<td>cytoplasmic protein, Fz-associated</td>
</tr>
<tr>
<td><em>diego</em></td>
<td>cytoplasmic protein, Fz-associated</td>
</tr>
<tr>
<td><em>prickle</em></td>
<td>cytoplasmic protein, Vang-associated</td>
</tr>
<tr>
<td><strong>Hippo pathway</strong></td>
<td></td>
</tr>
<tr>
<td><em>dachs</em></td>
<td>atypical myosin</td>
</tr>
<tr>
<td><em>discs overgrown</em></td>
<td>casein kinase I δ/ε</td>
</tr>
<tr>
<td><em>approximated</em></td>
<td>palmitoyltransferase</td>
</tr>
<tr>
<td><em>hippo</em></td>
<td>kinase</td>
</tr>
<tr>
<td><em>salvador</em></td>
<td>adaptor protein</td>
</tr>
<tr>
<td><em>warts</em></td>
<td>kinase</td>
</tr>
<tr>
<td><em>mats</em></td>
<td>adaptor protein</td>
</tr>
<tr>
<td><em>yorkie</em></td>
<td>transcriptional co-activator</td>
</tr>
<tr>
<td><em>scalloped</em></td>
<td>transcription factor</td>
</tr>
<tr>
<td><em>expanded</em></td>
<td>FERM-domain protein</td>
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</table>
1.3.2 The *Drosophila* eye and the Ft-Ds pathway

The *Drosophila* eye consists of ~800 clusters of photoreceptors called ommatidia. Ommatidia have a trapezoidal shape, and manifest PCP by consistently pointing upwards in the dorsal half of the eye, and downwards in the ventral half of the eye. The precise midline between the dorsal and ventral halves where ommatidial orientation abruptly switches is called the equator. By analogy, the dorsal and ventral extremes of the eye are called the poles. In either half, ommatidia normally point in the polar direction (Figures 5A,B).

Eye development in *Drosophila* initiates with a group of ~10 cells in the late embryo (Kumar, 2012). Eye tissue is specified by genes of the retinal determination network, some of which function in mammalian eye development as well, despite the evolutionary distance and structural and developmental dissimilarities between insect and mammalian eyes (reviewed in Tsachaki and Sprecher, 2012). Early dorsal-localized Wingless expression regulates selector genes of the Iroquois complex, which in turn set up a boundary of Fringe activity that results in Notch activation in a stripe along the dorso-ventral boundary, which establishes the equator (reviewed in Kumar, 2011).

Beginning with the third larval instar, a wave of apical constriction called the morphogenetic furrow (MF) initiates at the posterior midline and sweeps anteriorly across the eye disc. In the wake of the MF, previously undifferentiated epithelial cells start forming regularly spaced clusters and acquiring neural markers, indicating their transformation into photoreceptors. The formation of the MF and subsequent differentiation are a complex process, regulated by the JAK/STAT, Wingless, Dpp, Hedgehog, and Notch pathways (Kumar, 2011).
Each ommatidium consists of 8 photoreceptors, which are numbered according to their position. Photoreceptor 8 (R8) is the first to be specified following the MF (reviewed in Jenny, 2010). R2/5 and R3/4 are then specified as pairs. After another round of cell division, the remaining R1/6/7 cells are recruited, followed by ommatidial support cells. The R3/4 cells are initially identical, and ommatidial polarity is established through their specification: the photoreceptor closer to the equator is specified as R3, while the polar precursor is specified as photoreceptor R4. The ommatidium then rotates so that from the central axis of the ommatidium, rotation is towards R4. In this way, ommatidia in the dorsal and ventral halves of the eye acquire opposite polarities (Figure 5C). Ommatidial rotation is regulated by the Nemo kinase, and by classic cadherins: E-cadherin, which is present in all epithelial cells, promotes rotation, while N-cadherin, which is found specifically at the boundary between R3 and R4, inhibits rotation (reviewed in Tepass and Harris, 2007).

A key technique for analyzing planar polarity is analysis of clonal phenotypes. Clones are patches of cells mutant for a particular gene, which are surrounded by wild-type tissue. Mutant clones of PCP regulators can affect polarity in their genetically wild-type neighboring tissue (Gubb and Garcia-Bellido, 1982; Rawls et al., 2002; Yang et al., 2002). These are known as non-autonomous phenotypes. They suggest that the PCP regulator in question is involved not only in interpretation of PCP signals but also in long-range transmission of such signals, as the change in signal caused by a mutant clone is propagated across multiple cells.

*ft*, *ds*, and *fj* were first shown to act together to regulate PCP in the *Drosophila* eye and abdomen (Casal et al., 2002; Rawls et al., 2002; Strutt and Strutt, 2002; Yang et al., 2002; Zeidler et al., 1999). *ft* is expressed evenly in the developing eye, while *ds* and *fj* are expressed in complementary gradients: Ds levels are high near the poles, and Fj levels near the equator (Figure 5D). Importantly, PCP is disrupted both inside and outside clones with *ft* or *ds* mutations, and these disruptions can be quantified (Figure 5E).
The Ft-Ds pathway was found to regulate the R3/4 specification event that establishes ommatidial polarity. Loss of ft activity in one precursor specifies it as R4, while loss of ds specifies it as R3, regardless of position (Yang et al., 2002). ds ft double mutant precursors are specified as R4, suggesting that ft is epistatic to ds in this process, and that ds negatively regulates ft. If the polar precursor is misspecified as R3, or the equatorial precursor as R4, ommatidial polarity is reversed. If ft or ds are lost in both precursors, ommatidial polarity is randomized. While ds can specify photoreceptors in the absence of fj, fj cannot do so in the absence of ds, consistent with fj acting upstream. Phenotypes of Ft–Ds mutants outside mutant clones (non-autonomous phenotypes) are directionally specific: ft mutant clones disrupt wild-type polarity on the polar side, while ds mutant clones disrupt polarity on the equatorial side.
Figure 5. Planar polarity in the *Drosophila* eye.

**A.** An adult *Drosophila* eye. The dorso-ventral midline is the equator, and the opposite ends of the eye are the poles. **B.** An adult eye section. Dorsal ommatidia are marked with red arrows, and ventral ommatidia with green arrows. **C.** In the developing eye disc, ommatidia are recruited in clusters following passage of the morphogenetic furrow. After R3/4 fate specification, ommatidia rotate 90° towards R4 to acquire their final orientation. **D.** *fat (ft)* is expressed evenly throughout the eye disc, as is *atrophin (atro)*. *dachsous (ds)* is expressed highly at the poles, and less around the equator. *four-jointed (fj)* has the reverse gradient, with high levels around the equator and lower levels around the poles. **E.** Polarity disruptions can be quantified. For example, of the 10 ommatidia outside the polar border of this clone, 4 have reversed polarity, which is quantified as 40% reversal. Ommatidia that are dorsally oriented are represented as red arrows, and those that are ventrally oriented are represented as green arrows. Ommatidia in which both R3 and R4 are wild-type are represented as solid arrows, and all others are represented as hollow arrows.
Insect wing cells have actin-rich protrusions on the apical surface called hairs or trichomes. All wing hairs point in the distal direction. Loss of PCP signaling causes hair to misorient and form ‘swirling’ patterns, reflecting loss of long-range polarity, though short-range organization survives (Adler et al., 1998; Gubb and Garcia-Bellido, 1982). As in the eye, *ft* and *ds* mutant clones have both autonomous and non-autonomous polarity phenotypes. In particular, *ds* clones cause hairs outside the clone to point in toward it (most noticeable on the distal side of the clone), and *ft* clones cause hair outside the clone to point away from it (most noticeable on the proximal side of the clone) (Strutt and Strutt, 2002). Though wing PCP appears simple, its regulation is complex, with many different regional and temporal aspects. *ft* and *ds* clones show size dependence: small clones of *ds* and very small clones of *ft* have no polarity phenotype (Ma et al., 2003; Strutt and Strutt, 2002). *ft* clones affect hair polarity primarily in a central region roughly bounded by the anterior and posterior crossveins. *ds* clones, on the other hand, affect polarity throughout the wing (Strutt and Strutt, 2002). Although clonal polarity effects can occur in the distal wing, normal Ft-Ds signaling regulates hair polarity primarily in the proximal wing. This regulation occurs through controlling directed changes in cell shape, movement and oriented division, and microtubule dynamics upstream of Fz/PCP signaling (Aigouy et al., 2010; Harumoto et al., 2010; Hogan et al., 2011; Sagner et al., 2012).

The wing surface is folded in shallow peaks and troughs called cuticle ridges (Doyle et al., 2008; Hogan et al., 2011). These are also planar polarized: in the wing blade anterior to longitudinal vein 3 (L3), ridges are aligned along the antero-posterior (A-P) axis, and in the wing blade posterior to L3, ridges are aligned along the proximo-distal (P-D) axis. Ridge polarity in the posterior wing is established in an earlier phase of wing development, roughly prior to 18 hours after puparium formation (apf). From 18 to 32 hours apf, ridge polarity is established in the anterior wing, and this is also when hair polarity is established. In the early phase, *ds* and *fj* expression gradients that are symmetrical around L3 direct a form of Fz/PCP signaling that occurs through the Sple isoform of the LIM domain protein Prickle. In the late phase, Fz/PCP
signaling occurs through the Pk isoform of Prickle, and hair polarity is established in addition to anterior ridge polarity. In this phase, Fz/PCP signaling is directed by proximo-distal \( ds \) and \( fj \) gradients. As weak mutants and RNAi knockdowns of \( ft \), \( ds \), or \( fj \) disrupt ridge orientation to a greater degree than they affect hair polarity, the influence of Ft-Ds signaling on Fz/PCP in the wing is likely to be greater during the early phase than the late phase. In both phases, ridges are oriented orthogonally to the Fz/PCP signal, which is A-P oriented in the early phase and P-D oriented in the late phase.

Wing and eye polarity phenotypes of \( ft \) mutants can be partially rescued by loss of \( d \) (Mao et al., 2006). Asymmetric localization of D to the distal side serves as a marker of Ft-Ds polarity signaling. In the wing, clones that change Ft-Ds signaling, e.g. \( ds \) overexpression clones, repolarize the asymmetric distribution of D 4-5 cells away from the clone (Ambegaonkar et al., 2012; Brittle et al., 2012). This distance corresponds well with the degree of non-autonomy seen around \( ft \) and \( ds \) mutant clones. Ft and Ds are distributed asymmetrically in wing cells, with Ft localized to the proximal side of the cell, and Ds localized to the distal side, where it colocalizes with D (Ambegaonkar et al., 2012; Brittle et al., 2012). These data are consistent with the reduced localization of D on the side of greater Ft activity and with the propagation of this differential localization away from clone borders.

**1.3.4 Ft and Ds in the *Drosophila* abdomen and embryonic and larval epidermis**

The abdomen consists of repeated segments of anterior (A) and posterior (P) compartments, which maintain consistent hair and bristle polarity. \( ds \) and \( ft \) clones have directional non-autonomous polarity phenotypes that are the reverse of each other, though the direction depends on whether the clone is in the A or P compartment (Casal et al., 2002). Interestingly, \( ds ft \) double mutants have no clear non-autonomous polarity phenotype (Casal et al., 2006). Although \( ft \) is epistatic to \( ds \) in R3/4 fate specification in the eye (Yang et al., 2002), this seems not to be the
case in non-autonomous polarity regulation in the abdomen, as ds ft double mutant clones do not affect surrounding wild-type tissue.

The ventral epidermis of the *Drosophila* embryo and larva contains actin-rich cuticular projections called denticles, arrayed in six rows in each abdominal segment. The denticles of each row point either proximally or distally, thus manifesting PCP. Mutants of *ft* or *ds* have randomized polarity of denticle precursors in embryos (Marcinkevicius and Zallen, 2013), and denticles in larvae (Donoughe and DiNardo, 2011; Repiso *et al.*, 2010). Overexpression of Ds can repolarize denticles across multiple cells, suggesting that Ft-Ds signaling regulates long-range polarity in the larval epidermis (Donoughe and DiNardo, 2011; Repiso *et al.*, 2010). Interestingly, denticle polarity is not fixed at the beginning of the larval stages, but can instead be altered by changing Ds expression during larval development (Repiso *et al.*, 2010). In a process that seems to be distinct from denticle polarity establishment, Ft-Ds signaling also controls junctional remodeling in embryos, acting with members of the Hippo and Notch pathways (Marcinkevicius and Zallen, 2013). The Fz/PCP pathway has a minor, redundant role in controlling denticle polarity.

### 1.3.5 Ft and Ds in oriented cell division

Oriented cell division (OCD) refers to cell division in which the resultant daughter cells are aligned along a particular body axis. In combination with cell migration, OCD helps establish the shape of tissues and organs; for example, in *Drosophila* eyes and wings (Baena-Lopez *et al.*, 2005) and in vertebrate kidneys (Fischer *et al.*, 2006). The Ft-Ds pathway regulates OCD, as the orientation of spindle axes and subsequent mitosis is randomized in *Drosophila* mutants of the Ft-Ds pathway (Aigouy *et al.*, 2010; Li *et al.*, 2009; Sagner *et al.*, 2012). In mice, Ft-Ds signaling also regulates OCD in kidney development, and its loss leads to cystic kidneys (Mao *et al.*, 2011a; Saburi *et al.*, 2008; Saburi *et al.*, 2012). Recent results suggest that polarity signaling might not control spindle orientation directly but instead influence cell shape (Mao *et al.*, 2011b). Ft-Ds signaling localizes D to the distal side of wing cells, and D, acting as a myosin, is
proposed to induce tension at apical junctions along the proximo-distal (P-D) axis. This leads to elongation of cells along the P-D axis, and as mitotic spindles tend to orient along the long axis of cells (Théry et al., 2007), cell division, and consequently wing growth, occurs along the P-D axis. In the developing notum, D also acts with Ft-Ds signaling to establish junctional tension, but appears to regulate tissue shape through oriented cell rearrangements instead of oriented cell division (Bosveld et al., 2012).

1.3.6 Fat2 in the *Drosophila* ovary

The shape of the *Drosophila* oocyte is influenced by follicle cells of the ovary, which align their actin cytoskeletons perpendicular to the long axis of the ovary, thus forming a ‘molecular corset’, forcing the growing oocyte into a cylindrical shape. The cytoskeleton of follicle cells is planar polarized. Although mutations of *ft*, *ds* or Fz/PCP components do not affect this process, *fat2* mutants lose polarization of follicle cytoskeletons, and the resulting eggs are spherical. *fat2* mutant cells can also non-autonomously alter cytoskeletal polarity in genetically wild-type neighbors (Viktorinova et al., 2009). Interestingly, as long as less than 50% of cells are *fat2* mutants, the entire tissue can polarize correctly (Viktorinova et al., 2011).

1.3.7 Structure-function analysis of Ft-Ds and polarity regulation

Ft and Ds both have large extracellular domains (ECDs) with multiple cadherin repeats, and unique intracellular domains (ICDs) (Figure 1). In the *Drosophila* wing, membrane-bound versions of the Ft ICD and the Ds ECD are sufficient to rescue most aspects of growth control and PCP in *ft* or *ds* mutant backgrounds, respectively (Matakatsu and Blair, 2006). Overexpressing Ft ECD in a wild-type background results in a dominant-negative phenotype, resulting in overgrowth, similar to *ft* mutants (Matakatsu and Blair, 2006; Willecke et al., 2006). This indicates that the Ft ECD might interfere with normal *ft* function, perhaps forming
unproductive dimers with Ft or Ds. In the context of abdominal PCP, the Ft and Ds ECDs are more important for regulating polarity than the ICDs, as fusion proteins of the Ft ECD with the Ds ICD, or the Ds ECD with the Ft ICD behave like Ft ECD alone or Ds ECD alone, respectively (Casal et al., 2006).

Recent results suggest that the Ds ICD has signaling functions. In the eye, overexpression of Ds in a clone leads to increased activity of a fj-lacZ reporter around the clone boundary (Willecke et al., 2008). Expression of Ds ECD also has this effect. However, in a ds mutant background, overexpression of Ds in a clone leads to increased fj-lacZ reporter only inside the clone. The discontinuity at the clone border is thus detected only inside the clone, where ds is present. If the Ds ECD is expressed in a clone in the ds mutant background, no fj-lacZ reporter activity is observed. This suggests that while the Ds ECD is sufficient to create the boundary signal, the Ds ICD must be present to receive the signal (Willecke et al., 2008). The Ds ICD also has dominant-negative effects (Matakatsu and Blair, 2012).

Recent studies (Bossuyt et al., 2013; Matakatsu and Blair, 2012; Pan et al., 2013; Zhao et al., 2013) have conducted extensive structure-function analyses of Ft to identify regions important for Ft activity in different pathways (Figure 6). Matakatsu and Blair (Matakatsu and Blair, 2012) searched for the minimal regions required to rescue abdominal polarity and wing disc growth phenotypes of ft. They found that an 82-aa region of the Ft ICD just C-terminal to the transmembrane region was sufficient to mostly rescue polarity. They termed this region the PCP domain of the Ft ICD. They further found that two regions near the middle of the Ft ICD, Hippo N (62 aa) and Hippo C (82 aa), were sufficient to mostly rescue growth. A 41-aa region called the PCP-Hippo (PH) domain, located roughly between the PCP and Hippo N domains, had a minor role in regulating both polarity and growth. Bossuyt et al. (Bossuyt et al., 2013) found a 66-aa region termed the Hippo-interacting motif (HM) required for viability and imaginal disc growth regulation, that overlapped the Hippo N and Hippo C regions.
Pan et al. (Pan et al., 2013) examined growth and polarity changes in the adult wing. The authors found that a region of just 4 amino acids at the extreme C-terminus of Ft was important in regulating polarity. The region of Ft they found primarily responsible for growth regulation was also different and more C-terminal from those found by Matakatsu and Blair (Matakatsu and Blair, 2012). Zhao et al. (Zhao et al., 2013) looked at roles of Ft in regulating growth in the eye disc and polarity in the adult eye. They found that a 182 aa region relatively N-terminal in the Ft ICD was largely sufficient to rescue disc growth and lethality phenotypes of ft mutants. This region largely overlapped the regions found to be important for regulation of Hippo signaling by Ft in the work by Matakatsu and Blair, but was different from those presented in Pan et al. Intriguingly, Zhao et al. also found that the Ft ECD was important for regulating polarity in the eye, even though it appears largely dispensable for this purpose in the wing and abdomen (Matakatsu and Blair, 2006; Matakatsu and Blair, 2012).

What accounts for the discrepancies in the findings of these studies? It seems clear that different parts of Ft regulate PCP and growth in the eye, wing, and abdomen. Even within a tissue different parts have different requirements for signaling: a juxtamembrane region of the Ft ICD regulates hair polarity in the wing blade (Matakatsu and Blair, 2012), while the C-terminal region regulates hair polarity in the proximal costa (Pan et al., 2013). These differences could be due to the existence of varying downstream interactors in the different tissues. Importantly, while the Ft ECD is required for polarity regulation in the eye, it plays at most a minor role in regulating polarity in the wing and abdomen (Matakatsu and Blair, 2012), suggesting that Ft-Ds trans interaction is not required in all contexts. It should be noted that different methods of transgene expression were used in these studies. Bossuyt et al., Matakatsu et al. and Zhao et al. expressed transgenes with the Gal4-UAS system, with Bossuyt et al. and Matakatsu et al. using actin-gal4 or hh-gal4 as drivers, and Zhao et al. mainly using tub-gal4 as a driver. The amplification inherent in this system usually results in higher than endogenous levels of expression. This could result in researchers missing important sequences, as constructs without those sequences might still cause a degree of rescue, or conversely misidentifying sequences as negative-acting when their effects are instead caused by high protein levels. In contrast, Pan et al. used a genomic Bac clone that contained the whole ft gene and surrounding regulatory
regions. This would result in levels of Ft much closer to endogenous levels, and possibly more accurate identification of functional regions.

The binding sites of many Ft interactors such as Atrophin, Dco and Lowfat (Lft) are located near the C-terminus of Ft (Fanto et al., 2003; Feng and Irvine, 2009; Mao et al., 2009; Sopko et al., 2009). Surprisingly, a Ft construct (FtΔECDΔ6-C) lacking the binding sites for all these factors as well as the ECD was sufficient to rescue most abdominal polarity and wing disc growth phenotypes of ft mutant flies (Matakatsu and Blair, 2012). Similarly, many known interactor binding sites are dispensable for regulation of polarity by Ft in the eye (Zhao et al., 2013). Thus, when Ft constructs are overexpressed in these contexts, most of the known interactors of Ft are not needed for normal function. This, combined with the discovery that different parts of Ft are important in different contexts, implies the existence of a wide variety of as yet unknown Ft interactors.

In summation, both ECDs and ICDs of Ft and Ds have context-dependent roles in regulating polarity and growth control (Figure 6 and Table 2).
Figure 6. Functional regions of the Ft ICD.

Fat ICD domains

PCP (Matakatsu and Blair): 4520-4701
Growth (Zhao et al.): 4719-4895

PCP + Hippo (Matakatsu and Blair): 4734-4774
Hippo N (Matakatsu and Blair): 4775-4835

Hippo (Bossuyt et al.): 4834-4899
Hippo C (Matakatsu and Blair): 4839-4920

Atro binding (Fanto et al.): 4921-5069
Dco binding (Sopko et al.): 4945-4993

Growth (Pan et al.): 4975-4993
Ltt binding (Mao et al.): 5049-5147

Fat ICD sequence

PCP (Matakatsu and Blair): 4820-4701
YRFGKQKVKGSLASVVGVGLPPFVTGQVYDEVLVERL_WARNINGS

Growth (Zhao et al.): 4716-4896
RSP + Hippo (Matakatsu and Blair): 4734-4774

Hippo N (Matakatsu and Blair): 4775-4936

Hippo (Bossuyt et al.): 4834-4899
Hippo C (Matakatsu and Blair): 4839-4920

Atro binding (Fanto et al.): 4921-5069
Dco binding (Sopko et al.): 4945-4993

Growth (Pan et al.): 4975-4993
Ltt binding (Mao et al.): 5049-5147

Lowfat binding (Mao et al.): 5049-5147

PCP (Pan et al.): 5144-5147
Figure 6. Functional regions of the Ft ICD.

Functional regions of the Ft intracellular domain, as defined by structure-function studies and physical binding assays, are indicated. Numbering is based on full-length Ft (NCBI Reference Sequence: NP_477497.1). References: Matakatsu and Blair, 2012; Zhao et al., 2013; Bossuyt et al., 2013; Sopko et al., 2009; Pan et al., 2013; Fanto et al., 2003; Mao et al., 2009.
Table 2. Roles of Ft and Ds extracellular and intracellular domains.

<table>
<thead>
<tr>
<th>Domain</th>
<th>Function</th>
<th>Ft ECD</th>
<th>Ft ICD</th>
<th>Ds ECD</th>
<th>Ds ICD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Eye PCP</td>
<td>Required</td>
<td>Not tested</td>
<td>Not required</td>
<td>Dominant-negative</td>
<td></td>
</tr>
<tr>
<td>Eye growth</td>
<td>Not required</td>
<td>Required</td>
<td>Boundary effect on reporters</td>
<td>Required to receive boundary effect</td>
<td></td>
</tr>
<tr>
<td>Wing hair PCP</td>
<td>Minor role in most of the wing, but required in proximal regions</td>
<td>Required</td>
<td>Required</td>
<td>Not required</td>
<td></td>
</tr>
<tr>
<td>Wing growth</td>
<td>Not required</td>
<td>Required</td>
<td>Boundary effect on reporters</td>
<td>Dominant-negative effect on reporters</td>
<td></td>
</tr>
<tr>
<td>Abdomen PCP</td>
<td>Minor role</td>
<td>Required</td>
<td>Required for non-autonomous effect</td>
<td>Not required for non-autonomous effect</td>
<td></td>
</tr>
<tr>
<td>Abdomen growth</td>
<td>Not required</td>
<td>Required</td>
<td>Not tested</td>
<td>Not tested</td>
<td></td>
</tr>
</tbody>
</table>
1.3.8 Interaction between Ft and Ds and polarity regulation

Cells in culture that are singly transfected with Ft or Ds are unable to adhere to each other, but cells that are co-transfected with both, or that are singly transfected and then mixed, do adhere, showing that Ft and Ds can mediate heterophilic interactions between cells (Matakatsu and Blair, 2004). In imaginal discs, Ft and Ds promote each other’s membrane localization; loss of one leads to diffuse localization of the other (Strutt et al., 2002). Ds in wild-type cells immediately surrounding a ft mutant clone redistributes to the side of the cell away from the clone, i.e. where Ft is available for binding; the reverse also occurs (Ma et al., 2003). Ft molecules can also bind each other in cis, and Ds promotes this clustering (Sopko et al., 2009). In the wing, Ft localizes to the proximal sides of cells, while Ds is on the distal sides, suggesting that binding across cells promotes their asymmetric localization (Ambegaonkar et al., 2012; Brittle et al., 2012). In the mouse brain, loss of fat4 reduces membrane localization of Ds1, and vice versa, consistent with the data from flies (Ishiuchi et al., 2009). In contrast, Fat4 and Ds1 negatively regulate each other’s levels in the lung and kidney (Mao et al., 2011a). Thus, Ft and Ds interact with each other across cell boundaries, and in most contexts promote each other’s membrane localization.

Fj phosphorylates specific cadherin repeats in the Ft and Ds ECDs (Figure 1) (Ishikawa et al., 2008; Strutt et al., 2004). Biochemical and cell culture experiments show that phosphorylation by Fj increases the binding affinity of Ft for Ds, while decreasing the binding affinity of Ds for Ft (Brittle et al., 2010; Simon et al., 2010). The change in distribution of Ft and Ds around fj mutant or overexpression clones is consistent with these data. For example, loss of fj in a clone causes the Ds inside the clone to bind Ft with higher affinity, while the Ft inside the clone binds Ds with lower affinity; the Ds immediately inside the clone preferentially localizes to the clone border, where it can bind ‘higher-affinity’ Ft in the wild-type cells (Ma et al., 2003; Strutt and Strutt, 2002).

While ft is expressed ubiquitously in imaginal discs, ds and fj are expressed in complementary
gradients. $ds$ expression is high at the poles and low near the equator in the eye; high proximally and low distally in the wing; and high at the anterior-posterior compartment boundary and low at the segment boundary in abdominal segments. In each case, $fj$ expression is in the reverse gradient. In the eye, these gradients are established by Wingless, JAK/STAT, and Notch signaling (Yang et al., 2002; Zeidler et al., 1999), in the wing by Wingless and Dpp signaling (Cho and Irvine, 2004), and in the abdomen by Wingless and Hedgehog signaling (Casal et al., 2002; Lawrence et al., 2002). As $Ds$ binds $Ft$, and $Fj$ modulates $Ds$ and $Ft$ binding, the expression gradients of $ds$ and $fj$ combine to create a gradient of $Ft$-$Ds$ binding across the tissue. This modulates downstream signaling through $Ft$ and $Ds$, leading to asymmetric distribution of $Ft$, $Ds$, and $D$ in the wing (Ambegaonkar et al., 2012; Brittle et al., 2012; Mao et al., 2006). Indeed, the gradients act redundantly, as either one is sufficient to establish polarity. This is evident in the lack of polarity phenotype in $fj$ mutant flies (Zeidler et al., 1999), or when the expression gradients of either $fj$ or $ds$ are flattened (Simon, 2004). Only when both gradients are abolished, or when a sharp discontinuity is created through clones, is polarity disrupted. Interestingly, loss of $fj$ strengthens non-autonomous phenotypes of overexpressing $ft$ or $ds$ (Casal et al., 2006), suggesting that the presence of the $fj$ gradient helps suppress the non-autonomous effects of clones.

1.3.9 Interaction of $Ft$-$Ds$ signaling with $Fz$/PCP

The $Fz$/PCP pathway consists of the transmembrane proteins Frizzled ($Fz$), Van Gogh ($Vang$, also known as Strabismus, $Stbm$), and Flamingo ($Fmi$, also known as Starry night, $Stan$), as well as the cytoplasmic proteins Dishevelled ($Dsh$), Diego ($Dgo$), and Prickle ($Pk$). Current models (Bayly and Axelrod, 2011; Maung and Jenny, 2011; Struhl et al., 2012) for $Fz$/PCP function involve the formation of one complex by $Fz$, $Dsh$ and $Dgo$, and another complex by $Vang$ and $Pk$. $Fmi$ is part of both complexes. Each complex is localized to the apical cell membrane, but on opposite sides of the cell. For example, in the wing $Fz$/Dsh/Dgo is at distal boundaries whereas $Vang$/Pk is at the proximal borders. Each complex inhibits the other in its own cell, but recruits the other in the neighboring cell. This leads to establishment of polarity at the cellular level, and
also enables polarity information to propagate between cells, as seen by the observation that loss of one factor changes localization of its binding partner in the neighboring cell. The non-
autonomous phenotypes of Fz/PCP mutants result from propagation of this effect across several rows of cells (Chen et al., 2008; Strutt et al., 2011; Wu and Mlodzik, 2008). In the eye, Fz/PCP signaling biases Delta-Notch signaling to establish R3/4 fate (Cooper and Bray, 1999; Fanto and Mlodzik, 1999; Strutt et al., 2002; Tomlinson and Struhl, 1999).

How do the Ft-Ds and Fz/PCP pathways interact with each other? Fz/PCP signaling leads to asymmetric localization of its components. In the Drosophila eye disc, asymmetric localization of Fmi is maintained in ft mutant tissue, though its directional orientation is disrupted (Yang et al., 2002). Furthermore, mosaic analysis suggests that biasing of R3/4 fate specification by ft does not occur in tissue mutant for fz. These data suggest that in the eye, Ft-Ds signaling acts by biasing Fz/PCP asymmetry. However, other data suggest that Ds and Fz act in parallel during the early stages of polarity establishment in the eye. In particular, ds fz double mutant clones can cause non-autonomous polarity changes on both the polar side of the clone (as with fz mutants) and the equatorial side (as with ds mutants) (Strutt and Strutt, 2002), which would not be the case if fz was strictly downstream of ds.

The interaction between the two pathways also varies by tissue. In the Drosophila wing, Ft-Ds signaling is mostly upstream of Fz/PCP activity (Adler et al., 1998; Aigouy et al., 2010; Harumoto et al., 2010; Hogan et al., 2011; Ma et al., 2003; Matakatsu and Blair, 2004; Sagner et al., 2012). In the abdomen Ft-Ds and Fz/PCP act in parallel (Casal et al., 2006), with each pathway able to regulate polarity while the other is disrupted. In mice, loss of one copy of vangl2 enhances fat4 mutant phenotypes, suggesting that the two pathways have partially redundant functions in vertebrates (Saburi et al., 2008; Saburi et al., 2012).
1.4 Fat-Dachsous, growth control, and the Hippo pathway

1.4.1 Regulation of Hippo signaling by the Fat-Dachsous pathway

Proper development relies on tissues growing at an appropriate rate, and ceasing growth when they have reached the required size. A well-conserved kinase cascade signaling pathway called the Hippo pathway regulates this process (Figure 7) (Bao et al., 2011; Grusche et al., 2010; Halder and Johnson, 2011; Staley and Irvine, 2012). In Drosophila, the core of the Hippo pathway consists of the Ser/Thr kinases Warts (Wts) and Hippo (Hpo), and the adaptor proteins Salvador (Sav) and Mob as tumor suppressor (Mats). Signaling by the Hippo pathway results in phosphorylation of the transcriptional co-activator Yorkie (Yki), which promotes its binding to the 14-3-3 protein. 14-3-3 is localized in the cytoplasm, and so Hippo activity restricts Yki to the cytoplasm. In the absence of Hippo signaling, Yki is free to move to the nucleus. Transcriptional activation is based on targeted chromatin remodeling and recruitment of transcriptional machinery. Yki physically and genetically interacts with Moira, a subunit of the chromatin-modifying Brahma complex; with members of the Mediator complex, which links transcription factors and RNA polymerase; and with GAGMA factor (GAF), which is involved in both transcriptional machinery assembly and chromatin remodeling (Oh et al., 2013). In the nucleus Yki, acting with the transcription factor Scalloped (Sd), regulates the transcription of a variety of proliferation and anti-apoptosis effectors such as cyclinE and Diap1, and general growth promoters such as myc and wingless (Oh and Irvine, 2010).

Experiments in Drosophila showed that ft is an upstream activator of Hippo signaling (Bennett and Harvey, 2006; Cho et al., 2006; Silva et al., 2006; Willecke et al., 2006), and that ds, fj and d also regulate Hippo signaling (Cho and Irvine, 2004; Mao et al., 2006; Rogulja et al., 2008; Willecke et al., 2008). ft mutants phenocopy Hippo pathway mutants in causing imaginal disc overgrowth, and in upregulating growth promoting factors such as the cell cycle regulator Cyclin E and anti-apoptotic factors such as Diap1. Ft has complex interactions with another upstream
regulator of Hippo signaling, the protein Expanded (Ex), which is localized at the sub-apical membrane. *ft* and *ex* mutants phenocopy each other in their effects on growth and Hippo pathway targets, *ft* appears to regulate Ex membrane localization, and *ft* and *ex* single mutants and *ft ex* double mutants have a quantitatively similar increase in interommatidial cells in eye discs, leading to the suggestion that they act linearly to regulate Hippo signaling (Bennett and Harvey, 2006; Silva et al., 2006; Willecke et al., 2006). However, later work found that the *ft ex* double mutant phenotype is additive for clone size or overall tissue size, and that changes in Ex localization in *ft* mutants could occur as a consequence of cell shape change (Feng and Irvine, 2007), suggesting that *ft* and *ex* act in parallel. Ft is instead proposed to primarily promote Hippo signaling by inhibiting Dachs which in turn binds to and negatively regulates Wts levels (Cho et al., 2006).

Discs overgrown (Dco) is *Drosophila* casein kinase I δ/ε (Zilian et al., 1999), and it promotes Ft activity in the Hippo pathway. Dco binds and phosphorylates the C-terminus of Ft (Feng and Irvine, 2009; Sopko et al., 2009). This interaction is positively regulated by Ds, possibly through promoting formation of Ft homodimer clusters. Null mutants of *dco* have impaired growth of imaginal discs, reflecting its importance in multiple pathways. However, a hypomorphic allele of *dco*, *dco*3, which cannot phosphorylate Ft, has growth phenotypes similar to *ft* mutants, and upregulates the same Hippo target genes (Cho et al., 2006; Zilian et al., 1999). These effects can be rescued by overexpressing *wts* (Feng and Irvine, 2009).

Ft regulates Hippo signaling through D (Cho et al., 2006; Feng and Irvine, 2009; Mao et al., 2006), which negatively regulates *wts* levels genetically downstream of *ft* and *dco*. Gradients of *ds* and *ff* can modulate Hippo activity through Ft and D, and flattening both *ds* and *ff* gradients results in reduced growth (Rogulja et al., 2008; Willecke et al., 2008). Expression of Hippo pathway reporters is increased at boundaries of *ds* or *ff* clones, and *d* and *ft* are required for these effects.
Figure 7. A simplified version of the *Drosophila* Hippo pathway, focusing on the role of Ft.

Ft inhibits the atypical myosin Dachs, which in turn inhibits the kinase Warts (Wts). The palmitoyltransferase Approximated (App) promotes Dachs membrane localization. The kinase Discs overgrown (Dco) promotes Ft activity by phosphorylating the Ft ICD. Wts acts with the kinase Hippo (Hpo) and the adaptor proteins Salvador (Sav) and Mobs as tumor suppressor (Mats) to inhibit the transcriptional co-activator Yorkie (Yki). Expanded (Ex) inhibits Yki both directly and by acting on the Hpo kinase complex. Yki acts with the transcription factor Scalloped (Sd) to promote transcription of growth promoters such as *cyclinE* (*cycE*) and *diap1*. 
1.4.2 Neurodegeneration in ft and Hippo-pathway mutants

Loss of ft or overexpression of Atro in Drosophila eyes leads to degeneration of ommatidia through aberrant Hippo-mediated autophagy (Napoletano et al., 2011). This neurodegeneration can be reduced by loss of d or yki, while loss of wts or sav causes degeneration. atro and Hippo signaling both regulate autophagy (Dutta and Baehrecke, 2008; Nisoli et al., 2010), and degenerating atro-overexpressing or ft mutant eyes in show an increased number of autophagic vesicles.

1.5 Other members of the Ft-Ds pathway: Dachs, Atrophin, and Lowfat

1.5.1 Dachs

The unconventional myosin dachs (d) suppresses ft phenotypes in polarity and growth control (Cho and Irvine, 2004; Cho et al., 2006; Mao et al., 2006). ft positively regulates wts levels while d negatively regulates wts levels, and d physically interacts with wts (Cho et al., 2006). These data suggest that ft negatively regulates d, which in turn negatively regulates wts. This is supported by genetic epistasis studies placing d downstream of ft, ds, and dco, but upstream of wts (Cho et al., 2006; Feng and Irvine, 2007; Feng and Irvine, 2009; Mao et al., 2006; Willecke et al., 2008). Thus, d is proposed to repress wts activity and promote growth, unless this repression is suppressed by ft activity.
D colocalizes and physically interacts with Ds (Bosveld et al., 2012). Membrane localization of D is affected by changes in Ft-Ds signaling (Mao et al., 2006), and asymmetric localization of D serves as a readout of Ft-Ds gradient activity (Ambegaonkar et al., 2012; Bosveld et al., 2012; Brittle et al., 2012). D membrane localization is also influenced by approximated, a palmitoyltransferase with unknown targets that genetically interacts with ft (Matakatsu and Blair, 2004; Matakatsu and Blair, 2008). Loss of d partially rescues polarity inversions in ft and ds mutant abdomen and eyes (Brittle et al., 2012; Mao et al., 2006).

D is also involved in cross-talk between PCP and Hippo signaling. Overexpression of wts in ft mutant eyes and wings can partially rescue ft mutant polarity defects (Brittle et al., 2012; Feng and Irvine, 2007), and overexpression of yki can reverse polarity rescue in ft d double mutant eyes (Brittle et al., 2012). This suggests that regulation of yki activity by ft through d and wts influences planar polarity. How might this occur? Hippo signaling affects fj expression (Cho et al., 2006; Willecke et al., 2006), so a feedback loop might exist wherein changes in Hippo signaling alter Fj levels, which in turn modulates Ft and Ds binding to effect polarity. Rescue of ft and ds polarity phenotypes by d is incomplete, and ds overexpression can repolarize ommatidia in the absence of d (Brittle et al., 2012). Ft-Ds signaling thus likely also regulates polarity independently of d.

1.5.2 Atrophin

Another Ft-Ds PCP effector is the transcriptional co-repressor atrophin (atro). atro mutants have defects in multiple developmental pathways, including eye and wing polarity (Erkner et al., 2002; Zhang et al., 2002). atro phenocopies ft in regulating eye polarity, including non-autonomous polarity disruption on the polar side of mutant clones (Fanto et al., 2003), and in orienting cell division in response to cell death (Li et al., 2009). Atro physically interacts with the C-terminus of Ft, and, like ft, atro biases R3/4 fate specification towards the R3 fate. atro and ft mutants both upregulate expression of the fj-lacZ reporter. Atro protein is known to localize to
the nucleus (Erkner et al., 2002; Zhang et al., 2002). Thus, atro could be a d-independent Ft-Ds effector, transducing Ft-Ds signals to the nucleus.

1.5.3 Lowfat

lowfat (lft) is a conserved gene that shows only very mild polarity and growth phenotypes on its own, but genetically interacts with ft and ds in these processes (Mao et al., 2009). It also physically binds Ft and Ds, and post-transcriptionally promotes their protein levels without affecting their mRNA levels.

1.6 Ft-Ds signaling and vertebrate development

1.6.1 Vertebrate Ft

Vertebrate Fat homologs are involved in a variety of developmental processes, some not obviously linked to PCP. Fat1 binds actin cytoskeleton regulators Mena and Vasp, and influences cell-cell contacts and cytoskeletal polarity in cell culture (Moeller et al., 2004; Tanoue and Takeichi, 2004). fat1 mutant mice die soon after birth and do not form proper glomerular slit junctions in kidneys (Ciani et al., 2003). In the developing retina, interneurons called amacrine cells lose their normal unipolar morphology in fat3 mutant animals, and instead acquire multipolar morphology (Deans et al., 2011). fat3 genetically interacts with the vertebrate fj ortholog fjx1 in regulating this process.
fat4 mutant mice have extensive developmental defects and usually die at birth (Mao et al., 2011a; Saburi et al., 2008; Saburi et al., 2012). They have curly tails, small kidneys, lungs and intestines, and broader neural tubes and cochleae. Parts of their skeleton are wider and shorter. The growth and orientation of hair cells in the cochleae is disrupted. Their kidneys are cystic and show reduced ureteric epithelium branching and defects in oriented cell division. Their hearts have atrial septation defects. Mouse fat4 genetically interacts with other members of the Ft-Ds pathway in these processes (Saburi et al., 2008; Saburi et al., 2012). Partial loss of fat1 aggravates kidney cysts, cochlear widening and hair cell polarity changes of fat4 mutants. fat1 fat4 double mutant mice display exencephaly, a cranial neural tube morphogenesis defect. Partial loss of atn2l, the vertebrate ortholog of atro, aggravates kidney cysts and hair cell phenotypes of fat4 mutants. Loss of fat4 increases fjx1 expression, as is the case for Drosophila ft and fj.

fat4 also interacts with other pathways. Partial loss of vangl2, a member of the Fz/PCP pathway, aggravates kidney cysts, cochlear widening, and hair cell polarity changes of fat4 mutant mice (Saburi et al., 2008; Saburi et al., 2012). In the developing mouse cerebral cortex, Fat4 binds MUPP1 and Pals1, vertebrate homologs of Drosophila Patj and Stardust respectively. MUPP1 and Pals1 regulate apical cell membrane organization, and knocking down Fat4 disrupts apical architecture (Ishiuchi et al., 2009).

1.6.2 Vertebrate Ds

Loss of mouse Dachsous1 (dchs1) phenocopies loss of fat4 (Mao et al., 2011a). dchs1 mutant mice mostly die at birth with curly tails, small kidneys, lungs and intestines, broader neural tubes and cochleae, defects in their skeleton, abnormalities in some cochlear hair cells, and cystic kidneys. These phenotypes are similar to or slightly weaker than those of fat4 mutants. Notably, they are not stronger in fat4 dchs1 double mutants, suggesting that fat4 and dchs1 act in the same pathway, and that they interact the same way Drosophila ft and ds do.
1.6.3 Ft and Hippo signaling in vertebrates

Although Hippo signaling regulates organ size in vertebrates (Camargo et al., 2007; Dong et al., 2007; Heallen et al., 2011), there is so far no clear link between Ft-Ds and Hippo mammalian orthologs (Bossuyt et al., 2013). However, Ft and Hippo signaling interact in other vertebrates. In the chick neural tube, knockdown of fat4 increases proliferation in dp4-vp1 neural progenitor cells, which in turn differentiate into an increased number of dl4-v1 interneurons. Knockdown of fat4 results in decreased phosphorylation of the Yki vertebrate homolog Yap1, and knockdown of yap1 along with the sd vertebrate homolog tead4 rescues the increase in interneurons caused by fat4 knockdown (Van Hateren et al., 2011), indicating functional and possibly mechanistic conservation of the role of ft as a growth regulator. This role of fat4 in chick suggests that the broadened spinal cords in fat4 mutant mice (Mao et al., 2011a; Saburi et al., 2012) might also be caused by an increase in neural progenitors.

In zebrafish, knockdown of fat1 causes a cystic pronephros phenotype (Skouloudaki et al., 2009). Loss of fat4 in mice causes cystic kidneys (Saburi et al., 2008), and this effect is enhanced by removal of one copy of fat1 (Saburi et al., 2012), suggesting a generally conserved role for ft homologs in vertebrate excretory organ development. The fat1 knockdown phenotype in zebrafish is rescued by knockdown of yap1. Intriguingly, zebrafish Fat1 binds the adapter protein Scribbled (Scrib), and knockdown of scrib strengthened the effect of fat1 knockdown on pronephros cyst formation. As scrib links ft and the Hippo pathway in Drosophila (Doggett et al., 2011; Verghese et al., 2012), the link between Ft and Hippo signaling appears conserved in this context as well.
1.7 Questions in Ft-Ds signaling

Fat and Dachsous are critical for a variety of developmental processes in vertebrates and invertebrates. Ft-Ds signaling provides directional information to establish PCP and modulates Hippo signaling for appropriate growth control. However, important aspects of this pathway are still not understood. Does long range transmission of polarity information downstream of Ft-Ds signaling occur by a secreted signaling molecule or by changes in distribution of Ft, Ds, and D? What are the epistatic relationships between members of the Ft-Ds pathway? In which contexts are the enormous Ft and Ds extracellular domains functionally significant? What role do effectors such as D and Atro play in generating and transmitting the polarity signal? Below, I describe how I address these questions in my thesis.

1.8 Thesis overview

My research focuses on the roles of ft and ds and their interactors in regulating planar cell polarity, using the Drosophila eye as a model. Although their roles in polarity have been previously examined, a systematic in-depth quantitative study of clonal polarity phenotypes has not been previously performed for any pathway. In chapter 2 I present the results of quantitative analysis of polarity disruptions inside and outside mutant or overexpression clones of various members of the Ft-Ds pathway in the Drosophila eye. This analysis illuminates many aspects of polarity that were not evident in previous qualitative observations, and that are contrary to prevalent models of Ft-Ds signaling. Specifically, I show that polarity can be established in mutant tissue inside clones lacking Ft, Ds, or both, in contradiction to a model based on asymmetric protein localization of Ft or Ds constituting the polarity signal. Furthermore, I show that this polarity establishment inside clones is guided by polarity information transmitted from the clone borders, suggesting that Ft or Ds accumulation at clone borders generates a long-range secondary polarity signal. I find that the transcriptional co-repressor Atro primarily regulates polarity near the equator of the eye, suggesting the existence of other Ft polarity effectors near
the poles of the eye. I also show that Fj dampens clonal phenotypes of *ft*, *ds*, and *atro*. I find that the Ft extracellular domain is required for polarity establishment in the eye, and the Ds intracellular domain can regulate polarity in a Ft-dependent manner. Finally, I find that neither D nor the Ft homolog Fat2 are involved in PCP in the eye. My work thus shows, in contradiction to previous studies, that Ft-Ds signaling acts not through cascades of asymmetric protein distribution but by regulating a long-range polarity signal that directs further polarity establishment.

In chapter 3, I summarize these findings and discuss their implications for broader planar cell polarity research. I also discuss the many unanswered questions in this important and controversial field.
Chapter 2

Regulation Of Long-Range Planar Cell Polarity By Fat-Dachsous Signaling In The Drosophila Eye

This chapter is adapted from Sharma, P. and McNeill, H. (2013). Development. 140, 3869-3881.
2 Regulation of long-range planar cell polarity by Fat-Dachsous signaling in the Drosophila eye

2.1 Abstract

Fat (Ft) and Dachsous (Ds) are large cadherins that bind each other and have conserved roles in regulating Planar Cell Polarity (PCP). I quantitatively analyzed Ft-Ds pathway mutant clones for their effects on ommatidial polarity in the Drosophila eye. My findings suggest that the Ft-Ds pathway regulates PCP propagation independently of asymmetric cellular accumulation of Ft or Ds. I find that the Ft effector Atrophin has a position-specific role in regulating polarity in the eye, and that asymmetric accumulation of the atypical myosin Dachs is not essential for production and propagation of a long-range PCP signal. My observations suggest that Ft and Ds interact to modulate a secondary signal that regulates long-range polarity, that signaling by the Ds intracellular domain is dependent on Ft, and that ommatidial fate specification is genetically separable from long-range signaling.

2.2 Introduction

Development and function of many tissues requires coordination of cellular characteristics along a consistent direction. This coordinate organization is called Planar Cell Polarity (PCP). Most forms of PCP are regulated by two well-conserved pathways: the Frizzled(Fz)/PCP, and the Fat (Ft)-Dachsous (Ds) PCP pathway (reviewed in Axelrod, 2009; Goodrich and Strutt, 2011; Lawrence et al., 2007; Maung and Jenny, 2011; Thomas and Strutt, 2012). Ft and Ds are large cadherins (Bryant et al., 1988; Clark et al., 1995; Mahoney et al., 1991), whose binding is regulated by the kinase Four-jointed (Fj) (Brittle et al., 2010; Ishikawa et al., 2008; Simon et al., 2010). Ds and Fj are expressed in gradients in the eye, regulating Ft activity (Simon, 2004; Yang
et al., 2002) (Figure 5D). Ft, Ds, and Fj are also involved in the growth-regulatory Hippo pathway (reviewed in Grusche et al., 2010; Halder and Johnson, 2011; Lawrence et al., 2008; Staley and Irvine, 2012)). A critical question is how the orientation of neighboring cells is established and propagated across a tissue. Analysis of clones of cells lacking ft, ds, or fj reveals that mutant tissue inside clones and wild-type tissue adjacent to clones has altered polarity (Casal et al., 2002; Casal et al., 2006; Fanto et al., 2003; Ma et al., 2003; Rawls et al., 2002; Strutt and Strutt, 2002; Yang et al., 2002). The reorganization of polarity in wild-type tissue, called domineering non-autonomy (Krasnow et al., 1995; Vinson and Adler, 1987), can propagate over many cell lengths, indicating that the Ft-Ds pathway has a role in communicating polarity information across a tissue.

Recent studies have revealed that Ft, Ds, and the atypical myosin Dachs (D) are asymmetrically distributed (Ambegaonkar et al., 2012; Bosveld et al., 2012; Brittle et al., 2012; Mao et al., 2006; Rogulja et al., 2008). D localization redistributes several cells away from a border of Ds or Fj expression (Ambegaonkar et al., 2012; Bosveld et al., 2012; Brittle et al., 2012; Mao et al., 2006; Mao et al., 2011b). These data, along with previous studies, have led to a model in which polarity information is propagated from cell to cell by polarized distribution of Ft and Ds, which in turn reorganizes Ft, Ds, and D in adjacent cells (Casal et al., 2006; Ma et al., 2003; Matakatsu and Blair, 2006; Strutt and Strutt, 2002; Yang et al., 2002).

An alternative model to explain the propagation of polarity from Ft and Ds expressing cells is that Ft-Ds binding leads to production of a second signal, which is responsible for the propagation of polarity. The finding that the cytoplasmic domain of Ft binds to the transcriptional co-repressor Atrophin (Atro) led to the proposal that this second signal is transcriptionally regulated by Ft activity (Fanto et al., 2003). Loss of either ft or atro leads to increased expression of fj, providing a potential mechanism for regulation of PCP.
I reasoned that quantitative analysis of polarity disruptions caused by mutant clones of *ft*, *ds*, and *atro* could provide insight into the mechanisms underlying the propagation of polarity, and allow us to distinguish between these models. Using these analyses, I show that asymmetric subcellular localization of Ft and Ds cannot account for the propagation of polarity in the *Drosophila* eye. I further show that the cytoplasmic domains of Ft and Ds can generate a signal that can propagate across a tissue independent of changes in Ft, Ds or D localization. I demonstrate that *atro* regulates polarity only in regions near the equator, indicating that other as yet unknown polarity regulators mediate Ft signaling in polar regions. I show that loss of D does not block the propagation of polarity defects, and that increased expression of *fj* cannot account for polarity disruptions caused by loss of *ft* or *atro*. These data support a model in which long-range polarity propagation in the Ft-Ds pathway is mediated by a second signal regulated by Ft-Ds interactions.

### 2.3 Materials and methods

#### 2.3.1 Generation of mutant clones

Mutant clones were generated using the FLP/FRT system with *ey* or *hs* drivers (Xu and Rubin, 1993). This system uses Flp recombinase from the yeast 2µ plasmid, and its associated FRT target sequence. Fly lines have been constructed with FRT sites integrated into chromosomes near either side of the centromeres. This allows homologous recombination between chromosome arms on expression of the Flp recombinase. Initially, flies are generated with mutant loci or visible markers recombined by meiotic recombination onto chromosomes containing FRT sites. These flies can be crossed together to generate flies that are heterozygous for the mutant locus and the marker (Figure 8A). The marker chromosome itself is wild-type for the gene of interest. On expression of the Flp recombinase following S-phase, recombination occurs between the homologous chromosomes (Figure 8B), and the following segregation and cell-division results in wholly wild-type and mutant daughter cells (Figure 8C,D).
Figure 8. A schematic of mitotic recombination using the Flp/FRT system.

**A.** Cells are initially heterozygous for the mutant locus and for the visible marker (which is on a chromosome that is wild-type for the gene of interest). **B.** Recombination occurs between sister chromatids of homologous chromosomes following DNA replication in S-phase. **C-D.** Wholly mutant and wild-type cells result following segregation and cell division. These then divide normally to give rise to patches of mutant and wild-type tissue.
The MARCM system (Lee and Luo, 2001) was used for overexpression or RNAi-expressing clones. Adult clones were marked by loss of $w^+$ or expression of UAS-\textit{w-RNAi} (VDRC). The following fly stocks were used:

$ft^{G-rv}\ FRT40$

$ft^{Ed}\ FRT40$

$ds^{38k}\ FRT40$ (David Strutt)

$ds^{38k}\ ft^{G-rv}\ FRT40$

$ds^{UA071}\ ft^{G-rv}\ FRT40$

$atro^{35}\ FRT80$

$atro^{e46}\ FRT80$ (Manolis Fanto)

$GFP\ FRT40$

$GFP\ FRT80$

$GFP\ FRT40;\ UAS-Ft\Delta ECD$ (Seth Blair)

$GFP\ FRT40;\ UAS-Ds\Delta ECD$ (Seth Blair)

$GFP\ FRT40;\ UAS-fz-RNAi$ (TRiP)

$GFP\ FRT40;\ UAS-ds-RNAi$ (TRiP)

$GFP\ FRT40;\ UAS-\textit{atro-RNAi}$ (CC Tsai)

$d^{GCl13}\ FRT40$ (Ken Irvine and David Strutt)

$ft^{G-rv}\ d^{GCl13}\ FRT40$ (Ken Irvine and David Strutt)

$fat^{258D}\ FRT80$ (Christian Dahmann)

$ft^{G-rv}\ FRT40;\ UAS-Ft\Delta ECD$
\text{ft}^{G-rv} FRT40 ; UAS-fz-RNAi
\\ds^{38k} FRT40 ; UAS-Ds\Delta ECD
\\ft^{G-rv} FRT40 ; UAS-Ds\Delta ECD
\\ds^{38k} \; \ft^{G-rv} FRT40 ; UAS-Ds\Delta ECD
\\ds^{38k} FRT40 ; UAS-fz-RNAi
\\ds^{38k} FRT40 ; UAS-atro-RNAi
\\ft^{G-rv} FRT40 \; fj^{D1}
\\ds^{38k} FRT40 \; fj^{D1}
\\ds^{38k} \; \ft^{G-rv} FRT40 \; fj^{D1}
\\fj^{D1} ; atro^{35} FRT80
\\GFP FRT40 \; fj^{D1}
\\fj^{D1} ; GFP FRT80
\\ft^{G-rv} FRT40 ; fat^{258D} FRT80
\\ds^{38k} FRT40 ; fat^{258D} FRT80
\\GFP FRT40 ; fat^{258D} FRT80
\\text{ser-lacZ.II-9.5 (Maria Dominguez)}
\\ft^{G-rv} FRT40 ; ser-lacZ
\\ds^{38k} FRT40 ; ser-lacZ
\\ds^{38k} \; \ft^{G-rv} FRT40 ; ser-lacZ
2.3.2 Sectioning of adult eyes

Adult eyes were processed and sectioned according to a protocol slightly modified from Wolff, 2000:

1. Dissect fly head away from the neck with a clean #11 scalpel blade. Care must be taken to not make a dent in the eye by pressing it down on the cutting surface. Then, cut away one eye from the other, such that the proboscis stays attached to one eye. The piece with the attached proboscis will be used further. If both eyes need to be used, attempt to cut down the middle of the proboscis, such that half of it stays attached to each eye.

2. Put dissected tissue into microfuge tubes with 200 µl of 2% glutaraldehyde in 0.1 M NaPO₄, on ice - put each head into the fix as it is dissected. 2% glutaraldehyde is prepared in a fumehood, by adding 8 µl 50% glutaraldehyde (Electron Microscopy Sciences) to 192 µl 0.1 M NaPO₄, pH 7.2.

3. Once all desired eyes have been collected, move to a fumehood. Add 200 µl of 2% osmium tetroxide (Electron Microscopy Sciences) to each tube.

4. Incubate on ice for 30 minutes.

5. Replace the osmium/glutaraldehyde with 200 µl of fresh 2% osmium tetroxide and incubate for ~1.5 hours on ice. Liquid replacement in this and following steps should be done as rapidly as possible, to minimize exposure of eyes to air.

6. Rinse with 0.1 M NaPO₄.

7. Dehydrate by adding, on ice, 200 µl 30% ethanol for 10 minutes.

8. Replace with 50 % ethanol for 10 minutes. All eyes should settle to the bottom of the tube at this point. Those that don’t likely have air pockets trapped in them, and if they don’t settle they might not undergo dehydration properly.

9. Replace with 70 % ethanol for 10 minutes.

10. Replace with 90 % ethanol for 10 minutes.

11. Replace with 100 % ethanol for 10 minutes.

12. Replace again with 100 % ethanol for 10 minutes.

13. Replace ethanol with propylene oxide for 10 minutes at room temperature. Repeat three times.
14. Without removing the final propylene oxide, add an equal volume of Durcupan resin (Sigma Aldrich/Fluka) and mix well. Incubate overnight at room temperature. If the resin does not flow easily at room temperature it should not be used, and a fresh batch should be prepared.

15. Replace the resin/propylene mixture with resin. At this point it is safe to move out of the fumehood.

16. Incubate for at least 4 hours at room temperature.

17. At this point, it is helpful to cover working surfaces with aluminum foil, to make it easier to deal with any accidental resin drips. With the help of a large needle or toothpick, move eyes from the tubes to the molds.

18. Fill the molds with resin. Avoid over- or underfilling as it makes it hard to section the eyes later.

19. Position the eyes so that the part of the eye to be cut is facing the edge, with the cutting plane at as close to a tangent to the middle of the eye or clone of interest as possible. The proboscis is useful to orient the eye – it should generally be perpendicular to the long axis of the mold.

20. Bake the resin at 70° for at least 15 hours and no more than 36 hours. The orientation of the eyes should be checked and corrected if necessary within the first hour of baking. All containers and instruments that have come in contact with resin should also be baked before reuse or disposal.

21. Remove individual resin blocks from mold and place in airtight container. Store blocks at room temperature.

22. Trim excess resin from around the eye using a razor blade. If desired, some of the remaining resin can be fractured away, leaving the eye surface exposed, but care must be taken to not cut the eye or to remove so much resin that the mounting becomes unstable.

23. Make 1-1.5 µm thick sections using a microtome and a glass or diamond knife (a Leica EM UC6 microtome and a Diatome HI 5317 knife were used in this work).

24. Move sections to distilled water droplets on Superfrost Plus coated slides (Fisher Scientific) and allow the water to dry at room temperature or on a slide warmer.

25. Mount sections with DPX (Sigma Aldrich) and a cover slip.
26. Image with light microscopy (Nikon Eclipse 80i microscope with Nikon Ds-Ri1 camera) with a 40×-60× objective. A stitching function in the microscopy software (NIS Elements BR) makes it easy to examine a large section at high resolution in one image.

2.3.3 Dissection and staining of imaginal discs

Larval eye imaginal discs were dissected and stained as follows:

1. Place late third instar larvae in small pools of PBS on dissection dishes coated with Sylgard silicone elastomer (Dow Corning). PBS in this and following steps should be without Ca or Mg ions.
2. Using sharp forceps, grip tip of larval head on one side and the body approximately 1/3 from the end on the other side.
3. Pull larva apart slowly but firmly. The mouth hooks should come apart, bringing the brain and eye/antennal discs with them. Remove any cuticle.
4. Place each eye-brain complex in 100 µl PBS on ice as it is dissected.
5. Once all desired discs have been collected, add 100 of µl 8% paraformaldehyde (PFA) to each tube. 8% paraformaldehyde is made by adding 50 µl 16% PFA (Electron Microscopy Sciences) to 50 µl PBS. This is done to ensure rapid mixing of PFA in the final solution, and results in 4% PFA. Incubate for 30 minutes at room temperature. This and following incubation and washing steps should be performed on a nutator or rotator.
6. Rinse with PBS.
7. Incubate with 100 µl of 0.3% PBT (PBS with 0.3% Triton X-100) for 10 minutes at room temperature.
8. Incubate with 100 µl of 1% bovine serum albumin (BSA) in 0.1% PBT for 30 minutes.
9. Incubate with primary antibodies at desired concentration in 100 µl of 1% BSA in 0.1% PBT, overnight at 4°.
10. Wash 5× for 10 minutes in 0.1% PBT at room temperature.
11. Incubate with 0.25 µl of each secondary fluorescent antibody (1:400 concentration) in 100 µl of 0.1% PBT, for 1 hour at room temperature in the dark.
12. Wash 5× for 10 minutes in 0.1% PBT at room temperature in the dark.
13. Replace with 100 µl of 70% glycerol/30% PBS solution. Allow discs to settle to bottom at 4°, which should take about an hour, or very gently centrifuge (~2 second short spin) and wait about 15 minutes.
14. Place eye-brain complexes on a slide, and using fine needles dissect away the brain and mouth hooks. One needle can pin the tissue down while the other moves along it in a scissoring action to sever the antennal discs from the mouth hooks, and then the eye discs from the optic lobes.
15. Move the eye-antennal discs to a drop of Vectashield mounting medium on a new slide. If there are many discs arrange them in a grid to make it easier to find them under a microscope.
16. Gently place a coverslip to avoid disturbing the position of the discs, and seal with nail polish.
17. Image using fluorescent (Nikon Eclipse 80i microscope with Nikon Ds-Ri1 camera) or confocal microscopy (Nikon Eclipse 90i microscope with Nikon C1 confocal system). As eye discs are slightly curved and uneven, confocal images should be taken at multiple Z-heights so that the best signal can be acquired from different parts of the disc and merged together if required.

Antibodies used were rat α-Ft (McNeill lab, 1:1000), rabbit α-Ds (McNeill lab, 1:1000), rat α-Bar (McNeill lab, 1:100), mouse α-Elav (DSHB, 1:1000) and mouse α-β-gal (Promega, 1:1000).

2.3.4 Categorization of ommatidial genotypes

Photoreceptor mosaicism refers to photoreceptors in the same ommatidium having different genotypes. As ommatidial polarity determination occurs through photoreceptor R3/4 fate determination (Cooper and Bray, 1999; Fanto and Mlodzik, 1999; Fanto et al., 2003; Yang et al., 2002; Zheng et al., 1995), and it has been shown that R3/R4 mosaicism can affect ommatidial
polarity, I excluded ommatidia that were mosaic for R3/R4 from my analysis of long-range polarity. I considered ommatidia in which R3 and R4 cells were both wild-type as wild-type for the purpose of long-range polarity establishment, and those in which both were mutant as being genetically mutant, unless otherwise specified. Inside clones, I only quantified entirely mutant ommatidia for polarity analysis. No R3+/R4− or R3−/R4+ mosaic ommatidia were included in any of my quantification.

2.3.5 Data processing and analysis

Images were processed with Adobe Photoshop (www.adobe.com) and statistical analysis was done with GraphPad Prism (www.graphpad.com). Comparisons of phenotypic strength were done by unpaired t-tests, and size- and position-dependence were determined by linear regression.

2.4 Results

2.4.1 ft regulates long distance repolarization independent of changes in Ft levels or localization

To gain insight into the mechanisms underlying polarity propagation, I quantitatively analyzed alterations in polarity that occur in and around Ft-Ds pathway mutant clones. I found that ftG-rv mutant clones cause reversal of polarity in 34% of genetically wild-type ommatidia in the first row of ommatidia outside the polar border of the clone (n = 40 clones), while polarity outside the equatorial border was unaffected (Figure 9A,C).
Models in which polarity propagation by the Ft-Ds pathway is due to asymmetric distribution of Ft and Ds in one cell promoting asymmetric redistribution of Ds and Ft in adjacent cells predict that (1) polarity propagation in clones lacking Ft or Ds could only re-orient polarity in one row of cells inside mutant clones; (2) the degree of propagation in wild-type tissue outside clones depends on the relative levels and affinities of Ft and Ds, and; (3) inside mutant clones, ommatidia not contacting a wild-type cell should have randomized polarity. To test these predictions, I quantified the degree of polarity disruption inside ft clones. If polarity were randomized, quantification should show a polarity distribution of ~50% normally-oriented ommatidia and ~50% reverse-oriented ommatidia. A significant departure from this distribution would indicate the presence of polarity signaling in the absence of Ft and Ds redistribution.

I found that inside ft clones, 89% of entirely mutant ommatidia in the first row inside the polar border of the clone had reversed polarity (n = 36 clones) (Figure 9G). Polarity was almost completely rescued on the equatorial side of ft clones, as 93% of entirely mutant ommatidia had normal polarity (n = 32 clones) (Figure 9H). The rescue and reversal of polarity were statistically different from the 50% normal/50% reversed polarity distribution expected of randomized ommatidial orientation (both p < 0.0001). Significantly, and in further contrast to a redistribution model, in the interior of many ft clones polarity was normal on the equatorial side and reversed on the polar side over up to 4-5 ommatidial rows, in the complete absence of Ft protein (Figures 9A, 10A, 11A,B). Importantly, these rescue and reversal effects extended only a limited distance, and polarity was randomized in the middle of large ft clones. Another null ft allele, ft^{df} (Matakatsu and Blair, 2006), showed similar clonal polarity effects (Figures 9B, 12). These data strongly argue that polarity information can propagate ~10-40 cells in the absence of Ft, in contrast to the predictions of an asymmetric redistribution-based model.
Figure 9. Polarity changes propagate from ft clone borders.

<table>
<thead>
<tr>
<th>Polarity outside ( ft^{2-2} ) clones</th>
<th>Polarity inside ( ft^{2-2} ) clones</th>
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<tr>
<td>Reversal of ommatidial polarity outside ( ft^{2-2} ) polar clone borders</td>
<td>Reversal of polarity inside polar clone border</td>
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<tr>
<td>Correlation of polarity reversal with clone size</td>
<td>Change in polarity reversal with polar clone border alignment</td>
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<td>Correlation of polarity reversal with clone location</td>
<td>Rescue of polarity inside equatorial clone border</td>
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<td>% of ommatidia with reversed polarity</td>
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<td>Clone size along DV axis in ommatidial rows</td>
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<td>Distance of polar clone border from equator in ommatidial rows</td>
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<td>Border parallel to w.r.t. equator</td>
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Legend: n.s. = not significant; + = significant; ++ = highly significant; **** = extremely significant.
Figure 9. Polarity changes propagate from *ft* clone borders.

**A.** *ft*<sup>G-rv</sup> mutant clones. The area inside the box is shown in the middle and right panels. Mutant photoreceptors are marked by absence of pigment. Yellow marking refers to symmetrical (R3/R3 or R4/R4) ommatidium. Several wild-type ommatidia outside the polar border have reversed polarity. Inside the clone, many mutant ommatidia have reversed polarity on the polar side, and normal polarity on the equatorial side. These effects inside the clone extend over many ommatidial rows. **B.** An eye with *ft*<sup>fd</sup> mutant clones. Polarity is not disrupted outside this particular clone, but inside the clone many mutant ommatidia have reversed polarity on the polar side, and normal polarity on the equatorial side. (C-F) Quantification of polarity effects outside *ft*<sup>G-rv</sup> clones. Only R3<sup>+</sup>/R4<sup>+</sup> ommatidia are quantified. **C.** Polarity is reversed in an average of 34% of ommatidia in the first row outside the polar border (40 clones, 331 ommatidia). There is no x-axis on this and other scatterplots – data points are distributed for legibility. **D.** Plot of polarity reversal outside the clone against clone size, measured in ommatidial rows on the dorso-ventral axis (36 clones, 274 ommatidia). There is no significant dependence of polarity reversal on clone size. **E.** Plot of polarity reversal outside the clone against polar clone border distance from the equator, as measured in ommatidial rows on the dorso-ventral axis (37 clones, 282 ommatidia). There is no significant dependence. **F.** Scatterplots of polarity reversal outside the clone, according to clone border alignment relative to the equator (parallel: 36 clones, 215 ommatidia; slanted: 28 clones, 107 ommatidia). *ft*<sup>G-rv</sup> clones have significantly different polarity reversal between parallel and slanted clone borders. (G-H) Quantification of polarity effects inside *ft*<sup>G-rv</sup> clones. Only fully mutant ommatidia are quantified. **G.** 89% of ommatidia in the first row on the polar side have reversed polarity (36 clones, 153 ommatidia). **H.** 93% of ommatidia in the first row on the equatorial side have normal polarity (32 clones, 128 ommatidia).

Statistics: **D** and **E:** Deviation from zero (no dependence) of linear regression slope; **F:** unpaired *t*-test; **G** and **H:** one sample *t*-test against null hypothesis of randomized polarity (50% reversal).

Statistical significance indicators are: *p* > 0.05, not significant (n.s.); *p* < 0.05, *; *p* < 0.01, **; *p* < 0.001, ***; *p* < 0.0001, ****. Horizontal bars (solid) indicate mean. Trend lines (dashed) do not indicate significance.
Figure 10. Polarity information is transmitted inside *ft* and *ds* clones.
Figure 10. Polarity information is transmitted inside \(ft\) and \(ds\) clones.

**A.** \(ft\) clones have polarity rescued on the equatorial side, and reversed on the polar side. These effects are strongest near the borders and extend up to several rows. In the middle of the clones these effects are weak or non-existent, and polarity is randomized. **B.** \(ds\) clones have polarity rescued on the polar side, and reversed on the equatorial side. The reversal effect of \(ds\) clones is not as strong as that of \(ft\) clones, and rarely extends past one ommatidial row. However the rescue effect is strong, and can extend for multiple rows. The equator is not marked in eyes in which a clear boundary of wild-type dorsal and ventral ommatidia cannot be determined. **C.** Example of a \(ft\) clone indicating clone borders parallel (cyan line) and slanted (magenta line) relative to the equator. This clone shows the general trend of parallel borders to cause more reversals outside the clone than slanted borders.
Figure 11. Polarity information is transmitted for multiple rows in ft or ds mutant tissue.

A. Polarity is frequently rescued for 2 rows inside equatorial borders of ft clones, with consistently normal polarity occurring for up to 5 rows. B. Polarity is frequently reversed for 2 rows inside polar borders of ft clones, with consistently reversed polarity occurring for up to 6 rows. C. Polarity is frequently rescued for 1-2 rows inside polar borders of ds clones, with consistently normal polarity occurring for up to 4 rows. D. Polarity is frequently reversed for 1 row inside equatorial borders of ft clones, with consistently reversed polarity occurring for up to 2 rows.
Figure 12. \( ft^{fd} \) affects polarity similarly to \( ft^{G-rv} \).

\( ft^{fd} \) mutant clones cause polarity reversals in the eye qualitatively similar to \( ft^{G-rv} \) mutant clones.  

**A.** Polarity reversal of an ommatidium with seven wild-type photoreceptors (in box in picture, and marked with black outline) on the polar side of a \( ft^{fd} \) mutant clone. **B.** Scatterplot of polarity reversals outside polar borders of \( ft^{fd} \) clones. The mean strength is 14% \((n = 46 \) clones\), which is weaker than the 34% polarity reversals caused by \( ft^{G-rv} \) clones.
2.4.2 Clonal borders are the source of polarity changes in ft clones

To further understand the non-autonomous polarity effects of ft clones, I tested if the degree of reversals dependent on size, location or shape of the clones. I found that the degree of reversal did not vary with clone size along the D-V axis – clones that were 1-2 ommatidial rows in size caused reversals outside clones as often as those that were >5 rows in size (Figure 9D). I also examined effects of D-V position, and found that clones near the equator caused reversals as often as those near the poles ($p = 0.63$) (Figure 9E).

Analysis of the shape of clone borders indicated that boundary geometry is critical in determining the extent of polarity disruption. ft clone borders that were parallel to the equator induced more polarity reversals (43%) than clone borders slanted relative to the equator (10%) (Figures 9F, 10C), a significant difference ($p < 0.0001$). As different alignments of clone boundaries result in different degrees of polarity reversal, I conclude that boundary-specific signaling is likely to be important in the ft clonal polarity effect.

2.4.3 ds regulates long-range repolarization independent of changes in Ds levels or localization

I continued my examination of polarity effects caused by Ft-Ds pathway mutants by analyzing $ds^{38k}$ clones, and found that polarity can also propagate in the absence of Ds (Figures 13A,B). Polarity reverses in 31% of wild-type ommatidia in the first row of ommatidia outside the equatorial border of ds clones ($n = 40$ clones). Polarity outside the polar border was unaffected. Polarity information is also transmitted inside ds mutant clones. 66% of entirely ds mutant ommatidia in the first row inside the equatorial border of the clone have reversed polarity ($n = 29$ clones) (Figure 13G), a statistically significant degree of reversal ($p = 0.0046$). On the polar side
of \( ds \) clones, 95% of entirely \( ds \) mutant ommatidia in the first row have normal polarity \((n = 36\) clones\) (Figure 13F). As with \( ft \) mutant clones, polarity rescue extends for many ommatidial rows inside clones, though the reversal effect is weaker (Figure 10B, 11C,D). The degree of polarity reversal caused by \( ds \) clones does not vary with clone size \((p = 0.09)\) (Figure 13C), but does vary with position: \( ds \) clones near the poles cause more polarity reversals, \((p = 0.007)\) (Figure 13D). \( ds \) levels are higher near the poles, so the difference in levels inside and outside a mutant clone is greater there, which may be responsible for the stronger polarity effect. Unlike \( ft \) clones, \( ds \) clones do not show significantly different degrees of reversal along parallel or slanted borders \((p = 0.23)\) (Figure 13E). I observed changes in ommatidial orientation inside and outside \( ft \) and \( ds \) clonal borders in developing larval eye discs as well (Figure 14). Together, these data indicate that though Ft and Ds are asymmetrically distributed in wild-type tissue (Ambegaonkar et al., 2012; Brittle et al., 2012), polarity changes in the interiors of \( ft \) and \( ds \) mutant clones cannot be accounted for by a chain-reaction propagation of asymmetric Ft-Ds distribution.

\( ft \) is epistatic to \( ds \) in R3/4 fate determination (Yang et al., 2002). Quantitative analysis of \( ds ft \) clones revealed polar non-autonomous polarity effects similar to \( ft \) clones (Figure 15A). However, while \( ft \) clones cause this effect in 34% of ommatidia, only 3% of ommatidia outside \( ds ft \) clones \((n = 40\) clones\) have reversed polarity (Figure 15B). This effect was seen with two strong \( ds \) mutant alleles, \( ds^{38k} ft^{G-\text{rv}} \) and \( ds^{UAS071} ft^{G-\text{rv}} \) (Figures 15A, 16A), and also when \( UAS-ds-RNAi \) is expressed inside \( ds ft \) clones (Figure 16C), suggesting that the polarity effects of \( ds ft \) clones are not due to residual \( ds \) function. Thus loss of \( ds \) largely but not entirely suppresses the polar non-autonomous polarity effects caused by loss of \( ft \) in clones.

The polarity reversal observed inside the polar border of \( ft \) clones does not occur in \( ds ft \) clones (Figure 15F). However, 73% of entirely \( ds ft \) mutant ommatidia in the first row inside the equatorial border have normal polarity, which is statistically different from randomized polarity \((p < 0.0001)\) (Figure 15G). Thus, polarity information can be transmitted from clone borders in the absence of both Ft and Ds (Figure 17). The double mutant clonal phenotype is not size dependent \((p = 0.52)\) (Figure 15C), and only slightly position dependent, perhaps reflecting the
*ds* gradient ($p = 0.034$) (Figure 15D). *ds ft* clones do not show significantly different polarity effects along parallel or slanted borders ($p = 0.11$) (Figure 15E).

It was suggested that polarity phenotypes associated with *ft* mutant clones are due to generation of an additional equator inside the clones (Rawls *et al.*, 2002), leading to long-distance changes in ommatidial polarity. I did not find changes in the *serrate-lacZ* reporter (Figure 18), which marks regions of equator formation (Bachmann and Knust, 1998; Gutierrez-Avino *et al.*, 2009), in *ft* or *ds* clones. Furthermore, an ectopic equator would be expected to create a sharp polarity reversal, like the normal equator, in the middle of the clone. However, I find that consistent polarity, whether normal or reversed, is most strongly observed near clone borders, and is increasingly randomized farther inside clones (Figures 10A,B, 11, 17). I therefore conclude that apparent equators in clone interiors are a consequence of rescue and reversal of polarity at clone borders, and are not true equators, as observed, *e.g.*, in Wg pathway clones (Wehrli and Tomlinson, 1998).

Together these data indicate that accumulation of Ft and Ds at cell borders establishes a polarity signal, and that polarity propagation can occur independently of Ft and Ds.
Figure 13. Polarity changes propagate from ds clone borders.
Figure 13. Polarity changes propagate from ds clone borders.

A. ds$^{38k}$ mutant clones. Polarity is reversed inside and outside the equatorial border, and rescued inside the polar border. (B-E) Quantification of polarity effects outside ds$^{38k}$ clones. B. Polarity is reversed in an average of 31% of ommatidia in the first row outside the equatorial border (40 clones, 355 ommatidia). C. Polarity reversals do not vary with clone size (27 clones, 221 ommatidia). D. Polarity reversal increases with distance of clone equatorial border from equator (33 clones, 293 ommatidia). E. Polarity reversal does not vary with clone border alignment relative to equator (parallel: 37 clones, 284 ommatidia; slanted: 13 clones, 71 ommatidia). (F-G) Quantification of polarity effects inside ds$^{38k}$ clones. F. 95% of ommatidia in the first row on the polar side have normal polarity (36 clones, 153 ommatidia). G. 66% of ommatidia in the first row on the equatorial side have reversed polarity (29 clones, 179 ommatidia).

Statistics: C and D: Deviation from zero (no dependence) of linear regression slope; E: unpaired t-test; F and G: one sample t-test against null hypothesis of randomized polarity (50% reversal).
Figure 14. Polarity disruptions are evident during development in the eye imaginal disc.
Figure 14. Polarity disruptions are evident during development in the eye imaginal disc.

Elav staining marks all photoreceptors, and Bar staining marks photoreceptors 1 and 6. Loss of GFP marks mutant tissue. Mutant tissue is outlined in white in close-ups. A. *ft* clones cause polarity disruptions on the polar side of clones. B. *ds* clones cause polarity disruptions on the equatorial side of clones.
Figure 15. Polarity changes are reduced but present at $ds \ ft$ clone borders.

<table>
<thead>
<tr>
<th>Outside $ds^{58k}$ fi$^{G\gamma}$ clones</th>
<th>Inside $ds^{58k}$ fi$^{G\gamma}$ clones</th>
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<tbody>
<tr>
<td>Reversal of ommatidial polarity outside $ds^{58k}$ fi$^{G\gamma}$ polar clone borders</td>
<td>Reversal of polarity inside polar clone border</td>
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<tr>
<td>Correlation of polarity reversal with clone size</td>
<td>Correlation of polarity reversal with polar clone location</td>
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<tr>
<td>% of ommatidia with reversed polarity</td>
<td>Change in polarity reversal with polar clone border alignment</td>
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<tr>
<td>Clone size along DV axis in ommatidial rows</td>
<td>Border parallel to w.r.t. equator</td>
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<tr>
<td>Distance of polar clone border from equator in ommatidial rows</td>
<td>Border slanted to w.r.t. equator</td>
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<tr>
<td>% of ommatidia with reversed polarity</td>
<td>% of ommatidia with normal polarity</td>
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Figure 15. Polarity changes are reduced but present at *ds ft* clone borders.

**A.** An eye with *ds^{38k} ft^{G-rv}* mutant clones. Several genetically wild-type ommatidia outside the polar border have reversed polarity. Inside the clone, many mutant ommatidia have normal polarity on the equatorial side, but polarity is randomized on the polar side. (B-E) Quantification of polarity effects outside *ds^{38k} ft^{G-rv}* clones. **B.** Polarity is reversed in 3% of ommatidia in the first row outside the polar border (40 clones, 288 ommatidia). **C.** Polarity reversals do not vary with clone size. **D.** Polarity reversal increases slightly with distance of clone equatorial border from equator (36 clones, 267 ommatidia). **E.** Polarity reversal does not vary with clone border alignment relative to equator (parallel: 26 clones, 211 ommatidia; slanted: 17 clones, 77 ommatidia). (F-G) Quantification of polarity effects inside *ds^{38k} ft^{G-rv}* clones. **F.** 62% of ommatidia in the first row on the polar side have reversed polarity, which is statistically similar to randomized polarity (27 clones, 112 ommatidia). **G.** 73% of ommatidia in the first row on the equatorial side have normal polarity (30 clones, 195 ommatidia).

Statistics: **C and D:** Deviation from zero (no dependence) of linear regression slope; **E:** unpaired *t*-test; **F and G:** one sample *t*-test against null hypothesis of randomized polarity (50% reversal).
Figure 16. Residual ds function does not cause ds ft clonal polarity effects.
Figure 16. Residual $ds$ function does not cause $ds ft$ clonal polarity effects.

A. $ds^{UA071} ft^{G-rv}$ clones, marked by absence of pigment, show polarity reversals outside clones similarly to $ds^{38k} ft^{G-rv}$ clones. B. Clones expressing $UAS-ds$-RNAi, marked by absence of pigment, show polarity reversals outside equatorial borders of clones, confirming $ds$ knockdown. C. $ds^{38k} ft^{G-rv}$ clones expressing with $ds$ knockdown, marked by absence of pigment, show polarity reversals outside clones similar to $ds ft$ clones without $ds$ knockdown.
Figure 17. Polarity information is transmitted inside equatorial but not polar sides of ds ft clones.

A. Polarity is frequently rescued for 1 row inside equatorial borders of ds ft clones, with consistently normal polarity occasionally occurring for up to 8 rows. B. Polarity is largely randomized inside polar borders of ds ft clones. C. Polarity is frequently rescued for 1-2 rows inside equatorial borders of ds ft clones expressing UAS-ds-RNAi, with consistently normal polarity occasionally occurring for up to 4 rows. D. Polarity is largely randomized inside polar borders of ds ft clones expressing UAS-ds-RNAi.
Figure 18. Ectopic equators do not form in ft or ds clones.

Eye discs expressing the equator marker ser-lacZ, detected by anti-β-gal staining. No change is observed in Ft-Ds mutant clones, marked by loss of GFP. A-A’’: ft clones. B-B’’: ds clones. C- C’’: ds ft clones.
2.4.4 Expression of DsΔECD modulates long-range repolarization

What roles do the enormous extracellular domains of Ft and Ds play in regulating polarity? Expression of a version of Ft with most of the extracellular domain deleted (FtΔECD) can substantially rescue PCP when expressed ubiquitously in the wing (Matakatsu and Blair, 2006; Matakatsu and Blair, 2012). I found that expression of FtΔECD in clones resulted in rare disruptions in PCP within the clone, and no disruptions outside the clone (quantification in summary Figure 29C). In contrast to the ability of FtΔECD to rescue polarity in the wing, FtΔECD is unable to rescue ft clonal polarity defects in the eye (Figure 29D). This suggests that the Ft ECD is important for polarity in the eye, possibly via interaction with Ds. Recent work by Zhao et al. (Zhao et al., 2013) found that expression of FtΔECD in fully ft mutant eyes also does not rescue polarity, supporting this observation.

Expression of Ds lacking the entire extracellular domain (DsΔECD) in clones resulted in occasional non-autonomous polarity reversals outside the equatorial side of clones (Figure 29J). This effect, which is qualitatively similar to but weaker than the ds clone effect, suggests that the Ds intracellular domain (ICD) acts in a dominant-negative manner in non-autonomous polarity signaling. Strikingly, expression of DsΔECD in ds clones resulted in polarity disruptions stronger than those of ds clones alone, with polarity reversal in 48% of wild-type ommatidia in the first row of ommatidia outside the equatorial border of the clone (n = 18 clones) (Figures 29K and 19), significantly stronger than the 31% polarity reversal outside ds clones (p = 0.023).

Importantly, ft clones that express DsΔECD do not exhibit the equatorial polarity reversals seen when DsΔECD is expressed in wild-type tissue. They instead behave like ft clones with polar reversals (Figure 29L). Strikingly, ds ft clones expressing DsΔECD also have no polarity defects outside their equatorial borders (Figure 29M), in contrast to 48% equatorial polarity reversals induced by ds clones expressing DsΔECD. Thus, as DsΔECD is unable to bind Ft in neighboring cells, DsΔECD induced polarity changes must depend on the presence of Ft in cis.
Figure 19. Expression of DsΔECD strengthens ds clonal polarity effects.

**A-B.** ds mutant clones expressing UAS-DsΔECD. There is very strong polarity reversal outside equatorial borders.
2.4.5 Atro regulates polarity near the equator

What is downstream of Ft-Ds signaling? The transcriptional co-repressor Atro binds to the cytoplasmic domain of Ft, and, like ft, loss of atro results in changes in polarity (Erkner et al., 2002; Fanto et al., 2003; Zhang et al., 2002), but no quantitative analysis was previously performed. Quantification of polarity disruptions around atro$^{35}$ clones showed that 17% of wild-type ommatidia in the first row outside the polar border of atro clones have reversed polarity ($n = 38$ clones) (Figures 20A,C). Similarly to ft clones, the degree of polarity reversals induced by atro clones did not change with clone size ($p = 0.56$) (Figure 20D). However, it did change with clone position: clones near the equator caused more reversals and those near the poles caused significantly fewer reversals (Figure 20E).

Strong atro alleles cause neurodegeneration in mutant tissue, making polarity inside clones indecipherable (Figure 20A). To examine polarity inside atro clones, I analyzed a weaker allele, atro$^{e46}$. Clones of atro$^{e46}$ rarely cause non-autonomous effects, but do cause polar reversal and equatorial rescue inside clones, qualitatively similar to the effect of ft clones (Figure 20B).

Loss of atro leads to increased fj expression (Fanto et al., 2003). To test if increased expression of fj contributes to polarity disruptions caused by atro, I analyzed atro clones in fj$^{D1}$ null background. Strikingly, atro clones in fj$^{-}$ animals caused polarity reversals in 55% of wild-type ommatidia in the first row of ommatidia outside the polar border of the clone ($n = 13$ clones) (Figure 29S), significantly stronger than atro clones in a wild-type background ($p < 0.0001$, Figure 29R). Thus fj is not required for atro clones to affect polarity, and in fact loss of fj enhances atro non-autonomous polarity disruptions, suggesting that fj normally suppresses polarity disruptions caused by atro.
The degree of polarity reversal does not significantly differ along parallel or slanted *atro* clone borders (*p* = 0.08) (Figure 20F). Since *atro* suppresses *ft* expression in the adult eye (Napoletano *et al.*, 2011), one possible mechanism of *atro* activity is through regulating Ft accumulation, promoting polarity disruptions. However, in developing discs, loss of *atro* has no effect on Ft or Ds levels or localization (Figure 21). These data further argue against changes in Ft and Ds localization being responsible for propagation of polarity information, and suggest that a downstream signal establishes polarity.

*ds* and *atro* mutant clones have opposite polarity phenotypes, allowing examination of their epistatic relationship. *ds* and *atro* are on different chromosomes, so I could not generate double mutant clones. I instead made *ds* clones expressing *atro-RNAi*. These clones caused both polar non-autonomous polarity reversals (more frequently than *atro-RNAi*-only clones), and equatorial non-autonomous polarity reversals (less frequently than *ds* clones, Figures 29T,U and 22). This suggests that *ds* and *atro* might act in parallel to some degree. Additionally, I observed that mutant ommatidia inside clones were sometimes misrotated, and wild-type ommatidia on clone borders were occasionally misrotated.
Figure 20. *atro* primarily regulates polarity near the equator.
Figure 20. *atro* primarily regulates polarity near the equator.

A. *atro* \(^{35}\) mutant clones. Several genetically wild-type ommatidia outside the polar border have reversed polarity. B. *atro* \(^{e46}\) mutant clones. A wild-type ommatidium outside the clone has reversed polarity, while inside the clone, there is both reversal on the polar side and rescue on the equatorial side. (C-F) Quantification of polarity effects outside *atro* \(^{35}\) clones. C. Polarity is reversed in an average of 17% of ommatidia in the first row outside the polar border (38 clones, 362 ommatidia). D. Polarity reversals do not vary with clone size (38 clones, 362 ommatidia). E. Polarity reversal decreases with distance of clone equatorial border from equator (35 clones, 314 ommatidia). F. Polarity reversal does not vary with clone border alignment relative to equator (parallel: 34 clones, 302 ommatidia; slanted: 14 clones, 66 ommatidia).

Statistics: D and E: Deviation from zero (no dependence) of linear regression slope; F: unpaired \(t\)-test.
Figure 21. Ft and Ds levels and localization are not affected by loss of *atro.*
Figure 21. Ft and Ds levels and localization are not affected by loss of *atro*.

**A-A’**: *ft* mutant clones in eye discs, marked by loss of GFP, show loss of α-Ft staining. **B-B’**: *atro* mutant clones in eye discs, marked by loss of GFP, do not show changes in α-Ft staining. **C-C’**: *ds* mutant clones in eye discs, marked by loss of GFP, show loss of α-Ds staining. **B-B’**: *atro* mutant clones in eye discs, marked by loss of GFP, do not show changes in α-Ds staining.
**Figure 22.** *ds* clones expressing *UAS-atro-RNAi* cause polar and equatorial polarity reversals.

**A.** Clones expressing *atro-RNAi* occasionally cause polarity reversals in wild-type ommatidia on the polar side. **B.** Polarity reversals occur in wild-type ommatidia on the polar and, less frequently, equatorial sides of *ds* clones expressing *atro-RNAi*. No clear rescue or reversal of ommatidial polarity is evident inside the clones. Many mutant and a few wild-type ommatidia are misrotated.
2.4.6 Dachs and Fat2 do not contribute to the long-range repolarization signal

How do other members of the Ft-Ds pathway regulate polarity in the *Drosophila* eye? D is an unconventional myosin that acts downstream of Ft (Feng and Irvine, 2007; Mao et al., 2006). D subcellular localization is regulated by Ft and Ds in the wing and eye (Ambegaonkar et al., 2012; Brittle et al., 2012), and loss of *d* suppresses polarity randomization in *ft* and *ds* mutant eyes, likely through its effect on the Hippo pathway (Brittle et al., 2012). However, *d* <sup>GCI3</sup> mutant flies have extremely mild polarity defects in the eye (Mao et al., 2006 and Figure 23A). Of 45 wild-type ommatidia examined on the polar borders of 11 *d* mutant clones, only 1 ommatidium had reversed polarity. To examine the contribution of *d* to *ft*-dependent polarity propagation, I examined *ft d* clones and found that they were similar to *ft* clones in their effects on polarity both inside and outside clones (Figures 29G and 23B). *ft d* clones caused polarity reversals in 31% of wild-type ommatidia in the first row of ommatidia outside the polar border of the clone (*n* = 19 clones), similar to the effect of *ft* clones (34% polarity reversal, *p* = 0.72), and also display reversal and rescue inside clones. Thus D does not mediate production and propagation of the Ft-Ds long range PCP signal in the eye.

*fat2*, the only *ft* paralog in *Drosophila* (Viktorinova et al., 2009), regulates polarization of ovarian follicle cells. I found that neither eyes wholly mutant for *fat2<sup>58D</sup>* nor *fat2* mutant clones had polarity defects (Figures 24A,B). Furthermore, the non-autonomous polarity caused by *ft* and *ds* mutant clones were not altered by loss of *fat2* in the background (Figures 24C,D). *fat2* thus appears not to be involved in polarity in the *Drosophila* eye.
Figure 23. *d* does not regulate PCP in the eye.

**A.** *d* mutant clones do not disrupt polarity in the eye. As a clone overlaps the equator in this section, the position of the equator is not marked directly on the eye, but is indicated by the arrowhead. **B.** *ft d* double mutant clones affect polarity inside and outside clones similarly to *ft* mutant clones.
Figure 24. *fat2* does not regulate PCP in the eye.

Polarity effects of *ft* or *ds* clones are not changed by loss of *fat2* in the background.

### A
- Dorsal pole
- Ventral pole

### B
- Dorsal pole
- Ventral pole

### C
- % of ommatidia with reversed polarity
- *ft* clones in *fat2* mutant background
- *ft* clones
- *n.s.*

### D
- % of ommatidia with reversed polarity
- *ds* clones in *fat2* mutant background
- *ds* clones
- *n.s.*
Figure 24. *fat2* does not regulate PCP in the eye.

**A.** A *fat2* mutant eye. Polarity is normal. **B.** A *fat2* mutant clone, marked by absence of pigment. No changes in polarity are observed inside or outside the clone. **C.** *ft* mutant clones in a wild-type background do not differ in strength of polarity reversals outside clones from *ft* mutant clones in a *fat2* mutant background. **D.** *ds* mutant clones in a wild-type background do not differ in strength of polarity reversals outside clones from *ds* mutant clones in a *fat2* mutant background.
2.4.7 Fj dampens long-range repolarization by Ft and Ds

How does the Golgi-localized kinase Fj regulate polarity? Fj acts both upstream and downstream of Ft and Ds (Ishikawa et al., 2008; Strutt et al., 2004; Yang et al., 2002). fj expression is negatively regulated by ft and atro, and positively regulated by ds and d (Cho et al., 2006; Fanto et al., 2003; Mao et al., 2006; Yang et al., 2002). Fj phosphorylates Ft and Ds cadherin domains, regulating their binding (Brittle et al., 2010; Simon et al., 2010). fj is expressed in a gradient (Yang et al., 2002; Zeidler et al., 1999), and the graded modulation of Ft-Ds binding by Fj is sufficient for normal polarity establishment (Brittle et al., 2010; Simon, 2004; Simon et al., 2010). Loss of fj enhances polarity defects of ft and ds mutants in the wing and eye (Simon, 2004; Strutt et al., 2004), and of Ft and DsECD overexpression clones in the abdomen (Casal et al., 2006). I therefore examined the role of fj in ft and ds clonal polarity disruptions in the eye.

I found that in a fj null background, polarity reversals caused by ft and ds mutant clones increased. ft clones in a fj background caused polar polarity reversals in 63% of wild-type ommatidia (n = 9 clones) (Figure 29B), compared to 34% in a wild-type background. ds clones in a fj background caused equatorial polarity reversals in 68% of wild-type ommatidia (n = 23 clones) (Figure 29I), compared to 31% in a wild-type background. Similarly, ds ft clones in a fj background caused polar polarity reversals in 16% of wild-type (n = 6 clones) (Figure 29P), compared to 3% in a wild-type background. Thus, fj is not required for non-autonomous polarity of ft and ds clones, and loss of fj strengthens these effects, as with atro clones. Interestingly, even though both ds and fj gradients are absent inside ds mutant clones in a fj background, there is extensive rescue and reversal of polarity around clone borders (Figure 25).
Figure 25. Loss of fj strengthens ds clonal polarity reversals.

*ds* clones generated in a *fj* mutant background show extensive reversal of polarity outside, and reversal and rescue of polarity inside clones, which are missing both *ds* and *fj* gradients.
2.4.8 Interactions of ft and ds with fz

The relationship between The Ft-Ds and Fz/PCP pathways is complex and context-dependent (Adler et al., 1998; Aigouy et al., 2010; Casal et al., 2006; Hogan et al., 2011; Ma et al., 2003; Matakatsu and Blair, 2004; Sagner et al., 2012; Strutt and Strutt, 2002; Yang et al., 2002). ft clones and ds clones expressing fz-RNAi had no consistent rescue or reversal of polarity inside clones. Instead, polarity was randomized, with reversed, misrotated, and symmetrical ommatidia (Figure 26), similar to fz clones (Zheng et al., 1995). Wild-type ommatidia surrounding ft and ds mutant clones expressing fz-RNAi were often misrotated, complicating analysis. However, I detected polarity reversals in wild-type ommatidia on polar borders of both types of clones, characteristic of the fz phenotype (Figures 29E,F,N, 26A,B,C). Furthermore, I occasionally observed ommatidia with reversed polarity outside equatorial borders of ds clones expressing fz-RNAi. These reversed ommatidia were also usually misrotated (Figure 26D). Thus, ds clones expressing fz-RNAi have stronger polarity effects than fz-RNAi-only clones, and weaker equatorial polarity effects than ds mutant-only clones.
Figure 26. *ft* or *ds* clones expressing *UAS-fz-RNAi* cause polar polarity reversals.
Figure 26. *ft* or *ds* clones expressing $UAS$-$fz$-$RNAi$ cause polar polarity reversals.

**A.** Clones expressing $fz$-$RNAi$ cause frequent polarity reversals inside the clone, and occasional reversals in wild-type ommatidia on outside the polar border. **B.** *ft* clone expressing $fz$-$RNAi$. There is frequent polarity reversal outside the polar side, and polarity inside clones is disrupted to a greater degree than with *ft* clones, with many more misrotated and symmetrical ommatidia. **C-D.** *ds* clones expressing $fz$-$RNAi$. There is frequent polarity reversal outside the polar side but only rarely outside the equatorial side of these clones. Furthermore, reversals outside the equatorial side are often associated with misrotation. Polarity inside clones is disrupted to a greater degree than with *ds* clones, with many more misrotated and symmetrical ommatidia.
2.4.9 Polarity reversals in completely wild-type or mosaic ommatidia

To ensure a large sample size, and based on the critical role of R3 and R4 in determining polarity, in the data presented above I counted ommatidia in which both R3 and R4 cells were wild-type as being genetically wild-type. I tested this approach by measuring polarity effects in ommatidia in which all outer photoreceptors were wild-type, surrounding $ft$, $ds$, $ds ft$, and atro mutant clones. These showed similar trends as R3$^+/R4^+$ ommatidia (Figure 27), confirming the validity of my analyses. I note that R3/R4 mosaic ommatidia also tend to have reversed or rescued polarity according to their location in the clone, similar to fully mutant ommatidia (Figure 28).
Figure 27. Polarity reversals occur in fully wild-type ommatidia on clone borders.

- **A'** Reversal of ommatidial polarity outside polar $ft^{3 Ign}$ clone borders
- **B'** Reversal of ommatidial polarity outside equatorial $ds^{3Ma}$ clone borders
- **C'** Reversal of ommatidial polarity outside polar $ds^{3Ma} ft^{3 Ign}$ clone borders
- **D'** Reversal of ommatidial polarity outside polar $atro^m$ clone borders
Figure 27. Polarity reversals occur in fully wild-type ommatidia on clone borders.

Polarity reversals in ommatidia with at least seven wild-type photoreceptors. In this figure, these fully wild-type ommatidia are enclosed in boxes in the sections, and are represented as solid arrows with a black outline to distinguish them from ommatidia that are R3+/R4+ but mosaic for other photoreceptors, which do not have the black outline. A-A’. ft clones cause polarity reversals in 19% of fully wild-type ommatidia on the polar side (n = 40 clones/331 ommatidia). B-B’. ds clones cause polarity reversals in 12% of fully wild-type ommatidia on the equatorial side (n = 40 clones/355 ommatidia). C-C’. ds ft clones cause polarity reversals in 1% of fully wild-type ommatidia on the polar side (n = 40 clones/288 ommatidia). D-D’. atro clones cause polarity reversals in 6% of fully wild-type ommatidia on the polar side (n = 38 clones/362 ommatidia).
Figure 28. Polarity reversals occur in R3/4 mosaic ommatidia on clone borders.
Figure 28. Polarity reversals occur in R3/4 mosaic ommatidia on clone borders.

**A-B:** R3/R4 mosaic ommatidia are represented with stars over hollow arrows (R3+/R4-: cyan stars, R3-/R4+: magenta stars). **A.** Mosaic ommatidia on the polar border of a ftG-rv clone have reversed polarity, similarly to their fully mutant neighbors. **B.** Mosaic ommatidia on the polar border of a ftfd clone have reversed polarity, similarly to their fully mutant neighbors. **C.** R3/4 mosaic ommatidia polarity is affected similarly to fully mutant ommatidia. Numbers of each type of ommatidium observed are indicated. 94% of ft mosaic ommatidia have reversed polarity on the polar border, and 99% of ft mosaic ommatidia on the equatorial border have normal polarity on the equatorial border. 100% of ds mosaic ommatidia have normal polarity on the polar border, and 75% of ds mosaic ommatidia have reversed polarity on the equatorial border. 88% of ds ft mosaic ommatidia have normal polarity on the equatorial border. Polarity of ds ft mosaic ommatidia on the polar border is closer to randomized.
2.5 Discussion

My quantitative analyses of polarity changes caused by clones modifying Ft-Ds pathway components are summarized in Figure 29 and Table 3. What conclusions can be drawn from these data?

Figure 29. Summary of polarity reversal outside mutant clones.
Figure 29. Summary of polarity reversal outside mutant clones.

Diagrams and scatterplots of polarity reversal outside clones. B, I, P and S are in ff– background. Strengths of polarity reversal are: **A. ft–** clones: 34% (40 clones, 331 ommatidia); **B. ft–** clones in fβ– background: 63% (9 clones, 65 ommatidia); **C. FtΔECD expression clones:** 2% (16 clones, 81 ommatidia); **D. ft–** clones expressing FtΔECD: 26% (13 clones, 71 ommatidia); **E. fz-RNAi** clones: 4% (10 clones, 58 ommatidia); **F. ft–** clones expressing fz-RNAi: 40% (7 clones, 45 ommatidia); **G. ft– d–** clones: 32% (19 clones, 127 ommatidia); **H. ds–** clones: 31% (40 clones, 355 ommatidia); **I. ds–** clones in fβ– background: 68% (23 clones, 112 ommatidia); **J. DsΔECD expression clones:** 4% (26 clones, 169 ommatidia); **K. ds–** clones expressing DsΔECD: 48% (18 clones, 120 ommatidia); **L. ft–** clones expressing DsΔECD: 41% polar (7 clones, 71 ommatidia) and 0% equatorial (11 clones, 92 ommatidia); **M. ds– ft–** clones expressing DsΔECD: 6% polar (8 clones, 42 ommatidia) and 0% equatorial (11 clones, 72 ommatidia); **N. ds–** clones expressing fz-RNAi: 36% polar (14 clones, 84 ommatidia) and 4% equatorial (15 clones, 114 ommatidia); **O. ds– ft–** clones: 3% (40 clones, 288 ommatidia); **P. ds– ft–** clones in fβ– background: 16% (6 clones, 43 ommatidia); **Q. ds-RNAi** clones: 48% (4 clones, 40 ommatidia); **R. atro–** clones: 17% (38 clones, 362 ommatidia); **S. atro–** clones in fβ– background: 55% (13 clones, 121 ommatidia); **T. atro-RNAi** clones: 0.5% (9 clones, 69 ommatidia); **U. ds–** clones expressing atro-RNAi: 10% polar (29 clones, 168 ommatidia) and 4% equatorial (44 clones, 302 ommatidia). Statistical comparisons are in Table 3.
Table 3. Quantification and comparison of Ft-Ds polarity effects.

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</tr>
<tr>
<td>28</td>
<td>29I</td>
<td>ds' in ff background</td>
<td>68% equatorial reversal</td>
<td>23</td>
<td>112</td>
<td>Different from #15, p&lt;0.0001</td>
</tr>
<tr>
<td>29</td>
<td>29N</td>
<td>ds' with fzf-RNAi expression – polar border</td>
<td>36% polar reversal</td>
<td>14</td>
<td>84</td>
<td></td>
</tr>
<tr>
<td>30</td>
<td>29N</td>
<td>ds' with fzf-RNAi expression – equatorial border</td>
<td>4.2% equatorial reversal</td>
<td>15</td>
<td>114</td>
<td>Different from #15, p&lt;0.0001</td>
</tr>
<tr>
<td>31</td>
<td>15B, 29O</td>
<td>ds' ft</td>
<td>3.0% polar reversals</td>
<td>40</td>
<td>288</td>
<td>Different from #1, p&lt;0.0001</td>
</tr>
<tr>
<td></td>
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<td></td>
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<td></td>
</tr>
<tr>
<td>32</td>
<td>15F</td>
<td>ds' ft interior – polar side</td>
<td>38% rescue</td>
<td>27</td>
<td>112</td>
<td>Not different from random polarity, p = 0.084</td>
</tr>
<tr>
<td>33</td>
<td>15G</td>
<td>ds' ft interior – equatorial side</td>
<td>73% rescue</td>
<td>30</td>
<td>195</td>
<td>Different from random polarity, p&lt;0.0001</td>
</tr>
<tr>
<td>34</td>
<td>15C</td>
<td>ds' ft – DV size effect</td>
<td>Correlation: r = 0.11</td>
<td>38</td>
<td>277</td>
<td>No, p = 0.52</td>
</tr>
<tr>
<td>35</td>
<td>15D</td>
<td>ds' ft – DV position effect</td>
<td>Correlation: r = 0.36</td>
<td>36</td>
<td>267</td>
<td>No, p = 0.034</td>
</tr>
<tr>
<td>36</td>
<td>15E</td>
<td>ds' ft – parallel border</td>
<td>3.4% polar reversals</td>
<td>36</td>
<td>211</td>
<td></td>
</tr>
<tr>
<td>37</td>
<td>15E</td>
<td>ds' ft - slanted border</td>
<td>0.53% polar reversals</td>
<td>17</td>
<td>77</td>
<td>Not different from #36, p = 0.11</td>
</tr>
<tr>
<td>38</td>
<td>29P</td>
<td>ds' ft in ff background</td>
<td>16% polar reversals</td>
<td>6</td>
<td>43</td>
<td>Different from #31, p = 0.0004</td>
</tr>
<tr>
<td>39</td>
<td>29Q</td>
<td>ds-RNAi expression</td>
<td>48% equatorial reversals</td>
<td>4</td>
<td>40</td>
<td></td>
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</tr>
<tr>
<td>40</td>
<td>20C, 29R</td>
<td><em>atro</em>’</td>
<td>17% polar reversals</td>
<td>38</td>
<td>362</td>
<td>Different from #1, ( p = 0.0003 )</td>
</tr>
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<td>41</td>
<td>20D</td>
<td><em>atro</em>’ – DV size effect</td>
<td>Correlation: ( r = -0.11 )</td>
<td>38</td>
<td>362</td>
<td>No, ( p = 0.53 )</td>
</tr>
<tr>
<td>42</td>
<td>20E</td>
<td><em>atro</em>’ – DV position effect</td>
<td>Correlation: ( r = -0.39 )</td>
<td>35</td>
<td>314</td>
<td>Yes, ( p = 0.020 )</td>
</tr>
<tr>
<td>43</td>
<td>20F</td>
<td><em>atro</em>’ – parallel border</td>
<td>19% polar reversals</td>
<td>34</td>
<td>302</td>
<td></td>
</tr>
<tr>
<td>44</td>
<td>20F</td>
<td><em>atro</em>’ – slanted border</td>
<td>8.0% polar reversals</td>
<td>14</td>
<td>66</td>
<td>Not different from #43, ( p = 0.080 )</td>
</tr>
<tr>
<td>45</td>
<td>29S</td>
<td><em>atro</em> in ( f^f ) background</td>
<td>55% polar reversals</td>
<td>13</td>
<td>121</td>
<td>Different from #40, ( p &lt; 0.0001 )</td>
</tr>
<tr>
<td>46</td>
<td>29T</td>
<td><em>atro</em>-RNAi</td>
<td>0.46% polar reversals</td>
<td>9</td>
<td>69</td>
<td></td>
</tr>
<tr>
<td>47</td>
<td>29U</td>
<td><em>ds</em>’ with <em>atro</em>-RNAi expression – polar border</td>
<td>10% polar reversals</td>
<td>29</td>
<td>168</td>
<td>Different from #46, ( p = 0.033 )</td>
</tr>
<tr>
<td>48</td>
<td>29U</td>
<td><em>ds</em>’ with <em>atro</em>-RNAi expression – equatorial border</td>
<td>3.6% equatorial reversals</td>
<td>44</td>
<td>302</td>
<td>Different from #15, ( p &lt; 0.0001 )</td>
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2.5.1 Ft-Ds interaction and a long-range polarity signal

Ft and Ds can regulate each others’ subcellular localization, and changes in their levels in clones can induce redistribution outside clone boundaries (Ambegaonkar et al., 2012; Bosveld et al., 2012; Brittle et al., 2012; Ma et al., 2003; Matakatsu and Blair, 2004; Strutt and Strutt, 2002). It has recently been proposed that a redistribution cascade may be responsible for long range polarity propagation (Ambegaonkar et al., 2012; Brittle et al., 2012). The redistribution model can explain polarity changes in wild-type tissue, where Ft and Ds are both present. However, a redistribution cascade cannot occur inside ft or ds clones, and yet I observe very strong polarity reorganization well inside these clones. This can only occur if long-range signaling inside clones occurs through a mechanism other than Ft or Ds redistribution. Instead of invoking completely separate mechanisms to explain polarity changes inside and outside clones, it is simpler to propose a model in which Ft or Ds accumulation at the clone boundary result in aberrations in a long-range polarity signal that then propagate both inside and outside clones.

Why do ds ft clones cause polarity reversals that are weaker than those caused by ft clones? ft and ds single mutant clones cause strong accumulation of Ds or Ft, respectively, in mutant cells just inside the clone border. In ds ft double mutant clones, however, there would be no such strong accumulation at the border (Figure 30). There is only loss of signal within clones, which causes milder polarity disruptions.

Why are the border effects of ft and ds clones opposite in orientation? I speculate that Ft-Ds heterodimers form both in cis (in the same cell), and in trans (with neighboring cells). In wild-type cells both cis and trans forms exist. However, cells immediately inside ft clone borders cannot have Ds bound in cis to Ft, and cells inside ds clones cannot have Ft bound in cis to Ds. Cells in ft and ds mutant clones with only trans bound forms of Ds and Ft respectively could regulate downstream events differently from each other and from wild-type cells. This could lead to opposite regulation (i.e. promotion vs. suppression) in ft or ds clones of the same long-range
signaling molecule, or to regulation of two different signaling molecules. Although Ft and Ds are both cadherins, they have very different cytoplasmic domains, and may transmit information about binding in different ways.

I find that polarity propagation extends much further in the absence of Ft or Ds than in wild-type tissue. In \textit{ft} and \textit{ds} clones, rescue or reversal of polarity can in some cases extend to a distance of \~4-5 ommatidial rows, or \~40 cells along the D-V axis. The increased range of polarity reversals in the absence of Ft or Ds suggest that the normal distribution of Ft and Ds outside the clone provides a signal that counteracts and dampens the polarity propagation from the clone border.

Could Ft and Ds be involved in transmitting or interpreting the polarity signal by a mechanism other than redistribution? It is reasonable to ask if transmembrane proteins with large extracellular domains could in some way interact with, for example, a secreted, diffusible molecule. In such a model, either one of Ft or Ds would be sufficient to help transmit the aberrant signal (presumably still being generated at the clone border), but if both were absent, as in a double mutant clone, the signal would only travel a short distance (1-2 rows), which would also imply that Ft or Ds are not required for signal transmission, but enhance it. In this model, Ft and Ds could interact directly with the signal. They could either simply be required for the movement of the signal, or possibly help or hinder its movement in a specific way, so as to set up an aberrant gradient. Or, they could act as receptors for the signal. Alternatively, Ft and Ds could act as part of a pathway that is involved in transmission or interpretation of the signal. This model proposes that the aberrant signal being generated at the \textit{ds ft} clone border is similar in both direction and strength to that generated at a \textit{ft} clone border, but is transmitted or interpreted to a lesser degree in the double mutant clone than when Ds is present in a \textit{ft} clone. While there is no data available to disprove this alternative model, it is a less parsimonious explanation than my model of transmission of the signal occurring independently of Ft and Ds. Importantly, both models require generation of a signal at the clone border. The alternative model then requires Ft and Ds to either interact with the signal or be involved in another pathway that interprets the signal, and there is no evidence for such activity by Ft or Ds. The model I present, on the other hand, follows from the observed differences in Ft and Ds accumulation between single and
double mutant clone borders, and does not require any further function on the part of Ft and Ds. Thus, while the normal asymmetric distribution of Ft and Ds play a role in creating or orienting the polarity signal, it is not necessary for transmission of the signal.

At what time in development would the long-range polarity signal be established by Ft-Ds signaling, transmitted, and interpreted? Several lines of evidence suggest that Ft-Ds signaling is active relatively early in the eye disc, anterior to the furrow. *ds* regulates oriented cell division anterior to the furrow (Baena-Lopez, 2005), and asymmetric localization of Dachs and Ft can be observed anterior to the furrow as well (Brittle et al., 2012). Additionally, loss of *fz* function specifically prior to the furrow results in a polarity phenotype similar to loss of *ds* (Strutt and Strutt, 2002), suggesting that regulation of ommatidial polarity by Ft-Ds signaling occurs prior to the furrow, while the disc is still growing. This would suggest that clonal effects of *ft* and *ds* are established when clones are smaller than when observed in the adult, and that the range of clonal polarity effects is less than inferred from quantification in the adult. However, other evidence suggests that the critical phase of Ft-Ds signaling occurs after the furrow. Evident establishment of ommatidial polarity is preceded by the coming together of R3/4 precursors, followed by R3/4 fate specification and rotation of the cluster in the direction of R4. If polarity identity (i.e. equatorial-polar identity) of cells were specified before the furrow, mosaicism of R3/4 for, e.g., *ft*, would not be a strong determinant of fate specification and polarity establishment, as it is not known until the furrow passes which cells will join any given ommatidial cluster. Furthermore, cell division proceeds until the furrow, with some of these newly divided cells being incorporated into developing ommatidia. These cells, if created from a mutant mother cell, would not have been present for early Ft-Ds signaling. Yet, especially close to clone borders, these cells do acquire specific (i.e. non-random) polarity. This suggests that ommatidial polarity is not decided until ommatidia form and R3/4 precursors come into contact. I propose that Ft-Ds signaling can initiate before the furrow, yet cells do not read the polarity signal gradient until after the furrow passes, at the time of R3/4 precursor contact. This means that the aberrant gradient around clone borders is maintained until ommatidial specification, and can extend up to 4-5 rows, as seen for *ft* clones.
A surprising result was that ommatidia along borders of ft mutant clones that are parallel to the equator are more likely to be reversed than those along slanted borders. What is the basis of this difference? I speculate that this is due to different degrees of accumulation of Ft-Ds dimers at parallel and slanted borders. In the interior of a ft clone, Ds is diffusely distributed. At the border of the ft clone, all available Ds redistributes to bind Ft in wild-type cells touching the clone (Ma et al., 2003; Simon et al., 2010; Strutt and Strutt, 2002). In ft clones that have a border that spreads over several cell diameters along the A-P axis, parallel to the equator, this causes redistribution of Ds to only one cell border along the D-V axis. Along slanted borders, in contrast, Ds redistribution is spread out over both D-V and A-P sides of the cell, reducing the degree of accumulation (Figure 30).

I examined the effects of expression of FtΔECD and DsΔECD on non-autonomous polarity effects outside clones. I saw only subtle changes in polarity upon expression of FtΔECD. However, I saw clear non-autonomous polarity effects upon clonal expression of DsΔECD. Surprisingly, this caused a polarity change qualitatively similar to loss of ds in clones, suggesting that DsΔECD had a dominant negative effect on polarity signaling. Importantly DsΔECD signals in a Ft-dependent manner, as equatorial polarity disruptions are lost in ft clones. As there is no extracellular domain of Ds in DsΔECD, this suggests that Ds ICD interacts with Ft ICD in cis to regulate non-autonomous polarity propagation.

Could the specific alleles I used influence my interpretation of these data? I mainly used two ft and ds mutant alleles in this work: ft<sup>G-rv</sup> and ds<sup>38k</sup>. ft<sup>G-rv</sup> is a genetically strong allele of ft (Bryant et al., 1998), that has been molecularly characterized (Matakatsu and Blair, 2006). This mutant allele codes for a protein that has a stop site in the extracellular region, and is likely to result in an extracellular, secreted protein. Interestingly, another ft allele, ft<sup>fd</sup>, also has a premature stop site in the extracellular region (Matakatsu and Blair, 2006), resulting in a smaller secreted protein than the ft<sup>G-rv</sup> protein. ft<sup>fd</sup> shows weaker phenotypes than ft<sup>G-rv</sup> in growth regulation (Feng and Irvine, 2007) and PCP (my data), although ft<sup>fd</sup> has a stronger adult neurodegeneration phenotype than ft<sup>G-rv</sup> (Napoletano et al., 2011). What accounts for the different effects of these two alleles? It is possible that the larger ft<sup>G-rv</sup> protein acts in a dominant-negative manner, while the smaller
$ft^{Ed}$ protein does not, and represents a true null. Alternatively, the $ft^{Ed}$ allele could have lesions downstream of the stop site that reintroduce a start site and result in partial rescue of $ft$ function, while $ft^{G-rv}$ represents a true null. $ds^{38k}$ has not been molecularly characterized. Genetically, it is very strong allele (Rodriguez, 2004), though other strong alleles are also known (Clark et al., 1995). It is possible that there is some $ds$ function in this allele, which could also account for the $ds ft$ double mutant non-autonomous polarity effect. I consider this unlikely though, as another strong allele, $ds^{UA071}$, causes quantitatively similar polarity effects for single and $ds ft$ double mutants, and expression of effective $UAS-ds$-RNAi in these double mutant clones also does not abrogate the non-autonomous polarity effect. Thus, I believe that the $ft$ and $ds$ alleles I used are valid for performing loss-of-function analysis.
Figure 30. A model for Ft-Ds polarity signaling in the eye.
Figure 30. A model for Ft-Ds polarity signaling in the eye.

A. Ds accumulates on the borders of ft clones, leading to aberrant polarity signal and reversal of polarity on the polar border. On a slanted border, Ds accumulation is redistributed to both sides of cells, leading to a weaker aberrant signal and hence fewer polarity reversals. B. Ft accumulates on the borders of ds clones, leading to aberrant polarity signal and reversal of polarity on the equatorial border. C. There is no accumulation of Ft or Ds on a ds ft clone border. The aberrant polarity signal is weak, and few polarity reversals occur. D-E: Polarity inside the clone is established by aberrant polarity signal from clone borders, which can extend to several ommatidial rows. Polarity outside the clone is established by a combination of the aberrant clone border signal and the background polarity signal generated by wild-type tissue. D. ft clones cause polar reversals and equatorial rescue. E. ds clones cause equatorial reversals and polar rescue. F. ds ft clones cause a small amount of equatorial rescue, and very few polar reversals. Polarity is largely randomized inside clones. G. Loss of fj weakens the background polarity signal, allowing aberrant signaling from the ds clone border to reorganize polarity more extensively.
2.5.2 Ft-Ds signalling and Dachs

What is downstream of Ft-Ds signaling? The Ds intracellular domain is known to affect Hippo pathway targets in the wing (Matakatsu and Blair, 2012), and is required to receive boundary signals that regulate Hippo targets in the eye (Willecke et al., 2008). However, loss of Hippo pathway genes does not lead to non-autonomous polarity disruptions in clones (Blaumueller and Mlodzik, 2000; Harvey et al., 2003; Tapon et al., 2002; Udan et al., 2003; Wu et al., 2003). Although D localization can change in response to Ds levels (Ambegaonkar et al., 2012; Brittle et al., 2012), I find that d clones do not cause non-autonomous polarity disruptions, and ft d double mutant clones have similar polarity disruptions as ft clones. Thus, D is not a component of polarity signal propagation in the eye.

2.5.3 Ft-Ds signalling and Atrophin

Atro binds to Ft ICD and atro clones qualitatively phenocopy ft clones, (Fanto et al., 2003; Figure 20), suggesting Atro could mediate the Ft-Ds polarity signal. Loss of atro does not lead to changes in Ft or Ds levels or localization (Figure 21), indicating that atro does not affect polarity by changing Ft-Ds accumulation. Interestingly, I found that atro mainly functions near the equator, as loss of atro near the poles has little effect. This suggests atro functions downstream of Ft and Ds near the equator, with another unidentified polarity regulator acting as a Ft-Ds polarity effector near the poles.
2.5.4 Ft-Ds signaling and Four-jointed

Ds, Ft, and Fj interactions have been extensively studied in the Drosophila abdomen wing, eye, and larval denticles (Ambegaonkar et al., 2012; Bosveld et al., 2012; Brittle et al., 2012; Casal et al., 2002; Casal et al., 2006; Donoughe and DiNardo, 2011; Harumoto et al., 2010; Ma et al., 2003; Repiso et al., 2010; Simon, 2004; Strutt et al., 2004; Yang et al., 2002). However, the effects of loss of fj on non-autonomous polarity caused by ft and ds clones have not been examined. fj levels are altered in ft, ds, and atro mutant clones (Fanto et al., 2003; Yang et al., 2002). If changes in fj levels were essential for change in polarity signalling at ft, ds or atro clone borders, the phenotypes of these clones would be weaker in a fj- animal. Instead, I found that non-autonomous polarity disruptions of ft, ds or atro clones are enhanced in fj- animals. Thus, changes in fj expression cannot be responsible for their non-autonomous polarity effects. The strengthened polarity effects could be explained in terms of diminution of a standing polarity gradient in the wild-type cells surrounding the clone, as fj and ds establish complementary and redundant polarity gradients in the eye (Simon, 2004; Zeidler et al., 1999). Loss of fj strongly enhances polarity phenotypes of Ft, Ds and Ds-ECD overexpression, consistent with this conclusion (Casal et al., 2006; Simon, 2004; Strutt et al., 2004).

2.5.5 Genetic interactions of ds with atro and fz

ds mutant clones expressing atro-RNAi exhibit both polar and equatorial non-autonomous polarity reversals. Analysis of knockdown phenotypes is complicated, but observation of reversals on both sides suggests some parallel function between ds and atro. Analysis of ds clones expressing fz-RNAi suggests a similar, partially parallel relationship between ds and fz, consistent with previous observations in the eye (Strutt and Strutt, 2002).
ds clones expressing atro-RNAi cause misrotations in mutant and wild-type ommatidia to a small degree, and ds clones expressing fz-RNAi cause misrotations to a large degree. Ommatidial rotation occurs after chirality is established; ft, ds, or fj mutants cause misrotations only rarely (Simon, 2004 and this work), while Fz/PCP pathway mutants cause misrotations frequently, reflecting the requirement of this pathway in proper rotation subsequent to chirality establishment (Strutt and Strutt, 2002; Yang et al., 2002; Zheng et al., 1995). A variety of downstream effectors, including the Nemo kinase and members of the EGFR pathway (reviewed in Jenny, 2010), are involved in carrying out the rotation. The occurrence of misrotations in and around ds clones expressing atro-RNAi or fz-RNAi is likely due to a combination of two effects: first, the combined loss of ds and reduction in atro could cause disruptions in tissue architecture due to changes in, for example, oriented cell division or increased apoptosis, such that the mechanical forces involved in ommatidial rotation are improperly transmitted. This would lead to occasional misrotation. Second, the loss of ds and reduction in fz could also disrupt tissue architecture to some extent, and also cause aberrant regulation of the downstream effectors, thus leading to frequent misrotation.

In the next chapter, I summarize my work and discuss its implications for understanding planar cell polarity, and propose future experiments to address questions raised by my findings.
Chapter 3

Conclusion
3 Conclusion

3.1 A brief summary of the work

I have performed a thorough and comprehensive quantitative analysis of the regulation of planar cell polarity by the Ft-Ds pathway in the *Drosophila* eye. To summarize my results, I observe that (1) polarity information is transmitted to a large (but not unlimited) distance inside *ft* and *ds* clones, and (2) clone size does not affect phenotype but (3) clone shape affects PCP signal generation. These data, along with previously observed changes in localization of Ft and Ds at clone borders (Ma *et al.*, 2003; Matakatsu and Blair, 2004; Strutt and Strutt, 2002), suggest a model in which polarity signals from clone borders act in concert with changes in signaling inside mutant clones. I propose that normal Ft-Ds signaling, acting in a gradient due to graded *ds* and *fj* expression, results in a graded production of long-distance secondary signal. This creates a standing gradient of polarity information. Inside *ft* and *ds* clones, the gradient of polarity information is reduced or lost. These changes are reinforced by accumulation of Ds or Ft at clone borders. The resultant changes in secondary signal oppose the standing gradient in surrounding wild-type tissue, leading to non-autonomous polarity reversals outside the clone. Inside the clone, with no standing gradient to oppose it, the aberrant signal from clone borders leads to extensive reversal or rescue of polarity.

Thus, my data support a Ft-Ds signaling model in the *Drosophila* eye wherein different levels of Ft and Ds lead to modulation of a long-range polarity effector that directs further polarity signaling in the eye independently of changes in the asymmetric distribution of Ft and Ds. Ultimately, this signaling would bias Notch and Delta activity between polar and equatorial precursors, leading to R3/4 fate specification and ommatidial polarity establishment (Cooper and Bray, 1999; Fanto and Mlodzik, 1999; Strutt *et al.*, 2002; Tomlinson and Struhl, 1999).
3.2 Nature of the long-range polarity signal

Though my data strongly support the existence of a long-range signal, its identity is open to speculation. It could be a secreted molecule that acts as a ligand. Alternatively, the secreted molecule could modify another pathway’s ligand that is present in the extracellular medium. The signal could be a cell-cell relay as well, but it would have to be a rather robust one given the extent of regulated polarity I observe inside ft and ds mutant clones. Intriguingly, epithelial cells mutant for ft have disruptions in gap junctions (Bryant et al., 1988). Might the long-range signal normally travel between cells through such intercellular connections?

Signaling molecules that travel long distances and set up gradients are of course ubiquitous in development (Rogers and Schier, 2011). In the eye, the Wingless, Hedgehog, Decapentaplegic, and JAK/STAT pathways regulate long-range developmental processes (reviewed in Tsachaki and Sprecher, 2012). But these pathways all seem to act upstream of polarity establishment, for example in regulating morphogenetic furrow progression or overall dorso-ventral identity (as opposed to ommatidial polarity). Neither of these pathways fulfill the requirements of a long-range polarity signal in the wing or abdomen (discussed in Lawrence and Casal, 2013; Strutt, 2009).

Could the long-range signal in the eye be created by, or interact with mechanical forces during development? In the developing wing, mechanical forces play important roles in planar polarity establishment: Loss of ft, ds, and d alters cell shape (Bosveld et al., 2012; Harumoto et al., 2010; Mao et al., 2011b), and Dachs asymmetric localization depends on Ft and Ds activity (Ambegaonkar et al., 2012; Brittle et al., 2012); it is thus proposed that anisotropic constriction of junctions by Dachs myosin activity regulates oriented cell division, rearrangements, and movement (Aigouy et al., 2010; Bosveld et al., 2012; Mao et al., 2011b; Sagner et al., 2012). These cellular events generate coordinated tissue flow, which then orients downstream polarity regulators (Aigouy et al., 2010; Sagner et al., 2012), by generating mechanical tension that in
Turn orient microtubules along the cell axis and regulates asymmetric transportation of members of the Fz/PCP pathway (Harumoto et al., 2010; Shimada et al., 2006). Removal of mechanical tension by severing links between cells by laser ablation disrupts normal polarity establishment (Aigouy et al., 2010; Bosveld et al., 2012). Do similar events occur in the eye? Oriented cell division does occur in the eye disc (Baena-Lopez et al., 2005), and mechanical forces are known to be important in late-larval and pupal eye development (Hilgenfeldt et al., 2008; Käfer et al., 2007). Asymmetric localization of Ft-Ds-Dachs and Fz/PCP components is also present in the eye (Brittle et al., 2012; Yang et al., 2002). A direct role for mechanical tension in eye polarity establishment has not yet been tested. The morphogenetic furrow, a wave of apical constriction that passes over the eye, might serve as a source of oriented mechanical tension – intriguingly, changes in ft, ds, and fj alter normal patterns of cell division that are associated with passage of the furrow (Bennett and Harvey, 2006; Silva et al., 2006; Willecke et al., 2006; Willecke et al., 2008). However, regulation of junctional tension by Dachs is not necessarily required for polarity information transmission in the eye, as my data shows that polarity rescue and reversal occur inside ft dachs double mutant clones.

Could the differences in dependence of polarity effects on clone shape between ft and ds clones reflect differences in mechanical forces between these clones? Research in the cotton bug showed that differences in cell adhesion can orient cells and thus affect their planar polarity (Nübler-Jung and Mardini, 1990). It is possible that cell adhesion differs between ft and ds clone boundaries; alternatively, there might be differences in cell shapes, or orientations of cell division or rearrangement at these clone boundaries that result in different polarity effects.

What might mutant clones of the long-range signal be like? One possibility is that they would look very similar to ft or ds mutant clones, with organized polarity immediately inside the clone border, due to the signal traveling in from surrounding wild-type tissue, but randomized polarity near the middle. These mutant clones of the signal could show polar or equatorial non-autonomy, depending on whether Ft-Ds signaling positively or negatively modulates the signal. A double mutant of the putative signal and ft or ds would show clear epistasis, unlike ds ft double mutants.
3.3 Relationship between Ft-Ds and Fz/PCP pathways

The Fz/PCP pathway itself is a candidate for being the long-range polarity signal. In the eye, it seems to act mostly downstream of Ft-Ds signaling, though there are also data that point towards the pathways acting in parallel (Strutt and Strutt, 2002 and this work). However, it is not clear whether Fz/PCP can signal directionally over long distances without upstream input, as the long-range signal appears to do in ft and ds clones, and existing models of redistribution-based polarity changes caused by Fz/PCP pathway clones imply a range of no more than ~4 cells (Amonlirdviman et al., 2005). In other contexts, such as the abdomen, Fz/PCP and Ft-Ds are able to act independently of each other (Casal et al., 2006). Both pathways display intracellular asymmetry of their components, and each can maintain this asymmetry in the absence of the other (Ambegaonkar et al., 2012; Brittle et al., 2012; Yang et al., 2002). In both the wing and the eye, the relationship between Ft-Ds and Fz/PCP also changes at different times in development (Hogan et al., 2011; Strutt and Strutt, 2002). Their relationship is thus complex, and is unlikely to be resolved without determination of the actual mechanisms that comprise the largely phenomenological concepts of “ft activity” and “fz activity”.

3.4 Functional domains of Fat and Dachsous

It is interesting to consider how the same factors, Ft, Ds, and Fj, working towards the same goal – the establishment of tissue polarity – appear to work in different ways in different contexts (Casal et al., 2006; Matakatsu and Blair, 2012; Willecke et al., 2008). For example, in the abdomen, ds ft clones have no polarity effects outside clones, while there are clear, though limited, polarity disruptions outside ds ft clones in the eye. In contrast to my conclusion that polarity propagation is independent of Ft and Ds in the eye, Casal and coworkers argue that Ds and Ft are both needed in the receiving cell to propagate Ft and Ds polarity information in the abdomen (Casal et al., 2006).
A novel feature of our work is the quantitative analysis of polarity effects inside clones in the eye. Similar analyses conducted in the wing and abdomen would form the best test of whether the long-range signal model applies in other tissues. If, for example, polarity were completely randomized after 1 or 2 cells inside ft or ds mutant clones in the wing, it would support the redistribution model; if longer range rescue and reversal were observed, the long-range signal model would be a better explanation.

Considerable variation exists in the parts of the Ft ICD responsible for polarity and growth regulation in different tissues (Bossuyt et al., 2013; Matakatsu and Blair, 2012; Pan et al., 2013; Zhao et al., 2013). Meanwhile, FtΔECD cannot rescue polarity effects of ft clones in the eye (this work and Zhao et al., 2013), unlike in the wing and abdomen (Casal et al., 2006; Matakatsu and Blair, 2006; Matakatsu and Blair, 2012), which might suggest that Ft-Ds trans binding is more important for polarity in the eye than in other tissues.

Ft and Ds orthologs are highly conserved among metazoans, with a remarkable consistency in the exact number of extracellular cadherin repeats across hundreds of millions of years of evolution (Hulpiau et al., 2013). Ft and Ds might have established planar cell polarity in a simple ancestral epithelium. It is reasonable to think that as specialized epithelia evolved, with different pathways regulating their development and different functional requirements for planar polarity, so the upstream and downstream interactors of Ft and Ds changed. Ease of scientific study is not a goal in the evolution of biological processes, and it is not surprising that the details of interactions between Ft-Ds pathway components differ with developmental context. Continued structure-function analysis will further improve our understanding of Ft-Ds interactions.
3.5 Fat-Dachsous effectors

How do Ft and Ds control polarity? The atypical myosin Dachs (D) acts downstream of Ft to regulate the Hippo pathway (Cho and Irvine, 2004; Cho et al., 2006; Feng and Irvine, 2009; Mao et al., 2006; Willecke et al., 2008), and is also asymmetrically localized by Ft-Ds signalling (Ambegaonkar et al., 2012; Brittle et al., 2012). However, as I and others have shown D is not required for polarity establishment (Brittle et al., 2012). Thus, while D is a downstream interactor, it is not a Ft-Ds polarity effector.

Atrophin (Atro) interacts with Ft physically and genetically, affects polarity in the eye and wing, and seems to regulate the long-range polarity signal in the same direction as Ft (this work and Erkner et al., 2002; Fanto et al., 2003; Zhang et al., 2002). As Atro is a transcriptional co-repressor, a natural model of its function is that it modulates transcription of specific targets that further regulate polarity, perhaps including the long-range polarity signal, in response to Ft-Ds signalling. Importantly, changes in Atro levels, localization, or binding state correlated with changes in Ft or Ds levels or binding have not yet been observed. Thus this model, while reasonable, remains unproven.

I found that atro clones strongly affected polarity only near the equator of the eye. As ft clones affect polarity to the same degree near the poles and the equator, there is likely another effector of Ft-Ds signalling that performs the same function near the poles that atro does near the equator. It is possible that this effector might be the Ft ICD itself. Both Ft and Ds are post-translationally cleaved (Ambegaonkar et al., 2012; Feng and Irvine, 2009; Sopko et al., 2009). The Ft ICD might translocate to the nucleus to act with cofactors to modulate transcription in response to Ds binding. This model is similar to the nuclear translocation of the Notch ICD and subsequent transcriptional regulation in response to Delta/Serrate binding (Fortini, 2009).
3.6 Future directions

Some of the most striking findings of my work are: 1. There exists a long range polarity signal regulated by Ft-Ds signaling in the eye, as shown by polarity rescue and reversal inside mutant clones; 2. Ds does not just act as a repressive ligand for Ft, but it (a) can regulate Ft in the same cell, as shown by the Ft-dependent dominant-negative polarity effects of DsΔECD, (b) likely signals through its own cytoplasmic domain, as shown by the opposite polarity effects of ft and ds mutant clones, and (c) lacks the shape-dependent clonal polarity effect of ft clones; 3. Atro primarily functions near the equator, and one or more other molecules must perform the Ft-Ds effector function near the poles. I propose three projects to further explore each of these findings.

1. Identification of the long range polarity signal: It is likely that this signal is a secreted protein. Thus, proteins that are predicted to be secreted or cleaved extracellularly are candidates to be the signal, and an RNAi screen could be conducted with their genes as targets. Polarity could be examined in all tissues by using actin or tubulin drivers, just in the eye using an eyeless driver, or in and around clones, using FRT-flip out or MARCM. RNAi or deficiencies could also be used to uncover candidate genes in a sensitized background, such as fj mutant flies.

The signal could also be searched for using the effect of the Ft-Ds pathway on it. If Ft-Ds signaling affects transcription of the signal, it could be detected using microarrays comparing whole-genome expression patterns in wild-type and ft or ds mutant tissue. Whole-proteome analysis using mass spectrometry can be used to compare changes in protein levels or post-translational modifications in wild-type and Ft-Ds mutant tissue, or peptides present in supernatants of wild-type and Ft-Ds mutant tissue or knockdown cell culture. This could reveal the long range signal if Ft-Ds signaling regulates it by controlling its post-translational processing, or by affecting its secretion or endocytosis. If these approaches are unsuccessful in identifying the long range signal, broader methods
could be used, such as EMS or hybrid dysgenesis mutagenesis followed by screening for polarity effects, or screens examining clonal phenotypes using FRT chromosomes.

How could the identity of the long range signal be confirmed? The signal would have to be capable of diffusing or otherwise traveling across ~40 cell diameters, which could be tested using antibody staining around clone borders or GFP-labeling followed by live imaging. It would have to respond to and be oriented by Ft-Ds signaling. It would likely normally exist in a standing gradient, or possibly a dynamic dorso-ventral oriented flow. Its clonal phenotype would be clearly epistatic to ft and ds and, critically, its directed overexpression would establish polarity in the absence of ft and ds. Live imaging can also help decipher changing cell contacts in discs. Tracking of cells from before to after passage of the furrow over a particular spot could show, for example, if cells' DV orientation relative to each other is labile before differentiation, helping to clarify the timing of action of the long-range signal.

2. **Determination of Ds function:** Compared to Ft, Ds is poorly studied, and several approaches can be used to better understand its role in PCP. *Trans*-binding of Ft and Ds across different cells occurs in a Ca\(^{2+}\)-dependent manner, and is thus likely a form of cadherin dimer formation, a well-understood process. However, my work suggests that DsΔECD, which does not have any cadherin domains, could interact with Ft in the same cell, presumably through the transmembrane or cytoplasmic domains. This binding can be confirmed through immunoprecipitation of FtΔECD by DsΔECD and vice versa, using antibodies or tagged versions of the proteins. Once binding is confirmed, it can be better understood by examining the Ft-Ds bound structure using NMR spectroscopy or X-ray crystallography. Once the long range signal is identified, its regulation by binding of overexpressed DsΔECD to Ft in the same cell can also be observed.
My results suggest that Ds has downstream effectors independent of Ft. Although D physically interacts with Ds, it does not act as a polarity regulator. Thus, novel interactors of Ds should be identified. This could be done by immunoprecipitation of the Ds ICD followed by mass spectrometry, or a yeast two-hybrid screen. Once putative interactors are identified, their binding to different functional domains as defined by structure-function analysis can be determined. Structure-function analysis could also reveal sequences important for Ft-Ds cis binding. Transcriptional targets of Ds, especially those different from Ft targets, could be discovered by comparing microarrays of wild-type, ft mutant, and ds mutant tissue.

I previously discussed how different effects of clone shape on ft and ds clonal polarity effects could be due to differential accumulation of Ft and Ds on parallel and slanted borders. This could be examined by high-resolution imaging of tagged versions of Ft and Ds on clone borders that would allow quantification of the degree of Ft and Ds accumulation. However, it is also possible that the different effects of clone shape are due to different organization of cells in and around the clones. Ft-Ds signaling regulates many aspects of cell shape and movement in wing and notum development (Aigouy et al., 2010; Bosveld et al., 2012). Live imaging and quantification of cell shape and movement in and around ft and ds mutant clones could reveal changes important for causing different degrees of polarity reversal in the eye. For example, different effects of ft and ds on oriented cell division, cell adhesion, or cell movement could lead to ft clones being more circular when they are first created. The slanted borders of ft clones, not being orthogonal to the direction of the polarity signal gradient (which would be oriented along the dorso-ventral axis) would have less disruption of polarity. ds clones, on the other hand, could initiate with a flatter shape with the long axis along the antero-posterior axis, such that most of the clone border would be orthogonal to the polarity signal gradient, and only acquire a rounded shape after ommatidial chirality is established. In these clones, polarity effects would be similar around what eventually become parallel and slanted borders. Live imaging could reveal these dynamic changes in clone shape. Alternately, ft clones could disrupt transmission of the long-range signal in a directionally biased manner, while ds clones would not. Imaging could reveal if directionally biased
movement of the signal, once identified, occurs along slanted borders of $ft$ clones compared to parallel borders, or along both types of $ds$ clone borders.

3. **Determination of Atro function and identification of other effectors:** As previously discussed, Atro probably acts in response to changes in Ft-Ds signaling to regulate a downstream factor. It is possible that changes in Atro levels or nuclear localization in response to Ft-Ds signaling are subtle, and could be revealed with higher resolution and more sensitive imaging. It is also possible that Atro is post-translationally modified in response to Ft activity, and these changes could be discovered using mass spectrometry. On the other hand, despite their physical interaction, Atro might not be directly affected by Ft activity; instead, Ft-Ds signaling could regulate an interaction partner of Atro. Whatever the nature of regulation of Atro by Ft, it seems likely that this regulation applies mainly near the equator. Farther from the equator, one or more factors must serve the same function as Atro. They may or may not physically bind Ft, but should be able to affect the long range polarity signal, as should Atro. It is possible that the Ft ICD itself acts as a Ft-Ds signaling effector in part or all of the eye, or in other tissues. If a Ft binding partner is involved, such a protein could be discovered by techniques similar to those suggested for Ds above, and indeed a mass spectrometry-based search for Ft interaction partners is already underway in my lab.
Appendix 1

Fat4 and Cell Growth
Appendix 1: Fat4 and cell growth

Introduction

The Hippo pathway regulates tissue growth in invertebrates and vertebrates (Bao et al., 2011; Grusche et al., 2010; Halder and Johnson, 2011; Staley and Irvine, 2012). Changes in Hippo signaling can result in increased size of heart and liver in mice (Camargo et al., 2007; Dong et al., 2007; Heallen et al., 2011). Drosophila ft is an upstream activator of Hippo signaling (Bennett and Harvey, 2006; Cho et al., 2006; Silva et al., 2006; Willecke et al., 2006). The mammalian ortholog of Drosophila ft, fat4, regulates many aspects of development: fat4 mutants have defective kidneys, lungs, and intestines, along with broader neural tubes and cochleae, and do not survive past birth (Mao et al., 2011a; Saburi et al., 2008; Saburi et al., 2012). The fat4 paralog fat1 acts with yap1 in zebrafish to regulate pronephros development (Skouloudaki et al., 2009). fat4 acts with yap1 to regulate neural progenitor cell proliferation in the chick neural tube, and fat4 knockdown increases the number of mitotic cells, which is similar to the effect of loss of ft in Drosophila (Van Hateren et al., 2011). ft vertebrate homologs are involved in human cancers: fat4 was lost in induced mouse mammary tumors, and several human primary tumors and breast cancer cell lines (Qi et al., 2009) with loss of fat4 also leading to increased yap1 activity in cell culture. Loss of heterozygosity of fat1 is associated with astrocytic gliomas (Chosdol et al., 2009), and homozygous loss of fat1 is associated with oral cancers (Nakaya et al., 2007).

Changes in Hippo signaling in cultured mammalian cells can cause aberrant cell behavior, such as increased growth rates, loss of contact-inhibition of growth, and anchorage-independent growth (Lallemand et al., 2003; Overholtzer et al., 2006; Zhao et al., 2007). I asked whether cells mutant for fat4 display such aberrant behavior.
Methods

1. Isolation of mouse embryonic fibroblasts (MEFs):

1. Dissect out embryos from E13.5 pregnant mouse.
2. Place in PBS in petri dish.
3. Discard placenta and amniotic membrane.
4. Remove head, place in microfuge tube.
5. Excise and discard liver.
6. Wash rest of body, then place in cell culture plate/well containing 2 ml 0.05% trypsin/EDTA.
7. Homogenize tissue with 19 ga. syringe followed by 22 ga. syringe (pass up and down ~10 times).
8. Add 2ml growth medium (DME high glucose + 1/10 vol. FBS, 1x (2mM final) L-glutamine, 1x (100 U/ml final) penicillin/streptomycin (henceforth DMEM/10% FBS or growth medium).
9. Grow overnight at 37°.
10. Change medium (no wash required, add 4 ml).

2. MEF passage:

1. Aspirate medium.
2. Wash with 4 ml PBS.
3. Aspirate PBS.
4. Add 1 ml 0.25% Trypsin/EDTA. Incubate for 5 minutes at 37°.
5. Add 2-3 ml DMEM/10% FBS growth medium. Pipette well. Counting can be done at this step. Staining with trypan blue is recommended to exclude dead cells from the count.

6. Transfer desired number/dilution of cells to new wells (1.5×10^5 cells/well in a 6-well plate for growth rate measurement; all cells for contact-inhibition measurement). If necessary, add growth medium in new wells.

3. Soft agar assay

1. Boil 3.3% agar to dissolve. Allow to cool, then place all media and agar in 45° water bath for 10 minutes
2. In 50 ml tube mix 3 ml 3.3% agar, 5 ml 2x DMEM, 10 ml 1x DMEM, and 2 ml FBS
3. Pipet 2 ml/well into 6 well clusters
4. Allow to set without disturbing in hood for 30 minutes
5. Turn water bath to 41°
6. Boil 1.8% agar to dissolve; allow to cool
7. Transfer 2x DMEM, FBS and agar to 41° water bath for 10 minutes
8. Detach cells and dilute to 3.4×10^5 cells/ml in DMEM
9. In 50 ml tube mix 3 ml 1.8% agar, 6.7 ml 2x DMEM, 3.6 ml DMEM, and 1.7 ml FBS
10. Allow to cool for 20 minutes then add 2 ml cells
11. Carefully pipet 0.5 ml on top of base layer of agar and allow to set in hood for 30 minutes. Then transfer to incubator.

Results

I examined three aspects of fat4 mutant MEFs in comparison to wild-type MEFs. Growth rates were measured by passaging cells every 3 days – at each passage cells were counted, then replated at a set concentration (1.5×10^5 cells/well). A projected cumulative cell number was
calculated by taking into account the amount of growth at each passage. This would then give the number of cells that would be alive if they were allowed to grow with infinite medium and surface area. Cumulative cell numbers were essentially identical for two fat4+/− lines (each line is from a separate embryo) and one fat4−/− line (Figure A1).

Loss of contact-inhibition in growth was also measured by initially plating $5 \times 10^4$ cells/well, then passaging and counting them every 2 days. Unlike in growth rate measurement, all cells were replated in the same surface area without dilution. Counts from three separate fat4+/+ lines and three fat4−/− lines were averaged for final wild-type and mutant cell numbers. Growth arrested at similar times and densities for both wild-type and mutant cells (Figure A2).

Anchorage-independent growth is measured by plating cells in soft agar, where they exist in semi-solid suspension. Cells from three fat4+/+ lines and three fat4−/− lines were unable to display anchorage-independent growth.
Figure A1: Growth rates of fat4+/− and fat4−/− MEFs.

No significant difference is observed in growth rates of fat4+/− and fat4−/− MEFs.
Figure A2: Loss of contact inhibition in $fat4^{+/+}$ and $fat4^{-/-}$ MEFs.

Growth of both $fat4^{+/+}$ and $fat4^{-/-}$ MEFs arrests at high densities.
Discussion and future directions

My experiments found no significant differences in the growth characteristics of wild-type and fat4 mutant MEFs. However, Ben Pakuts in my lab examined other aspects of these cells and found several differences. Wild-type MEFs have two centrioles after S-phase, and one primary cilium. A significant number of fat4−/− MEFs have four centrosomes and two primary cilia per cell, indicating defects in centrosome maturation and ciliogenesis. Cilia in fat4−/− MEFs also tend to be more irregular. fat4−/− MEFs tend to have larger nuclei and a higher proportion of binucleate cells compared to wild-type MEFs. A larger proportion of fat4−/− MEFs were found to be in G2 compared to wild-type cells. fat4−/− MEFs arrested in G2 by nocodazole treatment occasionally showed an increase in proliferation based on BrdU/EdU incorporation. Of cells in mitosis, a larger proportion of fat4−/− MEFs compared to wild-type were in telophase, and these cells often had irregular nuclei. Thus, though fat4 does not seem to directly affect growth in MEFs, it is able to modulate aspects of cytoskeleton and cell cycle regulation. In particular, cilia act as signaling centers in mammalian cells, and understanding the effect of fat4 on ciliogenesis could reveal new links to other pathways.

Loss of fat4 affects growth in other contexts. Dr. Sakura Saburi in my lab used SV40 T-antigen expressing mice to generate immortalized renal epithelial cell lines from the Henle’s loop and collecting duct of developing E15.5 kidneys. She found that fat4−/− cells from both parts of the kidney grew less than corresponding wild-type cells. fat4−/− mouse kidneys are smaller than wild-type kidneys, and E11.5-11.75 fat4−/− kidneys have reduced proliferation and increased apoptosis compared to wild-type kidneys (Mao et al., 2011a), though these effects are not seen at E16.5 (Saburi et al., 2008). Thus, in at least some stages of kidney development, fat4 is necessary for growth. However, in other tissues fat4 acts to inhibit growth: loss of fat4 causes increased proliferation in chick neural progenitors (Van Hateren et al., 2011), and a similar process might be responsible for broadened spinal cords in fat4 mutant mice (Mao et al., 2011a; Saburi et al., 2012). Inhibition of fat4 promotes tumorigenesis in cells injected into mice (Qi et al., 2009).
fat4 thus seems to both positively and negatively regulate cell growth in different developmental contexts. This regulation is unlikely to occur directly through the Hippo pathway, as it does in flies (Bossuyt et al., 2013), though the pathways do interact in vertebrates (Skouloudaki et al., 2009; Van Hateren et al., 2011). It would be interesting to see if fat4 affects the vertebrate homologs of its growth-regulating targets in Drosophila, such as the diap1 homolog naip5 and the cyclin E homologs ccne1/2. The interaction of fat4 with other growth regulating pathways, such as TOR and IGF-1, can also be examined.

Clarification of the role of fat4 in regulating growth in mammals and other vertebrates will require examination of a variety of cell types, both in culture and in vivo. siRNA-mediated knockdown of fat4 is effective (Ishiuchi et al., 2009) and can allow rapid screening of many cell cultures and tissues to determine if any growth effects occur.
References


polarity via physical interactions with Atrophin, a transcriptional co-repressor. *Development* 130, 763–74.


