Characterization of Gf

a *Drosophila* trimeric G protein alpha subunit

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Abstract

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In the morphogenesis of tissue development, how coordination of patterning and growth achieve the correct organ size and shape is a principal question in biology. Efficient orchestrating mechanisms are required to achieve this and cells have developed sophisticated systems for reception and interpretation of the multitude of extracellular stimuli to which they are exposed. Plasma membrane receptors play a key role in the transmission of such signals. G-protein coupled receptors (GPCRs) are the largest class of cell surface receptors that respond to an enormous diversity of extracellular stimuli, and are critical mediators of cellular signal transduction in eukaryotic organisms. Signaling through GPCRs has been well characterized in many biological contexts. While they are a major class of signal transducers, there are not many defined instances where GPCRs have been implicated in the process of development to date. The Drosophila wing provides an ideal model system to elucidate and address the role of GPCRs in development, as its growth is regulated by a small number of conserved signaling pathways. In my thesis work, I address the role of a trimeric G alpha protein in Drosophila, Gαf, and what part it may play in development. In particular, I explore the role of Gαf as an alpha subunit of a trimeric complex, to determine what heptahelical receptors might act as its cognate receptor.
# Table of Contents

List of Abbreviations ........................................................................................................... x
List of Figures and Tables ...................................................................................................... v

Chapter I: General Introduction .......................................................................................... 1
1. Introduction to heterotrimeric G-protein signaling ....................................................... 2
  1.1 Overview .................................................................................................................... 2
1.2 G-protein Coupled Receptors (GPCRs) ...................................................................... 3
1.3 Heterotrimeric G-proteins ......................................................................................... 5
1.4 Architecture of a $G\alpha$ subunit ............................................................................... 8
1.5 *Drosophila* $G\alpha$f ............................................................................................... 11

2. Heterotrimeric G-Protein Signaling Overview ............................................................... 16
  2.1 Heterotrimeric G-Protein Signaling in Asymmetric Cell Division (ACD) .................. 16
  G-Protein Coupled Receptors (GPCRs) / 7-Transmembrane Receptors ....................... 18
  2.2 Wnt/Wingless Signaling Pathway ............................................................................ 18
  2.3 The Planar Cell Polarity (PCP) / Frizzled signaling pathway .................................... 20
  2.4 Frizzled as a GPCR ................................................................................................ 23
  2.5 The Hedgehog Signaling Pathway ........................................................................... 25
  2.6 Smoothened as a GPCR ......................................................................................... 26

3. The *Drosophila* Wing ................................................................................................... 28

Chapter II: Characterizing G alpha f ................................................................................. 50
Abstract ............................................................................................................................ 51

1. Introduction ................................................................................................................... 51

2. Materials and Methods ................................................................................................. 55
  2.1 *Drosophila* strains and maintenance .................................................................... 55
  2.2 Construction of fly lines ......................................................................................... 57
  2.3 Immunohistochemistry ......................................................................................... 58
2.4 Generation of a Gαf antibody...............................................................58
2.5 Histology................................................................................................59
3. Results........................................................................................................59
3.1 RNAi-mediated knockdown of Gαf in the Drosophila wing causes various patterning
defects............................................................................................................59
3.2 Little discernible phenotype produced with RNAi-mediated knockdown of Gαf in the
Drosophila eye..................................................................................................63
3.3 Ectopic expression of Gαf in the adult wing causes minor patterning defects..................63
3.4 Protein patterning in the wing disc....................................................................65
3.41 Characterization of the protein expression patterns correlated with knockdown of Gαf
in the wing disc..............................................................................................65
3.42 Induction of TRiP UAS-Gαf RNAi clones.......................................................67
3.43 Characterization of the protein expression patterns in the wing disc correlated with ectopic
Gαf expression..................................................................................................68
3.5 Genetic Interactions.......................................................................................69
3.51 Involvement of Gαf in Wnt/Wg signaling pathway..........................................69
3.52 Involvement of Gαf in Fz/PCP signaling pathway............................................70
3.53 Involvement of Gαf in Hh signaling pathway..................................................71
3.54 Involvement of Gαf in asymmetric cell division...............................................74
4. Discussion.....................................................................................................76
4.1 Gαf expression gives weak phenotypes that are characteristic of wg features.............76
4.2 Gαf expression may affect dpp........................................................................77
4.3 Gαf knockdown gives phenotype consistent with PCP signaling..........................78
4.4 Ectopic Gαf may affect hedgehog signaling......................................................78
4.5 Cell numbers decrease with Gαf downregulation..............................................79
4.6 Gαi and Gαf act redundantly in SoP cells.......................................................81
4.7 Continuing Characterization of Gαf..............................................................83
Chapter III: Description of Other Related Projects

1. Mis-localization of planar cell polarity proteins in the wing by adapting the Boss/Sevenless ligand/receptor proteins of the eye

Abstract

1.1 Introduction

1.2 Materials and Methods

1.3 Results

    Initial Strategy

    Second Strategy

    Third Strategy (‘Quick and Dirty’)

    Fourth Strategy

    Fifth Strategy

    Sixth Strategy

1.4 Discussion

2. Examination of the role of $G_\alpha_o$ and microtubules

Abstract

2.1 Introduction

2.2 Materials and Methods

2.3 Results

2.4 Discussion

3. Candidate Screen

Abstract

3.1 Introduction

3.2 Materials and Method

3.3 Results

3.4 Discussion
Chapter IV: General Discussion........................................................................................................169
Appendix........................................................................................................................................181
References....................................................................................................................................192
List of Figures and Tables

Chapter I: Figures and Tables

Figure 1. Heterotrimeric G-protein signaling ..........................................................32
Figure 2. Schematic of Gα protein ...........................................................................33
Figure 3. Structures of Gα .......................................................................................34
Figure 4. Alignment of Drosophila Gα Amino Acid Sequences ..............................35
Figure 5. Schematic diagram of the putative secondary structure of Gαf ..................37
Figure 6. Phylogenetic analysis of G-protein amino acid sequences ........................38
Figure 7. Schematic of SOP divisions and resulting sense organs .........................40
Figure 8. The Wnt/Wg Signaling Pathway ..............................................................41
Figure 9. The Planar Cell Polarity Pathway in Drosophila ......................................42
Figure 10. Planar Cell Polarity in Drosophila .........................................................43
Figure 11. Helical Structures of Frizzleds and Smoothened ....................................44
Figure 12. The Hedgehog Signaling Pathway in Drosophila .................................45
Figure 13. Wing Fate Map ......................................................................................46
Figure 14. Patterning of the wing ...........................................................................47
Table 1. Trimeric Gα subunits in Drosophila .........................................................49

Chapter II: Figures and Tables

Figure 15. The Gαf gene .........................................................................................86
Figure 16. Gαf knockdown phenotypes in the adult wing ......................................87
Figure 17. Overexpression of three separate RNAi lines ........................................89
Figure 18. Overexpression of three separate UAS-Gαf RNAi lines show similar phenotypes .............................................................91
Figure 19. Overexpression of TRiP UAS-Gαf RNAi line shows enhancement with Deficiencies .................................................................93
Figure 20. Overexpression of TRiP UAS-Gαf RNAi line shows enhancement with Deficiencies .................................................................95
Figure 21. Overexpression of TRiP UAS-Gαf RNAi with a non-overlapping UAS-Gαf RNAi line shows enhancement..........................................................................................................................97
Figure 22. Overexpression of TRiP UAS-Gαf RNAi with a non-overlapping UAS-Gαf RNAi line shows enhancement..........................................................................................................................99
Figure 23. Overexpression of TRiP UAS-Gαf RNAi with a non-overlapping UAS-Gαf RNAi line shows enhancement..........................................................................................................................101
Figure 24. Overexpression of TRiP UAS-Gαf RNAi in the wing and the eye...............................103
Figure 25. Gαf overexpression................................................................................................104
Figure 26. TRiP UAS-Gαf RNAi causes perturbation in Wg activity and downregulation of Vg....105
Figure 27. TRiP UAS-Gαf RNAi causes downregulation of Dll expression...............................107
Figure 28. TRiP UAS-Gαf RNAi causes minor effects on Sal expression.................................108
Figure 29. TRiP UAS-Gαf RNAi causes downregulation of Cut expression...............................109
Figure 30. Scalloped wings can be seen with clones of TRiP UAS-Gαf RNAi............................110
Figure 31. Overexpression of UAS-Gαf wild type with MS1096-G4 does not show any obvious defects........................................................................................................................................111
Figure 32. fz2 overexpression................................................................................................113
Figure 33. fz1 overexpression................................................................................................114
Figure 34. smo overexpression...............................................................................................115
Figure 35. UAS-Gαi RNAi appears to enhance TRiP UAS-Gαf RNAi phenotypes....................117
Figure 36. UAS-Gαi RNAi appears to enhance TRiP UAS-Gαf RNAi phenotypes....................119
Figure 37. UAS-Gαi RNAi appears to enhance TRiP UAS-Gαf RNAi phenotypes....................121
Figure 38. UAS-Gαi RNAi appears to enhance TRiP UAS-Gαf RNAi phenotypes....................123
Figure 39. Enhancement of defects observed with two non-overlapping Gαf RNAi expression..................................................................................................................................................127
Figure 40. Overexpression of Gαf..........................................................................................130
Figure 41. Reduction of ectopic bristle number corresponds with a reduction in Gαf transcript.................................................................................................................................................131
Table 25. Ectopic bristles in posterior compartment

Table 24. Genetic interaction of Gaf with Gai shows enhancement of the venation defect

Table 23. Genotype: UAS-dcr2/+; en-G4/+; Gaf TRiP/+ 

Table 22. Genotype: UAS-dcr2/+; en-G4/VDRC 

Table 21. Genotype: UAS-dcr2/+; en-G4/+; 12232R-1/+ 

Table 20. Genotype: MS1096-G4/+; UAS-dcr2/+; Gaf TRiP/+ 

Table 19. Genotype: MS1096-G4/+; Gaf TRiP/Gaf TRiP (No Dicer) 

Table 18. Genotype: MS1096-G4/+; UAS-dcr2/VDRC 

Table 17. Genotype: MS1096-G4/+; UAS-dcr2/+; 12232R-1/+ 

Table 16. Genotype: UAS-dcr2/en-G4; Gaf TRiP/12232R-1 

Table 15. Genotype: UAS-dcr2/en-G4; Gaf TRiP/Df(3L)Exel6130 

Table 14. Summary of Phenotypes induced by knockdown of Gaf with various wing GAL4 driver lines 

Table 13. Genotype: UAS-dcr2/en-G4; Gaf TRiP/Df(3L)ED223 

Table 12. Genotype: UAS-dcr2/en-G4; Gaf TRiP/DDf(3L)BSC561 

Table 11. Genotype: UAS-dcr2/en-G4; Gaf TRiP/Df(3L)Df4674 

Table 10. Genotype: UAS-dcr2/en-G4; Gaf TRiP/Df(3L)Exel6130 

Table 9. Genotype: UAS-dcr2/en-G4; Gaf TRiP/12232R-1 

Table 8. Genotype: UAS-dcr2/en-G4; Gaf TRiP/Df(3L)ED223 

Table 7. Genotype: MS1096-G4/+; UAS-dcr2/VDRC 

Table 6. Genotype: MS1096-G4/+; Gaf TRiP/Gaf TRiP (No Dicer) 

Table 5. Genotype: MS1096-G4/+; UAS-dcr2/+; Gaf TRiP/+ 

Table 4. Genotype: MS1096-G4/+; UAS-dcr2/+; 12232R-1/+; Gaf TRiP/+ 

Table 3. Genotype: UAS-dcr2/+; en-G4/VDRC 

Table 2. Genotype: UAS-dcr2/+; en-G4/+; Gaf TRiP/+ 

Figure 43. Genetic interaction of Gaf with Gai shows enhancement of the venation defect.
Table 26. Genotype: en-G4/+;UAS-Gaf/fz,fz2 .........................................................134
Table 27. Genotype: MS1096-G4/+;UAS-Gaf/fz,fz2 .........................................................134
Table 28. Genotype: en-G4,UAS-dcr2/+;Gaf TRIP/fz,fz2 .........................................................135
Table 29. Genotype: MS1096-G4/+;UAS-dcr2/+;Gaf TRIP/fz,fz2 .........................................................135
Table 30. Genotype: MS1096-G4/+;UAS-dcr2/+;Gaf TRIP+/ (control) .........................................................135
Table 32. Genotype: en-G4/smo3;UAS-Gaf .........................................................136
Table 33. Genotype: UAS-dcr2, en-G4/smo3;Gaf TRIP/+ .........................................................137
Table 34. Genotype: UAS-dcr2/en-G4/smo3;Gaf TRIP/+ .........................................................137
Table 35. Genotype: MS1096-G4/+;UAS-dcr2/smo3;Gaf TRIP/ + .........................................................138
Table 36. Genotype: MS1096-G4/+;UAS-dcr2/+;Gai RNAi/Gaf TRIP .........................................................139

Chapter III: Figures and Tables ..............................................................................157

Figure 44. Cross-section through developing ommatidia ..............................................158
Figure 45. Chimeric proteins bearing the extracellular domain of Sev fused to individual PCP proteins .................................................................159
Figure 46. Initial Strategy .............................................................................................160
Figure 47. blink.boss co-expression with hh > UAS-sev-gfp-Gaf* ..........................................161
Figure 48. ci.LexA used to drive LexO.boss ................................................................162
Figure 49. Aggregation of Boss- and Sev-chimera – expressing S2 cells ................................163
Figure 50. S2 cells expressing Sev-chimera stained for acetylated Tubulin .........................164
Figure 51. Aggregates stained for Tubulin ...................................................................165
Figure 52. Ectopic expression of boss causes a mwh phenotype .......................................166
Table 39. Candidate genes that were screened ..........................................................167
List of Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
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<tbody>
<tr>
<td>Ap</td>
<td>Apterous</td>
</tr>
<tr>
<td>ACV</td>
<td>Anterior cross vein</td>
</tr>
<tr>
<td>ACD</td>
<td>Asymmetric Cell Division</td>
</tr>
<tr>
<td>APC</td>
<td>Adenomatus Polyposis Coli</td>
</tr>
<tr>
<td>AP</td>
<td>Antero-posterior</td>
</tr>
<tr>
<td>AttB</td>
<td>Attachment B</td>
</tr>
<tr>
<td>β2-AR</td>
<td>β2-adrenergic receptor</td>
</tr>
<tr>
<td>Boss</td>
<td>Bride of Sevenless</td>
</tr>
<tr>
<td>C.elegans</td>
<td>Caenorhabditis Elegans</td>
</tr>
<tr>
<td>CT</td>
<td>Carboxy-tail</td>
</tr>
<tr>
<td>C-terminal</td>
<td>Carboxy-terminal</td>
</tr>
<tr>
<td>cAMP</td>
<td>cyclic AMP</td>
</tr>
<tr>
<td>Con A</td>
<td>Concavalin A</td>
</tr>
<tr>
<td>CDD</td>
<td>Conserved Domain Database</td>
</tr>
<tr>
<td>CE</td>
<td>Convergent extension</td>
</tr>
<tr>
<td>Ci</td>
<td>Cubitus interruptus</td>
</tr>
<tr>
<td>CRD</td>
<td>Cysteine-rich domain</td>
</tr>
<tr>
<td>Dcr-2</td>
<td>Dicer-2</td>
</tr>
<tr>
<td>Df</td>
<td>Deficiency</td>
</tr>
<tr>
<td>DI1</td>
<td>Distalless</td>
</tr>
<tr>
<td>dFz</td>
<td>Drosophila Frizzled</td>
</tr>
<tr>
<td>Dpp</td>
<td>Decapentaplegic</td>
</tr>
<tr>
<td>Dgo</td>
<td>Diego</td>
</tr>
<tr>
<td>Dsh</td>
<td>Dishevelled</td>
</tr>
<tr>
<td>DII</td>
<td>distalless</td>
</tr>
</tbody>
</table>
DV  Dorsal-ventral

*D. melanogaster*  *Drosophila melanogaster*

En  Engrailed

ER  Endoplasmic reticulum

Fmi  Flamingo

Fj  Four-jointed

Fz  Frizzled

G4  Gal4

GAP  GTPase activating protein

GFP  Green fluorescent protein

GDI  Guanine nucleotide dissociation inhibitor

GEF  Guanine Exchange Factor

GSK-3β  Glycogen Synthase Kinase-3β

GPCR  G-protein coupled receptor

GRK  G-protein coupled receptor kinase

hs  heat shock

Hh  Hedgehog

hSmo  Human Smoothened

HEK  Human embryonic kidney cells

I3  Third intracellular loop

LEF-1  Lymphoid enhancer binding factor-1

LRP  Low-density lipoprotein receptor-related protein

MT  Microtubule

mwh  Multiple wing hair

N-terminus  Amino-terminus

NB  Neuroblast

Omb  Optomotor-blind

Ptc  Patched
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Term</th>
</tr>
</thead>
<tbody>
<tr>
<td>PCV</td>
<td>Posterior cross vein</td>
</tr>
<tr>
<td>PD</td>
<td>Proximo-distal</td>
</tr>
<tr>
<td>PLC</td>
<td>Phospholipase C</td>
</tr>
<tr>
<td>PCP</td>
<td>Planar cell polarity</td>
</tr>
<tr>
<td>PTX</td>
<td>Pertussis toxin</td>
</tr>
<tr>
<td>Pk</td>
<td>Prickled</td>
</tr>
<tr>
<td>PKA</td>
<td>Protein Kinase A</td>
</tr>
<tr>
<td>Rfz1</td>
<td>Rat Frizzled-1</td>
</tr>
<tr>
<td>RNAi</td>
<td>RNA interference</td>
</tr>
<tr>
<td>7-TM</td>
<td>Seven-transmembrane</td>
</tr>
<tr>
<td>Sal</td>
<td>Spalt</td>
</tr>
<tr>
<td>S.E.M.</td>
<td>Standard Error of the Mean</td>
</tr>
<tr>
<td>Sev</td>
<td>Sevenless</td>
</tr>
<tr>
<td>Smo</td>
<td>Smoothened</td>
</tr>
<tr>
<td>SOP</td>
<td>Sensory Organ Precursor</td>
</tr>
<tr>
<td>Starry Night</td>
<td>Stan</td>
</tr>
<tr>
<td>Strabismus</td>
<td>Stbm</td>
</tr>
<tr>
<td>TCF</td>
<td>T-cell factor</td>
</tr>
<tr>
<td>TM</td>
<td>Transmembrane</td>
</tr>
<tr>
<td>TRiP</td>
<td>Transgenic RNAi Project at Harvard Medical School</td>
</tr>
<tr>
<td>ts</td>
<td>temperature-sensitive</td>
</tr>
<tr>
<td>Tub</td>
<td>Tubulin</td>
</tr>
<tr>
<td>Vang</td>
<td>Van Gogh</td>
</tr>
<tr>
<td>Vg</td>
<td>Vestigial</td>
</tr>
<tr>
<td>Vg</td>
<td>Wingless</td>
</tr>
<tr>
<td>WT</td>
<td>Wild Typ</td>
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Chapter I
General Introduction
1. Introduction to heterotrimeric G-protein signaling

1.1 Overview

It is critical for eukaryotic organisms to be able to respond to changes in their environment, to sense any external stimuli, to translate this signal and then to relay the information, which will trigger molecular events to produce their biological effects. The ability to respond to these stimuli occurs through the interaction of specific receptors dispersed on the outer surface of cells, as few of the signals actually enter the plasma membrane. The plasma membrane acts as barrier to the flow of information, and as Alfred G. Gilman, who shared the 1994 Nobel Prize for the discovery of G-proteins with Martin Rodell, once stated:

“Clearly, the cell membrane is a switch board of considerable complexity, taking in a diversity of signals, assessing their relative strengths and relaying the summed signals to second messengers that will assure the cell reacts appropriately to a changing environment.”

(Linder and Gilman, 1992)

One of the most abundant receptor families is the seven-helical membrane-spanning G-protein coupled receptor (GPCR). When GPCRs are activated, heterotrimeric G-proteins inside the cell engage GPCRs and transduce extracellular signals received by these receptors to a host of downstream effectors.

This basic knowledge and the beginning of the study of GPCRs and G-proteins evolved from pioneering work performed in the late 1950s by E. W. Sutherland and his colleague T. Rall, illuminating how the hormone epinephrine stimulates liver cells to release glucose (Rall & Sutherland, 1958; Sutherland & Rall, 1958). By revealing that a hormone, the first messenger (here, epinephrine), works in part by attaching to receptors in target cells which stimulates an effector (adenylyl cyclase) to produce a second messenger (cyclic AMP), the team opened the door to our understanding of cellular signaling and essentially how cells communicate with one another. One of the fundamental insights gleaned from work that followed in the 1970s came about separately by A. Gilman and M. Rodbell, whose studies collectively introduced the concept of an intermediate transducer that links specific receptors to a common effector (in this case, adenylyl cyclase) (Birnbaumer & Rodbell, 1969; Rodbell, et al., 1971; Ross & Gilman, 1977). Gilman identified the first heterotrimeric G-protein, \( \text{G}_\alpha_s \) (Ross & Gilman, 1977) that he describes here as
“the middlemen”:

“…most of the agents convey information through intermediaries. They issue orders by binding at the outer surface of target cells to proteins that serve as specific receptors. Then, in a process called signal transduction, the receptors, which span the cell membrane, relay the information to a series of intracellular middlemen that ultimately pass the order to the final executors.”

(Linder and Gilman, 1992)

One of these “middlemen”, a Drosophila G-protein (Gαf) is the focus of my thesis. I will first begin with an overview of G-protein coupled receptors and G-proteins (what is known in the context of vertebrate and invertebrates). I will also provide an overview of the structure of the fly wing, where most of the studies were conducted. In Chapter II, I will describe the main body of my thesis work, where I began initial characterization of this G-protein during development, particularly in the wing. In Chapter III, I will provide a brief description of the main thrust of my energies as a graduate student, albeit with futile results on another G-alpha, Gαo, which is also, perhaps aptly, called brokenheart in the fly.

1.2 G-protein Coupled Receptors (GPCRs)

GPCRs are predicted to share the same conserved arrangement of structural features despite their low sequence homology (Gurevich & Gurevich, 2008). This topology consists of seven α-helical transmembrane stretches (TM1-TM7) that form a characteristic bundle with an extracellular amino-terminus (N-terminus), three extracellular loops (EL1-EL3), three intracellular loops (IL1-IL3) and an intracellular carboxy-terminal tail (C-terminal). Based on phylogenetic analysis, GPCRs are divided into five main families labeled according to their hallmark member: glutamate, rhodopsin, adhesion, secretin and frizzled (Fredriksson, et al., 2003; Perez, 2003).

Although the precise mechanism of activation likely differs from individual family members, in the broadest terms the GPCR undergoes conformational changes when binding of a hormone, neurotransmitter, ion or other stimuli occurs and permits the activation of the heterotrimeric G-protein complex. Binding sites for heterotrimeric G-proteins on GPCRs take place mainly through two functional regions: the third intracellular loop and the C-terminal tail (Conklin, et al., 1993; Kostenis, et al., 1997; J.
Therefore, the cytoplasmic or intracellular surfaces of activated receptors determine the interaction with specific G-proteins, and consequently which possible signaling pathways are activated (Hawes, et al., 1994; Luttrell, et al., 1993).

Heterotrimeric G-proteins are proteins associated with the cell membrane, and consist of a GTP-binding α subunit (39-52kDa) and βγ subunits (β: 35-36kDa; γ: 7-9kDa) that are constitutively conjoint in vivo (Downes & Gautam, 1999). This dimer is essential for functional association of G-protein α subunits with GPCRs and is known to exert regulatory control over certain downstream effectors (Neer, 1995). Functionally the βγ subunits act as a monomer; and the two subunits cannot be dissociated except with harsh detergents (Neves, et al., 2002). The Gα subunit alternates between an inactive GDP-bound state, where it forms a tightly bound complex with the Gβγ dimer, and the active GTP-bound state, where it dissociates from Gβγ. In the classical model of signaling by heterotrimeric G-proteins, binding of an extracellular ligand to a heptahelical receptor activates the receptor, which, in turn, acts as a guanine nucleotide exchange factor (GEF) for Gα. GEFs catalyze the exchange of GDP for GTP and act as positive modulators. Thus, once an activating ligand binds to the GPCR, the GPCR acts as a catalyst for the release of GDP on the Gα subunit and subsequent binding of GTP (Gilman, 1987; Johnston & Siderovski, 2007; Oldham & Hamm, 2008). The exchange of GDP for GTP on the Gα subunit leads to the concomitant release of the Gβγ dimer, and to the dissociation of all three subunits from the receptor. Free GTP–Gα and Gβγ are considered active and can potentially trigger distinct signaling pathways. Upon their release, free Gα-GTP or Gβγ can influence various targets, which include adenyl cyclases, phosphodiesterases, phospholipases, and ion channels (Clapham & Neer, 1997; Kammermeier, et al., 2000; Kozasa et al., 1998; Lutz et al., 2007; Neves, et al., 2002; Rhee, 2001; Simon, et al., 1991; Simonds, 1999).

Inactivation of GPCRs occurs when bound GTP is hydrolyzed to GDP by the intrinsic GTPase activity of Gα. The intrinsic GTPase activity of Gα, however, is slow and other regulatory components serve to hasten the hydrolysis of GTP to GDP. These are known as GTPase activating proteins (GAPs), which facilitate the termination of signaling by promoting hydrolysis, and thereby permitting the reassociation of the GDP-bound Gα with the Gβγ subunit (forming the inactive heterotrimer) to return to
the receptor for another cycle of activation. (Figure 1) This serves as the key mechanism to the termination of GPCR signaling.

However, eukaryotic cells can regulate the strength and duration of GPCR signal transduction by other means in order adapt to changes in external conditions and avoid damage to cells through sustained signaling. Classically, two other families of protein have been highly correlated with GPCR signaling (G-protein coupled receptor kinases (GRKs and β-arrestins) and have been implicated as having a central role in the desensitization of G-protein activation by heptahelical receptors (Premont et al., 1999; Strasser et al., 1986; Stadel et al., 1983; Pitcher et al., 1998). This occurs at the receptor level, where GRKs phosphorylate activated GPCRs, on serines and threonines in the carboxy-terminal tail region and/or the third cytoplasmic loop (Hanks et al., 1991; Gurevich & Gurevich, 2008; Pitcher, et al., 1998). β-arrestin is subsequently recruited after GPCRs have been phosphorylated by GRKs in order to functionally uncouple the receptor, block the recoupling of heterotrimeric G-proteins and promote the internalization of seven-transmembrane receptors (7-TM), which consequently results in their desensitization (Wilden 1986; Menard et al., 1996; S. Yu, et al., 1993; Luttrell & Lefkowitz, 2002; Oakley et al., 2001). Although GRKs and arrestins preferentially interact with the active conformation of the receptor and are allosterically regulated by this interaction, they are also capable of initiating their own G-protein independent signaling cascades (Luttrell & Lefkowitz, 2002; Reiter & Lefkowitz, 2006).

1.3 Heterotrimeric G-proteins

Despite the size diversity and size of the GPCR family, a relatively few number of G-proteins serve to initiate their signaling cascade. As a result, each member of the heterotrimeric G-protein family must engage several different receptors, although some overlap also occurs. However, there are different classes of α subunits that have different functions and activate distinct signaling pathways. The βγ subunits are also able to regulate cellular effectors and have their own additional distinct signaling (Oldham & Hamm, 2008). Metazoan organisms express four classes of Gα subunits (Gαs, Gα1/ Gαo, Gαq, and Gα12/13) that are distinguished according to primary sequence similarity as well as functional similarities, which includes the ability to couple to specific GPCRs and to regulate distinct classes of enzymes and ion channels (Downes & Gautam, 1999; Oldham & Hamm, 2008; Wilkie et al., 1992).
Heterotrimeric G-proteins are a family of ubiquitously expressed proteins, and the repertoire of Gα subunits have been extensively characterized in human, mouse and two invertebrates, Drosophila melanogaster (D. melanogaster) and Caenorhabditis elegans (C. elegans). In total, 16 G-protein α subunits have been identified in humans, 5 genes encode Gβ subunits, and 12 genes encode Gγ subunits (Morishita et al., 1995; Watson, et al., 1994; Downes & Gautam, 1999; Wilkie, et al., 1992). The first characterized α subunits were the Gαs protein that stimulates adenylyl cyclases to produce cyclic AMP (cAMP) (Gilman, 1987; Ross & Gilman, 1977), and the Gα proteins that have an antagonistic inhibitory function (Houslay, 1983). Today the inhibitory class is termed the Gαi/o family, which contains Gαo, GαGust and Gαt besides the three Gαi isoforms (in mammals). These members of the inhibitory class share in common their sensitivity to ADP ribosylation by pertussis toxin (PTX), although they have several effector targets (Kaslow & Burns, 1992). PTX is a bacterial toxin derived from Bordetella pertussis and acts to irreversibly uncouple this specific group of modulators of G-protein signaling by blocking interaction with GPCRs (Kaslow & Burns, 1992). In contrast, Gαs is sensitive to another toxin, derived from Vibrio cholera that catalyzes ADP-ribosylation at a location that constitutively activates Gαs to promote continuous GPCR signaling (Casey and Gilman, 1988; Neer & Clapham, 1988). Members of the third class of Gα subunits are termed Gαq/1. This class activates phospholipases, which cleave PIP2 and converts it to PIP3 and diacylglyceride (Kusakabe et al., 1998). The proteins Gα12/13 form the fourth class of Gα subunits and activate Rho GTPases and Rho-dependent formation of stress fibers (Buhl, et al., 1995; Fukuhara, et al., 1999; Longenecker, et al., 2001; McCudden, et al., 2005).

In contrast to mammals, flies and worms encode only one ortholog of the closely related Gα genes, even though some of the invertebrate genes, such as Drosophila Gaq are also alternatively spliced (Talluri, et al., 1995). However, invertebrates also express Gα genes that have not been identified in mammals. For instance, while all four subcategories are represented in Drosophila (Table 1), there is considered to be an additional fifth subfamily of Gα subunit encoded in Drosophila, Gaf. Nematodes also express gpai, gpa2, and gpa3 that are distantly related Gα genes (Lochrie, et al., 1991). Another class of Gα subunit, Gav (for class V of Gα proteins) was also recently discovered (Oka, et al., 2009). Gav is not present in human in Drosophila, nor most other model organisms, but it is present and conserved throughout the remainder of the animal kingdom, including in zebrafish (Oka, et al., 2009). The authors
speculate that the absence of Gαv in most studied model organisms likely contributed to its delayed identification and why in some animals (for example marine sponge, sea urchin and red flour beetle) Gαv was mis-assigned to other classes (Oka, et al., 2009). They point out that Gαv orthologs occur in sponges, which are members of one of the earliest diverged phyla in the animal kingdom, suggesting that Gαv is as ancient as the other 4 classes. One explanation for why Gαv appears to be retained in some lineages and have disappeared in others, while two independent gene duplications (the sponge and jawed fish) are also observed in the Gαv class, is that Gαv conforms to a birth and death mode of evolution (Nei & Rooney, 2005). Most gene families are thought to be subject to concerted evolution, where all members of the gene family do not evolve independently but as a unit in concert by means of gene conversion or unequal crossing-over (Nei & Rooney, 2005). In the birth-and-death model of evolution, new genes evolve independently and are created by repeated gene duplication. Therefore, some of the duplicate genes are maintained in the genome for a long time while others are inactivated through deleterious mutations or are completely removed (Nei, et al., 1997; Ota & Nei, 1994). However, this still raises the question whether the genes represented in invertebrates, such as Gαf, have actually evolved in these particular invertebrates specifically. One can potentially invoke a third model of evolution here, divergent evolution, to explain their existence, whereby they may have emerged through selection pressures to acquire higher specificity and activity.

The Drosophila genome encodes only two Gγ and three Gβ genes subunits. Gβ76C and Gγ30A are likely specific for phototransduction as they are mostly expressed in the visual system (Dolph et al., 1994; Schulz, et al., 1999), which suggests that the others serve in multiple signaling pathways. Importantly, the Gα subunits provide the specificity both in regard to GCPR coupling and effector activation, even though the bγ dimer is capable of activating signal effectors (McCudden, et al., 2005). While multiple functions have been attributed to the various Drosophila Gα proteins, Gαf remains to be characterized.
1.4 Architecture of a $\text{G}_\alpha$ subunit

Many crystal structures of these $\text{G}_\alpha$ proteins have been resolved in a variety of conformations, and have provided the basis for how a G-protein functions (Coleman et al., 1994; Lambright, et al., 1994; Noel, et al., 1993; Oldham & Hamm, 2006; Sprang, 1997; Wall et al., 1995). The structures have revealed a conserved protein fold composed of two domains, a GTP-binding domain (the GTPase or Ras-like domain) and a helical insertion domain. (Figure 3)

At the core of every G-protein is the GTPase domain that is involved in binding and hydrolyzing GTP, and is structurally identical to the superfamily of GTPases, such as the monomeric G-protein p21ras (Sprang, 1997). The guanine nucleotide-binding domain consists of a central six-stranded $\beta$-sheet surrounded by $\alpha$-helices (Sprang, 1997) (Figure 3), which forms five polypeptide loops. These loops are the most highly conserved elements in this domain and bear five conserved amino acid motifs that define the G-protein superfamily (Wilkie & Yokoyama, 1994). They are designated G-1 through G5 and are referred to as G boxes. (Figure 2A) This domain also provides binding surfaces for $\text{G}_\beta\gamma$ dimer, GPCRs and effector proteins (Oldham and Hamm, 2007). (Figure 2B) Additionally, this domain possesses three flexible loops that are termed switches I, II (the loop preceding the $\alpha2$ helix, and the helix itself) and III (comprises the loop connecting helix $\alpha3$ to strand $\beta5$) which show extensive structural differences between the GDP-bound (Lambright, et al., 1994; Mixon et al., 1995) and GTP$\gamma$S-bound (Coleman, et al., 1994; Noel, et al., 1993) conformations of $G_\alpha$. The structural arrangement of these switch regions is critical for the protein-protein interactions of $G_\alpha$, and its conformation determines its binding partners, such as $G_\beta\gamma$ and effectors (Bohm, et al., 1997; Tesmer, et al., 1997; Wall, et al., 1998).

The mechanism of exchange of GDP for GTP that occurs via an activated GPCR remains poorly understood, although great strides have been made about the structural components that are important for this process. When the $G_\alpha$ subunit is inactive it binds GDP within a nucleotide-binding pocket that is circumscribed not only by the GTPase domain, but also by an all $\alpha$-helical domain that is unique to the $G_\alpha$ family (Oldham & Hamm, 2007). The helical domain is inserted into the GTP-binding domain before switch region I and is comprised of a structurally distinct six-helix bundle that forms a lid over the nucleotide-binding pocket, serving to bury the GTP in the core of the protein (A. J. Kimple, et al., 2011). While conserved within a class or family, the helical domain is a divergent region of G-proteins in general.
Although the functions of this domain have not been fully determined, several studies have shown it to have effects on GTPase activity, and to be involved in the interaction with GPCRs as well as regulator and effector proteins (Cherfils & Chabre, 2003; Krieger-Brauer, et al., 1999; W. Liu & Northup, 1998; Skiba, et al., 1999).

The alpha subunit possesses an extended N-terminal α-helix that is the site of lipid modification (for most) to regulate G-protein localization and protein–protein interactions (C. A. Chen & Manning, 2001; Wedegaertner, et al., 1995). Excluding Gαt, all Gα subunits are post-translationally modified by covalent attachment of the fatty acid palmitate at a cysteine (or cysteines) near the amino terminus (Linder et al., 1993; Veit et al., 1994; Wedegaertner, et al., 1993). Members of the Gαi class (αi, αi, αz, and αt) are N-myristoylated at Gly-2 as well (Met-1 is removed by posttranslational processing) (Gallego, et al., 1992; Wilson & Bourne, 1995). These lipid modifications help in targeting α subunits to the membrane surface, juxtaposing them to their cognate GPCRs and effector targets (Wedegaertner, et al., 1993). The addition and removal of the palmitoyl group is a dynamic process that appears to be receptor-mediated which may contribute to Gα recycling between the membrane and cytosolic compartments (Wedegaertner & Bourne, 1994). Apart from the Gαz, members of the Gαi family can be ADP-ribosylated by PTX at a cysteine residue four amino acids removed from the C-terminus, and consequently inhibiting interaction with their receptors (Freissmuth & Gilman, 1989; West, et al., 1985; Gilman, 1987). Additionally, Gαs and Gαt can be ADP-ribosylated at a conserved arginine within the G-2 box, permitting GTP binding but abolishing GTPase activity (Freissmuth & Gilman, 1989; Van Dop, et al., 1984).

The best characterized receptor contact site with G-proteins is the extreme C-terminus of Gα (in particular, its last five residues) (Conklin, et al., 1993; Hamm et al., 1988; Natochin, et al., 2000). (Figure 2) In this region, there have been several mutations shown to uncouple G-proteins from their cognate GPCRs (Sullivan et al., 1987; Blahos, et al., 1998; Conklin et al., 1996; Kostenis, Conklin, et al., 1997; Natochin, et al., 2000). This includes an arginine to proline mutation in Gαs, initially identified in murine lymphoma cells and later attributed to the pathology of Albright’s hereditary osteodystrophy (Osawa & Weiss, 1995; Schwindinger, et al., 1994; Sullivan et al., 1987). Other mutagenesis studies have shown that the C-terminal region on G alpha subunits binds specifically to the third intracellular loop of receptors (Kostenis, Conklin et al., 1997; Hamm, et al., 1998). This was shown with of M2-muscarinic receptor that
couples to Gαi (Kostenis, Conklin, et al., 1997; Hamm, et al., 1998). Therefore, this region provides a critical contact surface for the receptor. As mentioned, PTX can inhibit signaling through the Gai-coupled receptors through ADP-ribosylation of the C-terminal cysteine in this region. This particular cysteine can only be substituted with other hydrophobic residues (Aris et al., 2001), thereby providing evidence to suggest that the Gα protein binds to a hydrophobic pocket in the activated receptor.

In addition, this region dictates the selectivity of the GPCR-Gαβγ interaction and has been shown to be an important determinant of coupling specificity (Conklin, et al., 1993; Conklin, et al., 1996; Hamm, et al., 1988; Natochin, et al., 2000). Elegant studies with chimeric Gα subunits have been performed frequently to switch receptor-effector coupling (J. Blahos, 2nd, et al., 1998; Conklin, et al., 1993; Conklin, et al., 1996; Natochin, et al., 2000). For instance, when the C-terminus of Gαq is replaced with that of Gαi, the resulting chimera is activated by Gαi-coupled receptors while concomitantly stimulating phospholipase C (PLC), which is a Gαq effector (Conklin, et al., 1993).

There are as yet no available structures of the Gαβγ heterotrimer in a nucleotide-free state to mimic its conformation when coupled with a GPCR. Importantly, while the extreme C-terminus is considered to be the primary recognition domain, it is not the sole determinant (Kostenis, et al., 2005). However, there are other regions that have been determined to provide docking sites for GPCRs as well, suggesting that coupling selectivity likely involves cooperative interactions among various domains within Gα (Kostenis, et al., 2005; Slessareva et al., 2003). For instance, close inspection of the C-terminal 11 amino acids of Gαt and Gai indicate that they are nearly identical, yet the 5HT1B serotonin receptor activates only Gai, which occurs through a specific interaction with two amino acids in the α4 helix of this protein (Bae, et al., 1999). Other specificity determinants that have been implicated in receptor contact have included the N-terminal helix (Kostenis, et al., 1997; Kostenis, et al., 1998), the αN–β1 loop (Blahos et al., 2001), the α2–β4 loop (Lee, et al., 1995) and the α3–β5 loop (Grishina & Berlot, 2000; Oldham & Hamm, 2006). A larger region of the C-terminus (beyond the few residues at the terminus) has been implicated as well. Mutagenesis via alanine-scanning of Gαt and subsequent analysis of residues conserved in subclasses of Gα subunits identified a number of residues in the C-terminal 50 amino acids of Gαt that contact rhodopsin (Onrust et al., 1997). In addition, residues within the α4–β6 loop (Figure 3A) have been shown to contribute to the binding surface of receptors by a variety of experimental
approaches. These have included studies with chimeric Gα subunits (Bae et al., 1997; Bae, et al., 1999) sequence analysis of conserved amino acids among the Gα subclasses (Lichtarge, et al, 1996), chemical cross-linking (Cai, et al., 2001), and protection from tryptic proteolysis (Mazzoni, Artemyev, & Hamm, 1996). For example, in Gαt, arginine-310 at the α4–β6 loop is blocked from tryptic proteolysis in the presence of light-activated rhodopsin, implicating a role for the α4–β6 loop in receptor contact.

Other than interacting with the receptor, the Gα subunit also makes contact with the βγ dimer. The βγ subunit of a heterotrimeric G-protein binds selectively to and stabilizes the GDP-bound state, thereby serving as inhibitors of nucleotide release (Brandt & Ross, 1985). The dimer directly competes with effector binding by direct contacts with the N-terminus (Hepler et al., 1996) and switch II regions (Berlot & Bourne, 1992) of Gα. Two crystal structures of G-protein heterotrimers (Gαiβ1γ1 and Gα1β1γ2) reveal two sites of interaction between Gα and the Gβγ dimer (Lambright, et al., 1994; Wall, et al., 1995). A hydrophobic pocket formed by switches I and II serves as the primary interacting surface on GDP-bound Gα, while a smaller contribution comes from the N-terminal helix. These two structures do not provide any evidence for actual Gα–Gγ contacts, although the acylated N-terminus of Gα and C-terminus of Gγ are each used to insert into the plasma membrane and are found in close proximity (Seitz et al., 1999). Independently of the role these regions serve to tether the proteins to the membrane, these modifications may also increase the affinity of these trimeric proteins for each other (Iliri, et al., 1996; Iniguez-Lluhi, et al., 1992).

1.5 Drosophila Gαf

Gαf was identified as a unique Gα subunit based on the DNA and corresponding amino acid sequence of the cloned PCR fragments that were amplified from early Drosophila pupae cDNA (Quan, Wolfgang, & Forte, 1993). Gαf is predicted to encode a protein of 399 amino acids with a relative molecular weight of 46.2 kDa (Quan, et al., 1993). This is consistent within the range of a typical Gα protein (Downes & Gautam, 1999) compared to that of the Ras superfamily, for example, which is generally between 20-25kDa (Gilman, 1987). It is also predicted to be an alpha subunit of heterotrimeric G-proteins by virtue of high sequence identity to the conserved amino acids of other Gα subunits, as indicated by BLAST analysis using the Conserved Domain Database (CDD) from the National Center for
Judging from sequence analysis, Gαf appears to retain all basic biochemical characteristics of Gα proteins, as it is predicted to possess motifs conserved among heterotrimeric G-proteins (Oldham & Hamm, 2008; Quan, et al., 1993). The various domains also appear to be properly arranged in order in the protein. (Figure 4, 5) G-proteins typically contain a GTP-binding domain that have catalytic residues to promote GTP hydrolysis, a helical domain that is inserted between α-helix-1 and β-2 sheet of the GTPase domain, and switch regions that undergo the conformational change upon nucleotide binding (Oldham & Hamm, 2008). (Figure 5) BLAST analysis with CDD confirmed that all 5 characteristic G-box motifs that are the hallmark of the GTPase domain (Sprang, 1997) are present in Gαf. Moreover, amino acid sequence similarity between Gαf and the other Gα subunits are the highest in these regions, where boxes G-1, G-3 and G-4 are the most conserved and boxes G2 and G5 being more divergent. Nonetheless, multiple alignments between the Drosophila Gα subunits indicate that the universally conserved G-5 alanine, which is the only residue that is in direct contact with GTP (Bourne et al., 1991) as mutagenesis of it reduces the affinity of the Gα for GDP (Wall, et al., 1998), is also present in Gαf. Interestingly, a portion of the G-1 box of Gαf (GTAE) differs from sequences (GAGE) generally observed in other Gα subunits in the corresponding region, except those of the Gαq or Gαz subfamilies (Fong, et al., 1988; Matsuoka, et al, 1988; Strathmann & Simon, 1990). When point mutations are introduced in this portion of the G-1 box of Gαs, intrinsic GTPase activity of the subunit has been shown to be reduced (Graziano & Gilman, 1989; Masters et al., 1989). In agreement with this, both Gαq and Gαz have reduced rates of guanine nucleotide exchange and hydrolysis relative to Gαs and Gαi (Casey, et al., 1990; Pang & Sternweis, 1990). This would suggest that Gαf might also behave as a sluggish GTPase relative to that of Gαs.

Apart from the domains that typify a heterotrimeric Gα subunit, such proteins often undergo co- or posttranslational modifications, whereby covalent attachment of fatty acids to the protein occurs (Chen & Manning, 2001). Posttranslational acylation in the N-terminal region has been shown to play a role in the membrane localization of G proteins (Marrari, et al., 2007; Wedegaertner, et al., 1995). The amino terminus of Gαf appears to lack the consensus site for myristoylation that is present in several α subunits.
of the G\(\alpha\)i class (Buss et al., 1987; Magee & Hanley, 1988; Mumby et al., 1990; Simon et al., 1991).

However, there remains another corresponding acylation site that is predicted in the N-terminal region of this protein. This was determined by using a palmitoylation site predictor. (Appendix Figure 1) (http://csspalm.biocuckoo.org/online3.php). Palmitoylation is the covalent attachment of lipid moieties onto cysteine residues, which is believed to contribute to membrane association and is a reversible process (Chen & Manning, 2001). If G\(\alpha\)f is indeed palmitoylated, this would suggest that the protein is able to come on and off the membrane.

Certain GDP-bound G\(\alpha\) subunits can be blocked from transducing the signal of stimulated receptors when treated with PTX. Members of the G\(\alpha\)i class possess a cysteine residue 4 amino acids from the carboxy terminus and are sensitive to ADP-ribosylation by PTX (Freissmuth & Gilman, 1989; West, et al., 1985; Gilman, 1987; Stryer & Bourne, 1986). In contrast, other G\(\alpha\) subunits that do not bear this cysteine at this particular position, such as G\(\alpha\)s and G\(\alpha\)q, are not susceptible to PTX modification by this toxin (Casey, et al., 1990; Pang & Sternweis, 1990). Likewise, G\(\alpha\)f lacks a cysteine residue at this position, implying this subunit is predicted to be insensitive to PTX and is expected to mediate signal-transduction pathways that are refractory to PTX. (Appendix Figure 2) However, G\(\alpha\)f could be a substrate for cholera toxin, as the protein has an arginine in position 192, which is identical to that of G\(\alpha\)s in the corresponding site, that is subject to ADP-ribosylation by the toxin (Gilman, 1987; Stryer & Bourne, 1986). It should be noted, the sequences surrounding this residue in G\(\alpha\)f are significantly different from the corresponding region of G\(\alpha\)s. (Figure 4)

Various regions of the G\(\alpha\) protein have been implicated in contact with the receptor and determine coupling specificity to GPCRs (Oldham & Hamm, 2008; Conklin & Bourne, 1993). Importantly, studies have overwhelmingly implicated the extreme C-terminal domain for specific interactions with the receptor (Sullivan et al., 1987; Masters, et al., 1988; Kostenis, Conklin et al., 1997; Hamm et al., 1998). This is supported by identification of a mutation that is located within the same region of G\(\alpha\)s that, when present, causes decoupling of G\(\alpha\)s from their cognate receptors (Sullivan, et al., 1987). In addition, many elegant chimeric studies have revealed the specificity of this region to receptors and point to its relevance (Conklin et al., 1993; Blahos, et al., 1998; Kostenis, Conklin et al., 1997). Thus, it is likely that the
extreme C-terminus may play a role in coupling to a receptor. Interestingly, comparison of this region reveals that the last three residues are identical to those of Gαi and would suggest that Gαf might be able to couple to the same GPCRs. (Appendix Figure 2,3)

In summary, all elements characteristic for G-proteins are present in the Gαf protein, confirming its identification as a G alpha protein subunit. Thus, Gαf subunit shares considerable amino acid identity with other Gα subunits within the conserved domains. What sets each class of Gα proteins apart is the amino acid sequence outside of these regions, and in agreement with this, Gαf can be distinguished from the remaining classes of Gα subunits by the difference in sequence identity in these other regions.

Comparisons of amino acid sequences between previously identified vertebrate and Drosophila Gα proteins revealed Gαf to be an additional member of the Gα family, and unique from the four classes (Gαs, Gαi, Gαq, and Gα12) that are expressed in metazoans (Quan, et al., 1993). In general, the criterion for a member of one class is that it is <50% identical to members of the other classes. Furthermore, the relationships between members of a class exhibit similarities not only in their primary sequence homologies but also, to some extent, functional similarities. This is displayed, for example by both Gαs and Gαolf, which are members of the same class and can both couple to β2-adrenergic receptors to adenylate cyclase (Jones et al., 1990). Pairwise alignment with various vertebrate Gα proteins indicates that Gαf shares at most overall amino acid sequence identity of 40% with that of human Gαs, and therefore shares a limited degree of sequence identity with known subtypes. (Appendix Figure 4) Comparison with Drosophila homologs of the various Gα classes indicates the greatest sequence identity with that of Gαs, which is between 38-40%. As mentioned, this degree of identity, where less than 50% amino acid sequence identity is seen between subunits, is typically observed when members of different classes are compared. Consistent with this, when the other known Drosophila Gα proteins are compared with their vertebrate homologues, the proteins appear to be at least 70% identical (de Sousa, et al., 1989; Provost, et al., 1988; Quan, et al., 1989; Strathmann & Simon, 1990; Thambi, et al., 1989; Yoon et al., 1989). Since sequence comparisons between Gαf and known Gα proteins revealed no convincing relationship between the other families, Quan et al. (1993) determined that it must be distinct from previously described Gα proteins and therefore defines yet another class of Gα subunit.
Using a more stringent approach, BLAST searches and phylogenetic analysis were performed in order to independently determine whether \( \Gamma f \) defines a separate class of G alpha proteins. The first question that arises here is does this additional class exist in higher or other organisms or is this unique to *Drosophila*? BLAST searches to identify related proteins yielded putative orthologs in a range of sequenced arthropod genomes, including other *Drosophila* species, Anopheles, Tribolium and Daphnia. These top hits were followed by \( \Gamma s \) entries from a variety of species, indicating that \( \Gamma f \) may be an arthropod-specific paralog of \( \Gamma s \). To delineate with more clarity the phylogenetic affinities of \( \Gamma f \), I performed a cladistic analysis using Maximum Parsimony (MP) in MEGA 5.0 (Tamura et al., 2011), incorporating G alpha protein sequences from all subfamilies from a range of vertebrate and invertebrate taxa. Ras and Rheb sequences, which represent more distantly related paralogs of G alphas, were used as outgroups. All sequences were aligned in Clustal X (Thompson, et al., 1994). (Appendix Figure 4)

The resultant tree topology is shown in Fig 6. It is rooted using the Ras sequences, and shows strong support for the \( \Gamma \) clade, with a bootstrap percentage (BP) of 100%. First, we can conclude that \( \Gamma f \) is firmly embedded within this \( \Gamma \) clade, demonstrating clearly that it is indeed a \( \Gamma \) protein, and thereby independently supporting the hypothesis of the original authors. Second, phylogenetic analysis of \( \Gamma \) alignment indicates that \( \Gamma f \) does not cluster strictly with any of the well-established classes defined for mammals nor invertebrates. Within the G alphas, the various subfamilies cluster into 4 main clades: 1) \( \Gamma i \) \( \Gamma o \) 2) \( \Gamma q \) and Human \( \Gamma 14+\Gamma 15 \), 3) \( \Gamma 12+ \) Drosophila Cta, 4) \( \Gamma s \). It is with this latter clade that \( \Gamma f \) associates, albeit with modest support (BP = 55%). Importantly, \( \Gamma f \) does not emerge from the \( \Gamma s \) clade. This outcome, combined with the apparent absence of \( \Gamma f s \) in non-arthropod genomes as judged by BLAST searches, suggests two possible explanations for the evolutionary origin of \( \Gamma f \). Firstly, \( \Gamma f \) may be a \( \Gamma \) protein that appeared in an arthropod-specific duplication event of \( \Gamma s \). However, if this were so, one would expect \( \Gamma f \) and Drosophila \( \Gamma s \) to emerge as sister groups. That this is not the case may mean that \( \Gamma f s \) have experienced accelerated sequence evolution, obscuring their sister group relationship with arthropod \( \Gamma s \). Alternatively, the duplication event which produced \( \Gamma f s \) and \( \Gamma s \) may predate the vertebrate-arthropod split, and the presence of \( \Gamma f \) exclusively in arthropod genomes may be explained by the loss of \( \Gamma f s \) from non-arthropod genomes.
2. Heterotrimeric G-Protein Signaling Overview

Although roles for GPCRs and trimeric G-proteins have been well characterized in many biological processes, their role in the signaling undertaken during development remains poorly understood. While there are a number of heptahelical receptors that play critical roles in development, their role as G-protein linked remain controversial. Indeed, whether trimeric G-proteins play only limited roles in mediating developmental signals, or whether their roles are more extensive is not clear. If it is the latter, their roles have gone undetected, which may be due to extensive redundancy between them. As a result, loss of any one may not uncover dramatic developmental phenotypes; or their roles may be so subtle, which prevents their detection. Below, I review the developmental pathways in which G-protein signaling has been implicated.

2.1 Heterotrimeric G-Protein Signaling in Asymmetric Cell Division (ACD)

One means of generating cell type diversity in metazoan development, such as in mammalian neurogenesis, epidermal lineages, and regulating stem cell lineages, is through the process of asymmetric cell division (Yamashita et al., 2003; Bellaiche & Gotta, 2005; Betschinger & Knoblich, 2004). Failure of this process can give rise to congenital defects in mammals, with loss of differentiated cell types as with the disease Lissencephaly, when neuronal fates are not properly specified (Yingling et al., 2008), or cancer in the case of stem cell fate determination, when there is excess proliferation (Morrison & Kimble, 2006; Wodarz & Nathke, 2007).

Asymmetric cell division is a mechanism by which two daughter cells are generated with distinct developmental potential, possessing distinct cell fates or functions. This is achieved with differential positioning of subcellular components by polarizing the mother cell (Horvitz & Herskowitz, 1992). Consequently, cell fate determinants are differentially segregated into each daughter cell. Much of the mechanistic insight has come from studies conducted in the invertebrate model systems D. melanogaster and C. elegans, which rely heavily on asymmetric cell divisions. In Drosophila, two types of asymmetric divisions have been described in depth: in the central nervous system, where progenitor cells delaminate from the neuroectoderm in the apical/basal axis to specify neuroblasts (NBs); and in the peripheral nervous system, where the sensory organ precursor (SOP) cells divide asymmetrically in the plane of the
epithelium (in the anterior/posterior axis) to produce the four cells that make up the external sensory structures (Betschinger & Knoblich, 2004; Fichelson & Gho, 2003; Gho, et al. 1999; Hartenstein & Posakony, 1989). (Figure 7)

The four differentiated cell types that are produced to make up the adult mechanosensory bristle organ are the shaft, socket, sheath and neuron (Hartenstein & Posakony, 1989). Initially, the SOP divides asymmetrically to produce two daughter cells manifesting distinct cell fates, the anterior pIIb cell and the posterior pIIa cell. These two cells differ in morphology and developmental potential, and undergo another round of asymmetric cell division. The pIIb cell then gives rise to the sheath and neuron cell types (the internal structures of the sensory organ) as well as an apoptotic glial cell that does not become part of the organ, while the pIIa cell gives rise to the socket and shaft/hair (the external structures, visible on the cuticle of the adult).

These sensory organs, known as bristles or macrochaetes, which cover the body of the larvae and adults of *Drosophila*, serve to transduce and convert physical stimuli into electrical signals (Hartenstein & Posakony, 1989; Jan & Jan, 1994). The morphogenesis of macrochaetes follows a strict developmental pattern, as described previously, and serves as a convenient model for studying asymmetric cell division. Moreover, the proteins involved in ACD have been shown to have a high degree of homology and the mechanism to be conserved (Knoblich, 2001; Roegiers & Jan, 2004).

Heterotrimeric G-proteins have been implicated with various roles in directing asymmetric cell division (Knoblich, 2001). In *C.elegans*, for instance, RNA interference (RNAi) of two redundant G alpha subunits, GOA-1 and GPA-16, which regulate orientation of the mitotic spindle and generates the cortical pulling forces for spindle displacement, prevents asymmetric division of the zygote (Gotta & Ahringer, 2001). In contrast, RNAi of the Gβ subunit GPB-1 results in hyperactive spindle movements and increased pulling forces (Gonczy, 2008). In *Drosophila*, involvement of heterotrimeric G-proteins has been demonstrated in both neuroblasts and SOP cells (Katanaev & Tomlinson, 2006a; Schaefer, et al., 2001; Knoblich, 2001). Gαi subunit has been shown to localize asymmetrically in both types of cells (Schaefer, et al., 2001). The role of trimeric G-proteins in *Drosophila* was demonstrated by manipulation of βγ subunits (Fuse, et al., 2003; Gotta & Ahringer, 2001; Izumi, et al., 2004; Schaefer, et al., 2001; F. Yu, et al., 2003), which implicated the significance of Gαi in the formation of the asymmetric spindle as
well as the appropriate localization of various cell fate determinants. \(G_{\alpha o}\) has subsequently been shown to play a more pervasive role in asymmetric divisions (Katanaev & Tomlinson, 2006a). In SOP asymmetric divisions, \(G_{\alpha o}\) appears to function in both establishing asymmetric complexes and in the orientation of the asymmetry. Importantly, it is believed to play a role in all cell asymmetric divisions in *Drosophila* (SOP and NBs), as \(G_{\alpha i}\) (Katanaev & Tomlinson, 2006a; Schaefer et al., 2001; F. Yu et al.; 2003). Katanaev et al. (2006) demonstrated \(G_{\alpha o}\) had stronger phenotypes than the corresponding ones of \(G_{\alpha i}\), and proposed that \(G_{\alpha o}\) may have a greater role than \(G_{\alpha i}\). Moreover, in SOP divisions both Frizzled (Fz) receptors (Fz1 and Fz2) appeared to act as exchange factors for \(G_{\alpha o}\).

### G-Protein Coupled Receptors (GPCRs) / 7-Transmembrane Receptors

#### 2.2 Wnt/Wingless Signaling Pathway

The highly conserved Wingless (Wg)/Wnt pathway has been extensively studied for the past two decades, as it governs many critical developmental processes, such as cell proliferation and cell fate specification (Nelson & Nusse, 2004). For instance, in the *Xenopus laevis*, Wnt signaling affects body axis formation (McMahon & Moon, 1989). In *Drosophila* the Wnt ortholog, Wingless, is essential at specific developmental stages: in the embryo, for the proper establishment of parasegmental boundaries (Nusslein-Volhard & Wieschaus, 1980); and in the imaginal discs, for growth and patterning (Neumann & Cohen, 1997; Struhl & Basler, 1993; Wilder & Perrimon, 1995). In the adult, the *wg* mutation results in loss of wings. More recent studies also implicate Wnt signaling in maintaining the pluripotency of stem cells in various tissues (Reya & Clevers, 2005; Sato, et al., 2004; Song, et al., 2002) as well as involvement in neural differentiation of embryonic stem cells (Miyabayashi et al., 2007; Otero, Fu, Kan, Cuadra, & Kessler, 2004). Furthermore, uncontrolled signaling of the pathway has been shown to play a key role in degenerative diseases as well as cancer, leading to increased interest in the study of this signaling pathway (Brack et al., 2007; H. Liu et al., 2007; Uematsu et al., 2003; van de Wetering et al., 2002).

Seminal work on the Wnt signal transduction research was conducted in the late 1980s and early 1990s, when the mouse *Int-1* gene (subsequently renamed *Wnt-1*, which is a combination of *int-1* and *wg*) and the *Drosophila wingless* genes were established as part of the same group of extracellular
secreted glycoproteins (Rijsewijk et al., 1987). Wnt-1 gene was first identified as an oncogene that facilitated the formation of primary mammary tumors, in response to the mouse mammary tumor virus (Nusse & Varmus, 1982). Subsequent isolation of the Drosophila wg revealed it to be homologous to Int-1 (Baker, 1987; Cabrera, et al., 1987; Rijsewijk, et al., 1987). Since then, there have been multiple wnts that have been identified: 5 in C.elegans, 7 in Drosophila, and 19 in the mouse (Mus musculus) and Humans (Nusse web page: http://www.stanford.edu/group/nusselab/cgi-bin/wnt/). Other components of the pathway were later identified in Drosophila in forward genetic screens, and epistasis analyses further enabled investigators to functionally map the general pathway for Wg signal transduction (Cadigan & Nusse, 1997; Siegfried, et al., 1992; Wodarz & Nathke, 2007). These findings have also been largely corroborated in other systems (C.elegans, Xenopus and in mouse).

In a simplified model of the ‘canonical’ Wnt signal transduction pathway, secreted Wg/Wnt ligand associates with the Frizzled family of transmembrane receptors when they reach their target cells (Bhanot et al., 1996; C. M. Chen & Struhl, 1999; Yang-Snyder, et al., 1996). It is believed by some that an extracellular portion of the Fz receptor, called the cysteine-rich domain (CRD), confers binding to Wnt (Bhanot, et al., 1996; Cheng, et al., 2010; Dann et al., 2001), although there is some debate as to whether this holds true in vivo (C. M. Chen, Strapps, et al., 2004). Different groups have attempted to address the role for the CRD in Wnt signaling, but their results have yielded conflicting outcomes. Initial findings suggested that the CRD of the Fz receptor, which consists of 120-125 residues with ten conserved cysteines to form disulfide bonds, is necessary for Wnt binding (Bhanot, et al., 1996; Dann, et al., 2001; Hsiehet al., 1999; C. H. Wu & Nusse, 2002). In support of this, CRD expressed alone acts as a dominant negative, which implies that this region of Fz receptor can bind Wnt ligands to limit the available pool for endogenous receptors (Cadigan, et al., 1998). Challenging the notion that CRD acts as the sole determinant of Wnt binding, and asserting CRD to be dispensable in Wnt signaling, came from in vivo experiments (C. M. Chen, et al., 2004). Here, Chen et al. (2004) showed that fz transgenes lacking CRD were still able to function in Wnt signaling. Following these studies, work by (Povelones & Nusse, 2005), based on both cell culture and in-vivo manipulations using CRD mutations, suggest that Fz CRD is indeed required for efficient Wnt signaling to occur. Fz transgenes carrying CRD mutations were shown to have compromised signaling especially in vitro, but in vivo the group noted that signaling was less
dependent on the presence of the CRD and that its absence is likely compensated for by other factors. Thus, they hypothesized that the function of the CRD is to bring the ligand in close proximity to the receptor for efficient signaling to occur.

This binding of the ligand to receptor then mediates a signal that regulates β-catenin (Armadillo in *Drosophila*) levels, which, in turn, activates T-cell factor/lymphoid enhancer binding factor-1 (TCF/Lef-1) transcription factors in the nucleus, and activates gene expression (Riggleman, et al., 1990; van de Wetering, et al., 1991). In the absence of Wnt/Wg stimulation, β-catenin activity is inhibited and its levels kept low, as it remains bound to a scaffolding complex that includes Axin, Adenomatous Polyposis Coli (APC) (Rubinfeld et al., 1993) and Glycogen Synthase Kinase-3β (GSK-3β, Shaggy in *Drosophila*) and is targeted for ubiquitination after phosphorylation by GSK-3β (Jiang & Struhl, 1998; Yanagawa et al., 2002; Yost et al., 1996). Therefore, Wg binding to the Frizzled receptor (in *Drosophila* Fz1 and F2) and coreceptor, the low-density lipoprotein receptor-related protein (LRP; Arrow in *Drosophila*) (Pinson, et al., 2000; Tamai et al., 2000; Wehrli et al., 2000), prompts the phosphorylation of the cytoplasmic protein Dishevelled (Dvl; Dsh in *Drosophila*) (Klingensmith, et al., 1994; Noordermeer, et al., 1994), which propagates the Wnt/Wg signal and ultimately leads to the stabilization of β-catenin. (Figure 8)

2.3 The Planar Cell Polarity (PCP) / Frizzled signaling pathway

In addition to the canonical pathway, there also exists a non-canonical pathway, known as the planar cell polarity (PCP) or frizzled signaling pathway, which is mediated by molecules distinct from the canonical pathway. (Figure 9) Introduced by Nübler-Jung (1987) to describe the organization of polarized structures of bristles on the insect cuticle, planar polarity refers to the alignment of a population of cells within a single epithelial plane that is orthogonal to the perpendicular axis. Initially studied in *D. melanogaster* (Adler, et al., 1997; Gubb & Garcia-Bellido, 1982), PCP has come to be recognized as a fundamental property conserved in tissues of many higher organisms thereafter. PCP appears to regulate diverse outputs, such as the coordinate organization of scales in fish, feathers in birds, hair in mammals and cochlear stereocilia in the inner ear (Dabdoub & Kelley, 2005; Fanto & McNeill, 2004; Guo, et al., 2004; J. Wang et al., 2005). Thus, establishing the appropriate orientations of these structures is essential for the ability of the individual organs to function. For instance, disruption of the orientation of
the inner hair cells can result in hearing loss, such as in Usher syndrome or in Ames waltzer hearing loss (Alagramam, Murcia, et al., 2001; Alagramam, Yuan, et al., 2001; Axelrod & McNeill, 2002). In addition to the orientation of specialized epithelial structures, PCP also plays a significant role in coordinating cell migration and tissue morphogenesis, as observed with convergent extension movements during gastrulation and neurulation (Bastock & Strutt, 2007; Curtin et al., 2003; Fanto & McNeill, 2004; Kibar et al., 2001; Wallingford & Harland, 2002).

In *Drosophila*, PCP has been well characterized and several tissues display planar polarity, wherein an (inferred) extracellular gradient is decoded by the epithelial cells to direct their cytoskeletal organization (Klein & Mlodzik, 2005; Lawrence, et al., 2004; Ma, et al., 2003). These include posteriorly projecting hairs and bristles on the thorax and abdomen, distally pointing hairs on the wing blade and the uniform orientation and chirality of each ommatidium in the eye. Polarization in these tissues is subject to control by a group of PCP genes, which, when mutated impair coordinate planar organization (Adler, 2002; Fanto & McNeill, 2004; Lawrence, et al., 2008; Seifert & Mlodzik, 2007; H. Strutt & Strutt, 2005). However, these mutant cells still retain their normal apical-basal polarity and their overall structure (Fanto & McNeill, 2004). While some of these genes play a tissue-specific role, there is a cassette of genes, known as the core PCP genes, which appear to affect polarity in all of these tissues.

In the *Drosophila* wing specifically, each of the approximate 30,000 epithelial cells produce a single cellular extension called a trichome or hair, containing both actin and microtubules (Eaton, et al., 1996; Turner & Adler, 1998). All of the hairs point distally and together form a near perfectly decorated parallel array. (Figure 10) When PCP genes are mutated in the wing epithelial cells, several key regulatory aspects are affected, including hair outgrowth from the distal vertex of the cell, hair orientation and the number of hairs produced by each cell (Axelrod & McNeill, 2002; Mihaly, et al. 2005).

Two of the core group of PCP proteins of the PCP/Fz signaling pathway are also involved in Wnt/Wg signaling (Fanto & McNeill, 2004; Klingensmith, et al., 1994; D. Strutt, 2003; Vinson, et al., 1989). These are the seven-pass transmembrane receptor Frizzled (Vinson, et al., 1989) and the cytoplasmic signal transducer, Dishevelled (Klingensmith, et al., 1994; Theisen et al., 1994). In Wnt signaling Fz and fz2 act as redundant receptors, where either one is able to transduce the signal. However, in PCP, Fz alone acts as the receptor; and as such, it can transduce both the PCP and Wnt signals where there is a
dedicated Wnt receptor. Divergence of the two pathways occurs at the level of Dsh, which, in the
noncanonical pathway involves a set of core genes. The other four, thus far, have only been implicated in
the PCP pathway, and consist of the cytoplasmic protein Prickle (Pk)/Spiny leg (Gubb et al., 1999), the
atypical cadherin Flamingo (Fmi)/Starry night; Stan) (Usui et al., 1999), the four-pass transmembrane
protein Strabismus (Stbm)/Vang Gogh (Vang) (Taylor, et al., 1998; Wolff & Rubin, 1998) and Diego (Dgo)
(Feigun, et al., 2001).

An exciting development in the study of PCP was the finding that the core gene products undergo
dramatic relocalization in the polarizing cells. This has been best described in Drosophila wing epithelial
cells. Just prior to the formation of the polarised structures (hairs in the wing, for example), these proteins
become localized to one or other ends of the cell (Lawrence, et al., 2008; D. Strutt, 2003). Initially, there
is a uniform distribution of the core proteins. In response to a directional cue, PCP core proteins,
recruited to the apicolateral membrane, relocalize to specific membrane domains of the cells: Fz and Dsh
accumulate at the distal edge of the wing cell, Stbm and Pk localize to the proximal side and Dgo and Fmi
to both proximal and distal sides of the apical membrane (Adler & Lee, 2001; Mihaly, et al., 2005; D.
Strutt, 2003). Whether these localizations are necessary for the polarization of these structures (and
actually direct prehair location in the wing, for instance) is still under debate (Lawrence, et al., 2008).

Even though the PCP cascade has only been partially characterized, what is understood is that
Fz rests at the top of the hierarchy (Katanaev & Tomlinson, 2006b; Klein & Mlodzik, 2005). It is believed
that an initial global polarity cue is set up, which feed into the core PCP cassette through Fz. To date, the
nature of the polarity signal in the wing remains elusive, as well as the mechanism of activation of Fz-
PCP signaling. In addition, the final read-out of the Fz/PCP cascade is tissue-dependent. In the wing,
cytoskeletal reorganization is the main response. One could speculate that a gradient of Wg/Wnt ligand
would be a likely candidate to initiate the gradient of Fz activity, similar to its role as a ligand in
convergent extension in vertebrates. CE is a complicated morphogenetic process that occurs during
gastrulation and neural tube closure in vertebrate embryos. In both of these processes, a Wnt signal
appears to be required (Heisenberg et al., 2000; Rauch et al., 1997). However, while Fz is capable of
binding the Wnt family of ligands, its role in Fz- PCP is distinct from its activity as a Wg receptor in the
Wnt canonical signaling pathway. Importantly, Wg itself plays no role in planar polarity in Drosophila. All
the *Drosophila* Wnts have been tested by genetic analysis and have yet to produce a PCP phenotype (Barrow, 2006). Furthermore, *Drosophila* Wnts are not expressed at the appropriate times and places to initiate PCP signaling in the tissues (Barrow, 2006). Therefore, the general perception is that the polarity signal induces a bias in Fz activity along the proximal/distal axis of the wing. Consequently, Fz signaling is higher on one side of the cell, which in turn generates molecular asymmetry within target cells to trigger cytoskeletal reorganization.

### 2.4 Frizzled as a GPCR

Fz proteins are membrane receptors that possess the signature seven-transmembrane organization characteristic of GPCRs, with an extracellular N-terminal domain and cytoplasmic C-terminus (Vinson, et al., 1989; Y. Wang et al., 1996). (Figure 11A) Such proteins typically associate with trimeric G-protein complexes to orchestrate downstream signaling events, although whether Fz behaves as a GPCR remains in question. Evidence to suggest that Fz is coupled to G-proteins has been growing in both canonical and noncanonical Fz signaling (Ahumada et al., 2002; W. Chen et al., 2003; Katanaev, et al, 2005; T. Liu et al., 2001). Indeed, sequence analysis has led some to place Frizzled (and the related protein Smoothened) into another category of GPRC family (Kristiansen, 2004).

Lending support to the role that Frizzleds may function as GPCRs arose from early data demonstrating that Wnt signaling is sensitive to pertussis toxin (PTX) (Slusarski, et al., 1997). Data to bolster the GPCR premise came from T.Liu et al. (T. Liu, et al., 2001), who constructed a chimeric receptor incorporating the extracellular transmembrane segments from the β2-adrenergic receptor (β2AR) fused to the cytoplasmic domains from rat Frizzled-1 (Rfz1). Using mouse F9 cells that expressed the chimera (β2AR-Rfz1), they demonstrated activation of a β-catenin-sensitive promoter, upon stimulation with the β-adrenergic agonist isoproterenol. In addition, this response was shown to be blocked by PTX and also by depletion of Gαq and Gαo. This implicated G-proteins in Wnt/Frizzled signaling, and offered indirect support for the involvement of G-proteins.

More recent work in the laboratory offers further compelling evidence that G-proteins are involved in Fz and PCP signaling (Katanaev, et al., 2005). There are several hundred GPCRs and six Gα subunits in the fly genome (Katanayeva, et al., 2010; Malbon, 2005). While PTX works by catalyzing the transfer
of ADP-ribose to a cysteine present in the Gα family of Gα subunits), the fly Gα has no cysteine present at the C-terminus. Upon examination of the sequence of the six Gαs that exist in Drosophila, only one had a cysteine present 4 amino acids from the terminus, which was Gao. The laboratory demonstrated that PCP phenotypes caused by Fz overexpression in Drosophila eyes could be suppressed by pertussis toxin, thereby signifying the existence of a PTX-sensitive G-protein functioning in the Fz-PCP pathways (Katanaev, et al., 2005). Importantly, insensitivity to the toxin does not preclude other G-proteins from roles in the process.

The role of the fly Gao subunit was examined in Fz (Wg and PCP) signaling. Overexpression of wild type Gao activated signaling in both pathways; and the constitutive active form (Gao-GTP) also activated both pathways. Overexpression of Gao was shown to increase protein levels of Wg target genes in the embryo and imaginal discs. Further, loss and gain of function clones in the wing lead to typical PCP defects (multiple wing hairs and orientation defect). Since 7-TM receptors generally behave as exchange factors for trimeric G-proteins, the wild type form of Gao would necessitate the presence of an exchange factor to load GTP, whereas the activated Gao (Gao-GTP) is receptor independent. Thus, if Fz acted as a GEF, then it would follow that the Gao-GTP would not be Fz dependent. Indeed, rescue of Wg signaling was observed when frizzled receptors were removed in cells concomitantly expressing Gao-GTP. Thus, Gao is likely part of a trimeric G-protein complex that directly transduces Fz signals from the membrane to downstream components in both pathways, and Fz is likely acting as the exchange factor. Nevertheless, direct biochemical evidence demonstrating the coupling of G-proteins to Frizzled is still lacking. Moreover, as noted previously, this does not rule out roles for other G-protein family members in the Wnt/Fz signaling pathway.

Frizzled may possess other similar characteristics with 7-TM receptors, particularly with regard to GPCR regulation. For example, the regulator of G-protein signaling protein RGS4, which is known to associate with Gαq subunits, can inhibit Wnt-8-induced axis duplication in Xenopus embryos (C. Wu, et al., 2000). In classical GPCR signaling, after activation of the receptor, a mechanism to desensitize the receptor involves its internalization by β-arrestins after it is phosphorylated by GRKs. One evidence of this was shown with Frizzled-4 in human embryonic kidney cells (HEK). In the presence of Wnt5A, Dishevelled and activated protein kinase C, Fz-4 was shown to be internalized (W. Chen, et al., 2003).
The C-terminal fragment of *Drosophila* Fz1 was also shown to be phosphorylated *in-vitro* by protein kinase C (Djiane, et al., 2005), although similar effects on full-length Frizzleds have not been reported.

### 2.5 The Hedgehog Signaling Pathway

The Hedgehog (Hh) signaling pathway mediates numerous important aspects of tissue patterning in metazoan development and its deregulation has been implicated in a variety of congenital disorders and cancer (Jiang & Hui, 2008; Toftgard, 2000). Most of what is known about this evolutionarily conserved pathway has come from studies in the *Drosophila*. Indeed, the various key proteins that were found to regulate Hh signaling were originally identified in a genetic screen for patterning defects carried out in *Drosophila* (Nusslein-Volhard & Wieschaus, 1980), where it is important for establishing the basis of the fly body plan.

While the *Drosophila* possesses one *hh* gene, three exist in mammals: Desert Hedgehog, Indian Hedgehog and Sonic Hedgehog. In the fly, where the intracellular cascade is best described, signaling occurs in response to the binding of the secreted Hh ligand, to a 12-transmembrane protein, Patched (Ptc), which acts as receptor (Y. Chen & Struhl, 1996; Marigo, et al., 1996). Epistasis experiments placed the 7-TM protein Smoothened (Smo) downstream of Ptc (Hooper, 1994); and further analysis revealed that the binding of Hh relieves Ptc-mediated inhibition of Smoothened, thus permitting signaling from Smo (Alcedo, et al, 1996). Intracellularly, de-repression of Smo allows for activation of the pathway through a large protein complex consisting of a kinesin like protein Costal2 (KIF7 in vertebrates)(Robbins et al., 1997; Sisson, et al., 1997), the serine/threonine kinase Fused (Fukuhara, et al., 1999) and the transcription factor Cubitus interruptus (Ci; Gli in vertebrates) (Domínguez, Brunner, Hafen, & Basler, 1996). When the pathway is inhibited, additional cytoplasmic components, which includes Protein Kinase A (PKA), Casein Kinase 1 and Glycogen Synthase Kinase 3, are recruited to form the signaling complex that promotes the constitutive cleavage of Ci (from a full length to a lower molecular mass repressor form: Ci-155 to Ci-75) (Aza-Blanc, et al., 1997; Y. Chen & Struhl, 1998). In the presence of an activating signal, Ci is stabilized (Ci proteolysis is inhibited) and can translocate to the nucleus to activate transcriptional targets. (Figure 12)
2.6 Smoothened as a GPCR

Similar to Fz, topographically, Smo bears a GPCR-like architecture: it is an integral membrane protein with seven membrane-spanning alpha helices, a lengthy N-terminal extracellular peptide and an intracellular C-peptide (Alcedo, et al., 1996). (Figure 11B) Structurally, its closest relative is the Wnt receptor Frizzled family of GPCRs, bearing a 31% identity and 52% similarity to the heptahelical region of *Drosophila* Fz (dFz) (Alcedo, et al., 1996). The N-terminal CRD of dFz and *Drosophila* Smo also bear a high homology (Alcedo, et al., 1996). However, unlike Fz, which is able to bind to Wg within its CRD (Hsieh, et al., 1999; C. H. Wu & Nusse, 2002), Smo has no known natural ligand. Thus, the role of the Smo CRD is even less clear than that of dFz.

While there are several lines of evidence linking Frizzled signaling to G-proteins, any evidence to support Smo coupling with G-proteins has been limited and controversial. Nonetheless, as a result of its molecular architecture, Smo has been long suspected to behave as a GPCR and engaging heterotrimeric G-proteins (Hammerschmidt & McMahon, 1998). Importantly, there have been recent finding in *Drosophila* and vertebrate systems that provide strong evidence that Smo may function as a GPCR.

Most of the studies that indicate Smo might couple with G-proteins in vertebrates come from work in cell culture (Ayers & Therond, 2010). Experiments on *Xenopus* melanophores using human Smo (hSmo) demonstrated that hSmo signaled through the Gαi subunit; and signaling was inhibited by pertussis toxin (DeCamp, et al., 2000). Further detailed studies, done with insect Sf9 cells (which provides a good assay for GDP-GTP exchange) as well as with mammalian fibroblast cells, showed the ability of Smo to activate Gαi (Riobo, et al., 2006). Thus, full activation of Gli transcription factors by Smo-Gαi-coupled signaling in fibroblasts was shown to be repressed by PTX. Likewise, Smo activation of Gαi could be stimulated by Smo agonists and repressed by the Smo antagonist cyclopamine (Riobo, et al., 2006). Whether these findings are simply cell-type specific results or whether they can be generalized remains unclear. Importantly, Smo signaling mediated via G-protein has not been demonstrated convincingly in-vivo in vertebrates. (Riobo, et al., 2006) showed in fact that Smo can signal to Gli by a mechanism independent of G-protein as well; their data suggests that signaling through Smo occurs through two signals (one involving Gαi and one independent of Gαi). In addition, while injection of PTX into zebrafish embryos caused phenotypes resembling partial loss of hedgehog signaling.
(Hammerschmidt & McMahon, 1998), overexpression of a constitutively active Gαi protein did not have any profound effects on Hh-dependent neural cell specification in the chick neural tube, where spinal cord patterning was unperturbed (Low et al., 2008).

As in vertebrates, whether Smo acts through heterotrimeric G-proteins in Drosophila has been hard to resolve and the evidence has been controversial. For example, when a large-scale RNAi screen in the Cl-8 cell line (cells originating from the Drosophila wing imaginal disc) was undertaken (where heterotrimeric G-protein subunits (α or γ) were targeted), the investigators did not observe any activation of Ci by Hh (Lum et al., 2003). Yet, more recently, more convincing data has come to light by Ogden et al. (2008) with the use of a combination of cell based assays and Drosophila genetics, showing the requirement of Gαi in Smo-mediated signaling. Specifically, they showed that reduction of Gαi in Cl-8 cells in the presence of Hh, caused an elevation of cAMP levels. Similarly, overexpression of a constitutively active Gαi (GαiQ205L) in flies gives rise to ectopic pathway signaling (ectopic veins in the wings were observed, which is a Hh gain of function phenotype), as well as increased Hh target gene expression; ectopic expression of Hh target gene decapentaplegic (dpp) was observed in the wing disc. The constitutively active Gαi could also rescue a loss of Hh signaling, overriding the SmoA5 strain, which is a transgenic dominant negative Smo line. Consistent with this, amount of Dpp was shown to be lower in flies harboring a deletion of the Gαi gene or Gαi hypomorphs. Ogden, et al.(2008) suggest that Hh signaling in Drosophila is transduced via Gαi, which lowers intracellular cAMP, thereby causing inactivation of PKA, which is believed to be the kinase essential for priming Gli/Ci transcription factors for cleavage and the termination of Hh signaling. However, in previous studies in Drosophila, several groups, using a cAmp-independent PKA, demonstrated that cAMP does not play a critical role in regulating Hh signaling, since low levels of cAMP-independent PKA can substitute for loss of normal PKA function (Jiang & Struhl, 1995; Ohlmeyer & Kalderon, 1997).

While the weight of evidence does implicate G-protein with some form of Smo signaling, it appears that another aspect of GPCR signaling, specifically phosphorylation-dependent binding to Arrestin, may also be critical for Smo activity. G-protein-coupled receptor kinases and β-arrestins are involved in the termination of G protein-dependent G protein-coupled receptor signaling (Lefkowitz & Shenoy, 2005). It is now understood that GRKs and β-arrestins can also act as triggers for endocytosis of
GPCRs and can engender signaling of G protein-independent signaling complexes (Beaulieu et al., 2005). There is evidence to support that GRKs facilitate Hh signaling by interaction with Smo in cells and in animals (W. Chen et al., 2004; Meloni et al., 2006; Philipp et al., 2008). In *Drosophila*, the two GRK orthologs (Gprk1 and 2) are partially redundant to one another in the Hh signaling pathway (S. Cheng, et al., 2010). When Smo is activated, it cannot induce high level of expression of target genes without the presence of Gprk2 (Molnar, et al., 2007). Consistent with this, a GRK enhancement of Hh signaling in flies, Hh signaling has also been shown to associate with GRKs in vertebrates (Philipp, et al., 2008). Additionally, in mammalian systems, HEK cells were used to demonstrate GRK2 to be a major kinase in activation-dependent Smo phosphorylation (W. Chen, et al., 2004). Another line of evidence that reinforces the case for Smo functioning as a GPCR, includes the fact that Gβγ, which is essential for full GRK2 activation, is required for Smo phosphorylation by GRK2 in cells (Molnar, et al., 2007; Philipp, et al., 2008). Lastly, β-arrestin has also been determined as significant to Smo-mediated zebrafish development (Wilbanks et al., 2004). Morpholino depletion of β-arrestin2 in zebrafish embryos produces phenotypes resembling partial loss of function of Smo (Wilbanks, et al., 2004). Studies in mammalian cells have also produced results consistent with the case for β-arrestin2 involvement with Smo activity (W. Chen, et al., 2003).

3. The *Drosophila* Wing

The *Drosophila* wing provides as an excellent model to address developmental and cell biological questions, since the formation of the wing entails numerous processes, including cell growth, proliferation, pattern formation, and differentiation. The wing also makes an ideal system for study owing to several characteristics, such as its constant size, shape, pattern, and sensitivity to genetic manipulations. Furthermore, it is amenable to molecular and cell biological approaches.

The adult wing develops from a single cell-layered epithelial sheet, known as the wing imaginal disc. Imaginal disc cells are a cluster of cells that are set aside during embryogenesis, which proliferate during larval development and differentiate into adult structures. The wing imaginal disc is made up of a single folded layer of undifferentiated epithelial cells that proliferates to roughly 50,000 cells during the larval stages, and acquires its complete size and cell number during the initial hours of pupal
development (Bate & Arias, 1991; F. J. Diaz-Benjumea & Cohen, 1993). Thus, the adult wing is a flat sheet of cells (single epithelial layer), which is essentially folded over once to form the dorsal and ventral sides. These sides are separated by a cell-lineage restriction called the dorsal-ventral (DV) compartment boundary. The *Drosophila* wing disc is also subdivided into separate domains within the proximo-distal (PD) axis, which ultimately forms part of the thorax or notum (proximal), the wing hinge (central) and the wing blade (distal). In addition, the disc is divided into anterior and posterior compartments where the cells of the two compartments do not intermix. (Figure 13)

During this stage of development and growth in the imaginal disc, the activities of three pathways subdivide the wing epithelium into domains of gene expression, which correspond to specific wing territories: the Decapentaplegic (TGFβ in vertebrates), Hedgehog and Wnt/Wg pathways (Lawrence & Struhl, 1996; Zecca, et al., 1995). All of these pathways have secreted ligands (known as morphogens) that act at a distance from the source of secretion to modulate downstream targets and pattern the *Drosophila* wing disc (Struhl & Basler, 1993). The cells of the posterior compartment express Hh protein that gets secreted into the anterior compartment and activates the cells at the edge of the anterior-posterior compartment (AP) border (Basler & Struhl, 1994; Tabata et al., 1992). The level of Hh expression patterns the central region of the wing disc. Hh also induces expression of target genes, which includes *dpp* that is expressed as a stripe of anterior cells at the AP compartment border. Expression of *dpp*, and therefore its pathway, in turn specifies cell fate along the AP boundary in a broad domain of cells (Basler & Struhl, 1994; Nellen, et al., 1996; Tsuneizumi et al., 1997); and Dpp will proceed to activate targets, such *spalt* (*sal*) and *optomotor-blind* (*omb*) (Lecuit et al., 1996; Nellen, et al., 1996). (Figure 14B) The DV boundary, which is established after the subdivision of the wing in the AP compartments, is initiated with the expression of the *apterous* (*ap*) gene in the dorsal compartment that, in turn, activates expression of *Notch* at the DV boundary (Cohen, et al., 1992; F. J. Diaz-Benjumea & Cohen, 1993). *Notch* activity is then required for the expression of *wg* (Baonza & Garcia-Bellido, 1999). *Wg* expression occurs at the DV boundary that will give rise to the wing margin; and it is the gradient of *Wg* expression that acts on different target genes, such as *vestigial* (*vg*) and *distalless* (*dll*) at various distances from the DV boundary (de Celis, et al., 1996; F. J. Diaz-Benjumea & Cohen, 1995; Micchelli, et al., 1997; Rulifson & Blair, 1995; Struhl & Basler, 1993; Zecca, et al., 1995). (Figure 14A)
At the pupal stage, cells of the wing secrete cuticle that develops into hairs, sensory bristles, trichomes or naked cuticle, which together make up the wing blade containing the wing margin, hinge and much of the dorsal thorax (http://cistron.ca/1_2_wing_morphology.shtml). These arise from the border between the dorsal and ventral cell layers. The anterior wing margin is made up of a triple row of bristles, while the posterior wing margin is made up of an alternating dorsal and ventral row of non-innervated hairs. Within the wing blade, each cell produces a hair or trichome at the distal edge of the cell, at a position that is determined by intercellular signaling of the Frizzled receptor (Eaton, et al., 1996).

The cells of the adult wing epithelium are packed in a hexagonal pattern, each of which is decorated with a hair that points distally. Other significant features of the adult wing include five major longitudinal veins (L1-L5) that cross the length of the entire wing and two cross veins (an anterior one, ACV; and a posterior one, PCV), forming a stereotypical pattern. The veins L3, L5 and distal part of L4 are dorsal wing veins and are located on the dorsal side of the wing (Blair, 2007; De Celis & Diaz-Benjumea, 2003). The remaining veins are considered ventral wing veins.
Chapter I

Figures and Tables
Figure 1. Heterotrimeric G-protein signaling. When a ligand activates the GPCR, it catalyzes the guanine nucleotide exchange on the $G\alpha$. This causes the release of the heterotrimer from the receptor, allowing free $G\alpha$ and $G\beta\gamma$ to trigger different signaling cascades.
Figure 2. Schematic of Gα protein. (A) Linear (1° Structure) Model of Gα: locations of conserved sequences in G regions are shown. Regions designated G1-G5 are the conserved sequence motifs that form the guanine nucleotide-binding pocket (Bourne, et al., 1991). Residues that are subject to N-linked palmitoylation, N-linked myristoylation, or are sensitive to cholera toxin (ctx) or pertussis toxin (ptx) are indicated. (B) Putative contact sites of Gα with βγ, receptor (R), and effector (E) are shown (Adapted from Conklin & Bourne, 1993). The best characterized contact sites are the N-terminus region for binding βγ-subunits and the extreme C-terminus in specific receptor recognition.
Figure 3. Structures of Gα. (A) Schematic diagram of the secondary structure of Gα showing α-helices as boxes and β-sheets as arrows. The helical domain is comprised of lettered α-helices; the GTPase domain is comprised of numbered α-helices and β-sheets (Adapted from Slessareva, et al., 2003). (B) Tertiary structure of a Gα protein. Ribbon drawing of a peptide (red) in contact with a Gα protein (Gαi1). Gα subunits are organized into two domains (the helical and Ras-like/GTPase domain) that surround the guanine nucleotide (magenta). Switch regions are also shown (blue) (Adapted from R. J. Kimple et al., 2002).
Figure 4. Alignment of *Drosophila* Gα Amino Acid Sequences
Figure 4. Alignment of *Drosophila* Gα amino acid sequences. Residues identical to those of Gαf are indicated (*). Similar residues (:) and weakly similar residues (.) are also indicated. A potential site for ADP-ribosylation by cholera toxin is shown (Arrow). Circle above the sequence indicates a putative palmitoylation site. Sequences were aligned and compared using EMBL-EBI multiple sequence alignment program. Putative nucleotide binding sequences are also indicated (G1-G5), and were determined using CDD of NCBI (Gαf NP_524118.1; Gαo NP_788307.1; cta EAA46036.2; Gαs NP_477505.1; Gαl NP_725191.1; Gαl NP_477502.1).
Figure 5. Schematic diagram of the putative secondary structure of Gαf. Secondary structure is shown below the Gαf sequence with boxes indicating helices and arrows for beta sheets. G-boxes (orange boxes) and switch regions (black bars) are indicated. Linkers 1 and 2 connect the helical and GTPase domains of Gα. Circle above the sequence indicates a putative palmitoylation site. The secondary structure of the full-length Gαf protein was predicted with Geno3D (Combet, et al., 2002) using default parameter settings.
Figure 6. Phylogenetic analysis of G-protein amino acid sequences
**Figure 6. Phylogenetic analysis of G-protein amino acid sequences.** Phylogenetic tree with G alpha proteins sequences of the four main G alpha classes from a range of vertebrate and invertebrate taxa. G\(_\alpha\)f shows strong support for the G\(_\alpha\) clade, with a bootstrap percentage of 100%. Statistical evaluation of the parsimony tree was performed using the bootstrap method (Felsenstein, 1985), with 500 replicates. Ras and Rheb sequences were used as outgroups. Scale bar shows amino acid substitution rate.

(assisted by J.Parker)
Figure 7. Schematic of SOP divisions and resulting sense organs. Double-headed arrows indicate axes of division (Katanaev and Tomlinson 2006).
Figure 8. The Wnt/Wg Signaling Pathway. In the canonical pathway Wnt binding to the Frizzled receptor (in *Drosophila* Fz1 and F2) and co-receptor, LRP (Arrow in *Drosophila*), prompts the phosphorylation of the cytoplasmic protein Dvl (Dsh in *Drosophila*), that ultimately leads to the stabilization of β-catenin, which activates gene transcription in the nucleus.
Figure 9. The Planar Cell Polarity Pathway in *Drosophila*. Frizzled is at the top of the cascade. The canonical and noncanonical pathways (likely) split at the level of Dsh.
Figure 10. Planar Cell Polarity in *Drosophila*. (A) Adult wing. Inset shows the uniform alignment of hairs in the distal direction. (B) Uniform alignment of hairs correlates with the asymmetry of the core proteins, which occurs during the pupal stage of development (Adapted from W. S. Chen et al., 2008).
Figure 11. Heptahelical Structures of Frizzleds and Smoothened. (A) Frizzleds (Adapted from Wodarz & Nusse, 1998). (B) Smoothened (Structural info derived from Ayers & Therond, 2010).
Figure 12. The Hedgehog Signaling Pathway in Drosophila. (A) In the absence of Hh, Ptc inhibits Smo. Ci, the downstream transcription factor of the Hh pathway is silenced by cleavage. (B) In the presence of Hh, Ptc can no longer repress Smo, allowing Smo to be phosphorylated by kinases. This allows activation of the signaling complex and results in Ci-155 stabilization that can travel to the nucleus and function as a transcriptional activator.
**Figure 13. Wing Fate Map**

(A) Scheme of a late third instar wing imaginal disc. The proximal domain of the wing disc (notum) gives rise to the thorax; the middle section gives rise to the hinge; and the center wing pouch gives rise to the wing blade of the adult fly. Imaginal discs are divided into developmental compartments, shown by the blue line which separates the anterior (A) and posterior (P) compartments, and by the dotted red line indicating the boundary between dorsal (D) and ventral (V) compartments. Positions of longitudinal vein (LV) proveins in late-third-instar wing disc are also indicated.

(B) Scheme of the adult wing. Veins are indicated. Both subpanels (A) and (B) use the L1–L6 nomenclature for the LVs. In (B) ACV, PCV denote the anterior, posterior crossveins, respectively.

(Adapted from Blair, 2007).
Figure 14. Patterning of the wing
**Figure 14. Patterning of the wing.** (A) Wg and its target genes. Wg (green) is produced along the DV border and induces target genes, such as *dll* (purple) *vg* (red); the resulting effects are observed in the adult wing margin. (B) Hh produced in the posterior compartment patterns the central domain of the wing, and induces expression of *dpp* (purple); Dpp induces expression of *sal* (green) and *omb* (red) (Adapted from Tabata and Takei, 2004).
### Table 1

**Trimeric Gα subunits in *Drosophila***

<table>
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<th>G protein subunit</th>
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<th>synonyms</th>
<th>human ortholog (% identity)</th>
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<td>G protein αi subunit 65A</td>
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<td>G protein α 49B</td>
<td>Gαq(77%)</td>
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<tr>
<td>concertina</td>
<td>Gα12/13</td>
<td>cta</td>
<td>Gα13(55%)</td>
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<td>Gαf</td>
<td>G protein α 73B</td>
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</table>

Sequence alignments provided in Appendix Fig. 4. (Adapted from Katanayeva et al., 2010)
Chapter II

Characterizing $G \alpha f$
Abstract

The classic form of signal transduction by heptahelical receptors occurs through trimeric G-proteins. This has been extremely well characterized in many biological contexts. In development however, there are few examples of a defined role for GPCRs. Several receptors that are potential GPCRs mediate at least two of the major signaling pathways, but how these receptors signal and to what extent they use trimeric G-proteins is unclear. Here I describe the investigations of Gaf, an alpha subunit of a trimeric complex. Gaf was first identified by Quan et al. (1993) as being expressed primarily in embryonic, larval, and early pupal stages of Drosophila development in a spatially and transiently restricted manner. It is also expressed as a maternal transcript, leading the authors to suspect that it may play a role in events accompanying the formation of embryonic position and polarity. However, there is little evidence for any functional role for this gene. Here, I show that RNAi knockdown of Gaf appeared to have numerous effects in the Drosophila wing, indicative of the signaling pathways that utilize potential GPCRs. There were PCP and Wnt phenotypes, which are consistent with transduction through Frizzled receptors; and there were phenotypes that appeared similar to those related to the Smoothened receptor. However, overexpression and epistasis analyses were inconclusive. Thus, it remains unclear exactly which pathways are affected with RNAi knockdown of Gaf. Strikingly, a strong synergy was detected between Gaf and another alpha subunit (Gai) when two corresponding RNAi lines were co-expressed, which affected the formation of sensory bristles; and these results suggest that Gaf and Gai may function redundantly in the mechanism of asymmetric cell division.

1. Introduction

GPCRs comprise a large class of transmembrane receptors and they typically mediate signal transduction into cells through activation of a heterotrimeric G-protein complex. This complex consists of a GDP bound Gα subunit and a Gβγ-dimer. Agonist binding facilitates activation of the heterotrimer, as the GPCR serves as a guanine nucleotide-exchange factor for Gα, to catalyze the exchange of GDP for GTP, which leads to the disassociation of the complex, releasing the now separate Gα-GTP and Gβγ-dimer, both of which are free to signal to downstream effectors.
Fz receptors are heptahelical transmembrane proteins that transmit extracellular signals in both the Wnt and PCP pathways. These receptors appear to bear the topology of a GPCR. Whether these proteins are indeed G-protein linked has been controversial. Although a large body of evidence now exists for the association of G-proteins with Fz signaling, both in Wnt and PCP pathways, it is still unclear whether these receptors function as classical GPCRs. There are two issues that may be relevant to this question. First, it is possible that Fz proteins function both as GPCRs and as also in a G-protein independent manner. For instance, Dsh is known to be an immediate downstream effector of Fz, and signaling through Dsh may possibly signal in a G-protein independent manner. As a result, there may be two signals transduced from the Fz receptors, one that involves coupling to a trimeric G-protein, and another that occurs through Dsh. If true, then experiments with G-proteins will not disturb all Fz signaling, and consequently yield results that confound from confirmation as to their function as GPCRs. The second issue that relates to this problem involves the possible redundancy of G-proteins. If Fz does behave as a GPCR, and if this activity is mediated by more than one G-protein, then loss of one would not necessarily reveal any phenotype.

Another heptahelical-like receptor is Smo, and it is closely related to Fz. Smo plays a critical role in the Hh signaling pathway, and whether it has a ligand is unknown. As with Fz, Smo has been associated with trimeric G-protein signaling. Whether it functions as a classic GPCR remains unclear.

Asymmetric cell division shares common features with PCP; and in both processes, cellular polarization involves proteins segregating to opposite ends of the cells. Interestingly, while Fz is thought to play a role in ACD to direct the axis of polarization, the general process is perceived to be GPCR independent. Yet the actions of trimeric G-proteins have been clearly implicated in asymmetric cell division, although their defined roles and how significant those roles are remain to be resolved.

Collectively, the available information on trimeric G-proteins in relation to these mechanisms (Wnt, PCP, Hh signaling and asymmetric cell division) suggests that they are strongly associated. However, how trimeric G-proteins function in these different processes and the extent of their redundancy remains unclear.

There are six identified Gα subunits in the Drosophila genome, and there may be more as yet unrecognized. Two of these have been associated with Fz and Smo signaling: Gαo in Frizzled signaling
(Katanaev, et al., 2005) and Gαi in Smo signaling (Ogden et al., 2008) and both Gαo and Gαi in asymmetric cell division (Katanaev & Tomlinson, 2006a; Schaefer, et al., 2001; F. Yu, et al., 2003). The question raised is whether these are the only two G-proteins relevant to these processes, or if more are present but have yet not been implicated.

In Chapter three I describe a genetic screen to identify proteins that likely mediate GPCR signaling, and this screen identified Gαf (f for fly; (Quan, et al., 1993) as having a potential role in PCP and other related signaling pathways. Gαf was originally identified in Drosophila by Quan, et al. (1993) who described its expression in the embryo, larval and early pupal stages and they surmised that it is likely to play a role in specific early developmental events. Gαf protein shares a 40% homology to vertebrate Gαs (40%) (Katanayeva, et al., 2010; Quan, et al., 1993).

More recently, Gαf was identified among a subset of genes in a screen for genes that modulate Rho signaling (one of the downstream components of the PCP pathway), albeit in cytokinesis (Gregory et al., 2007), using a gain-of-function approach. The group used an assay strain that drives expression of a dominant-negative form of the Pebble protein (a Rho pathway activator), (Pebble^ΔDH; (Prokopenko et al., 1999) under the control of an eye-specific promoter (GMR>Gal4; UAS>pebble^ΔDH) to give a sensitized phenotype in the eye. Using this overexpression eye phenotype as their background, they performed a screen with a random subset of the GeneSearch collection of EP insert strains (Seong, et al., 2001). Among the modulators of the phenotype, Gαf emerged as a moderate suppressor.

As Gαf has remained almost relatively uncharacterized in terms of which pathways it functions, and my preliminary experiments suggest that it may function in multiple critical pathways, we decided to examine the gene in more detail.

Wnt1 ortholog is represented in the fly by Wg, and this protein is critically required (among a host of other requirements) for the growth and patterning of the wing blade (Morata & Lawrence, 1977; Williams, et al., 1993). Here it is expressed as a thin strip of cells along the DV compartment boundary, and without it, the wing discs lose their ability to develop wing blade structures. Later in development, Wg is required in bristle patterning along the wing margin (Phillips & Whittle, 1993). Therefore, any misregulation of this secreted protein can cause a loss of wing margin bristles as well as notches in the wing margin. Wg also acts to restrict its own expression in cells immediately adjacent to the dorsoventral
compartment boundary. When Wg signaling is blocked (such as with a \textit{wg} mutant or \textit{axin} mutant), this restriction is alleviated causing ectopic expression of Wg. Ectopic Wg expression can induce formation of ectopic margin bristles or ectopic wing margin. Thus, the wing and its development provide an excellent tissue in which to examine effects on Wg signaling and its misregulation.

The wing is also an excellent tissue in which to study the three other mechanisms, which can aid in patterning formation, such as PCP, Hedgehog, and asymmetric cell division. The wing is made from many hundred cells, each carrying a single hair, all of which are coordinately aligned. Defects in the coordination, or other PCP phenotypes such as the secretion of more than one hair per cell (the so called multiple wing hair (mwh) phenotype), are readily evident. PCP is also evident in the bristles of the anterior wing margin in the manner in which they all point to the distal end of the wing.

Distinguishing between phenotypes of those manifested by Frizzled is problematic, as both PCP and Wg phenotypes are transduced through Fz receptors. Fz1 and Fz2 act as redundant receptors for Wg signaling (C. M. Chen & Struhl, 1999), but Fz1 separately and independently from Fz2 transduces the PCP pathway. When \textit{fz1} is removed (\textit{fz}) only PCP phenotypes emerge, and when \textit{fz2} alone is removed Wnt and PCP signaling is transduced normally. But in double mutants where both receptors are removed (\textit{fz,fz2}) Wnt signaling is lost, and no wing emerges (C. M. Chen & Struhl, 1999). Although both these receptors redundantly transduce the Wnt signal, the fact that Fz1 is a PCP receptor becomes evident when it is overexpressed. \textit{Fz1} overexpression produces phenotypes overwhelmingly of the PCP type: swirling patterns of the hairs in the wing result and mwh emerge (Wong & Adler, 1993). Overexpression of \textit{fz2} in contrast produces phenotypes that are largely similar to \textit{wg} phenotypes, and these include ectopic margin bristles and wing notches (Cadigan et al., 1998; Boutros et al., 2000). Thus although the transduction of the two pathways are closely related, the phenotypes of their gain and loss of functions are clearly separable.

The bristles of the anterior wing margin represent a group of cells that are generated from an asymmetric division process, whereby polarization in the mother cell directs segregation of determinants to daughter cells. When this process is aberrant, cells become mis-specified and many phenotypes can emerge, including the loss and duplication of various elements of the cell group. These features are
readily apparent in the anterior wing margin usually as absent bristles, or duplicated bristle shafts or empty/duplicated sockets.

The patterning of the wing disc is also regulated by Hh, which is a key regulator of several developmental processes, including patterning of the wing disc. Hh is secreted by the engrailed-expressing (en) cells in the posterior compartment (Tabata et al., 1992; Zecca et al., 1995; Basler & Struhl, 1994). The posterior cells do not respond to Hh because of the presence of En (Zecca et al., 1995). In contrast, the cells of the anterior compartment do not express en, and therefore do not secrete Hh but do respond to Hh. Thus, Hh diffuses from the posterior compartment into the anterior compartment. The compartment border receives the highest concentration of Hh and responds in a number of ways, including transcribing dpp (Basler & Struhl, 1994; Zecca et al., 1995). Dpp is expressed as a stipe of cells in the anterior compartment that lies adjacent to the compartment border. From this position, Dpp diffuses bilaterally into both the anterior and posterior compartments to direct their growth and patterning (Basler and Struhl, 1994; Zecca et al., 1995; Lecuit et al., 1996; Nellen et al., 1996). In the adult wing, the compartment border runs between veins L3 and L4, and overexpression of Hh (high activation of the pathway) in the more lateral regions of the anterior compartment will drive them towards the fate of the cells found between L3 and L4 veins. As the posterior cells do not respond to Hh signaling, the hallmark of this pathway is that only the anterior compartment shows responses. As a consequence, when experiments show effects in the anterior compartment but not in the posterior, this is a strong indication that it may be the Hh pathway that is affected.

Because the phenotypes of these different signaling pathways are readily apparent in the wing, it served as an ideal system to systematically characterize the effects of \( G_{\alpha f} \), both in knockdown and gain of function experiments, in order to determine in which signaling pathway it may function.

2. Materials and Methods

2.1 Drosophila strains and maintenance

Flies were maintained on a standard cornmeal medium and all crosses were performed at 25°C. Wildtype samples used were Canton-S flies or yw lab stocks.
**Drosophila Gal4 drivers**

We used the GAL4/UAS system (Brand & Perrimon, 1993) for tissue-specific expression of transgenes. Gal4 (G4) drivers used were as follows: MS1096-G4 (Brand and Perrimon, 1993) was a gift from M. Zecca; sev-G4, GMR-G4 (Hay et al., 1997), apterous-G4 (Calleja et al., 1996), distalless-G4, omb-G4 (Calleja et al., 1996) came from the lab stock; en-G4 was a gift from the Johnston lab; and ci-G4 (Croker et al., 2006) from the Mann lab. Other stocks used in the studies included tub>CD2,y+>G4 (from G. Struhl) and act>CD2>G4, UAS-GFP (from J. Parker) for making gain-of-function clones.

**Deficiencies**

Deficiencies of the Gαf used for the study were obtained from the Bloomington stock center (stock number in parentheses): Df(3L)Exel6130 (#7609); Df(3L)ED223 (#8079); Df(3L)ED4674(#8098); Df(3L)BSC561 (#25123).

**Insertions and mutations**

The insertion of the gene used came (Gαf) from the Bloomington stock center: Gα73BMB10610 (# 29157) (Bellen et al., 2004). Mutants used for the experiments came from the lab stock: fz;fz2 double mutant (fzP21, fz2C1: K. H. Jones et al., 1996; Chen and Struhl, 1999) and smo3 (Y. Chen & Struhl, 1996). UAS-fz (Strapps and Tomlinson, 2001), UAS-fz2 (Cadigan et al., 1998) and UAS-smo (Ingham et al., 2000) were kind gifts from G. Struhl. UAS-Gαf WT (Schaefer et al., 2001) came from the lab stock. Other stocks used for the RNAi experiments included (Bloomington stock number in parentheses): UAS-dicer2 (#25706; 25752). UAS-dicer-2 (dcr-2) transgene (Dietzl et al., 2007) was used to enhance RNAi potency.

The Gαf RNAi strain used throughout most of this study, targeted the C-terminus of the Gαf gene (forming a hairpin length of 587 nucleotides), was obtained from the TRiP collection at Harvard Medical School (Bloomington #25930) and inserted on chromosome 3 (Ni et al., 2008; Dietzl et al., 2007). A second Gαf RNAi strain, (Transformant ID 17056, targeted a partially overlapping region to the TRiP line, against nucleotides 807-1191) from the “GD” P-element library, inserted on chromosome 2 was from the Vienna Drosophila RNAi Center (VDRC and was a gift from V. Katanec), and gave a considerably weaker
phenotype (Dietzl et al., 2007). A third independent Gαf RNAi line (Stock ID 12232R-1, targeted the N-terminal domain, against nucleotides 290-789; and non-overlapping region to the TRiP line), from the Fly Stocks of National Intitutes of Genetics (NIG-FLY) on chromosome 3, was used as a specificity control (kind gift from J.Carlson); this strain also caused identical phenotypes to the first two RNAi lines, although it was also weaker than the TRiP Gαf RNAi line. A fourth Gαf RNAi line was also obtained and checked for similar phenotype from VDRC (Transformant ID 17054).

For the genetic interaction studies, a Gαo RNAi line was obtained from VDRC (Transformant ID 19124) inserted on chromosome 3; and a Gai RNAi line was also obtained from VDRC (Transformant ID 28150), inserted on chromosome 3. An independent Gαo line (Bloomington # 28010), from the TRiP collection, inserted on chromosome 3, was also used to validate the results. Recombined stocks of Gαo (VDRC 19124) and Gai (VDRC 28150) lines with the TRiP Gαf (#25930): yw; GαoRNAi, GαfRNAi/TM2 and yw; GαiRNAi, GαfRNAi/TM2 (made by A.Tomlinson) were also used in the genetic studies.

2.2 Construction of fly lines

The following Drosophila stocks were generated for these studies: UAS-Gαf (wild type), UAS-GαfGTP, UAS-GαfGDP cloned into the UAS-attB vector (Bischof, et al., 2007). The Gαf cDNA (CG 1223R1) was generated by GeneScript (Piscataway, NJ) in pCU57. The UAS-Gαf (wild type) cDNA was cloned into a modified version of the UAS-Attb vector (obtained from the Struhl lab) using EcoRI and HindIII sites. The following primers were used to introduce Ascl and Xho I sites, in order to subsequently clone the GDP and GTP versions of Gαf:

Gffor1 5’ – GGCAGCCATTCAAGACATGAAATT – 3’
Gfrev1 5’ – CTGAGGGATCTGGACTAGAATAGGCC – 3’

Primers used to generate point mutations in order to make the activated form of Gαf (GαfGTP: Q225L) were:

Gffor2 5’ – CAAATGTAGATGTTGGGTGCTTATTG – 3’
Gfrev2 5’ – CCTTAGCCACCACCATCGTACATTG – 3’
Primers used to generate the GDP version of $G\alpha f$ ($G\alpha f^{GDP}$: G224A) were the following:

Gffor3  5’ TATACAAATGTACGATGTGG GTGCCCAAAGG 3’
Gfrev3  5’ TATACCTTTGGGCACCCACATCGTACATT T 3’

All constructs were confirmed by sequencing, and transgenic flies were generated by Bestgene (Chino Hills, CA). Some were also made in the lab. All constructs were injected into phage attachment site (attP) [at 86Fb] embryos.

2.3 Immunohistochemistry

Third-instar larvae were dissected in PBS, and wing discs were fixed in 4% formaldehyde in PBS for 20 min at room temperature. Samples were blocked for 30 min at room temperature with 10% normal donkey serum + 0.5% NP-40 in PBS. After a quick wash with PBS, followed by two more washes with 0.3% Triton X-100 in PBS (PBST), the samples were incubated with primary antibody at their appropriate dilution overnight at 4 °C. After 5 washes with 0.3% PBST for 10 minutes each, the samples were incubated with secondary antibody. Labeled secondary antibodies (AlexaFluor488, AlexaFluor555 AlexaFlruo647 conjugates) were from Molecular Probes and used at a 1:500 dilution for 2 hours at room temperature. After staining, the samples were washed 5 more times with 0.1% PBST for 20 minutes each, then mounted on glass slides in Vectashield Mounting Medium (Vector Laboratories), with or without DAPI or Hoechst to stain DNA. Preparations were then imaged by confocal microscopy.

The following primary antibodies were used: guinea pig anti-Vg (gift of G. Struhl) at 1:500; guinea pig anti-DII at 1:500 (Estella et al., 2008) and guinea pig anti-Sal (Xie et al., 2007) at 1:1000 (gifts of R.Mann). Mouse antibodies against Cut (1:100), and Wg (1:30) were from Developmental Studies Hybridoma Bank.

2.4 Generation of a $G\alpha f$ antibody

Antibody reagent specific for Drosophila $G\alpha f$ was generated against a synthetic peptide (DITQEPFKRHRNQVD), corresponding to amino acids 341-356 of $G\alpha f$ by Pi Proteomics (Huntsville, AL). However, the crude rabbit antisera at various dilutions failed to work in our immunostaining studies.
2.5 Histology

Adult Eye Dissections

Eyes were processed for sectioning and analysis following Tomlinson & Ready (1987).

Mounting of Adult wings

Adult wings were removed from ≥ 1 day old female (unless indicated) flies, dehydrated in ethanol and mounted on glass microscope slides in Gary’s Mounting Medium or mounted using DPX.

3. Results

3.1 RNAi-mediated knockdown of $G_{\alpha f}$ in the *Drosophila* wing causes various patterning defects

I investigated the role of $G_{\alpha f}$ in *Drosophila* wing development and patterning using an UAS-RNAi transgenic fly from the Harvard Transgenic RNAi Project (TRiP) collection (which I will from herein refer to as the TRiP UAS-$G_{\alpha f}$ RNAi line) (Ni et al., 2008; Dietzl et al., 2007). The TRiP UAS-$G_{\alpha f}$ RNAi used in most of this study targeted a 587 bp nucleotide fragment corresponding to the C-terminal region of $G_{\alpha f}$. (Figure 15C) Driving TRiP UAS-$G_{\alpha f}$ RNAi in the posterior compartment of the wing blade using the en-G4 driver, in the presence of UAS-dcr2 (UAS-dcr2/+;en-G4/+;TRiP $G_{\alpha f}$ RNAi/+), resulted in polarity phenotypes including multiple wing hairs, hairs pointing in the wrong direction and bald areas, compared to the wild type wing. (Figure 16E, F; Table 2) Occasional loss of the wing margin was observed (some notching). (Figure 16B; Table 2) Notably, these phenotypes were not fully penetrant; these defects were present in the wing, but they were not visible throughout the compartment where the RNAi line was expressed. The most prominent phenotype observed when driven by en-G4 were the venation defects: collapse or merging of the L4 and L5 veins at the site of the posterior cross vein; incomplete veins, such as with the anterior cross vein; or the presence of ectopic vein material. (Figure 16A-A’, B; Table 2) I also drove this line in the dorsal compartment across the wing blade with the MS1096-G4 driver (MS1096-G4/+;UAS-dcr2/+;TRiP UAS-$G_{\alpha f}$ RNAi/+), where venation defects and polarity defects (mwh and hairs in different direction) were observed as well. (Table 5, 6) Occasional ectopic bristles were visible as well. (Figure 16G; Table 5, 6) Strikingly, I also observed
defects on the anterior wing margin (stout bristle defects: missing bristles or extra bristles originating from one socket). (Figure 16C, D; 17E; Table 5, 6)

To further corroborate these results, I used other RNAi lines and drove them under the same conditions. A second independent RNAi line (obtained from the Vienna collection: #17056, which I will refer to as VDRC UAS-\(G\alpha\)f RNAi ) (Dietzl et al., 2007), targeted the central portion of the gene, (Figure 15C) When expressed in the posterior compartment of the wing with en-G4, no visible effects were observed (UAS-dcr-2/+;en-G4/VDRC UAS-\(G\alpha\)f RNAi). (Table 3) Similarly, when expressed with MS1096-G4 (MS1096-G4/++;UAS-dcr2/VDRC UAS-\(G\alpha\)f RNAi), this line gave much weaker phenotypes when compared with the TRiP UAS-\(G\alpha\)f RNAi line. (Figure 17B, C, E; Table 7) However, when two copies of this RNAi line were driven with MS1096-G4 (MS1096-G4/+;UAS-dcr2,VDRC UAS-\(G\alpha\)f RNAi/VDRC UAS-\(G\alpha\)f RNAi), it appeared to show nearly the same level of penetrance as one copy of the TRiP line, although I failed to generate enough wings to quantify this observation. (Figure 18B, C)

To further ensure that these phenotypes were not an off-target effect of the RNAi line, I used a third independent RNAi line against \(G\alpha\)f (from the NIG-FLY: #12232R-1) that targeted the N-terminus of the gene under the same conditions. (Figure 15C) The phenotypes observed with this line were also weak when this line was expressed by both en-G4 (UAS-dcr2/+;en-G4/++;UAS \(G\alpha\)f RNAi #12232R-1/+) (Table 4) and MS1096-G4 (MS1096-G4/+;UAS-dcr2/+;UAS \(G\alpha\)f RNAi #12232R-1/+). (Figure 17D, E; Table 8) Interestingly, when two copies of this RNAi line were driven by MS1096-G4 (MS1096-G4/+;UAS-dcr2/+;UAS \(G\alpha\)f RNAi #12232R-1/++UAS \(G\alpha\)f RNAi #12232R-1), similar phenotypes to the TRiP UAS \(G\alpha\)f RNAi were observed. (Figure 18D). Ultimately, I obtained a fourth RNAi line (the second from the Vienna collection), targeting the same central portion of \(G\alpha\)f but inserted on a different chromosome. The phenotype was again weak with one copy of this line, driven by MS1096-G4.

To fully address whether the TRiP UAS-\(G\alpha\)f RNAi line used was targeting the \(G\alpha\)f gene directly, I also performed the same experiments in the presence of a Deficiency (Df) line for the gene. The \(G\alpha\)f gene is localized in a small genomic region on position 73B5 on the right arm of the third chromosome (Quan, et al., 1993). The assumption was that the RNAi in the presence of a deficiency for the region of the gene would reduce the transcript number by half, thereby increasing the effect of the RNAi. I looked
at flies that harbored the following genotype: en-G4/UAS-dcr2; TRiP UAS-Gαf RNAi/Deficiency (for Gαf region) to assess for any modulation of the phenotype, and specifically for any enhancement of the phenotypes observed with the TRiP UAS-Gαf RNAi line alone.

There are four available stocks carrying deficiencies in the region of the Gαf gene: Df(3L)Exel6130, Df(3L)ED4674, Df(3L)ED223, Df(3L)BSC561, which represent four unique deficiencies containing different breakpoints where the gene is presumably absent. As these deficiencies have not been accurately mapped and accordingly their specific breakpoints have not been determined (we did not know which of these Dfs actually removed the gene), I used all four available Dfs to conduct my experiments. As control, I first verified whether the Df lines did correspond to the noted regions in the literature by crossing each of the Df lines against each other. The expectation was that if the deficiencies were from the same region and overlapped, then there would be no adult survivors. This was indeed the case, as none of the progeny survived to adulthood and provided confidence that they were against the same region.

All four Df lines were then crossed to the TRiP UAS-Gαf RNAi line. As expected, inclusion of the deficiency of the gene with the RNAi line enhanced the RNAi phenotypes, although to different degrees. (Figure 19, 20; Table 10-13) When TRiP UAS-Gαf RNAi line was expressed in conjunction with all the Dfs, the most prominent venation defect observed when driven with en-G4 (collapse of the L4 and L5 veins at the site of the PCV) appeared to increase from 76% to 98%-100% of the flies. (Figure 20F; Table 10-13). Moreover, closer inspection of this venation defect, showed an enhancement of this defect; the venation defects appeared to expand. (compare brackets in Figures 19, 20) In addition, blistering over this venation defect that is generally not observed with TRiP UAS-Gαf RNAi alone, appeared with the presence of all four Dfs, although to varying degrees. (Figure 20F; Table 10-13) The notching of the wing margin that was observed only in 7% of the fly wings in the TRiP UAS-Gαf RNAi line, increased to about 20%, in the presence of one Df line: Df(3L)ED4674.

I also crossed the Df lines independently against a reported line with an insertion into the Gαf gene, as there is no reported mutant of Gαf available. This insertion line (Gα73BMB10810; Bellen et al., 2004) alone did not display any of the phenotypes that I observed with the TRiP UAS-Gαf RNAi or other
\( \text{G} \alpha \text{f RNAi lines. When crossed with the Df lines to produce a G} \alpha \text{f}^{73} \text{BMB10810}/Df(3L) \text{ heterozygote, I still did not observe any phenotypes that were reminiscent of the G} \alpha \text{f RNAi lines. It is likely that the insertion line is not an appropriate mutant of the G} \alpha \text{f gene or is too weak to be of any value.}

Aside from utilizing the Df lines to enhance the RNAi phenotypes, I also confirmed the RNAi phenotypes by using two of the non-overlapping RNAi constructs jointly. Therefore, when I crossed one copy of TRiP UAS-\( \text{G} \alpha \text{f RNAi} \) line with one copy of the #12232R-1 UAS-\( \text{G} \alpha \text{f RNAi} \) line, for instance, they gave similar phenotypes and exhibited an enhancement of the TRiP UAS-\( \text{G} \alpha \text{f RNAi} \) line phenotypes. For instance, when simultaneously expressed in the posterior compartment, the venation defect increased from 76\% to 100\% of the flies. (Figure 20F, 21, 22, 39; Table 9) Likewise, when driven by MS1096-G4, the observed defects appeared more pronounced (particularly the venation defects). (Figure 23) We can, as a result, infer that the phenotypes do indeed correspond to a knockdown of the gene function.

In addition to driving the TRiP UAS-\( \text{G} \alpha \text{f RNAi} \) with \( \text{en-G4} \) and MS1096-G4 drivers, I performed similar experiments with a panel of wing Gal4 drivers. (Table 14) Interestingly, the phenotypes observed (while mostly similar overall) varied with the different driver lines used. For example, \( \text{en-G4} \) produces polarity defects, venation effects and possible effects on the wing margin. \( \text{Nubbin-G4} \) (used to drive only in the wing blade) however, changed the shape and the size of the wing, causing it to be narrower relative to the wild type, as though it were affecting a morphogen or a growth control mechanism. Driving the TRiP UAS-\( \text{G} \alpha \text{f RNAi} \) line with \( \text{ap-G4} \) (expressed in the dorsal domain of the wing) produced few viable flies. However, the effects in those flies were even more severe than with any of the other drivers: the thorax of the fly was severely affected; and the wings were diminished in size, with even greater multiple wing hairs and polarity defects observed. Driving with \( \text{dll-G4} \) (expressed in a diffuse pattern from the wing margins into the wing blade) showed a pronounced effect in the anterior wing margin only, with many bristles missing. (Table 14; Figure 24A-A')

Taken together, these findings indicate potential multiple roles for \( \text{G} \alpha \text{f} \) in wing development, and we suspect \( \text{G} \alpha \text{f} \) may be affecting several pathways. The mwh and hairs pointing in the wrong direction indicate a possible role in planar cell polarity. The few instances we observed with the loss of wing margin also indicate a possible role in the Wg pathway. Lastly, the defects in the anterior wing margin, with the bristle defects, point to a possible role in asymmetric cell division.
3.2 Little discernible phenotype produced with RNAi-mediated knockdown of \(G_{\alpha f}\) in the 
\textit{Drosophila} eye

I also tested for any effects in the \textit{Drosophila} eye with overexpression of the \textit{TriP UAS-}\(G_{\alpha f}\) \textit{RNAi} line using three different Gal4 drivers. I looked for any roughness on the surface of the eye, which is often an indicator of any effect taking place. The eye-specific Gal4 drivers, \textit{GMR-G4} (is expressed in all photoreceptors behind the morphogenetic furrow) and \textit{sev-G4} (expressed in the R3,R4 and R7 photoreceptors and in the cone cells) did not have any pronounced effect on the eye compared to that of the wild-type. Overexpression with \textit{ci-G4} (expressed throughout the developing eye disc; \textit{ci-G4/UAS-dcr2;TriP UAS-}\(G_{\alpha f}\) \textit{RNAi/TRiP UAS-}\(G_{\alpha f}\) \textit{RNAi}) showed some roughness, which were subsequently sectioned. Cross-section of the retinas did not appear to have any pronounced effects, and the associated rough eye phenotype was likely associated with the merging of some ommatidial structures. (Figure 24B) In addition, I also performed crosses with the Df lines that produced more severe phenotypes in the wings (\textit{ci-G4/UAS-dcr2;TriP UAS-}\(G_{\alpha f}\) \textit{RNAi/Df(3L)}). The resulting adult eyes appeared rougher, but analyses of tangential sections of these eyes did not reveal anything reminiscent of phenotypes consistent with polarity defects. Importantly, we do not currently know where \(G_{\alpha f}\) is expressed. Since the eye sections did not reveal any polarity defects to complement the effects observed in the adult fly wing, I can only speculate that \(G_{\alpha f}\) likely subserves specific functions in specialized cell types, such as the epithelial cells of the wing.

3.3 Ectopic expression of \(G_{\alpha f}\) in the adult wing causes minor patterning defects

\(G_{\alpha}\) subunits exchange GDP for GTP when their cognate serpentine receptors are activated. Thus, I made three forms of \textit{UAS-}\(G_{\alpha f}\) and transformed each into flies: \textit{UAS-}\(G_{\alpha f}\) in its wild type form, one in which \(G_{\alpha f}\) was maintained in its GTP bound state (a GTPase-deficient mutant form which maintains it in the activated form) and one in which \(G_{\alpha f}\) was in its GDP bound state (an inactive form that is tightly associated with its \(\beta\gamma\) subunit, and is bound to the receptor).

When all three constructs are expressed with either \textit{en-G4} (\textit{en-G4/+;UAS-}\(G_{\alpha f}/+\)) or \textit{MS1096-G4} (\textit{MS1096-G4/+;UAS-}\(G_{\alpha f}/+\)) lines they show only infrequent or weak phenotypes. We expected lines
homozygous for the constructs would be much stronger than one copy alone in the presence of the Gal4 driver. However, two copies of all three versions of the UAS-Gαf reporters, driven with en-G4 (en-G4/+; UAS-Gαf/UAS-Gαf), did not produce any notable results. (Appendix Table 1-3)

The GTP form of the Gα construct should not interact with the βγ subunits, in contrast to the wildtype form. The idea was that if I observed the same phenotypes with both constructs, then we could infer that the phenotype was a result of activation of a pathway(s), rather than due to sequestration of βγ-subunits or attributable to other dominant negative effects. Interestingly, when driven under the control of the X-linked MS1096-G4 driver, two copies of UAS-Gαf lines (MS1096-G4/Y;UAS-Gαf/UAS-Gαf or females expressing MS1096-G4;UAS-Gαf) showed clear phenotypes. These included venation defects, wing margin problems (effects on the bristles of the anterior margin), and narrowing of the wing, particularly along the AP axis. (Figure 25, 40) Since these were the prominent phenotypes observed, only these were quantified. (Table 15-17) Nevertheless, there were no discernible polarity defects observed with these constructs that would implicate planar polarity signaling, as there had been with the RNAi lines. Even with expression of two copies of each of these constructs, the observed phenotypes were not pronounced. However, the phenotypes were consistent.

Conversely, we expected the GDP transformant to illuminate any dominant negative effects, including any effects resulting from possible sequestration of the βγ subunit from another Gα subunit. The observed effects on the wings of these flies appeared no different from those of the wild type and GTP forms. It should be noted that all three variants did appear to have a similar percentage of flies with anterior margin defects (where both gaps in the margin as well as extra bristles were quantified under one phenotype). While I did not quantify the two observed effects on the anterior margin separately, visually, the wings of the GDP transformant did strikingly appear to be frequently accompanied by anterior margin defects where at least two bristles emerged from a single socket under the same conditions. (Figure 25D'; Appendix Figure 6B) I also observed this phenotype with merely one copy of the GDP variant driven by MS1096-G4. (Appendix Figure 6D) Further, quantification should be pursued to verify this in the future. If this observation holds, this suggests that the observed phenotype could be a consequence of βγ depletion. It could also be due to the fact that it is receptor-bound, which might prevent the receptor from other interactions.
3.4 Protein patterning in the wing disc

3.41 Characterization of the protein expression patterns correlated with knockdown of Gαf in the wing disc

The various effects of the RNAi lines in the adult wing suggest that Gαf may be acting in multiple signaling pathways. To begin to address which particular pathway(s) Gαf may participate in or signal through, we attempted to make an antibody against Gαf, which would ideally allow me to trace the expression pattern of the protein in vivo. Unfortunately, the rabbit antibody I obtained, made against a synthetic peptide corresponding to the 341-356 amino acids of Gαf, failed to work in my immunostaining studies.

Additionally, I examined the expression profiles of various wing patterning genes, which correspond to induction of various signaling pathways. In order to visualize clearly the effects of Gαf downregulation in the wing with the TRiP UAS-Gαf RNAi line, I used the most potent arrangement that I could engineer for my immunostaining studies. I used stocks harboring two copies of the TRiP UAS-Gαf RNAi in the presence of UAS-dcr2, driven with ci-G4, which is expressed exclusively in the anterior compartment of the developing wing. When I attempted to do the same with en-G4, which drives in the posterior compartment, I was unable to obtain any larvae with two copies of the TRiP UAS-Gαf RNAi line. To circumvent this issue, I can potentially use this driver (en-G4) and generate clones in the compartment in the discs to assess for disparities in staining patterns; or I can incorporate a temperature-sensitive (ts) Gal80, which would permit conditional control of the TRiP UAS-Gαf RNAi expression (McGuire et al., 2003). However, for the sake of time and availability of reagents, I performed my immunostaining studies using the ci-G4 driver only (ci-G4/UAS-dcr2; TRiP UAS-Gαf RNAi/TRiP UAS-Gαf RNAi). As the RNAi line is expressed in the anterior compartment, the posterior compartment could serve as my control for all the antibody stainings that I carried out in an attempt to identify which signaling pathway(s) where Gαf may participate.

Two orthogonal systems of short and long range signaling proteins help to organize the development of the wing by regulating growth and gene expression (Neumann & Cohen, 1997; Serrano & O'Farrell, 1997). These signaling proteins include Wg and Dpp, which are made by cells along the dorso-
ventral and antero-posterior compartment boundaries that induce the expression of several target genes in the developing wing disc. In a first step toward elucidating the molecular mechanism by which \( G_{\alpha f} \) may regulate wing patterning, I began by examining the expression of Wg and its direct targets: distalless and vestigial.

Wg is involved in imaginal disc development and in the wing it is expressed in the pouch as well as in a narrow strip, demarking the wing margin, where it induces cell proliferation (Neumann & Cohen, 1997). In addition, \( wg \) is expressed in the notum (Cadigan, et al., 1998). When Wg function is diminished or is absent, the adult wing can lack a defined margin with its associating bristles or can lack wings altogether (Couso, et al., 1994). When I examined third instar larval wing discs for Wg protein, Wg staining consistently showed some perturbation corresponding with a downregulation of \( G_{\alpha f} \) in the anterior compartment. (Figure 26) While not entirely convincing, this could implicate \( G_{\alpha f} \) in Wg signaling.

Low levels of Wg are sufficient to activate dll, which is expressed throughout the wing pouch in the wing imaginal disc (Neumann & Cohen, 1997). In addition to dll, Wg activates vg, which is a selector protein for wing identity and development. Vg expression is also induced by dpp expression (Kim et al., 1996; Williams, et al., 1993; Williams, et al., 1994). It is expressed along the dorso-ventral boundary, which will become the wing margin and is expressed in the entire developing wing pouch (Halder et al., 1998). In accordance with my Wg result, there was a clear reduction in the protein expressions for the two obligate Wg targets –Dll and Vg – as a consequence of downregulation of \( G_{\alpha f} \) in the wing disc. (Figure 26, 27) Although Vg expression appears to show more diminished level of expression in the anterior compartment, both proteins appear to show a reduction compared to the posterior compartment. (Figure 26H'; 27B-B') As a result, this data provides additional data that \( G_{\alpha f} \) could act in the Wg signaling pathway.

Dpp also induces the spalt gene in a boxlike pattern centered along the Dpp stripe (Lecuit et al., 1996; Nellen, et al., 1996). As spalt gene expression is the most sensitive to reduction in Dpp signaling, I monitored its expression in the anterior compartment and found there to be predominantly intact staining with a possible slight reduction in expression in the anterior compartment. This effect was not pronounced, but it does not rule out whether \( G_{\alpha f} \) is involved in Dpp (TGF\( \beta \)) signaling. (Figure 28)
Lastly, to monitor effects of Notch activity (and indirectly Wg activity, as it requires both Notch and Wg inputs) (Neumann & Cohen, 1997), cut expression was also examined. Cut is expressed in all of the external sensory organs of the wing and the non-innervated bristles of the wing margin (Jack, Dorsett, Delotto, & Liu, 1991). In the third instar larvae cut is expressed as a band in the position of the presumptive wing margin, as well as into the thoracic portion of the disc. As a result of Gαf down-regulation, I also observed downregulation of Cut staining. (Figure 29) This could mean that as an indirect target of the Wg pathway, Cut protein expression is downregulated as a result of impacting the Wg pathway. Another possibility is that as a direct target of Notch, manipulation of Gαf is affecting Notch signaling.

I also counterstained these discs with Hoechst in order to monitor the nuclei in the region where I observed any effects. (Figure 28, 29) There appears to be less cells in the compartment expressing the two copies Gαf RNAi. However, it is difficult to determine whether this is due to cell death, as the cells do not appear to be unhealthy.

3.42 Induction of TRiP UAS-Gαf RNAi clones

One model proposed to explain planar polarity is that it results from a concentration gradient of polarizing molecules (where the highest gradient is in the proximal region and the lowest in the distal region of the wing epithelium) that are transmitted across the epithelium, which may be important at the tissue level. This model has been invoked to explain interesting observations with at least one gene associated with this phenomenon, four-jointed (fj). When there is global loss of this protein, only subtle defects can be detected, which suggests that fj may be redundant in this process. In contrast, loss-of-function clones produce very striking phenotypes in the wing hairs (Zeidler, Perrimon, & Strutt, 2000). Similarly, when fj is expressed uniformly throughout the developing wing using the actin promoter, very few polarity phenotypes arise. However, clones ectopically expressing fj can cause repolarization of hairs in the wing (Zeidler, et al., 2000).

Taking into consideration that the wing tissue could be sensitive to disparities in protein levels, we believed making clones, and thereby making local inversions of any gradients, might uncover a possible
role in planar polarity with the TRiP UAS-Gαf RNAi line. Unfortunately, there were relatively few flies that survived to adulthood with the appropriate genotype (clones were driven by tubulin-G4, a ubiquitous driver: tub-G4/UAS-dcr2;TRiP UAS-Gαf RNAi/+), and I suspected many were dying prior to the pupal stage. Of those that survived, some showed notching and/or had missing bristles along the anterior wing margin. (Figure 30) I did not, however, observe any aberrations within the wing blade, corresponding with possible clones within the wing.

Examination of the wing discs stained for the same set of proteins as before did not reveal anything conspicuously different from that of a wild type disc. I subsequently determined that I would need to make clones driving two copies of the TRiP UAS-Gαf RNAi construct, in order to see any perceptible changes in protein expression accompanying knockdown of Gαf. However, I was unable to produce larvae harboring the necessary genotype. This limitation can be potentially overcome by using the Gal4/Gal80ts system (McGuire et al., 2003) to delay the expression of the TRiP UAS-Gαf RNAi, and suppress the lethality that appeared to be occurring.

3.43 Characterization of the protein expression patterns in the wing disc correlated with ectopic Gαf expression

To test the effects of Gαf overexpression in the developing wing, I examined wing discs from homozygous constructs driven by MS1096-G4 (MS1096-G4;UAS-Gαf WT and UAS-Gαf-GTP), since these flies showed some phenotypes in the adult wing compared to those driven with the en-G4 driver (en-G4;UAS-Gαf). In these flies, I observed minor effects on wing veins, narrowing of the entire wing and occasional effects with the anterior wing margin (bristles spaced out more sparsely) of the adult wing. However, in the disc, the wild type form of Gαf and Gαf-GTP did not show any obvious effects when stained for Wg, Dll, Vg, Cut and Spalt (Figure 31), although I would have to stain a greater number of wings to be certain of these results. In addition, I was unable to obtain a homozygous stock of Gαf-GDP in time to make any valid comparison to the other two forms. Given the negative results from these stainings, it is difficult to assess whether conversion of Gαf-GDP to Gαf-GTP is key to a role in patterning wing development.
3.5 Genetic Interactions

Since Gαf is a trimeric G-protein, it likely mediates signaling from a seven-transmembrane receptor. Given the various phenotypes with the Gαf RNAi lines, I proceeded to test Gαf in concert with three receptors (Fz1 and Fz2 for the Wg pathway, Fz1 for the polarity pathway and Smo for the Hedgehog pathway) to determine which signaling pathway(s) through which Gαf may signal. Thus, I sought to ascertain whether overexpressing these receptors would enhance or suppress the phenotypes of the UAS-Gαf constructs, or of the RNAi line. Additionally, I investigated whether there was any enhancement or reduction of the effects of the RNAi construct, or of the UAS-Gαf constructs upon reduction of the receptor expressed.

3.51 Involvement of Gαf in Wnt/Wg signaling pathway

Gα proteins are often coupled to 7-TM receptors and modulate their signaling. To investigate which 7-TM receptor Gαf might couple to while addressing which pathway it may play a role in, I first looked at the Frizzled receptors. Frizzled2 receptor functions to propagate the Wg pathway, while Frizzled1 (described in the subsequent section) signals in both Wg and planar polarity.

If Gαf is the transducer of the Wg pathway, where Fz2 serves as its guanine exchange factor, we expected co-expression of TRiP UAS-Gαf RNAi with overexpression of Fz2 to show a decrease in Wg signaling. This did indeed appear to occur. When the Fz2 receptor is overexpressed alone in the adult wing with en-G4 (en-G4/+;UAS-fz2/+ ) (Figure 32B-B’) or with MS1096-G4 (MS1096-G4/+;UAS-fz2/+), ectopic bristles are observed consistently in all of the wing blades examined. When UAS-fz2 is co-expressed with TRiP UAS-Gαf RNAi, either with en-G4 (en-G4,UAS-dcr2;UAS-fz2/TRiP UAS-Gαf RNAi) (Figure 32D-D’) or MS1096-G4 (MS1096-G4/+;UAS-dcr2/+;UAS-fz2/TRiP UAS-Gαf RNAi), there appears to be a reduction in the percentage of wings bearing ectopic bristles. Importantly, I observed phenotypes resembling that of fz2 overexpression (ectopic bristles), as well as those of the TRiP UAS-Gαf RNAi line (venation defects). However, upon closer inspection, only 55% of flies (as opposed to a 100% of UAS-fz2 flies) of flies appeared to have wings with obvious ectopic bristles when co-expressed in the posterior compartment of the wing with en-G4 (en-G4,UAS-dcr2;UAS-fz2/TRiP UAS-Gαf RNAi).
(Table 18). When the bristle numbers within the wing blade in the posterior compartment were counted, there appeared to be a discernible decrease in bristle number as well; although greater number of wings will have to be generated for this result to be more compelling. (Figure 41). Similarly, when the TRiP UAS-G\(\alpha\)f RNAi line was co-expressed with UAS-fz2 using MS1096-G4, there appeared to be a small decrease in flies with ectopic bristle numbers present (86\% as compared to all flies expressing only UAS-fz2). (Table 19). Thus, it appears as though the fz2 overexpression phenotype was suppressed.

In the converse experiment, where I overexpressed the fz2 receptor with the UAS-G\(\alpha\)f forms (en-G4/+/UAS-G\(\alpha\)f/UAS-fz2 or MS1096-G4/+;UAS-G\(\alpha\)f/UAS-fz2), I expected to observe a possible upregulation of the Wg signaling pathway, by observing enhancement of the phenotype visible with fz2 overexpression. In other words, I expected to observe wings with more ectopic bristles, or ‘hairier’ wings. When UAS-fz2 was co-expressed with the three UAS-G\(\alpha\)f variants (WT, GDP and GTP forms), only ectopic bristles were observed, as is recognized with fz2 overexpression, but there were no apparent differences between the three variants co-expressed with UAS-fz2 as compared to UAS-fz2 alone. (Figure 42; Table 20-21) It is possible that by increasing the dosage of G\(\alpha\)f when co-expressed with fz2 might yield the expected results, (wings with increased ectopic bristles), which would definitively implicate G\(\alpha\)f in Wg signaling.

3.52 Involvement of G\(\alpha\)f in Fz/PCP signaling pathway

Overexpression of TRiP UAS-G\(\alpha\)f RNAi revealed phenotypes that are reminiscent of polarity phenotypes (mwh and hairs not uniformly aligned in the proximal direction). Therefore, the next step was to determine if G\(\alpha\)f might interact genetically with the fz1 receptor, which propagates both the polarity and Wg pathways. We surmised that if G\(\alpha\)f is the transducer of the PCP/Fz pathway, and Fz1 acts as its GEF, then a reduction of G\(\alpha\)f transcript by overexpressing TRiP UAS-G\(\alpha\)f RNAi should cause a reduction in PCP signaling. In theory, a reduction of both the Wg and PCP signaling should be observed, but as noted previously, overexpression of fz1 phenotypes are generally overwhelming features consistent with PCP. (Figure 33B-B’)

As before, I co-expressed UAS-fz1 with TRiP UAS-G\(\alpha\)f RNAi, using either en-G4 (UAS-dcr2/+;en-G4/+;UAS-fz1/TRiP UAS-G\(\alpha\)f RNAi) or MS1096-G4 (MS1096-G4/+;UAS-dcr2/+;UAS-fz1/TRiP
UAS-Gαf RNAi) drivers. Contrary to our expectations, I did not observe a reduction of the UAS-fz1 phenotype, which is a swirling pattern of the hairs in the adult wing. Rather, phenotypes unique to the TRiP UAS-Gαf RNAi line as well as to fz1 overexpression were observed. (Figure 33; Table 22, 23)

Next, I did the converse experiment. I expected an upregulation of the Fz pathway (increase in the swirling pattern or mwh) if Gαf transduces the Fz signal, when I overexpressed UAS-fz1 with UAS-Gαf. Again, I drove all three UAS-Gαf constructs with either en-G4 or MS1096-G4 drivers, together with UAS-fz1 (en-G4+/UAS-Gαf/UAS-fz1 or MS1096-G4+/UAS-Gαf/UAS-fz1), but I only observed the fz1 phenotype. There was no enhancement of the fz1 phenotype. Specifically, I saw no increase in the swirling patterns of hair in the dorsal wing compartment, although it should be noted that I did not generate enough wings to perform these experiments. Furthermore, I did not obtain results for all of the UAS-Gαf variants in this study. (Table 24,25)

I then tested whether Gαf was Fz dependent. If the Fz receptors act as the GEF for Gαf, then there would be disparities in the phenotypes observed between the three forms of UAS-Gαf when overexpressed in the wing with a reduction (by half) of Fz receptors present by crossing them to fz,fz2 mutant flies (MS1096-G4+/UAS-Gαf WT/GDP/GTP/fz,fz2). I would expect the Gαf WT form would need Fz to signal, while Gαf-GTP would not. Therefore, in this scenario Gαf WT and Gαf-GDP would transduce less of the Fz signal and would exhibit the same phenotype, compared to the Gαf-GTP. Unfortunately, I did not observe any striking differences in the adult wing between these constructs. (Table 26,27)

Lastly, I also examined wings of flies generated from crossing theTRiP UAS-Gαf RNAi line with the fz,fz2 mutant. There was no apparent effect of reducing for the level of Fz1 and Fz2 receptor in conjunction with knockdown of the Gαf protein. (Table 28-31) The results from these set of experiments did not provide any compelling evidence for a direct role for Gαf in the planar polarity pathway.

3.53 Involvement of Gαf in Hh signaling pathway

Finally, following the same methodology, I looked at smoothened (the 7-TM receptor involved in the Hh signaling pathway) to assess whether Gαf may mediate Hedgehog signaling. As before, we
expected that if Smo is the GEF for Gαf, overexpression of smo with TRiP UAS-Gαf RNAi should reduce Hh signaling. Conversely, overexpression of Gαf-GTP compared to that of Gαf WT (which is receptor dependent) or Gαf-GDP (which is receptor bound) should be able to signal in the absence of Smo; or the activity of the Gαf WT and Gαf-GDP should be reduced with a reduction in the expression of Smo, compared to that of Gαf-GTP activity.

Importantly, as previously mentioned, Hh is a secreted ligand that diffuses from the posterior compartment to the anterior compartment and acts on the central region (the AP border) of the wing, in order to activate dpp. When UAS-smo, the signaling receptor, is overexpressed in the posterior compartment (en-G4/+;UAS-smo/+), there are no visible phenotypes discernible in the adult wing because en-expressing cells do not respond to Hh. However, when driven by MS1096-G4 (MS1096-G4/+;UAS-smo/+), there is a consistent effect in the anterior compartment (ectopic venation effect due to activation of dpp and effects on the spacing in the compartment), which is striking in the male because MS1096-G4 is X-linked (MS1096-G4/Y;UAS-smo/+). Since MS1096-G4 is X-linked and Drosophila achieves dosage compensation by doubling the transcript levels of X-linked genes in males, one copy of the transgene shows different levels of effects in males and females. Thus, in males a more pronounced effect is observed, compared to their female (heterozygote) sibling. As a result UAS-smo driven by MS1096-G4 in the female exhibits a minor effect in the anterior compartment but a venation defect is still observed in this compartment (MS1096-G4/+;UAS-smo/+), as in the male wing.

Female wings were examined for the following set of studies, as any enhancement was readily apparent. (Figure 34A) To test whether Smo is a GEF for Gαf, the TRiP UAS-Gαf RNAi line was crossed with UAS-smo flies, driven by MS1096-G4 (MS1096-G4/+;UAS-dcr2/+;TRiP UAS-Gαf RNAi/UAS-smo). If Gαf transduces the Smo signal, then the effect on the wing (ectopic vein material) would be expected to be reduced. Contrary to this expectation, the anterior compartment appeared to be affected, but was even more pronounced. (Figure 34;Table 32) Nonetheless, this indicated an effect on Hh signaling.

If Smo is the GEF for Gαf, the expectation would be that overexpression of Gαf with smo should increase Hh signaling. It has already been demonstrated that another Gα protein (Gαi) can behave in such a fashion. Overexpression of constitutively active Gαi phenocopies overexpression of smo when
driven by another Gal4 driver in wing (Ogden et al., 2008). When I overexpressed the wild type form of Gαi along with Smo, the venation defect observed between the L2 and L3 veins in the central compartment of the wing appeared to be enhanced. (Figure 34G) Thus, I tested to see if I would observe a similar phenomenon. When the UAS-Gαf constructs were overexpressed in concert with UAS-Smo (MS1096-G4/+;UAS-Gαf/UAS-smo), it was hard to determine if there was any enhancement or suppression of this venation defect, although it appeared at times as there might be a slight enhancement. This observed phenotype was not pronounced, nor consistent; and I did not always observe this in all of the wings that I examined, although I did not necessarily generate enough wings from each of the Gαf variants to quantify them. (Figure 34C, E; Table 33)

To test whether Gαf was Smo dependent, I crossed the UAS-Gαf constructs with flies mutant for smo3 (en-G4/+;smo3+/+;UAS-Gαf/+) . Smo3 is is a null allele of the smo gene that blocks the reception of Hh signaling (Alcedo, et al., 1996; Y. Chen & Struhl, 1996), and consequently a homozygous fly for this gene shows reduced spacing between the L3 and L4 veins. Here, if Gαf transduces the Hh signal, we would expect that reduction of the receptor (by half) would also reduce the signal of the Gαf WT form, compared to Gαf-GTP (which does not require a receptor). Thus, if Gαf transduces the Hh signal, I would have expected the Gαf-GTP form to overcome this reduction in receptor number. As I was unable to make the cross with the Gαf-GTP with smo3, whether Gf transduces the Hh signal through Smo could not be definitively addressed. However, the Gαf WT and Gαf-GDP forms did not show any alteration in the spacing of the central compartment of the wing to indicate a prominent role in this signaling. (Table 34) As previously explained, the UAS-Gαf variants do not exhibit any pronounced effects when overexpressed in the wing as one copy. Consequently, these crosses did not yield any informative results.

Lastly, I also expressed the TRiP UAS-Gαf RNAi line with the smo3 mutant and examined the wings of the flies generated from the cross. If Gαf transduces the Hh signal through Smo, I would have expected a greater reduction in the spacing of the central region of the wing, above the AP border. I did not observe any remarkable effects from the resulting wings generated from the crosses. (Table 35, 36) I also took note of the standard phenotypes observed with the TRiP UAS-Gαf RNAi line, although those results were problematic in interpreting, as I had difficulty genotyping the flies that were expressed with
the en-G4 driver. The flies were often so severely blistered that it was difficult to discern whether they were actual cyo flies or flies that were blistered as a result of the cross, and therefore harbored the correct genotype.

Thus, overall, the initial results with the overexpression of smo and TRiP UAS-Gαf RNAi provide some compelling data that Gαf may have some potential role in Hh signaling.

3.54 Involvement of Gαf in asymmetric cell division

One phenotype has consistently appeared in the adult wing with both downregulation of Gαf through RNAi, as well as with overexpression of Gαf, albeit weakly. When the TRiP UAS-Gαf line is driven with various Gal4 drivers, such as MS1096-G4 and dll-G4 defects in the alignment, spacing and number of bristles of the anterior wing margin are observed. Likewise, when UAS-Gαf is overexpressed with MS1096-G4, there appears to be effects on the bristles of the anterior wing margin. Such phenotypes in the adult wing have been observed previously with another G alpha subunit (Gαo).

Subsequently, Gαo was demonstrated to participate in asymmetric cell division (Katanaev & Tomlinson, 2006a). Hence, these observations with Gαf in the adult wing led us to believe that Gαf too might play a role in asymmetric cell division. I proceeded to perform additional experiments using other available RNAi lines against the two Gα subunits known to participate in asymmetric cell division (Gαo and Gαi) (Katanaev & Tomlinson, 2006a; Schaefer, et al., 2001; F. Yu, et al., 2003), and began preliminary experiments to confirm any synergy with the other two Gα subunits; and I sought to determine if there was an enhancement of this effect on the anterior margin using RNAi lines against two other Gα subunits. Using both en-G4 and MS1096-G4, I first overexpressed UAS-Gαo RNAi and UAS-Gαi RNAi lines (UAS-dcr2/+;en-G4/+;UAS-Gα RNAi+ or MS1096-G4/+;UAS-dcr2/+;UAS-Gα RNAi/+). Even though I used two separate RNAi lines for Gαo, I did not observe any striking phenotypes with the UAS-Gαo RNAi lines, either in the wing blade with MS1096-G4 (MS1096-G4/+;UAS-dcr2/+;UAS-Gαo RNAi+) or solely in the posterior compartment of the wing with en-G4 (UAS-dcr2/+;en-G4/+;UAS-Gαo RNAi+) of the wing.

When I co-expressed the UAS-Gαo RNAi with TRiP UAS-Gαf RNAi, I did not observe any enhancement
or suppression of the \( \text{G} \alpha \text{f} \) phenotypes, using either \textit{en-G4 (UAS-dcr2/+;en-G4/+;TRiP UAS-\text{G} \alpha \text{f} RNAi/UAS-\text{G} \alpha \text{o} RNAi)} \) or \textit{MS1096-G4 (MS1096-G4/+;UAS-dcr2/+;TRiP UAS-\text{G} \alpha \text{f} RNAi/UAS-\text{G} \alpha \text{o} RNAi)}.

I had only one RNAi line against \( \text{G} \alpha \text{i} \) for my experiments. When \( \text{G} \alpha \text{i} \) was driven with \textit{en-G4 (UAS-dcr2/+;en-G4/+;UAS-\text{G} \alpha \text{i} RNAi/+)}), there was no apparent visible phenotype. (Figure 36C) However, when I co-expressed the \( \text{G} \alpha \text{f} \) and \( \text{G} \alpha \text{i} \) RNAi lines using \textit{en-G4 (UAS-dcr2/+;en-G4/+;TRiP UAS-\text{G} \alpha \text{f} RNAi/UAS-\text{G} \alpha \text{i} RNAi)}), I observed a robust enhancement of the venation defect I had previously observed with the TRiP \( \text{UAS-\text{G} \alpha \text{f} RNAi} \) line alone. (Figure 35, 36A-F, 43; Tale 37) Whereas 76\% of the wings of the TRiP \( \text{UAS-\text{G} \alpha \text{f} RNAi} \) line exhibits venation defects, those expressed in concert with the \( \text{UAS-\text{G} \alpha \text{i} RNAi} \) line appear to all share this venation phenotype. Indeed, when two copies of the TRiP \( \text{UAS-\text{G} \alpha \text{f} RNAi} \) line are co-expressed with \( \text{UAS-\text{G} \alpha \text{i RNAi}} \), I start to see effects on the entire wing morphology. (Figure 36G-I; Table 37) While I did not generate sufficient wings to definitively state that this occurs in all cases, but it appears that the effects on the posterior compartment must creep into the anterior compartment to cause such severe effects on the contour of the wing (to produce smaller wings and occasional effects on the anterior wing margin). (Figure 36I')

When driven with \textit{MS1096-G4 (MS1096-G4/+;UAS-dcr2/+;UAS-\text{G} \alpha \text{i} RNAi/+)}), \( \text{G} \alpha \text{i} \) did indeed show some phenotypes on its own. (Figure 37B-B') As I had observed with \( \text{G} \alpha \text{f} \), I saw extra bristles in the anterior wing margin. Notably, I did not observe this in as many of the wings expressing \( \text{UAS-\text{G} \alpha \text{i RNAi}} \), as I consistently observed with TRiP \( \text{UAS-\text{G} \alpha \text{f RNAi}} \). (Table 38)

Strikingly, when I co-expressed the \( \text{G} \alpha \text{f} \) and \( \text{G} \alpha \text{i} \) RNAi lines with \textit{MS1096-G4 (MS1096-G4/+;UAS-dcr2/+;TRiP UAS-\text{G} \alpha \text{f} RNAi/UAS-\text{G} \alpha \text{i} RNAi)}), I observed a robust enhancement of the bristle phenotypes I had noted with the \( \text{G} \alpha \text{f} \) and \( \text{G} \alpha \text{i} \) RNAi lines alone. (Figure 37C-C", 38; Table 38) The effects of simultaneous expression of both TRiP \( \text{UAS-\text{G} \alpha \text{f RNAi}} \) and \( \text{UAS-\text{G} \alpha \text{i RNAi}} \) on the wing margin were clearly apparent, with many bristles missing as well as extra bristles emerging from one socket. There appeared to be a clear synergistic effect with co-expression of the two lines (100\% of the flies harbored this phenotype), which was not identical to two copies of the TRiP \( \text{UAS-\text{G} \alpha \text{f RNAi}} \) driven with \textit{MS1096-G4} in the adult wing. This can be attributed to either enhancement of the \( \text{G} \alpha \text{i} \) phenotype or to that of the \( \text{G} \alpha \text{f} \)
phenotype. Remarkably, increasing the copies of **TRiP UAS-Gαf RNAi** with one copy of **UAS-Gαi RNAi**, enhanced the phenotype even further (**MS1096-G4/+;UAS-dcr2/+;TRiP UAS-Gαf RNAi/UAS-Gαi RNAi, TRiP UAS-Gαf RNAi**). (Figure 37D-D"; Table 38) These results suggest that Gαf could be playing a redundant role to Gαi in asymmetric cell division, which already has a well established role in ACD.

4. Discussion

4.1 Gαf expression gives weak phenotypes that are characteristic of wg features

Downregulation of Gαf with RNAi (using TRiP UAS-Gαf RNAi) resulted in phenotypes which were consistent with misregulation of Wg: partly scalloped wings and ectopic bristles were observed, albeit infrequently. Given this evidence and that G alpha subunits generally couple to heptahelical receptors, I first looked at a possible interaction with the Fz receptors. However, overexpression of Gαf rarely produced an ectopic bristle in the wing blade, which is commonly observed with overexpression of **UAS-fz2** and a feature of the Wg transduction pathway.

In the disc, with overexpression of the TRiP UAS-Gαf RNAi, we consistently observed perturbations in Wg expression, clear downregulation of obligate Wg targets Dll and Vg, as well as a reduction in Cut (indirect target of Wg). These demonstrated that Wg signaling is impacted by a reduction of Gαf.

I was unable to show that the converse – an upregulation of Wg and its targets - is true with ectopic expression of Gαf in the adult wing. There appeared to be no remarkable difference in expressions of the Wg targets between ectopic expression of Gαf (WT and GTP forms) in contrast to a wildtype disc. Additionally, I was unable to perform any immunostainings on the wing imaginal discs of the **UAS-Gαf-GDP** flies (**MS1096-G4; UAS-Gαf-GDP**), where I observed effects on the wing margin, that we believe are likely due to dominant negative effects of Gαf-GDP. If so, I would expect similar results to those with the TRiP UAS-Gαf RNAi line (downregulation of Wg and its targets). Nevertheless the phenotypes of GDP-Gαf is subtle, and it is possible that I would not observe anything.

Given the results seen with overexpression of the two **UAS-Gαf** variants compared to those observed with immunostainings from the TRiP UAS-Gαf RNAi line, how do we reconcile these
disparities? One possibility that needs to be considered is that the RNAi lines do indeed have off-target effects. The best way to validate these effects as true Gαf effects is to make a working antibody against Gαf. Reduction in protein expression in the disc with overexpression of the RNAi lines will confirm these effects to be attributable directly to Gαf. Although the possibility remains that the RNAi line against Gαf may downregulate Gαf expression as well as have off-target effects. Nevertheless, it must be noted that I used several RNAi lines, as well as deficiencies against the Gαf gene, in order to ensure that the phenotypes resulting from the TRiP UAS-Gαf RNAi line that I used predominantly in these experiments were due to Gαf downregulation. Therefore, we believe that overexpression of Gαf may not have yielded as potent phenotypes as reducing its protein expression because Gαf is likely not a limiting reagent.

Additionally, while I did observe reduction of Wg protein and its targets in the larval discs, which provides compelling evidence for a role of Gαf in Wg transduction, it must be noted that I only did so by using the most potent arrangement of the TRiP UAS-Gαf RNAi line. That is, while I observed striking phenotypes in the adult wing with only one copy of this RNAi line, it took two copies at the larval stage to observe any apparent disparities and make any assessments accordingly. Therefore, it is possible that Gαf acts at a later developmental stage than in the third instar larva; and may, in effect, function at the pupal stage when metamorphosis is taking place.

Importantly, the knockdown results with the TRiP UAS-Gαf RNAi line when co-expressed with UAS-fz2, also showed a reduction in ectopic bristles and therefore also implicated a possible role for Gαf in the Wg transduction pathway. Hence, overall, it remains unclear whether it is critically involved in Wg transduction, but some of the data provide hints that it might play a role in this signaling pathway.

4.2 Gαf expression may affect dpp

Spalt staining did not appear to be reduced dramatically with RNAi against Gαf. A slight decrease in expression was perceptible, which would indicate that dpp signaling might also be affected by reduction of Gαf protein. As there were clear venation defects and narrowing of the wing along the anterior-posterior axis with the overexpression constructs and similar venation defects (expansion) with the TRiP UAS-Gαf RNAi line, there may be effects on dpp. Thus, looking at expressions of other Dpp
regulated genes, such as *omb* is worth exploring. In addition, while staining with Spalt did not show perceptible difference from that of wild type alone, perhaps greater numbers need to be generated and compared for effects.

4.3 *Gαf* knockdown gives phenotype consistent with PCP signaling

Knockdown of *Gαf* produced what appeared to be a PCP-like phenotype. However, overexpression with *UAS-fz1* with the RNAi line suggested that the mwh and polarity defects were not a result of interaction with *UAS-fz1* directly. There was no suppression of either the swirling pattern of hairs from Fz1, which would indicate a reduction of the PCP signaling pathway. Moreover, while the ectopic expression of the *UAS-Gαf* constructs was weak, there were no PCP-like phenotypes produced when over-expressed in the wing. While there is no evidence of an interaction with the Fz1 receptor, and that *Gαf* probably does not couple to Fz1 to mediate signaling, the PCP phenotypes that occur in the *Gαf* knockdown are compelling. Hence, the results suggest the *Gαf* acts in PCP signaling but not as an immediate transducer of Fz.

4.4 Ectopic *Gαf* may affect hedgehog signaling

Overexpression of *UAS-smo* in the wing with the *MS1096-G4* driver shows effects on the anterior compartment (with none in the posterior), which is the hallmark of the Hh pathway (which serves to activate *dpp* in the anterior cells adjacent to the anterior-posterior border). Since *MS1096-G4* is X linked, and *Drosophila* dosage compensate for the sex chromosomes by hyperactivating transcription of genes on the X, the males and females with one copy of this transgene show different levels of effects. The single copy (hemizygous) males show much stronger phenotypes than their single copy (heterozygous) female siblings. In the males, overexpression of *smo* using *MS1096-G4* leads to an expansion of the spacing between veins L3 and L4, and a striking venation phenotype (ectopic vein material between veins L2 and L3). Heterozygote females produce a weak but discernible phenotype in the anterior compartment, which includes a small ectopic venation defect. I looked at only female wings, as modulation of this phenotype was easier to note. Interestingly, co-expression of the *UAS-Gαf* constructs with *UAS-smo* and *MS1096-G4* driver sometimes mildly enhanced these affects in wings of heterozygote
females. Similar but pronounced effects were shown with UAS-Gαi GTP (Ogden, et al., 2008). From this data, one might conclude that Gαf acts to weakly transduce Hh signaling and Smo might act as a GEF for Gαf. However, reduction of Gαf with TRiP UAS-Gαf RNAi, while co-expressing also UAS-smo potently enhanced these anterior phenotypes, suggesting that Gαf may antagonize Smo activity. These anterior venation defects were not observed to the same degree with knockdown of Gαf alone. Although we have to be cautious in drawing a quick conclusion, these results point to a possible role in the Hh signaling pathway.

If true, further experiments will need to be conducted. I have already shown that the wild type form of UAS-Gαi when co-expressed with UAS-smo, driven with MS1096-G4 in heterozygotes exhibit a more pronounced effect on the anterior compartment than when UAS-Gαf is co-expressed with UAS-smo. Thus, any role that Gαf plays in this signaling pathway does not appear to be as significant as the role of Gαi. However, subsequent tests should still be performed to determine what effect (if any) occurs with co-expression of UAS-Gαi and UAS-Gαf with UAS-smo. In the wing discs, targets (such as Ci, for instance) of this pathway would have to be examined for any modifications (such as variations in its processing compared to the wild type) to confirm these initial findings. For instance, overexpression of Gαi-GTP in the wing disc causes an overgrowth of the wing pouch (readily observed in the adult), which results in an expansion of the activator form of Ci, which in turn triggers an expansion of dpp (Ogden et al., 2008). As my results with reduction of the smo receptor were inconclusive, using a more sensitized background such as a dominant negative form of the smo transgene (UAS-Smo5A) with the three UAS-Gαf variants, might yield more definitive results as to whether specifically Gαf is able to modulate the effects of Ci, and overall if it is involved in Hh signaling. Thus, more data will need to be collected in order to ascribe a role for Gαf in hedgehog signaling.

4.5 Cell numbers decrease with Gαf downregulation

When two copies of Gαf RNAi were expressed in the anterior compartment there appeared to be less nuclear staining, indicating the presence of less cells in that domain in contrast to the posterior compartment. There are two possible explanations for this observation. Either knockdown of Gαf
transcript promotes cell death or that it affects cell cycle progression. If cell death were a result of Gαf RNAi expression, this might explain the reduction of protein expression of the Wg targets, as well as reduction of other protein markers. However, these cells did not appear to be smaller, which would denote obvious chromatin condensation. To directly test whether the decrease in cell number as shown with Hoechst or DAPI staining is a consequence of cell loss, I would first analyze the levels of apoptosis in TRiP UAS-Gf RNAi expressing tissues. To this end, I would stain wing discs expressing TRiP UAS-Gf RNAi with activated caspase-3 or label with TUNEL, and determine if the cells were positive for either of these markers of apoptosis. If so, simultaneously crossing these flies with UAS-p35 should rescue the decreased cell numbers in the anterior compartment.

The other implication for the phenotypes manifested by TRiP UAS-Gαf RNAi expressing tissue is that it has an effect on cell cycle progression. To test whether the decreased cell number is due to cells in G1 arrest or Gαf RNAi expressing cells exit the cell cycle before the wild type cells, I would stain TRiP UAS-Gαf RNAi expressing wing discs with markers of cell cycle progression to visualize the effect on proliferation. These would include using the antiphospho-histone H3 mitosis marker as well as BrdU to examine cells in the S phase of the cell cycle. For further corroboration, I would perform flow cytometry analysis on cells from dissociated wing discs that are co-expressed with UAS-GFP (UAS-drc2/++;en-G4, UAS-GFP/++;TRiP UAS-Gαf RNAi/+), in order to quantify the DNA content and measure the proportions of cells in the G1, S and G2 phases. If Gαf is indeed involved in cell cycle progression, the expectation would be that overexpression of Gαf would promote extra cell divisions in the wing and therefore induce an overgrowth in the adult wing. However, that does not appear to be the case, as ectopic Gαf (flies harboring two copies of Gαf) appears to have narrower wings along the AP compartment when driven by MS1096-G4. Therefore, if Gαf is involved at all in inducing proliferation, it is possible that such extra divisions are uncoupled from cell growth.
4.6 Gαi and Gαf act redundantly in SOP cells

Strikingly, one phenotype observed with the RNAi line driven by MS1096-G4 was the effect on the stout bristles on the anterior wing margin. There were defective bristles where several shafts appeared from one socket, or shafts were missing from their sockets. Interestingly, ectopic expression of the GDP-bound Gαf also produced similar phenotypes (two bristles from one socket) when two copies were expressed by MS1096-G4. Katanaev & Tomlinson (2006) demonstrated previously by overexpression of Gαo-GTP that such phenotypes correspond to defects in asymmetric cell division.

Asymmetric cell divisions have been described in two contexts in Drosophila: one type by neural precursors or neuroblasts (NBs) and the other by the SOP cells. We decided to pursue this phenomenon further by testing the two previously described Gα (Gαo and Gαi) subunits alongside the Gαf subunit (in the context of SOPs). Using RNAi lines against all three, I was able to show that expression of the TRiP UAS-Gαf RNAi line enhances the Gαi phenotype. The number of gaps between sockets as well as the number of bristles emerging from one socket (as opposed to one bristle per socket) increased substantially. In contrast, UAS-Gαo RNAi did not suppress nor enhance the Gαf phenotype. This could be due to an ineffective RNAi line (it does not knockdown Gαo at all or far too weakly). Overexpression of Gαo-GTP has been shown to have an effect on the bristles (Katanaev & Tomlinson, 2006a) and as such, we expected the RNAi line against Gαo to have an effect with the TRiP UAS-Gαf RNAi line. However, only the TRiP UAS-Gαf RNAi phenotype was observed (neither enhancement nor suppression). Before ruling out any interaction between Gαf and Gαo, the RNAi lines against Gαo would have to be tested for a reduction in Gαo protein expression first. Staining the wing disc for Gαo protein (as there is a commercially available antibody) while driving the RNAi line in one compartment (UAS-dcr2+/;en-G4i+/;UAS-Gαo RNAi/+ ) should be able to reveal if they are effective lines. As with my other two Gαf RNAi lines, the UAS-Gαo RNAi line might have a more potent effect if two copies are used to assess for any effects.

Gαo has been implicated in both contexts of asymmetric cell division (SOPS and NBs) and has been shown to function in a receptor-dependent fashion; it is dependent on the Frizzled receptor and its 'active' form is the GTP version, thereby consistent with transducing its signal via the canonical model of
heterotrimeric G protein signaling (Katanaev & Tomlinson, 2006a). In contrast, Gαi, which has been extensively studied in NBs has been shown to be receptor independent (Schaefer, et al., 2001; F. Yu, et al., 2003). In the canonical model, the GTP variant is considered to be the active form. However, it has not yet been determined whether the GTP or GDP-bound form is the active form in the receptor-independent model where Gαi has been described. Indeed, Schaefer et al. (2001) contend that the active form is the GDP-bound form of Gαi, since the wild type form produced phenotypes while the GTP-variant failed to have any effect on asymmetric cell division in their studies. In the receptor-independent mechanism, heterotrimeric G-proteins can be activated by cytosolic proteins, which contain GoLoco/GPR domains that interact with GDP-Gα and compete with Gβγ for GDP-Gα binding (F. Yu et al., 2003). While these cytosolic proteins act as guanine nucleotide dissociation inhibitors (GDIs) to dissociate GDP-Gα from Gβγ dimer, the question remains as to what may act as a GEF in the absence of GPCRs to convert GDP-Gαi to GTP-Gαi. Ric-8 has been proposed to act as a GEF for Gαi (David et al., 2005; Hampoelz, et al., 2005; H. Wang et al., 2005).

If Gαf is Fz-receptor dependent (akin to Gαo), overexpression of the UAS-Gαf constructs by crossing them to the fz,fz2 mutant, thereby reducing the receptor number by half, might have elicited some effects on the wing margin. That is, overexpression of the UAS-Gαf-GTP form would be expected to display an effect on the bristles of the wing margin with a reduction of the receptor (MS1096-G4/+;UAS-Gαf/fz,fz2), compared to the wild type variant. Neither construct exhibited any differences. However, as noted previously, these constructs do not show any effects when driven as one copy. Thus, repeating the same experiments with two copies might be another means to address this problem (MS1096-G4/+;UAS-Gαf/fz,fz2,UAS-Gαf). Furthermore, it may be necessary to completely remove the receptors (in clones, entirely mutant for fz,fz2 with overexpression of the various UAS-Gαf constructs). Hence this scenario, in the complete absence of the receptor, may yield more discernible differences; and therefore, help to address whether Gαf acts in a receptor dependent or independent fashion (similar to Gαi) to exert its effects.
Overall, the results of the genetic interaction studies with the RNAi lines against Gαi and Gαo indicate that Gαf, which has not been described in this role before, may act redundantly with Gαi in asymmetric cell division.

4.7 Continuing Characterization of Gαf

So far, the most compelling results came from using the RNAi lines (primarily TRiP UAS-Gαf RNAi line). The other RNAi lines and use of Dfs indirectly confirmed that downregulation of Gαf affects wing patterning, and were thus indirect measures of knockdown of Gαf transcript. Nevertheless, more direct evidence would solidify the results that I have obtained. This would include doing RT-PCR on the wings expressing the various RNAi lines against Gαf versus wild type, which ought to show a direct correlation with a decrease in transcript. The different RNAi lines against Gαf showed varying degrees of penetrance. Therefore, the RNA levels observed ought to reveal these differing levels. Another independent means of demonstrating that Gαf transcript levels are indeed playing a direct role in the phenotypes manifested by the TRiP UAS- Gαf RNAi and other Gαf RNAi lines is via in-situ hybridization of the larval wing discs. Lastly, the antibody raised about Gαf may not have been sensitive for immunostaining purposes. However, western blots could not be performed in time to test the effectiveness of the antibody biochemically. Therefore, if this antibody ‘worked’, by taking embryos overexpressing the various RNAi lines against Gαf, along with wild type for control, as well as embryos overexpressing Gαf (the three variants), I would anticipate detecting correlating protein levels, which would validate the results obtained in this thesis. Following the mentioned analyses, it will be prudent to revisit the genetic experiments with the 7-TM receptors, where only adult wings were analyzed and examine those genotypes at the larval stage, staining for the same markers as before (Wg, Vg, Dll, Cut and DAPI).

Since the work on this chapter was conducted over a brief period, greater depth of studies could not be applied, many questions have been raised and the previous ones remain. The most critical question is does Gαf behave as a canonical trimeric G-protein?
To begin to shed further light on this problem and characterize this protein, I began making GFP-labeled constructs *UAS-Gαf* so that we may observe its subcellular localization. There are hints that it may localize to the membrane, which would be an indicator that it might behave as a trimeric G-protein in the canonical sense. Any findings of co-localization with a membrane-bound receptor (7-TM) would help us gain more insight with respect to which signaling pathway it may play a part in.

When Gαf was initially characterized, preliminary data in the blastoderm suggested that it is involved in some developmental context. Our recent data suggests that Gαf is likely involved in a basic biological process that is critically required for various signaling pathways to operate. Thus, one possible mechanism that might account for the various effects observed, implicating the different signaling pathways, is that it may be required for receptor recycling. As such, it may not transduce the receptors in the classical sense, but may be involved in the machinery that is required to remove the receptors (such as Fz and Smo) off/on the membrane. For example, it is believed re-localization of the receptors from endosomes to the plasma membrane is required for signaling to occur. It is possible that, if Gαf acts as a positive regulator in the signaling pathway, it participates in this process by bringing the receptor to the membrane to promote signaling. If the converse is true, where Gαf acts as a negative regulator (which could be the case with Smo), then it might play a role similar to β-arrestin to bring about ‘desensitization’. 
Chapter II
Figures and Tables
Figure 15. The Gaf gene

Male wings. (A) Wild type. (B) UAS-dcr2/+;en-G4/+;TRiP UAS-Gaf RNAi/+. The posterior compartment of the wing has been affected: venation defects are visible, where the L4 vein appears to be collapsing into the L5 vein at the site of the PCV (bracket); as well as an incomplete ACV. (C) Scheme of full length Gaf, depicting the approximate sites of three individual UAS-Gaf RNAi lines against the Gaf gene: (#1) TRiP UAS-Gaf RNAi (587bp); (#2) VDRC #17056 UAS-Gaf RNAi (382bp); (#3) NIG-FLY #12232R-1 UAS-Gaf RNAi (500bp).
Figure 16. Gαf knockdown phenotypes in the adult wing
Figure 16. Gaf knockdown phenotypes in the adult wing. (A) UAS-dcr2/+;en-G4/+;TRiP UAS-Gaf RNAi/+ (male wing). Venation defects are observed. Collapse or merging of the L4 and L5 veins, at the site of the PCV (arrow) occurs in the posterior compartment when TRiP UAS-Gaf RNAi is expressed by en-G4. (A’) Inset. Venation defect: incomplete ACV (arrow). (B) Notching or scalloping of the wing margin (arrow). More venation defect: L4 and L5 merging (arrow head). (C) Anterior margin defects gap (bracket) between stout bristles are observed when TRiP UAS-Gaf RNAi is driven by MS1096-G4. (D) Anterior margin defect: extra bristles emerging from an individual socket (arrow). (E) Polarity defects (arrow head), where hairs are misaligned (not uniformly oriented towards the distal direction) and appearance of mwh (arrows) are observed. (F) Areas of balding (circle) within the wing blade. (G) Ectopic bristles (arrows).
Figure 17. Overexpression of three separate RNAi lines against Gaf
Figure 17. Overexpression of three separate RNAi lines against $G_{af}$. Male wings. (A) Wild type. (B) $MS1096$-$G4/Y; UAS-dcr2/+;TRiP UAS-$G_{af}$ RNAi/+. The contour of the wing appears to be affected, and other effects are observed (listed and quantified in E). (C) A second RNAi line against $G_{af}$ driven with $MS1096$-$G4$ ($MS1096$-$G4/Y; UAS-dcr2/VDRC UAS-$G_{af}$ RNAi$). Any visible effects on the wing are minimal, which are indicated in (E). (D) A third RNAi line was also used: $MS1096$-$G4/Y; UAS-dcr2/+;#12232R-1 UAS-$G_{af}$ RNAi/+. The effects are minimal, which are indicated in (E). (E) Quantitative analysis of the phenotypes observed with the three RNAi lines. When one copy of each of the three separate $G_{af}$ RNAi lines is driven with $MS1096$-$G4$, only the TRiP UAS-$G_{af}$ RNAi line appears to show the most pronounced effects. $Gf$ TRiP/+$ (n=109) is shown compared to one copy of the other two lines, VDRC/+$ (n=56)$ and $12232R-1/+(n=93)$. Error bars represent the standard error of the mean (S.E.M). Wing blades were quantified according to phenotypes that appear commonly when the RNAi lines are driven with $MS1096$-$G4$: venation defect (the presence of ectopic vein material, incomplete veins, and/or collapse of veins); polarity defects (if there are any areas on the wing blade where hairs are not uniformly arranged); balding (any areas where hairs appear to be missing or appear to be just sprouting from the wing blade); ectopic bristles (presence of ectopic bristles in the wing blade); mwh (presence of two of more hairs emerging where only one hair should be present); anterior wing margin (presence of gaps where stout bristles should appear and/or extra bristles emerging from one socket). Quantification was done according to whether a phenotype was visible or present, but the level of penetrance (whether something such as polarity defects or mwh were visible throughout the wing blade or in a small patch) was not quantified.
Figure 18. Overexpression of three separate UAS-Gaf RNAi lines show similar phenotypes
Figure 18. Overexpression of three separate UAS-Gαf RNAi lines show similar phenotypes.

Male wings. When two copies of the other Gαf RNAi lines are expressed, they show similar phenotypes as one copy of the TRiP UAS-Gαf RNAi line. Effects on wing morphology can also be observed. (A) Wild type. (B-B") One copy of TRiP UAS-Gαf RNAi: (B) MS1096-G4/Y;UAS-dcr2/+; TRiP UAS-Gαf RNAi/+. (B') Mwh are observed within the wing blade (circle). (B") Effect on the anterior wing margin: extra stout bristles emerging from one socket (arrow). (C-C") Two copies of VDRC UAS-Gαf RNAi: (C) MS1096-G4/Y;UAS-dcr2,VDRC UAS-Gαf RNAi/VDRC UAS-Gαf RNAi). (C') Appearance of mwh (circle). (C") Extra bristles on the anterior wing margin (arrow). (D-D") Two copies of #12232R-1 UAS-Gαf RNAi: (D) MS1096-G4/Y;UAS-dcr2/+;#12232R-1 UAS-Gαf RNAi/#12232R-1 UAS-Gαf RNAi. (D') Appearance of mwh (circle). (D") Effects on the anterior wing margin: extra bristles (arrow).
Figure 19. Overexpression of TRiP UAS-Gafl RNAi line shows enhancement with Deficiencies
Figure 19. Overexpression of TRiP UAS-Gaf RNAi line shows enhancement with Deficiencies. Male wings. (A) Wild type. (B-C) Representative samples of UAS-dcr2/+;en-G4/+
TRiP UAS-Gaf RNAi/+ wings. (B) Collapse or merging of the L4 and L5 veins, at the site of the PCV (bracket). (C) Near collapse of the L4 vein towards the L5 vein (bracket). Incomplete veins: ACV (arrow) and L4 are visible. (D-F) Representative samples of UAS-dcr2/en-G4;TRiP UAS-Gaf RNAi/Df 3(L)Exel6130. There was clear enhancement observed with the venation defects in the posterior compartment. Expansion of the venation defect is observed (bracket). Incomplete ACV (arrow), or thickening at site of ACV. Notching is also sometimes observed (arrow head).
Figure 20. Overexpression of TRiP UAS-Gaf RNAi line shows enhancement with deficiencies
Figure 20. Overexpression of TRiP UAS-Gaf RNAi line shows enhancement with Deficiencies. Male wings. (A) Wild type. (B-C) Representative samples of UAS-dcr2/+;en-G4/+; TRiP UAS-Gaf RNAi/+ wings. (B) Collapse or merging of the L4 and L5 veins, at the site of the PCV (bracket). (C) Near collapse of the L4 vein towards the L5 vein (bracket). Incomplete veins: ACV (arrow) and L4 are visible. (D-E) Representative samples of UAS-dcr2/en-G4;TRiP UAS-Gaf RNAi/Df 3(L)ED4674. There was clear enhancement observed with the venation defects in the posterior compartment. Expansion of the venation defect is observed (bracket) in (D) & (E). Incomplete ACV (arrow) in (D) & (E). Sometimes a blister is observed directly over the venation defect that occurs at the site of the collapse of L4 and L5 veins, as observed in (D). (E) Other venation defect where there is ectopic vein material close to the L4 vein (arrow). Notching is also sometimes observed (arrow head). (F) Quantification of the enhancement observed with expression of TRiP UAS-Gaf RNAi when supplemented with another Gaf RNAi line or in the presence of Deficiencies. Expression of Gf TRiP/+ (n=58) alone is compared with Gf TRiP/12232R-1 (n=67), as well as when combined with Dfs: Gf TRiP/Df(3L)ED4674 (n=20); Gf TRiP/Df(3L)BSC561 (n=59); Gf TRiP/Df(3L)ED223 (n=20); Gf TRiP/Df(3L)Exel6130 (n=57). Error bars represent S.E.M. Wing blades were quantified according to phenotypes that appear commonly when the RNAi lines were driven with en-G4: venation defect (primarily collapse of the L4 and L5 veins); venation enhancement (if the venation defect appears to expand further, as noted by brackets in the images); blistered (if there are any blisters present above the venation defect); notching (presence of scalloping at the wing margin).
Figure 21. Overexpression of TRiP UAS-Gαf RNAi with a non-overlapping UAS-Gαf RNAi line shows enhancement
Figure 21. Overexpression of TRiP UAS-Gαf RNAi with a non-overlapping UAS-Gαf RNAi line shows enhancement. Male wings. (A) Wild type. (B-C) Representative sample UAS-dcr2/+; en-G4/+; TRiP UAS-Gαf RNAi/+ wings. (B) Collapse or merging of the L4 and L5 veins, at the site of the PCV (bracket). (C) Near collapse of the L4 vein towards the L5 vein (bracket). Incomplete veins: L4 and ACV (arrows) in (B-C). (D-F) Representative sample UAS-dcr2/+; en-G4/+; TRiP UAS-Gαf RNAi / #12232R-1 UAS-Gαf RNAi wings. There was clear enhancement observed of the venation defects in the posterior compartment. Expansion of the venation defect is observed as indicated (bracket). Incomplete ACV (arrow in E & F), or thickening at site of PCV (arrow) in (D-F). Blistering is also observed in (E) and (F) above the venation defect.
Figure 22. Overexpression of TRiP UAS-\(G_{af}\) RNAi with a non-overlapping UAS-\(G_{af}\) RNAi line shows enhancement
Figure 22. Overexpression of TRiP UAS-Gαf RNAi with a non-overlapping UAS-Gαf RNAi line shows enhancement. Female wings. (A) Wild type. (B-C) Representative sample UAS-dcr2/+;en-G4/+;TRiP UAS-Gαf RNAi/+ wings. Collapse or merging of the L4 and L5 veins at the site of the PCV (bracket). Incomplete veins: ACV (arrows). (D-F) Representative sample UAS-dcr2/+;en-G4/+;TRiP UAS-Gαf RNAi/#12232R-1 UAS-Gαf RNAi wings. There was clear enhancement observed of the venation defects in the posterior compartment. Expansion of the venation defect is observed as indicated (bracket), when compared with TRiP UAS-Gαf RNAi/+ alone. Incomplete ACV (arrow). Blistering is also observed in (E) above the venation defect.
Figure 23. Overexpression of TRiP UAS-Gαf RNAi with a non-overlapping UAS-Gαf RNAi line shows enhancement.
Figure 23. Overexpression of TRiP UAS-Gaf RNAi with a non-overlapping UAS-Gaf RNAi line shows enhancement. Enhancement of defects are also observed with the TRiP UAS-Gaf RNAi line when driven with MS1096-G4. Female wings. (A) Wild type. (B-C) Representative sample MS1096-G4/+;UAS-dcr2/+;TRiP UAS-Gaf RNAi/+ wings. The wings adopt a smaller size than that of wild type wings, and occasionally display minor venation defects (B: arrows). (D-E) Representative sample MS1096-G4/+;UAS-dcr2/+;TRiP UAS-Gaf RNAi/#12232R-1 UAS-Gaf RNAi wings. Expression of a non-overlapping Gaf RNAi line with TRiP UAS-Gaf RNAi causes the wings to decrease further in size. Areas of balding is observed (F). The venation defects are far more pronounced (arrows: G, H). The anterior wing margins are also affected (ectopic bristles: arrows).
Table 14
Summary of Phenotypes induced by knockdown of Gαf with various wing GAL4 driver lines

<table>
<thead>
<tr>
<th>Gal4 line</th>
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<th>mwh</th>
<th>balding</th>
<th>anterior wing margin</th>
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<td>+</td>
<td>+</td>
<td>+</td>
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<tr>
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<td>+++</td>
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<td>++</td>
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<tr>
<td>nubbin</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

Expression of scored levels: - indicates not observed; + some expression; ++ moderate levels; +++ high levels of expression.

(wing margin here refers to effects on all margins: either notching or effects on bristles)

Figure 24. Overexpression of TRiP UAS-Gαf RNAi in the wing and the eye. (A) dll-G4/UAS-dcr2;TRiP UAS-Gαf RNAi/+ (female). (A’) Inset. Pronounced effects are observed in the anterior wing margin where multiple bristles are often missing (brackets). (B) Tangential section of the adult eye expressing ci-G4i/UAS-dcr2;TRiP UAS-Gαf RNAi/TRiP UAS-Gαf RNAi. Minor effects are observed (arrows), with some merging of ommatidia.
Figure 25. Gaf overexpression. Female wings. Ectopic expression of Gaf in the wing shows narrowing of the wing, particularly along the AP axis when two copies of Gaf are driven in the wing with MS1096-G4 (MS1096-G4;UAS-Gaf). Venation defects are also observed (arrows). (A) Wild type. (B-B’) MS1096-G4;UAS-Gaf wild type. (B’) Effects on the wing margin also occur: gap (bracket). (C-C’) MS1096-G4;UAS-Gaf GTP. (C’) Anterior margin is commonly affected: gap (bracket). (D-D’) MS1096-G4;UAS-Gaf GDP. (D’) Anterior wing margin is affected. Here, two effects are shown: gap (bracket) and an extra bristle emerging from one socket (arrow).
Figure 26. TRIP UAS-\(G_\alpha f\) RNAi causes perturbation in Wg activity and downregulation of Vg
Figure 26. TRiP UAS-\(G\alpha_f\) RNAi causes perturbation in Wg activity and downregulation of Vg. Third instar wing discs expressing \(ci-G4/UAS-dcr-2;TRiP\) UAS-\(G\alpha_f\) RNAi/\(TRiP\) UAS-\(G\alpha_f\) RNAi. In these discs, two copies of the RNAi line are driven in the anterior compartment with \(ci\), and the posterior compartment which does not express the RNAi line, serves as wild type control. (A-C): (A) \(ci-G4, UAS-GFP\) denotes where the TRiP UAS-\(G\alpha_f\) RNAi line is expressed. (B) Wg staining in the anterior compartment indicates some perturbation (arrows) with downregulation of \(G\alpha_f\), when compared to the posterior (control) compartment. (C) Merge. (D-H) A second wing of the same genotype stained for both Wg and Vg. (D) \(ci-G4, UAS-GFP\) denotes where the TRiP UAS-\(G\alpha_f\) RNAi line is expressed. (E,H) Vg expression in the compartment appears greatly reduced (arrow) when compared to the adjacent posterior compartment. (F-F’) Counterstaining for Wg indicates a weaker effect here, with a slight perturbation in Wg expression (arrows). (G) Merge of D-F. (H’) Vg staining magnified shows reduced levels of expression with expression of \(TRiP\) UAS-\(G\alpha_f\) RNAi in the compartment.
Figure 27. TRiP UAS-Gaf RNAi causes downregulation of Dll expression. Third instar wing disc expressing ci-G4/UAS-dcr-2;TRiP UAS-Gaf RNAi/TrIP UAS-Gaf RNAi. (A) ci-G4, UAS-GFP denotes where the TRiP UAS-Gaf RNAi line is expressed. (B-B’) Dll staining shows a clear reduction in the anterior compartment compared to the posterior compartment (arrow). (C-C’) Merge. Overlap of the GFP indicates the site of reduction corresponding with expression of TRiP UAS-Gaf RNAi.
Figure 28. TRIP UAS-Gαf RNAi causes minor effects on Sal expression. Third instar wing disc expressing ci-G4/UAS-dcr-2;TRiP UAS-Gαf RNAi/TRiP UAS-Gαf RNAi. (A) DAPI shows more intense staining the posterior (control) compartment. (B) ci-G4, UAS-GFP denotes where the TRiP UAS-Gαf RNAi line is expressed. (C) Merge of Dapi and Sal (D) staining. Sal staining shows slight reduction in the anterior compartment (D’: arrows). (E-E’) Merge of GFP and Sal, shows areas of reduced Sal staining (arrow).
Figure 29. TRiP UAS-Gαf RNAi causes downregulation of Cut expression. Third instar wing disc expressing ci-G4/UAS-dcr-2;TRiP UAS-Gαf RNAi/TrIP UAS-Gαf RNAi. (A) ci-G4, UAS-GFP denotes where the TRiP UAS-Gαf RNAi line is expressed. (B) DAPI shows more intense staining the posterior (control) compartment. (C-C’) There is clear downregulation of Cut in the anterior compartment (arrow). (D) Merge of Dapi and Cut, shows a reduction in staining of both in the anterior compartment. (E-E’) Merge of GFP and Cut shows the reduction of Cut where GFP (knockdown of Gαf) is expressed (arrow). Interestingly, the reduction appears to be somewhat of a distance from the AP boundary.
Figure 30. Scalloped wings can be seen with clones of TRiP UAS-\textit{Gaf} RNAi. Male wings. (A) Wild type. (B-F) Wings of separate flies: \textit{tub} >\textit{y}+>\textit{G4} were used to make clones of TRiP UAS-\textit{Gαf} RNAi in the adult wing. Not many flies survived. Of those that did, many showed notching phenotypes (arrow). It was difficult to observe any effects within the wing blade.
Figure 31. Overexpression of UAS-Gaf wild type with MS1096-G4 does not show any obvious defects
Figure 31. Overexpression of UAS-Gaf wild type with MS1096-G4 does not show any obvious defects. (A-C) Third instar wing discs expressing MS1096-G4 > UAS-Gaf wild type. (A) Wg staining appeared slightly weak, but its expression is consistent with wild type patterns. (A') Counterstaining for Vg appeared to show no effects. (A'') Merge of Wg and Vg. (B) Cut and (B'') Dll staining showed no effects with ectopic Gaf wild type. (B') Merge of Cut and Dll. (C) Counterstaining for DAPI showed no visible effects of Gaf wild type overexpression. (C') Sal staining expression also showed no effects. (C'') Merge of Dapi and Sal.
Figure 32. **fz2 overexpression.** Female wings. (A) Wild type. (B) *en-G4/UAS-fz2*. (B’) Closer magnification of the posterior compartment indicates the presence of ectopic bristles that are present predominantly, although not exclusively, in the margin (arrows). (C) Knockdown of *Gαf* (*UAS-dcr2/+;en-G4/+;TRiP UAS-Gαf RNAi+*) in the posterior compartment shows a prominent venation phenotype: collapse or merging of the L4 and L5 veins at the region of the PCV (arrow). (D) Overexpression of *fz2* and TRiP *UAS-Gαf RNAi* (*UAS-dcr2,en-G4;UAS-fz2/TRiP UAS-Gαf RNAi*) shows expression of both phenotypes. Venation defects are still observed (arrows) with the appearance of ectopic vein material at the end of the L4 vein, and incomplete ACV. At the site of the merging of the L4 and L5 veins, there appears to be thickening of the PCV. (D’) Magnification of the posterior region indicates presence of ectopic bristles.
Figure 33. *fz1* overexpression. Female wings. (A) Wild type. (B) *en-G4/UAS-fz1*.

(B’) Magnification of the posterior compartment shows swirling hairs and presence of mwh (circle), consistent with the *fz1* phenotype. (C) Knockdown of Gαf (*UAS-dcr2/+;en-G4/++;TRiP UAS-Gαf RNAi/+*) in the posterior compartment shows a prominent venation phenotype: collapse or merging of the L4 and L5 veins at the region of the PCV (arrow). (D) Overexpression of *fz1* and TRiP *UAS-Gαf RNAi* (*UAS-dcr2;en-G4;UAS-fz1/TRiP UAS-Gαf RNAi*) shows expression of both phenotypes. Venation defects are still observed (arrows), which appears to be enhanced with the thickening of the L5 vein end and PCV. (D’) Magnification of the posterior region indicates presence of the *fz1* phenotype (swirling hair pattern and mwh (circle)) as well.
Figure 34. smo overexpression
**Figure 34. Smo overexpression.** Female wings. (A) MS1096-G4/+;UAS-smo/+ wing. Overexpression of smo with MS1096-G4 shows a minor effect in the anterior compartment, between the L2 and L3 vein, above the ACV. A small but consistent ectopic vein material is observed (arrow). (B) Overexpression of TRiP UAS-Gαf RNAi with MS1096-G4 (MS1096-G4/+; UAS-dcr2/+; TRiP UAS-Gαf RNAi/+) also shows some minor venation defects (arrows). Knockdown of Gαf in conjunction with smo overexpression (MS1096-G4/+; UAS-dcr2/+; UAS-smo/ TRiP UAS-Gαf RNAi ) shows a very prominent venation phenotype (arrow), that appears to be an expansion of the smo overexpression phenotype in (D). (C, E) Overexpression of two Gαf variants: UAS-Gαf GDP (C) and UAS-Gαf GTP (E) in conjunction with smo overexpression appear to have the same venation defect between the L2 and L3 veins (arrow) as with overexpression of smo alone. This defect sometimes appears slightly more enhanced than when smo is expressed in the dorsal wing blade alone with MS1096-G4. However, those defects are not nearly as pronounced as when smo is overexpressed in the wing together with UAS-Gαi wild type (arrow), as shown in (G), indicating the synergistic effect between Gαi and Smo. These venation defects are not present when UAS-Gαi wild type is ectopically expressed in the wing alone (F).
Figure 35. *UAS-Gαi RNAi* appears to enhance TRiP *UAS-Gαf RNAi* phenotypes
**Figure 35.** *UAS-Gαi RNAi* appears to enhance TRiP *UAS-Gαf RNAi* phenotypes. Male wings.

(A) Wild type. (B) Wings expressing *Gαf* RNAi in the posterior compartment: *UAS-dcr2/+;en-G4/+; TRiP UAS-Gαf/+.* Venation defects are observed: collapse or merging of the L4 and L5 veins (bracket) and incomplete ACV (arrow). (C-F) Expression of *Gαi* RNAi in conjunction with *Gαf* RNAi appears to enhance the venation defects (bracket) in the wing, as well as affect the overall morphology.
Figure 36. *UAS-Gαi RNAi* appears to enhance TRiP *UAS-Gαf RNAi* phenotypes
Figure 36. *UAS-Gai RNAi* appears to enhance TRiP *UAS-Gaf RNAi* phenotypes. Female wings. (A) Wild type. (B) Overexpression of TRiP *UAS-Gaf RNAi* in the posterior compartment (UAS-dcr2/+;en-G4/+;TRiP UAS-Gaf RNAi/+ ) exhibits prominent venation phenotypes: merging of the L4 and L5 veins at the site of the PCV (bracket) and ectopic vein material can be seen in the blade (arrow). (C) Overexpression of Gai RNAi in the posterior compartment (did not reveal any defects (UAS-dcr2/+;en-G4/+;UAS-Gai RNAi/+ ). However, when co-expressed with TRiP UAS-Gai RNAi, (UAS-dcr2/+;en-G4/+;UAS-Gai RNAi/TRiP UAS-Gaf RNAi ) there was clear enhancement of the venation phenotypes observed (D-F). Collapse of the L4 and L5 veins, as well as expansion of the vein material are observed (bracket: D-F). Ectopic venation material is observed around the region of the ACV (arrow: D, E). Blistering is observed in some (F). The wings also appear to be adopting a slightly smaller size (D-F). Increasing expression of TRiP UAS-Gaf RNAi (UAS-dcr2/+; en-G4/+;UAS-Gai RNAi, TRiP UAS-Gaf RNAi/TRiP UAS-Gaf RNAi) appears to affect the anterior compartment, as well as the morphology of the wing (G-I). (G) The venation defects appear to be similar to that of TRiP UAS-Gaf RNAi alone: minor affects of the merging of the L4 and L5 vein at the site of the PCV (bracket) is present, as well as effects around the region of the ACV (arrow, where there appears to be thickening of the vein material). However, the wing appears to be considerably smaller than that of wild type and even that of wings expressing TRiP UAS-Gaf RNAi in the posterior compartment. (H). Aside from and expansion of the venation defect (bracket) and incomplete ACV (arrow), defect on the anterior margin is also evident (arrow head). Similarly, in (I) the wing is considerably smaller than that of wild type, and the anterior margin is also affected. (I’) Magnification of the affected margin where there is a gap and a loss of the stout bristles (arrow head).
Figure 37. UAS-Gαi RNAi appears to enhance TRiP UAS-Gαf RNAi phenotypes
Figure 37. *UAS-Gαi RNAi* appears to enhance TRiP *UAS-Gαf RNAi* phenotypes. Female wings. Overexpression of *UAS-Gαi RNAi* with TRiP *UAS-Gαf RNAi* in the dorsal compartment of the wing with MS1096-G4 shows a clear enhancement of the anterior margin defects observed with TRiP *UAS-Gαf RNAi* alone. (A) TRiP *UAS-Gαf RNAi* (MS1096-G4/+;UAS-dcr2+/;TRiP *UAS-Gαf RNAi*/+). Anterior margin displays two defects commonly with TRiP *UAS-Gαf RNAi* expression: extra bristles emerging from an individual socket (A’; arrow) and/or a gap where bristles should be present (A’’; bracket). (B) Overexpression of the *UAS-Gαi RNAi* line (MS1096-G4/+;UAS-dcr2+/;UAS-*Gαi RNAi*/+) also exhibits similar effects on the anterior wing margin. Ectopic bristles are evident in (B’; arrows). (C) Overexpression of the two RNAi lines (MS1096-G4/+;UAS-dcr2+/;UAS-*Gαi RNAi*/ TRiP *UAS-Gαf RNAi*) together has a synergistic effect that is evident by the increase in the defects on the anterior wing margin. (C-C’’) Anterior wing margin show an increase in the presence of gaps (bracket) and extra stout bristles (arrows) emerging from one socket. (D) With two copies of the TRiP *UAS-Gαf RNAi* in conjunction with *UAS-Gαi RNAi* MS1096-G4/+;UAS-dcr2+/;UAS-*Gαi RNAi*, TRiP *UAS-Gαf RNAi*/ TRiP *UAS-Gαf RNAi*), this effect increases further. The anterior wing margin appears to have an even greater number of extra bristles (D-D’).
Figure 38. UAS-Gαi RNAi appears to enhance TRiP UAS-Gαf phenotypes. Female wings. (A) MS1096-G4/+;UAS-dcr2/+;UAS-Gα RNAi. Graphical representation of the effects on the anterior wing margin. Gf TRiP/+ (n=33) alone was compared with Gi/+ (n=29). In addition, the former were compared to wings of flies expressing Gi/Gf TRiP (n=23) and Gi,Gf TRiP/Gf TRiP (n=10). Error bars represent S.E.M. With increasing presence of Gαf and Gαi RNAi lines, increasing percentage of fly wings are affected at their anterior margin. Gap represents areas in the wing margin where bristles are expected to be present, as shown in the image (B) below (bracket). The other effect on the wing margin include extra bristles emerging from a single socket (arrows).
Table 2
Genotype: UAS-dcr2/+; en-G4/++; Gαf TRiP/+  

<table>
<thead>
<tr>
<th>Phenotype</th>
<th>Male</th>
<th>Female</th>
<th>Total wings scored</th>
</tr>
</thead>
<tbody>
<tr>
<td>Venation defect</td>
<td>26(93%)</td>
<td>18(60%)</td>
<td>44(76%)</td>
</tr>
<tr>
<td>Polarity</td>
<td>26(93%)</td>
<td>22(73%)</td>
<td>48(83%)</td>
</tr>
<tr>
<td>Notching</td>
<td>2(7%)</td>
<td>2(6.7%)</td>
<td>4(6.9%)</td>
</tr>
<tr>
<td>Balding</td>
<td>13(46%)</td>
<td>14(47%)</td>
<td>27(47%)</td>
</tr>
<tr>
<td>Ectopic bristles</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Mwh</td>
<td>19(68%)</td>
<td>5(17%)</td>
<td>24(41%)</td>
</tr>
<tr>
<td>n</td>
<td>28</td>
<td>30</td>
<td>58</td>
</tr>
</tbody>
</table>

Table 3
Genotype: UAS-dcr2/+; en-G4/VDRC  

<table>
<thead>
<tr>
<th>Phenotype</th>
<th>Male</th>
<th>Female</th>
<th>Total wings scored</th>
</tr>
</thead>
<tbody>
<tr>
<td>Venation defect</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Polarity</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Notching</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Balding</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Ectopic bristles</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Mwh</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>n</td>
<td>11</td>
<td>8</td>
<td>19</td>
</tr>
</tbody>
</table>

Table 4
Genotype: UAS-dcr2/+; en-G4/+; 12232R-1/+  

<table>
<thead>
<tr>
<th>Phenotype</th>
<th>Male</th>
<th>Female</th>
<th>Total wings scored</th>
</tr>
</thead>
<tbody>
<tr>
<td>Venation defect</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Polarity</td>
<td>11(22%)</td>
<td>0</td>
<td>11(10%)</td>
</tr>
<tr>
<td>Notching</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Balding</td>
<td>20(39%)</td>
<td>11(18%)</td>
<td>31(28%)</td>
</tr>
<tr>
<td>Ectopic bristles</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Mwh</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>n</td>
<td>51</td>
<td>61</td>
<td>112</td>
</tr>
</tbody>
</table>

(phenotypes are described in detail in Figure 17)
### Table 5
**Genotype: MS1096-G4/+; UAS-dcr2/+; Gaf TRiP/+**

<table>
<thead>
<tr>
<th>Phenotype</th>
<th>Male</th>
<th>Female</th>
<th>Total Wings Scored</th>
</tr>
</thead>
<tbody>
<tr>
<td>Venation Defect</td>
<td>46(96%)</td>
<td>26(43%)</td>
<td>72(66%)</td>
</tr>
<tr>
<td>Polarity</td>
<td>48(100%)</td>
<td>50(82%)</td>
<td>98(90%)</td>
</tr>
<tr>
<td>Notching</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Balding</td>
<td>47(98%)</td>
<td>39(64%)</td>
<td>86(79%)</td>
</tr>
<tr>
<td>Ectopic Bristles</td>
<td>6(12.5%)</td>
<td>1(1.6%)</td>
<td>7(6.4%)</td>
</tr>
<tr>
<td>Mwh</td>
<td>45(94%)</td>
<td>13(21%)</td>
<td>58(53%)</td>
</tr>
<tr>
<td>Anterior Wing Margin</td>
<td>44(92%)</td>
<td>29(48%)</td>
<td>73(67%)</td>
</tr>
<tr>
<td><strong>n=48</strong></td>
<td><strong>n=61</strong></td>
<td><strong>n=109</strong></td>
<td></td>
</tr>
</tbody>
</table>

### Table 6
**No Dicer**

**Genotype: MS1096-G4/+; Gaf TRiP/Gaf TRiP**

<table>
<thead>
<tr>
<th>Phenotype</th>
<th>Male</th>
<th>Female</th>
<th>Total Wings Scored</th>
</tr>
</thead>
<tbody>
<tr>
<td>Venation Defect</td>
<td>4(40%)</td>
<td>5(63%)</td>
<td>9(50%)</td>
</tr>
<tr>
<td>Polarity</td>
<td>8(80%)</td>
<td>6(75%)</td>
<td>14(78%)</td>
</tr>
<tr>
<td>Notching</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Balding</td>
<td>9(90%)</td>
<td>7(88%)</td>
<td>16(89%)</td>
</tr>
<tr>
<td>Ectopic Bristles</td>
<td>1(10%)</td>
<td>2(25%)</td>
<td>3(17%)</td>
</tr>
<tr>
<td>Mwh</td>
<td>2(20%)</td>
<td>3(38%)</td>
<td>5(28%)</td>
</tr>
<tr>
<td>Anterior Wing Margin</td>
<td>6(60%)</td>
<td>8(100%)</td>
<td>14(78%)</td>
</tr>
<tr>
<td><strong>n=10</strong></td>
<td><strong>n=8</strong></td>
<td><strong>n=18</strong></td>
<td></td>
</tr>
</tbody>
</table>

(Phenotypes are described in detail in Figure 17)

**MS1096 > Gaf RNAi.** TRiP UAS-Gaf RNAi also shows the effects of downregulation of Gaf in the absence of UAS-dcr2, but requires at least two copies of TRiP UAS-Gaf RNAi to exhibit any effect.
### Table 7
**Genotype:** *MS1096-G4/+; UAS-dcr2/VDRC*

<table>
<thead>
<tr>
<th>Phenotype</th>
<th>Male</th>
<th>Female</th>
<th>Total Wings Scored</th>
</tr>
</thead>
<tbody>
<tr>
<td>Venation defect</td>
<td>4(12%)</td>
<td>4(17%)</td>
<td>8(14%)</td>
</tr>
<tr>
<td>Polarity</td>
<td>20(59%)</td>
<td>12(50%)</td>
<td>32(55%)</td>
</tr>
<tr>
<td>Notching</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Balding</td>
<td>23(68%)</td>
<td>22(92%)</td>
<td>45(78%)</td>
</tr>
<tr>
<td>Ectopic bristles</td>
<td>2(6%)</td>
<td>0</td>
<td>2(3.4%)</td>
</tr>
<tr>
<td>Mwh</td>
<td>4(12%)</td>
<td>1(4%)</td>
<td>5(9%)</td>
</tr>
<tr>
<td>Anterior wing margin</td>
<td>4(12%)</td>
<td>0</td>
<td>4(7%)</td>
</tr>
<tr>
<td><strong>N=34</strong></td>
<td><strong>N=24</strong></td>
<td><strong>N=58</strong></td>
<td></td>
</tr>
</tbody>
</table>

### Table 8
**Genotype:** *MS1096-G4/+; UAS-dcr2/+; 12232R-1/+*

<table>
<thead>
<tr>
<th>Phenotype</th>
<th>Male</th>
<th>Female</th>
<th>Total Wings Scored</th>
</tr>
</thead>
<tbody>
<tr>
<td>Venation defect</td>
<td>3(7.5%)</td>
<td>0</td>
<td>3(3%)</td>
</tr>
<tr>
<td>Polarity</td>
<td>36(90%)</td>
<td>43(81%)</td>
<td>79(85%)</td>
</tr>
<tr>
<td>Notching</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Balding</td>
<td>25(63%)</td>
<td>49(92%)</td>
<td>74(80%)</td>
</tr>
<tr>
<td>Ectopic bristles</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Mwh</td>
<td>3(7.5%)</td>
<td>1(2%)</td>
<td>4(4.3%)</td>
</tr>
<tr>
<td>Anterior wing margin</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td><strong>N=40</strong></td>
<td><strong>N=53</strong></td>
<td><strong>N=93</strong></td>
<td></td>
</tr>
</tbody>
</table>
Table 9
Genotype: UAS-dcr2/en-G4;Gαf TRiP/12232R-1

<table>
<thead>
<tr>
<th>phenotype</th>
<th>male</th>
<th>female</th>
<th>Total wings scored</th>
</tr>
</thead>
<tbody>
<tr>
<td>venation defect</td>
<td>39(100%)</td>
<td>28(100%)</td>
<td>67(100%)</td>
</tr>
<tr>
<td>venation enhancement</td>
<td>24(62%)</td>
<td>18(64%)</td>
<td>42(63%)</td>
</tr>
<tr>
<td>blister over vein</td>
<td>4(10%)</td>
<td>5(18%)</td>
<td>9(13%)</td>
</tr>
<tr>
<td>n=39</td>
<td>n=28</td>
<td>n=67</td>
<td></td>
</tr>
</tbody>
</table>

Figure 39. Enhancement of defects observed with two non-overlapping Gαf RNAi expression.

Quantitative analysis of phenotypes observed with Gf TRiP/+ (n=58) expression in the posterior compartment of wings compared to that of Gf TRiP/12232-R1 (n=67). Venation defect here denotes primarily collapse of the L4 and L5 veins, as well as incomplete cross-veins and/or ectopic venation material in the posterior compartment. Venation enhancement denotes specifically expansion of the venation defect observed with the collapse and merging of the L4 and L5 veins at the PCV. Blisters are often observed directly above the L5 vein, that is not generally observed when TRiP UAS-Gαf RNAi is expressed alone. Plot shows total wings scored from Tables 2 and 9. Error bars represent S.E.M.
### Table 10
**Genotype: UAS-dcr2/en-G4;Gaf TRiP/Df(3L)Exel6130**

<table>
<thead>
<tr>
<th>phenotype</th>
<th>male</th>
<th>female</th>
<th>Total wings scored</th>
</tr>
</thead>
<tbody>
<tr>
<td>venation defect</td>
<td>34(97%)</td>
<td>22(100%)</td>
<td>56(98%)</td>
</tr>
<tr>
<td>venation enhancement</td>
<td>27(77%)</td>
<td>22(100%)</td>
<td>49(86%)</td>
</tr>
<tr>
<td>blister over vein</td>
<td>4(11%)</td>
<td>5(23%)</td>
<td>9(16%)</td>
</tr>
<tr>
<td>notching</td>
<td>2(5.7%)</td>
<td>0</td>
<td>2(3.5%)</td>
</tr>
<tr>
<td></td>
<td>n=35</td>
<td>n=22</td>
<td>n=57</td>
</tr>
</tbody>
</table>

[Breakpoint: 73B5;73D1]

### Table 11
**Genotype: UAS-dcr2/en-G4;Gaf TRiP/Df(3L)ED223**

<table>
<thead>
<tr>
<th>phenotype</th>
<th>male</th>
<th>female</th>
<th>Total wings scored</th>
</tr>
</thead>
<tbody>
<tr>
<td>venation defect</td>
<td>2(100%)</td>
<td>18(100%)</td>
<td>20(100%)</td>
</tr>
<tr>
<td>venation enhancement</td>
<td>2(100%)</td>
<td>18(100%)</td>
<td>20(100%)</td>
</tr>
<tr>
<td>blister over vein</td>
<td>0</td>
<td>11(61%)</td>
<td>11(55%)</td>
</tr>
<tr>
<td>notching</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>n=2</td>
<td>n=18</td>
<td>n=20</td>
</tr>
</tbody>
</table>

Note: rarely obtained male adults
[Breakpoint: 73A1;73D5]

### Table 12
**Genotype: UAS-dcr2/en-G4;Gaf TRiP/Df(3L)BSC561**

<table>
<thead>
<tr>
<th>phenotype</th>
<th>male</th>
<th>female</th>
<th>Total wings scored</th>
</tr>
</thead>
<tbody>
<tr>
<td>venation defect</td>
<td>47(100%)</td>
<td>12(100%)</td>
<td>59(100%)</td>
</tr>
<tr>
<td>venation enhancement</td>
<td>47(100%)</td>
<td>12(100%)</td>
<td>59(100%)</td>
</tr>
<tr>
<td>blister over vein</td>
<td>46(98%)</td>
<td>9(75%)</td>
<td>37(63%)</td>
</tr>
<tr>
<td>notching</td>
<td>3(6.4%)</td>
<td>0</td>
<td>3(5%)</td>
</tr>
<tr>
<td></td>
<td>n=47</td>
<td>n=12</td>
<td>n=59</td>
</tr>
</tbody>
</table>

[Breakpoint: 73A2;73C1]

### Table 13
**Genotype: UAS-dcr2/en-G4;Gaf TRiP/Df(3L)ED4674**

<table>
<thead>
<tr>
<th>phenotype</th>
<th>male</th>
<th>female</th>
<th>Total wings scored</th>
</tr>
</thead>
<tbody>
<tr>
<td>venation defect</td>
<td>8(100%)</td>
<td>12(100%)</td>
<td>20(100%)</td>
</tr>
<tr>
<td>venation enhancement</td>
<td>8(100%)</td>
<td>12(100%)</td>
<td>20(100%)</td>
</tr>
<tr>
<td>blister over vein</td>
<td>5(63%)</td>
<td>1(8%)</td>
<td>6(30%)</td>
</tr>
<tr>
<td>notching</td>
<td>4(50%)</td>
<td>0</td>
<td>4(20%)</td>
</tr>
<tr>
<td></td>
<td>n=8</td>
<td>n=12</td>
<td>n=20</td>
</tr>
</tbody>
</table>

[Breakpoint: 73B5; 73E5]
### Table 15
**Genotype:** *MS1096-G4;UAS-Gaf WT*

<table>
<thead>
<tr>
<th>Phenytype</th>
<th>Male</th>
<th>Female</th>
<th>Total wings scored</th>
</tr>
</thead>
<tbody>
<tr>
<td>Venation defect</td>
<td>1(8.3%)</td>
<td>10(100%)</td>
<td>11(50%)</td>
</tr>
<tr>
<td>Anterior margin</td>
<td>5(42%)</td>
<td>9(90%)</td>
<td>14(64%)</td>
</tr>
<tr>
<td>Narrowing of wing (AP axis)</td>
<td>0</td>
<td>10(100%)</td>
<td>10(45%)</td>
</tr>
<tr>
<td>Total</td>
<td>n=12</td>
<td>n=10</td>
<td>n=22</td>
</tr>
</tbody>
</table>

### Table 16
**Genotype:** *MS1096-G4;UAS-Gaf-GTP*

<table>
<thead>
<tr>
<th>Phenytype</th>
<th>Male</th>
<th>Female</th>
<th>Total wings scored</th>
</tr>
</thead>
<tbody>
<tr>
<td>Venation defect</td>
<td>2(100%)</td>
<td>17(85%)</td>
<td>19(86%)</td>
</tr>
<tr>
<td>Anterior margin</td>
<td>0</td>
<td>14(70%)</td>
<td>14(64%)</td>
</tr>
<tr>
<td>Narrowing of wing (AP axis)</td>
<td>2(100%)</td>
<td>15(75%)</td>
<td>17(77%)</td>
</tr>
<tr>
<td>Total</td>
<td>n=2</td>
<td>n=20</td>
<td>n=22</td>
</tr>
</tbody>
</table>

### Table 17
**Genotype:** *MS1096-G4;UAS-Gaf-GDP*

<table>
<thead>
<tr>
<th>Phenytype</th>
<th>Male</th>
<th>Female</th>
<th>Total wings scored</th>
</tr>
</thead>
<tbody>
<tr>
<td>Venation defect</td>
<td>2(50%)</td>
<td>27(96%)</td>
<td>29(91%)</td>
</tr>
<tr>
<td>Anterior margin</td>
<td>3(75%)</td>
<td>15(54%)</td>
<td>18(56%)</td>
</tr>
<tr>
<td>Narrowing of wing (AP axis)</td>
<td>n/a</td>
<td>n/a</td>
<td>n/a</td>
</tr>
<tr>
<td>Total</td>
<td>n=4</td>
<td>n=28</td>
<td>n=32</td>
</tr>
</tbody>
</table>

Venation defects corresponds to ectopic vein material or thickening or collapsing of the veins in the wing.

Effects on the anterior margin correspond to gaps between bristles and/or extra stout bristles, emerging from one socket. Narrowing of the wing corresponds to the wing appearing more constricted along the AP axis. (n/a – not available)
Figure 40. Overexpression of Gaf. Quantitative analysis of phenotypes observed with Gf WT (n=22) compared to Gf-GTP (n=22) and Gf-GDP (n=32). Similar effects on the anterior margin and venation defects are seen with all three variants. Plot shows total wings scored from Tables 15,16 and 17. Error bars represent S.E.M.
Table 18
Genotype: en-G4,UAS-dcr2/UAS-fz2;Gαf TRiP/+  

<table>
<thead>
<tr>
<th>phenotype</th>
<th>male</th>
<th>female</th>
<th>Total wings scored</th>
</tr>
</thead>
<tbody>
<tr>
<td>venation defect</td>
<td>13(87%)</td>
<td>12(100%)</td>
<td>25(93%)</td>
</tr>
<tr>
<td>ectopic bristles</td>
<td>8(53%)</td>
<td>7(58%)</td>
<td>15(55%)</td>
</tr>
<tr>
<td>n=15</td>
<td>n=12</td>
<td>n=27</td>
<td></td>
</tr>
</tbody>
</table>

Table 19
Genotype: MS1096-G4/+;UAS-dcr2/UAS-fz2;Gαf TRiP/+  

<table>
<thead>
<tr>
<th>phenotype</th>
<th>Total wings scored</th>
</tr>
</thead>
<tbody>
<tr>
<td>venation defect</td>
<td>7(50%)</td>
</tr>
<tr>
<td>anterior margin</td>
<td>11(79%)</td>
</tr>
<tr>
<td>gap</td>
<td>11(79%)</td>
</tr>
<tr>
<td>extra</td>
<td>2(14%)</td>
</tr>
<tr>
<td>ectopic bristles</td>
<td>12(86%)</td>
</tr>
<tr>
<td>balding</td>
<td>14(100%)</td>
</tr>
<tr>
<td>polarity</td>
<td>13(93%)</td>
</tr>
<tr>
<td>mwh</td>
<td>7(50%)</td>
</tr>
<tr>
<td>n=14</td>
<td></td>
</tr>
</tbody>
</table>

Examined females only.

Figure 41. Reduction of ectopic bristle number corresponds with a reduction in Gαf transcript. (en-G4,UAS-dcr2/UAS-fz2/TRiP;UAS-Gαf/+). Comparison of ectopic bristle number in wings expressing UAS-fz2 (n=5) versus wings expressing UAS-fz2/Gf TRiP (n=15). Flies from the experiment in Table 18 were scored for ectopic bristles found in the posterior compartment. (A) anterior; (P) posterior.
Table 20
Genotype: en-G4/+;UAS-fz2/UAS-Gαf

<table>
<thead>
<tr>
<th>phenotype</th>
<th>UAS-Gf WT</th>
<th>UAS-Gf-GTP</th>
<th>UAS-Gf-GDP</th>
</tr>
</thead>
<tbody>
<tr>
<td>effects apart from fz2 phenotype</td>
<td>0</td>
<td>n/a</td>
<td>0</td>
</tr>
<tr>
<td>fz2 phenotype</td>
<td>n=47</td>
<td>n/a</td>
<td>n=18</td>
</tr>
</tbody>
</table>

(n/a – not available)

fz2 phenotype: ectopic bristles

Table 21
Genotype: MS1096-G4/+;UAS-fz2/UAS-Gαf

<table>
<thead>
<tr>
<th>phenotype</th>
<th>UAS-Gf WT</th>
<th>UAS-Gf-GTP</th>
<th>UAS-Gf-GDP</th>
</tr>
</thead>
<tbody>
<tr>
<td>effects apart from fz2 phenotype</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>fz2 phenotype</td>
<td>n=12</td>
<td>n=45</td>
<td>n=12</td>
</tr>
</tbody>
</table>

Figure 42. Ectopic bristles in posterior compartment. (MS1096-G4/+;UAS-fz2/UAS-Gαf).

Comparison of the number of bristles of wings expressing UAS-fz2/+ (n=19), UAS-fz2/UAS-Gf WT (n=12), UAS-fz2/UAS-Gf GTP (n=45) and UAS-fz2/UAS-Gf GDP (n=12). There is no discernible trend in ectopic bristle number. Ectopic bristles only in the posterior compartment were counted. Plot shows total wings scored from Table 21. Error bars represent S.E.M. (A) anterior; (P) posterior.
Table 22
Genotype: en-G4,UAS-dcr2/+;Gaf TRiP/UAS-fz1

<table>
<thead>
<tr>
<th>phenotype</th>
<th>male</th>
<th>female</th>
<th>Total wings scored</th>
</tr>
</thead>
<tbody>
<tr>
<td>venation defect</td>
<td>15(94%)</td>
<td>44(100%)</td>
<td>59(98%)</td>
</tr>
<tr>
<td>venation enhancement</td>
<td>2(12.5%)</td>
<td>30(68%)</td>
<td>32(53%)</td>
</tr>
<tr>
<td>blister over vein</td>
<td>2(12.5%)</td>
<td>23(52%)</td>
<td>25(42%)</td>
</tr>
<tr>
<td></td>
<td>n=16</td>
<td>n=44</td>
<td>n=60</td>
</tr>
</tbody>
</table>

fz1 phenotype (swirling hair pattern with mwh present) was observed in all.

Table 23
Genotype: MS1096-G4/+;UAS-dcr2/+;Gaf TRiP/UAS-fz1

<table>
<thead>
<tr>
<th>phenotype</th>
<th>Total wings scored</th>
</tr>
</thead>
<tbody>
<tr>
<td>venation defect</td>
<td>16(94%)</td>
</tr>
<tr>
<td>anterior margin</td>
<td>16(94%)</td>
</tr>
<tr>
<td>gap</td>
<td>12(71%)</td>
</tr>
<tr>
<td>extra</td>
<td>10(59%)</td>
</tr>
<tr>
<td></td>
<td>n=17</td>
</tr>
</tbody>
</table>

Examined females only. fz1 phenotype was observed in all. Wings are often smaller and rounder.
Table 24
Genotype: en-G4/+; UAS-fz1/UAS-Gαf

<table>
<thead>
<tr>
<th>phenotype</th>
<th>UAS-Gf WT</th>
<th>UAS-Gf-GTP</th>
<th>UAS-Gf-GDP</th>
</tr>
</thead>
<tbody>
<tr>
<td>effects apart from</td>
<td>0</td>
<td>n/a</td>
<td>0</td>
</tr>
<tr>
<td>fz phenotype</td>
<td>n=12</td>
<td>n/a</td>
<td>n=9</td>
</tr>
</tbody>
</table>

Table 25
Genotype: MS1096-G4/+; UAS-fz1/UAS-Gαf

<table>
<thead>
<tr>
<th>phenotype</th>
<th>UAS-Gf WT</th>
<th>UAS-Gf-GTP</th>
<th>UAS-Gf-GDP</th>
</tr>
</thead>
<tbody>
<tr>
<td>effects apart from</td>
<td>n/a</td>
<td>n/a</td>
<td>0</td>
</tr>
<tr>
<td>fz phenotype</td>
<td>n/a</td>
<td>n/a</td>
<td>n=14</td>
</tr>
</tbody>
</table>

fz1 phenotype: swirling hair pattern. And mwh (n/a – not available)

Table 26
Genotype: en-G4/+; UAS-Gαf /fz,fz2

<table>
<thead>
<tr>
<th>phenotype</th>
<th>UAS-Gf WT/fz,fz2</th>
<th>UAS-Gf-GTP/fz,fz2</th>
<th>UAS-Gf-GDP/fz,fz2</th>
</tr>
</thead>
<tbody>
<tr>
<td>venation or polarity defects</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>n=48</td>
<td>n=47</td>
<td>n=8</td>
</tr>
</tbody>
</table>

Table 27
Genotype: MS1096-G4/+; UAS-Gαf/fz,fz2

<table>
<thead>
<tr>
<th>phenotype</th>
<th>UAS-Gf WT/fz,fz2</th>
<th>UAS-Gf-GTP/fz,fz2</th>
<th>UAS-GF-GDP/fz,fz2</th>
</tr>
</thead>
<tbody>
<tr>
<td>venation or polarity defects</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>n=45</td>
<td>n=8</td>
<td>n=10</td>
</tr>
</tbody>
</table>
Table 28
Genotype: en-G4,UAS-dcr2/+;Gαf TRiP/fz,fz2

<table>
<thead>
<tr>
<th>phenotype</th>
<th>Gf TRiP/+</th>
<th>Gf TRiP/fz,fz2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total wings scored</td>
<td></td>
<td></td>
</tr>
<tr>
<td>venation defect</td>
<td>0</td>
<td>21(70%)</td>
</tr>
<tr>
<td></td>
<td>n=27</td>
<td>n=30</td>
</tr>
</tbody>
</table>

n=27
n=30
n=41

Table 29
Genotype: MS1096-G4/+;UAS-dcr2/+;Gαf TRiP/fz,fz2

<table>
<thead>
<tr>
<th>phenotype</th>
<th>Gf TRiP/+</th>
<th>Gf TRiP/fz-</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total wings scored</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>n=26</td>
<td>n=48</td>
</tr>
<tr>
<td></td>
<td>n=26</td>
<td>n=48</td>
</tr>
</tbody>
</table>

MS1096-G4/+; UAS-dcr2/+;fz,fz2/+ - no defects were observed

Table 30
Genotype: MS1096-G4/+;UAS-dcr2/+;Gαf TRiP/+ (control)

<table>
<thead>
<tr>
<th>phenotype</th>
<th>male</th>
<th>female</th>
<th>Total wings scored</th>
</tr>
</thead>
<tbody>
<tr>
<td>venation defect</td>
<td>14(100%)</td>
<td>22(65%)</td>
<td>36(75%)</td>
</tr>
<tr>
<td>anterior margin</td>
<td>14(100%)</td>
<td>26(76%)</td>
<td>40(83%)</td>
</tr>
<tr>
<td>gap</td>
<td>14(100%)</td>
<td>21(62%)</td>
<td>35(73%)</td>
</tr>
<tr>
<td>extra</td>
<td>8(57%)</td>
<td>12(35%)</td>
<td>20(42%)</td>
</tr>
<tr>
<td>ectopic bristles</td>
<td>2(14%)</td>
<td>1(2.9%)</td>
<td>3(6.3%)</td>
</tr>
<tr>
<td>balding</td>
<td>14(100%)</td>
<td>22(65%)</td>
<td>36(75%)</td>
</tr>
<tr>
<td>polarity</td>
<td>14(100%)</td>
<td>30(88%)</td>
<td>44(92%)</td>
</tr>
<tr>
<td>mwh</td>
<td>9(64%)</td>
<td>4(12%)</td>
<td>13(27%)</td>
</tr>
<tr>
<td></td>
<td>n=14</td>
<td>n=34</td>
<td>n=48</td>
</tr>
</tbody>
</table>

Table 31
Genotype: MS1096-G4/+;UAS-dcr2/+;Gαf TRiP/fz,fz2

<table>
<thead>
<tr>
<th>phenotype</th>
<th>male</th>
<th>female</th>
<th>Total wings scored</th>
</tr>
</thead>
<tbody>
<tr>
<td>venation defect</td>
<td>30(100%)</td>
<td>20(38%)</td>
<td>50(60%)</td>
</tr>
<tr>
<td>anterior margin</td>
<td>29(97%)</td>
<td>46(87%)</td>
<td>75(90%)</td>
</tr>
<tr>
<td>gap</td>
<td>26(87%)</td>
<td>35(66%)</td>
<td>61(73%)</td>
</tr>
<tr>
<td>extra</td>
<td>18(60%)</td>
<td>21(40%)</td>
<td>39(47%)</td>
</tr>
<tr>
<td>ectopic bristles</td>
<td>5(17%)</td>
<td>0</td>
<td>5(6%)</td>
</tr>
<tr>
<td>balding</td>
<td>30(100%)</td>
<td>28(53%)</td>
<td>58(70%)</td>
</tr>
<tr>
<td>polarity</td>
<td>30(100%)</td>
<td>44(83%)</td>
<td>75(90%)</td>
</tr>
<tr>
<td>mwh</td>
<td>25(83%)</td>
<td>6(11%)</td>
<td>31(37%)</td>
</tr>
<tr>
<td></td>
<td>n=30</td>
<td>n=53</td>
<td>n=83</td>
</tr>
</tbody>
</table>
Table 32
Genotype: **MS1096-G4/+;UAS-dcr2/+;Gaf TRiP/UAS-smo**

<table>
<thead>
<tr>
<th>phenotype</th>
<th>Total wings scored</th>
</tr>
</thead>
<tbody>
<tr>
<td>venation defect</td>
<td>24(100%)</td>
</tr>
<tr>
<td>enhancement</td>
<td>19(79%)</td>
</tr>
<tr>
<td></td>
<td>n=24</td>
</tr>
</tbody>
</table>

Examined females only

Table 33
Genotype: **MS1096-G4/+;UAS-smo/UAS-Gaf**

<table>
<thead>
<tr>
<th>phenotype</th>
<th>UAS-Gf WT</th>
<th>UAS-Gf-GTP</th>
<th>UAS-Gf-GDP</th>
</tr>
</thead>
<tbody>
<tr>
<td>venation defect</td>
<td>n/a</td>
<td>n/a</td>
<td>4(14%)</td>
</tr>
<tr>
<td>enhancement</td>
<td>n/a</td>
<td>n/a</td>
<td>1(3.4%)</td>
</tr>
<tr>
<td></td>
<td>n/a</td>
<td>n/a</td>
<td>n=29</td>
</tr>
</tbody>
</table>

Venation defect corresponds to extra vein material between L1 and L2 veins. Enhancement refers to thickening and/or expansion of this ectopic vein material. (n/a – not available)

Table 34
Genotype: **en-G4/smo3;UAS-Gaf/+**

<table>
<thead>
<tr>
<th>phenotype</th>
<th>UAS-Gf WT/+</th>
<th>UAS-Gf-GTP/+</th>
<th>UAS-Gf-GDP/+</th>
</tr>
</thead>
<tbody>
<tr>
<td>venation defect</td>
<td>0</td>
<td>n/a</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>n=20</td>
<td>n=34</td>
<td>n/a</td>
</tr>
<tr>
<td></td>
<td>0</td>
<td>n/a</td>
<td>n=12</td>
</tr>
</tbody>
</table>
Table 35  
Genotype:  *UAS-dcr2, en-G4/smo3; Gaf TRiP/+*  

<table>
<thead>
<tr>
<th>phenotype</th>
<th>male</th>
<th>female</th>
<th>Total wings scored</th>
</tr>
</thead>
<tbody>
<tr>
<td>venation defect</td>
<td>23(100%)</td>
<td>62(94%)</td>
<td>85(96%)</td>
</tr>
<tr>
<td>venation enhancement</td>
<td>15(65%)</td>
<td>56(85%)</td>
<td>71(80%)</td>
</tr>
<tr>
<td>blister over vein</td>
<td>15(65%)</td>
<td>54(82%)</td>
<td>59(66%)</td>
</tr>
<tr>
<td></td>
<td>n=23</td>
<td>n=66</td>
<td>n=89</td>
</tr>
</tbody>
</table>

Note: there was difficulty in discriminating between cyo flies and blistered flies, and therefore determining the genotype in this set of experiments

Table 36  
Genotype:  *MS1096-G4/+; UAS-dcr2/smo3; Gaf TRiP/+*  

<table>
<thead>
<tr>
<th>phenotype</th>
<th>Total wings scored</th>
</tr>
</thead>
<tbody>
<tr>
<td>venation defect</td>
<td>4(14%)</td>
</tr>
<tr>
<td>anterior margin</td>
<td>23(82%)</td>
</tr>
<tr>
<td>gap</td>
<td>19(68%)</td>
</tr>
<tr>
<td>extra</td>
<td>10(36%)</td>
</tr>
<tr>
<td>ectopic bristles</td>
<td>3(11%)</td>
</tr>
<tr>
<td>balding</td>
<td>24(86%)</td>
</tr>
<tr>
<td>polarity</td>
<td>27(96%)</td>
</tr>
<tr>
<td>mwh</td>
<td>5(18%)</td>
</tr>
<tr>
<td></td>
<td>n=28</td>
</tr>
</tbody>
</table>

Examined females only
Table 37
Genotype: UAS-dcr2, en-G4/+; Gi RNAi/Gf TRiP

<table>
<thead>
<tr>
<th></th>
<th>Gi/+</th>
<th>Gf TRiP/+</th>
<th>Gi RNAi/Gf TRiP</th>
<th>Gi RNAi,Gf TRiP/Gf TRiP</th>
</tr>
</thead>
<tbody>
<tr>
<td>phenotype</td>
<td>Total wings scored</td>
<td>Total wings scored</td>
<td>Total wings scored</td>
<td>Total wings scored</td>
</tr>
<tr>
<td>venation defect</td>
<td>0</td>
<td>57(90%)</td>
<td>85(100%)</td>
<td>8(100%)</td>
</tr>
<tr>
<td>enhancement</td>
<td>-</td>
<td>-</td>
<td>13(15%)</td>
<td>4(50%)</td>
</tr>
<tr>
<td>blister over vein</td>
<td>0</td>
<td>17(27%)</td>
<td>4(4.7%)</td>
<td>4(50%)</td>
</tr>
<tr>
<td>n=34</td>
<td>n=63</td>
<td>n=85</td>
<td>n=8</td>
<td></td>
</tr>
</tbody>
</table>

Note: The only time blisters were observed with the TRiP UAS-Gαf RNAi driven by en-G4 was this one occasion, which I attribute to incubator trouble. All experiments above were performed side by side, however, and the trend should still hold.

Figure 43. Genetic interaction of Gαf with Gαi shows enhancement of the venation defect.

Quantitative analysis of phenotypes observed in wings expressing Gf TRiP/+ (n=63) compared to Gi/+ (n=34), Gi/Gf TRiP (n=85) and Gi,Gf TRiP/Gf TRiP (n=8) in the posterior compartment. Plot shows total wings scored from Table 37. Error bars represent S.E.M.
<table>
<thead>
<tr>
<th>phenotype</th>
<th>Gi/+ Total wings scored</th>
<th>Gf TRiP/+ Total wings scored</th>
<th>Gi RNAi/Gf TRiP Total wings scored</th>
<th>Gi RNAi,Gf TRiP/Gf TRiP Total wings scored</th>
</tr>
</thead>
<tbody>
<tr>
<td>anterior wing margin</td>
<td>25(86%)</td>
<td>31(94%)</td>
<td>23(100%)</td>
<td>10(100%)</td>
</tr>
<tr>
<td>gap</td>
<td>19(66%)</td>
<td>31(94%)</td>
<td>20(87%)</td>
<td>10(100%)</td>
</tr>
<tr>
<td>extra</td>
<td>15(52%)</td>
<td>13(39%)</td>
<td>19(83%)</td>
<td>10(100%)</td>
</tr>
<tr>
<td>n=29</td>
<td>n=33</td>
<td>n=23</td>
<td>n=10</td>
<td></td>
</tr>
</tbody>
</table>
Chapter III

Description of Other Related Projects
1. Mis-localization of planar cell polarity proteins in the wing by adapting the Boss/Sevenless ligand/receptor proteins of the eye

Abstract

Planar cell polarity is the process whereby cells of an epithelium uniformly, orient their hairs (in the wing) in the same direction. Each hair is secreted from the distal end of each cell in the wing. This uniform position is predefined by an asymmetric localization of a set of proteins in the cells, known as the core planar polarity group. While some of these proteins localize to the distal ends of the cell where the hair will grow, others localize to the proximal end of the cells (opposite to where the hair will grow); and yet others localize to both positions (Lawrence, et al., 2008; Seifert & Mlodzik, 2007; H. Strutt & Strutt, 2005). Although there is a striking correlation between the asymmetry of the protein localizations with the subsequent positioning of the hair, there is some debate as to how critical these protein localizations are. To address this issue, we sought to mis-localize various members of the core group proteins within the polarizing cells, in order to determine whether this could dominantly re-polarize the position from which the hairs are secreted.

1.1 Introduction

In the adult wild-type wing, PCP determines the direction of the individual hairs or trichromes that extend from the distal vertex of each cell. Prior to the initiation of these hairs, the key core group of proteins undergoes asymmetric distribution, which occurs gradually over time. Between 18 and 32 hours after puparium formation, the transmembrane protein Fz accumulates on the distal side of the individual cells, along with the cytosolic protein Dsh while the transmembrane protein Vang accumulates on the proximal side with Prickled. Flamingo accumulates on both sides. When some of these core group of genes (fz, dsh, fmi, dgo, stbm, pk) are absent, all other core protein asymmetric distribution is lost, and distal wing hair outgrowth is affected, leading to irregular hair orientations (Lawrence, et al., 2008; Seifert & Mlodzik, 2007; H. Strutt & Strutt, 2005). Importantly, while there is a striking correlation between the asymmetric protein distributions and the subsequent position of the hair outgrowth, analysis of another core group protein, Prickled, calls into question this view. In prickle mutants, even though the localization of the core group of proteins is disrupted, the hairs remain coordinately aligned (Lawrence, et al., 2004).
Therefore, it is unclear as to what extent the asymmetric localizations of the core group proteins are critical for PCP.

In PCP mutants (such as \( fz \), and \( dsh \)) the vast majority of the cells effectively polarize. That is, they still produce a single focus for hair outgrowth. What is lost is the coordination of the polarizations, such that the hairs project randomly rather than project distally and all in the same direction. A trimeric G-protein alpha subunit (\( G_\alpha \)) is interesting in this regard, since weak mutants induce the standard PCP phenotype (randomly oriented single hairs) but strong mutations prevent the formation of the single foci for hair outgrowth. Instead, multiple wing hairs between two or more are observed. Thus \( G_\alpha \) appears to function not only in the PCP mechanism that directs the axis of polarization of the cells, but also in the intrinsic mechanism of polarization itself (Katanaev & Tomlinson, 2006b). Furthermore, the very nature of \( G_\alpha \)'s enrichment on the proximal end of the cell makes it appear to function as a core group protein. Katanaev and Tomlinson (2005) argued a role for \( G_\alpha \) as the alpha subunit of a trimeric G-protein complex that transduces Fz signaling in both the PCP and Wnt transduction pathways. They presented a model of how PCP sees Fz/G\( _\alpha \) signaling as the primary reader of extracellular polarizing information. We infer that Fz initially ‘reads’ a graded extracellular signal and activates \( G_\alpha \) in a correspondingly graded manner, which may then proceed to reorganize the apical microtubule web once activated (see Section 2 below). Consequently, Fz proteins become redistributed to one end of the cell while \( G_\alpha \) molecules are sent to the opposite end.

The experiments in this body of work were designed overall to determine the consequences of mis-localization of PCP proteins. Specifically, these experiments were also designed to determine whether the localization of \( G_\alpha \) could domineeringly direct the redistribution of the other core group proteins.

### 1.2 Materials and Methods

**Drosophila strains and Maintenance**

Gal4 drivers used in this section included, \( \text{sev-G4}, \text{hh-G4} \) (Tanimoto et al., 2000) (both from the lab stock); and \( \text{en-G4} \) (obtained from the Johnston lab). The initial UAS-constructs used in the experiments were the following: \( \text{UAS-sev-gfp} \) (3\(^{rd}\) chromosome), \( \text{UAS-sev-gfp-G}_\alpha \) (WT, 3\(^{rd}\) chromosome), \( \text{UAS-sev-} \).
gfp-\(\Gamma\alpha^\ast\) (activated, 2nd chromosome), UAS-sev-gfp-\(\Gamma\alpha\) (GDP, 3\textsuperscript{rd} chromosome); they were made in the lab and injected into \(yw\) flies. I then made the UAS-sev-fz chimeras (UAS-sev-gfp-fz, UAS-sev-fz-gfp, UAS-sev-fz), which were also injected into \(yw\) flies. Later, I took all of the above UAS-sev constructs and moved them into the AttB system [86Fb] embryos, and generated a UAS-sev-gfp-dsh fly stock. In addition, I generated the following chimeras: blink.boss, ci.boss, ci.LexA and LexO.boss fly stocks. LexO.gfp was obtained from the Mann Lab. LexO is another binary expression system, which is UAS independent (Lai & Lee, 2006).

Constructs

All of the initial P-element-based constructs (UAS-sev-gfp/pW8Rx, UAS-sev-gfp-\(\Gamma\alpha\) (WT)/pW8Rx, UAS-sev-gfp-\(\Gamma\alpha\) (GDP)/pW8Rx, UAS-sev-gfp-\(\Gamma\alpha^\ast\) (activated)/pW8Rx, UAS-sev-gfp-fz/pUAST, UAS-sev-fz-gfp/pUAST, UAS-sev-fz/pUAST) were engineered as Asp718 to EcoRI fragments. The above constructs as well as UAS-boss and UAS-sev-gfp-dsh were subsequently moved into the AttB vector (Bischof, et al., 2007). They were also moved into a modified AttB vector, containing an internal FRT site (made by SK in the Struhl lab). Blink.boss, ci.boss, ci.LexA and LexO.boss constructs were also cloned into another modified AttB vector, where the original Gal4 binding sites and multiple cloning site were removed, and replaced with a new modified multiple cloning site (by A.Tomlinson). Once all these constructs were generated, they were transformed into the same AttB sites as described above.

Histology

Histological preparations of the adult eye were performed as described in Tomlinson & Ready (1987).

Immunostaining

Imaginal discs were stained as described in the previous section. Mouse anti-Boss (Kramer et al., 1991) (1:200) was from the Zipursky lab.
1.3 Results

Experimental Design

The key idea for the various strategies that I undertook in this section was to mis-localize $G_{\alpha}o$ and other PCP proteins by utilizing a non-diffusible ligand/receptor system present in the *Drosophila* eye, to tether the proteins of interest to one side of the cell in the *Drosophila* wing. Bride of sevenless (Boss) and Sevenless (Sev) are a membrane tethered ligand and receptor system used in the *Drosophila* for the specification of the R7 photoreceptor (Reinke & Zipursky, 1988). Sev is a receptor tyrosine kinase, which upon binding to Boss (in the neighboring cell) becomes activated and triggers transduction of the Ras-MAPK pathway. Three of the developing photoreceptor cells (R3, R4 and R7) express sev and abut the boss-expressing R8 cell (Tomlinson, et al., 1987). At the point of contact (on the membrane portions) of these cells with the R8 cell, there is heavy staining for Sev, (Figure 44) which indicates Sev accumulation. Hence, Boss caps Sev. The approach here was to remove the cytoplasmic portion of Sev and replace it with various PCP proteins, and then use the expression of Boss in a neighboring cell to cause the accumulation of the chimera specifically to one side of the cell. (Figure 45) Each of these constructs were engineered and transformed into flies under UAS transcriptional control.

Our goal was to have one cell expressing the sev-chimera adjacent to a neighboring boss expressing cell. Since the chimeras were to be expressed under UAS control, the mis-expression of boss in neighboring cells needed to be under another promoter system. *Blink* is an enhancer from the *decapentaplegic* locus that is expressed in a narrow stripe at high levels in the anterior cells immediately adjacent to the posterior compartment of the wing (Morimura, et al., 1996). Our initial approach was to construct a blink-boss transformant and thereby ectopically express Boss protein in the cells of the anterior wing compartment directly adjacent to the compartment border; and to express the sev-chimeras throughout the posterior compartment (using *hh*-G4). At the compartment border, cells expressing Boss would be directly adjacent to cells expressing the Sev-chimeras. Importantly, we expected the Boss/Sev capping mechanism to localize the Sev-chimeras to the side of the cell facing Boss. (Figure 46A)

Once these flies were engineered, I expected to first examine the orientation of the hairs in the posterior cells immediately adjacent to the Boss expressing cells, and determine whether their polarity was disturbed. Then, I would assess protein localization by dissection of the pupal wings and
immunostaining with antibodies of interest. Most of the constructs were designed to also have GFP labels, so that I could visualize protein localization. Lastly, once the technique was working, I expected to make Sev-chimeras of other core group proteins and assess their abilities to domineeringly alter PCP. Subsequently, epistasis experiments could be performed. One question we would address was if the Sev-Gα chimera could domineeringly change the polarization of the cell, then could it still achieve this when other core group proteins were absent, such as in dsh, fz, or vang mutant tissue?

As documented below, this strategy did not work. A subsequent series of approaches were undertaken, each of which is documented below.

**Initial Strategy:** Using *blink boss* to ectopically express *boss* and limit the expression of the Boss protein to a stripe in the anterior compartment immediately adjacent to the posterior margin of the adult wing; and using the UAS/Gal4 system to drive all the *sevenless-chimeras* in the posterior compartment with the *hh-G4* driver. (Figure 46)

First, I conducted preliminary experiments to confirm that the UAS constructs (in pw8 and pUAST vectors) would work. Various sev-gfp-Gα constructs and fly transformants had been generated previously. The initial data that I gathered suggested that this system was likely to work (Figure 46D). I began by looking in the adult eye by crossing *UAS-sev-gfp-Gα* to sev-Gal4. This would ensure the chimeras were ectopically expressed in the R3, R4 and R7 photoreceptors, and would interact with the endogenous Boss ligand present on R8.

If it did, we expected the overexpression of this chimera to act as a dominant negative to the endogenous Sevenless protein and the resulting phenotype would be similar to the sev mutant. (Figure 46C) It further indicated that the chimera is able to reach the apical membrane, which is a critical feature of PCP protein localization (they reach the level of the adherens junction). This was also confirmed by examination of the eye imaginal discs to determine if GFP expression was apical. Strikingly, in the homozygous flies, many ommatidia appeared to have completely lost their polarity (symmetrical ommatidia circled in red). (Figure 46D)

After confirming the functionality of the chimera in the eye, I crossed the sev-chimeras with *hh-G4* driver (as well as other drivers in the wing) to determine if there was corresponding GFP expression in the
posterior compartment of the wing imaginal disc. There was GFP present in the discs; and the corresponding adult wings appeared no different to wild type wings.

Before the blink.boss constructs were made, the UAS-boss flies were tested in the wing and driven with hh-G4 (and other drivers). Interestingly, we observed multiple wing hairs in the adult wings, which is reminiscent of the PCP phenotype. Nevertheless, we decided to continue to pursue this Sev/Boss receptor/ligand system despite this observation. We noted that we could use this as a reference or marker for Boss expression in the adult wing.

Two versions of blink.boss constructs were made. One contained the full length blink promoter, which showed little evidence of Boss expression in the wing imaginal disc or adult wing. When I looked at the wing imaginal discs of 3rd instar larvae to ascertain whether any Boss expression is present with antibody against Boss, there appeared to be only faint staining visible. The other construct was a multimerized form of blink (it had three copies of the shortened enhancer introduced directly in front of boss), which showed limited expression in both the disc and the adult wing. In the disc, this showed some Boss staining though it was not as we had expected (the expression was about a third the length of the strip along the margin). (Figure 47B-B’) With this construct, we observed mwh in adult wings as we had seen with UAS-boss driven with various wing Gal4 drivers, but it seemed to go beyond the expected ‘strip’ and the margin; it also went into the posterior compartment.

Despite the limited and unpredictable expression of blink.boss, I went ahead and performed some experiments to determine if the cells with UAS-sev-chimeras would be adjoined against the blink.boss expressing cells, in the imaginal wing disc. They did not abut. (Figure 47C) Generally, there appeared to be one or more cells in between the two cells (one expressing Boss and the other expressing the chimera). The failure of the two cell types to directly and cleanly abut prevented the experiment from being adequately performed, and this approach was discontinued.

Second Strategy: Using ci.boss to drive boss in the anterior compartment of the wing, while driving the sev-chimeras with the hh-G4.

In this new strategy, we hoped that by driving boss with another construct (ci.boss), the Boss-expressing cells would now sit directly adjacent to the Sevenless-expressing chimeras. C1 is expressed
in the anterior compartment of the wing imaginal disc (Q. T. Wang & Holmgren, 1999), and a construct was made in which the ci enhancer element was used to drive expression of boss (ci.boss).

Unfortunately, when the wing imaginal discs of 3rd instar larvae were checked for Boss expression by staining the discs with the antibody raised against Boss, I found only weak expression. The mwh phenotype we observed with Boss overexpression was also not present. We determined that that the fusion constructs were too weak and decided to pursue another modified strategy, rather than try to make multiple copies of ci.boss, as was done with blink. The ci enhancer element is much larger than that of blink, and multimierizing it did not appear a feasible approach to follow.

**Third Strategy (‘Quick and Dirty’):** Using the tubulin-flip-out cassette to drive boss in clones in the wing, while driving the sevenless-chimeras with the hh-Gal4 driver.

An additional construct had to be made to attempt this new strategy. We made the following: tubulin> w+> boss in AttB, which would allow us to make gain-of-function clones of boss in the fly, by induction of heat shock to third instar larvae at 37°C. The sev-chimeras were driven in the wing with the hh-G4 driver as before. The idea was to randomly induce clones of Boss expression. We expected to induce some clones in the anterior compartment that would grow to and then respect the compartment border, as well as line up against the posterior cells expressing the Sev-chimeras, which we could assess for any effects.

First, the new construct had to be tested on its own. The larvae were heat-shocked for an hour at 37°C, and the eyes and wings of the adults that emerged were examined for clones. The adult eyes indeed appeared to have clones (areas with less pigmentation than others, as the flip-out cassette was marked by the presence of the white gene). When these eyes were dissected and sectioned extra R7s were present in the clones, which is consistent with the ectopic expression of Boss. In the wing however, this level of Boss protein expression did little. In the adult wing, there was no induction of the multiple wing hairs in the adult. Likewise, in the third instar disc clones Boss expression was detected, but at a very low level.
Fourth Strategy: Using the LexO/LexA system to drive boss in the anterior compartment, with blink.LexA to drive boss.

A blink.LexA transgene showed strong expression of LexO.GFP in the domain of blink expression (J. Parker, personal communication). Here, I constructed and transformed a LexO.boss transgene in hopes of driving high levels of Boss expression in the correct domain (in the anterior cells next to the compartment border). But when I crossed these flies to the flies containing the blink.LexA driver, only a weak Boss expression was detected. Again, this approach was discontinued.

Fifth Strategy: Using the LexO/LexA system to drive boss in the anterior compartment of the wing using ci.LexA.

In this strategy, I made ci.LexA to drive LexO.boss in the entire anterior compartment of the wing, while the sev-chimeras were expressed in the posterior compartment with the hh-G4 driver. While there were regions where the two domains of expression directly abutted, it appeared to be restricted only to the hinge regions of the wing. (Figure 48) This would prevent adequate assessment of the polarity of the hairs and would not be a viable approach. Why the two domains did not generally directly abut, was not clear. I then tried en-G4 to drive the sev-chimeras in the posterior compartment, instead of hh-G4. In this scenario, the two domains of expression frequently overlapped, again preventing the frank assessment of the effects of expressing the sev-chimeras in cells directly abutting other cells expressing boss.

Sixth Strategy: Twin Spot experiments to drive expression of boss and sev-chimeras in adjacent clones.

Having tried multiple strategies without success, I tried one last approach, which involved inducing clones of boss and the sev-chimeras in adjacent clones. In order to carry out this strategy, I transferred the boss and sev-chimera constructs to a new AttB vector which contained an FRT to facilitate somatic recombination between the sister chromosome arms. The constructs were transformed into AttP sites at position 86Fb on 3R. However, unintentionally, this arrangement placed the FRT in the wrong position relative to the centromere and the gene coding sequences, which prevented the induction of clones. Having discovered this, we next recombined FRTs onto these chromosomes to allow the experiment to be performed. To drive expression of the constructs we used en-G4, and clones were
generated using standard heat shock (hs-flip) techniques. Although there was evidence that this method may have been feasible, time constraints made this impractical, and the approach was dropped.

1.4 Discussion

By generating a system where the core PCP proteins are ectopically expressed and mis-localized, we would be able to establish the significance of cell autonomous effects of the asymmetry of planar polarity proteins exhibited prior to hair initiation in the wing. This would address long-held generalizations about the localizations of these proteins, as there remains clearly a debate as to whether the localizations are simply an output of planar polarity or whether they direct planar polarity. However, after many attempts and employing different strategies, I finally opted to discontinue this project, as there were far too many technical difficulties to overcome.

2. Examination of the role of $G_\alpha_o$ and microtubules

Abstract

Previous work indicated that PCP proteins are redistributed within the cells in a microtubule-dependent manner (Shimada, et al., 2006). Since $G_\alpha$ had been implicated in the PCP process, and $G_\alpha$ proteins in many systems are known to regulate microtubule dynamics, I sought to investigate whether Drosophila $G_\alpha_o$ may regulate microtubule polymerization and organization.

2.1 Introduction

As described previously, many proteins become asymmetrically localized as the PCP mechanism proceeds in the Drosophila wing. Shimada et al. (2006) proposed that the redistribution of Fz and Fmi occurs through trafficking on intracellular vesicles along elements of the apical microtubule web (Eaton, et al., 1996; Shimada, et al., 2006). Recently, the same group showed that a small but significant subset of non-centrosomal microtubules (MT) bearing +ends in the proximal region, which align along the proximal-distal axis, (that occurs at the time of redistribution of the core PCP proteins to generate an asymmetry in MT growth), does so via the activation of atypical cadherins; dachsous and fat, and are in part mediated by PAR-1 (Harumoto et al., 2010).
One can infer the same machinery may also be utilized for the remaining components of the PCP proteins. For example, \( \text{G} \alpha \text{o} \) also becomes localized to the proximal ends of wing cells, and in mammalian PC12 (neuronal cells) \( \text{G} \alpha \text{o} \) colocalizes with MTs (Sarma, et al., 2003). Further, the authors demonstrated that re-localization of \( \text{G} \alpha \text{o} \) to newly forming processes in PC12 cells could be reversibly blocked by nocodazole (a MT depolymerizing drug), which implies that \( \text{G} \alpha \text{o} \) re-localization in these cells depends on the integrity of MTs. Thus it is likely that \( \text{G} \alpha \text{o} \) is trafficked to its proximal position in \textit{Drosophila} wing cells by the apical MT network.

Many mammalian G alpha subunits directly bind Tubulin polymers and dimers (N. Wang, Yan, & Rasenick, 1990; H. C. Wu, et al., 2001), which are the building block of MTs. Among the \( \text{G} \alpha \) family of proteins, \( \text{G} \alpha \text{i1} \) (inhibitory G-protein subunit of adenylyl cyclase) as well as \( \text{G} \alpha \text{s} \) (stimulatory G-protein subunit of adenylyl cyclase) have been shown to bind with high affinity to Tubulin (Rasenick, et al., 1981; N. Wang, et al., 1990). In contrast, other classes such as \( \text{G} \alpha \text{t} \), show no binding (N. Wang, et al., 1990). Our laboratory has also shown that \textit{Drosophila} \( \text{G} \alpha \text{o} \) can bind Tubulin as well (Katanaev & Tomlinson, 2006b). Binding of \( \text{G} \alpha \text{o} \) to Tubulin has been also been shown to stimulate GTP hydrolysis of \( \beta \)-Tubulin – removing the GTP cap and triggering rapid de-polymerization (Roychowdhury, Panda, Wilson, & Rasenick, 1999). When \textit{Drosophila} \( \text{G} \alpha \text{o}-\text{GTP} \) was over-expressed in neuroblasts, the mitotic spindle was shown to dramatically reduce in size (Katanaev & Tomlinson, 2006b), which suggests a possible role for a MT depolymerizing activity for \textit{Drosophila} \( \text{G} \alpha \text{o} \). Furthermore, GTP-loaded \( \text{G} \alpha \text{o} \) pulled down Tubulin from fly extracts, as did a peptide of the ten C-terminal amino acids of the \( \text{G} \alpha \text{o} \) protein (Katanaev & Tomlinson, 2006b). Given these latter direct interactions, and the indications that \( \text{G} \alpha \text{o} \) may regulate Tubulin de-polymerization, we suspect that \( \text{G} \alpha \text{o} \) may not simply be passively trafficked along MT, but may also be active in organizing these structures. Using the chimeric receptors (made in the previous section), we hoped to investigate the relationships between \( \text{G} \alpha \text{o} \) and the MT cytoskeleton in vivo. Failing to make the system work, I attempted, without success, a cellular approach utilizing Schneider (S2) cells and sought to visualize any cytoskeletal reorganization that might take place, by aggregating cells, which separately express the ligand and the chimeric receptors.
2.2 Materials and Methods

Constructs
Generations of the UAS-sev constructs were described in the previous section. The boss gene was cloned in as an Asp 718 to XbaI fragment into the UAS-AttB vector (Bischof, et al., 2007). The heat-shock Gal4 (hs-G4) construct was kindly provided by T.Lieber.

Cell Culture
S2 cells were cultured and grown at room temperature in Schneider medium (Invitrogen) supplemented with glutamate, 10% fetal bovine serum, 5mg/ml penicillin-streptomycin, and 2.5mg/ml Bacto Peptone.

Cell Aggregation assay
S2 cells were transiently co-transfected with UAS constructs and hs-G4, using Cellfectin according to the manufacture’s protocol (Invitrogen). Expression from UAS-AttB vectors was induced by transiently co-transfecting S2 cells with the following constructs: hs-G4, UAS-boss; hs-G4, UAS-sev-gfp; hs-G4, UAS-sev-gfp-\(G_\alpha\)o-wt; hs-G4, UAS-sev-gfp-\(G_\alpha\)oGTP; hs-G4, UAS-sev-gfp-fz. Then the cells were incubated at room temperature, given a brief heat pulse (37°C for 30min.), and were allowed to recover at room temperature for 2 hours. Aggregation, of the sev and boss expressing cells was performed by following the original sev/boss aggregation study (Kramer, et al., 1991).

Immunostaining
For fixation and staining, aggregates were allowed to settle in concavalin A (Con A) coated coverslips. In order to facilitate attachment of the cells to the coverslips for immunofluorescence, I treated them with Con A, according to Rogers, et al. (2002). The spherical nature of S2 cells is not ideal for microscopy, and with con A treatment, the cells acquire a more flattened morphology, which provides for better visualization of the cytoskeleton. The cells were then fixed at −20°C with a 90% methanol, 3.2% formaldehyde, blocked with 10% normal donkey serum in PBST (0.1% Triton X-100 in PBS) and incubated with 1:200 anti-Boss, 1:1000 anti-\(\alpha\)-Tubulin (DM1\(\alpha\), Sigma, or YL, Serotec) at 4°C overnight. Cells were incubated with secondary antibodies (AlexaFluor-488, AlexaFluor-555 all at a 1:500 dilution,
Invitrogen) for 1 hour washed in PBST and mounted in ProLong Gold with DAPI mounting media (Invitrogen) to stain DNA.

2.3 Results

The initial goal here was to test in-vivo whether the polarity of the wing cells could be changed by directing various PCP proteins to inappropriate sides of the cells. If this occurred, and if the apical MT cytoskeleton is critically required, then in the repolarized cells a correspondingly repolarized MT network should be observed. For example, if localizing G\(\alpha\)o to the anterior (rather than the proximal) side of the cell is sufficient to direct the hair out growth to the posterior (rather than the distal) side, we wondered whether the MTs would be oriented to the anterior (G\(\alpha\)o) side, and most of the (+) end-MTs to the posterior side.

Given that the transformants from the previous section could not be used for this study, I chose to address the interaction between G\(\alpha\)o and the cytoskeleton by initiating aggregation assays with Drosophila cells. Specifically, adhesion of cultured S2 cells, expressing either UAS-boss or the various UAS-sev-chimeras (under conditional control by hs-G4), was performed to test whether cytoskeletal changes (MT or actin) that might occur with the binding of UAS-boss expressing cells to cells expressing UAS-sev-G\(\alpha\)o. (Figure 49A, A') Unfortunately, while I was able to perform the aggregation assay (Figure 49B-C), it was difficult to determine if there were any true cytoskeletal changes occurring. (Figure 50-51)

There were several technical issues. For instance, I could see the sharp border between the binding of the boss-expressing cells with the sev-expressing cells, which indicated that the aggregation had worked. Moreover, just as in the original studies (Kramer, et al., 1991), I observed punctae within the sev-expressing cells that appeared to be internalization of Boss into endocytic vesicles within the sev-cells. However, when I looked at the cytoskeleton in the sev-chimera expressing cells (delineated by the GFP-expressing cells), it was very difficult to visualize the cytoskeletal structures. This was in part because only the cells that touched the bottom of the coverslips within an aggregate flattened, while the remaining aggregate stayed mainly spherical in morphology. The relatively small size of the cells also proved to be somewhat of a detriment.
In order to look at MT reorganization specifically, I used two separate antibodies to look at both stable and dynamic MT. Our rationale was that if G\(_{\alpha_o}\) has any impact on MT reorganization, that it might impact specifically dynamic MT. However, S2 cells also have an unusual or greater number of stable MT, which might hamper the ability to draw any conclusions, using this approach.

2.4 Discussion

The experiments were ultimately abandoned because of the various technical difficulties. Nevertheless, the question of the relationship between G\(_{\alpha_o}\) and MT dynamics and how it may contribute to the asymmetric localization of PCP proteins remains valid and relevant. Clearly, other approaches need to be employed to address this, such as using electron microscopy or utilizing specifically epithelial cells from another animal system.

3. Candidate Screen

Abstract

Boss is the membrane-bound ligand for Sevenless (Reinke & Zipursky, 1988). But it possesses a heptahelical structure that appears in general topology similar to a GPCR (Hart, et al., 1990). Overexpression of boss in the wing robustly induced a multiple wing hair phenotype, in which each cell produced a clump of disordered hairs. This mwh phenotype is observed at a low frequency in fz and dsh mutants, and when G\(_{\alpha_o}\) is over-expressed, and is strongly associated with downstream effectors of the PCP pathway such as fuzzy, inturned and multiple wing hairs (Collier & Gubb, 1997; Wong & Adler, 1993; Yan et al., 2008). Given that boss overexpression phenocopied the mwh phenotype, and since Boss may act as G-protein linked receptor, we reasoned that although Boss is not normally involved in PCP, its over-expression may inappropriately activate a downstream trimeric G protein pathway that is closely linked to PCP. We therefore sought to detect any proteins that mediated the effects of this Boss overexpression.
3.1 Introduction

Despite a growing body of information on PCP including components of the pathway, additional modulators of the pathway tying the core group to the downstream genes remain to be identified. In order to fully understand, appreciate, and attain a complete picture of the Fz/PCP signaling pathway, further identification of additional components that are active in the pathway is required. Therefore, to effectively isolate additional genes, including redundant ones that are difficult to identify with traditional screens (such as with loss of function screens or with ethyl methane sulfonate mutagenesis), I employed a novel and alternative screening strategy, using forward genetics. The approach undertaken permitted me to directly target the wing tissue by utilizing specific Gal4 drivers, and enabled me to bypass any early lethal effects. Other instances where an overexpression approach was effectively utilized to screen for new components of the PCP pathway include the identification of the core PCP gene diego (Feiguin, et al., 2001) and more recently dGIPC (Drosophila GAIP interacting protein, C terminus) (Djiane & Mlodzik, 2010).

The adult wing provided a convenient screening tool to initiate the screen, since any alteration can be readily scored. In addition to the uniform arrangement of the hairs described previously, the wing also has specific landmarks where the veins join the margin, as well as where they intersect, thereby making any perturbations readily visible.

Although Boss is not thought to be involved in PCP, its overexpression causes a robust mwh phenotype. (Figure 52) Since Boss might act as a GPCR, we hypothesized that high levels of Boss may inappropriately activate a trimeric G-protein pathway to induce the mwh phenotype. We therefore performed genetic screens to identify the proteins that may lie downstream of Boss in this pathway.

3.2 Materials and Methods

**Drosophila strains and Maintenance**

The UAS-boss stock was generated in the laboratory by injecting the construct into AttB J5 [86Fb] embryos. All stocks utilized in the screen (as listed by the CG numbers) were obtained from Bloomington stock center. MS1096-G4 was obtained from M. Zecca. For the RNAi experiments, the following stocks
were used: MS1096-G4;UAS-dicer2 and UAS-dicer2;en-G4 (from Bloomington), and all RNAi lines were obtained from the TRiP at Harvard Medical School.

**Constructs**

Generation of the UAS-boss construct was described in the previous section.

**Histology**

Histological preparation of the adult wings was performed as described.

### 3.3 Results

These screens were initiated to quickly generate any novel components of the PCP pathway. Had I failed to identify any new genes, I would have performed a thorough and unbiased screen. However, as it turned out, I was able to identify at least one gene that could potentially participate in the pathway. I performed the screen in the sensitized boss background by selecting putative candidates according to certain criteria. Since Boss is a potential G-protein coupled receptor, I began with proteins that are known to be associated with trimeric G-proteins and their downstream effectors. In addition, I examined genes associated with both PCP and those associated with cytoskeletal organization.

Candidate genes were examined with whatever tools were available, including the following: over expression constructs, dominant negative constructs, RNAi lines, and mutations in the gene themselves. A summary of the lines screened is shown in Table 39. The wings were subsequently screened for any changes in the mwh phenotype. However, if the resulting crosses exhibited wing phenotypes other than mwh, such as venation defects, changes in wing morphology, they were also noted.

Among my findings here was the identification of a Gαf RNAi line (TRiP UAS-Gαf RNAi). This decreased the mwh of the ectopically expressed boss, and also showed other interesting features. For example, when driven by en-G4, in an otherwise wild type wing, the Gαf RNAi line induced moderate polarity defects. These included orientation and mwh phenotypes, as well as prominent venation defects and occasional wing notching (a loss of the wing margin). Since the driver line here was en-G4, all these defects were restricted to the posterior compartment. When MS1096-G4 (which drives expression
throughout the wing blade) was used, polarity defects were again observed. Additionally, notable defects in the anterior wing margin bristles were observed. These observations led to the major work contained within this thesis.

3.4 Discussion

One of the limitations of having performed a non-systematic (biased) screen with a defined set of genes is that many relevant genes are likely to have been missed. Nevertheless, together with the RNAi screen, as well as exploiting the ectopic boss genotype (MS1096-G4 > UAS-boss), I obtained one gene (Gαf) that might be implicated in the planar pathway. The mwh phenotype alone was not affected. I also observed changes in wing venation, some polarity defects in the hairs, which could implicate this gene in other pathways. Further and preliminary characterization of Gαf is described in the main body of this thesis.

As previously noted, Boss is an orphan receptor that bears a 7-TM topology. Among its known function as a ligand for the Sev receptor, it has also been implicated in functioning as a glucose responding receptor in metabolic regulation (Kohyama-Koganeya, et al., 2008) in Drosophila. Indeed, in a reporter gene assay Kohyama-Koganeya et al. (2008) demonstrated that Boss can respond to glucose, suggesting that this occurs through a heterotrimeric G-protein, specifically through Gαq to stimulate calcium release.

Here, by creating an artificial system by overexpressing boss in the wing, we might have also uncovered its potential role as a possible GPCR, wherein Gαf may be involved. Knockdown of Gαf appeared to reduce our mwh phenotype, which suggests that the two may interact. Further experiments such as overexpression of both boss and Gαf, and overexpression of Gαf in a mutant background for boss would have to be performed to begin to address if Gαf might be a trimeric G-protein candidate for Boss.
Chapter III

Figures and Tables
Figure 44. Cross-section through developing ommatidia. (A) Cross-section at the level of the adherens-type junction. Photoreceptors are numbered. At the early symmetrical cluster stage, staining occurs in R7, R3, and R4 and is localized in each of these cells at their points of contact with R8, shown by dark staining on the left and in yellow in the cartoon on the right. (Adapted from Tomlinson, et al., 1987). (B) Cartoon of the developing ommatidia. The capping of Sevenless protein to Boss protein is shown in yellow.
Figure 45. Chimeric proteins bearing the extracellular domain of Sev fused to individual PCP proteins. (A-D) The extracellular domain of the Sev protein, in addition to its transmembrane domain, was fused directly to GFP (which also harbors an HA epitope tag). The proteins of interest were attached to the C-terminus of GFP. (A) Control. (B) Full length Gαo (wild type, GDP, GTP versions were made). (C) Frizzled. Fz sequences beginning immediately after its first transmembrane domain was fused. (D) Full length Dishevelled.
Figure 46. Initial Strategy. (A) Schematic of initial Strategy: drive UAS-sev chimeras in posterior compartment with hh-G4, and co-express blink.boss in the same wing. (B-B’) Cross-section of a wild-type eye (adapted from Tomlinson & Struhl, 1999). Shown are the R1-R7 photoreceptors. These bear a trapezoidal shape. (C-C’) When the sev or boss gene is removed, the eyes adopt the same phenotype: R7 photoreceptor is missing (adapted from Reinke & Zipursky, 1988). We expected our chimeras to adopt the same phenotype. (D-D’) Cross-sections of sev-G4 > UAS-sev-gfp-Gαo* eyes. (D) Ectopic expression of one copy of UAS-sev-gfp-Gαo* has a dominant negative effect. The R7 photoreceptor is missing, as in (C-C’). (D’) Two copies of UAS-sev-gfp-Gαo* shows symmetric ommatidia which is reminiscent of planar cell polarity phenotype in the eye.
Figure 47. *blink.boss* co-expression with *hh > UAS-sev-gfp-Gαo*+. (A-C) Third instar wing discs. (A) *hh > UAS-sev-gfp-Gαo*+ in green, expressed in the posterior compartment. (B) *blink.boss* (red) is expressed only as a small strip in the anterior compartment, and is not expressed along the entire length of the disc as expected. (C-C‴) Co-expression of the two constructs show that the two types of cells (sev-chimera and Boss expressing cells) do not abut. (C) Entire wing disc is shown. (C′) Cells within the wing blade appear to be in close proximity. However, in (C″-C‴) inset from C′, closer inspection reveals only two cells appear to abut.
Figure 48. **ci.LexA used to drive LexO.boss.** (A) Schematic of Strategy: drive UAS-sev-chimeras in posterior compartment with *hh-G4*, co-expressed with *ci.LexO* to drive *boss* in the anterior compartment of the wing. (B-B’) Third instar larval disc expressing *hh > UAS-sev-gfp-Gαo*, *ci.LexA > LexO.boss*. Gαo is shown in green and Boss is in red. (B’) The area where the cells appear to abut corresponds to the hinge of the wing disc.
Figure 49. Aggregation of Boss- and Sev-chimera – expressing S2 cells. (A-A’) Cartoon of aggregates. (A) hs >boss-expressing cell (red) aggregating to a control sev-gfp-expressing cell (green). (A’) hs > boss -expressing cell (red) aggregating to a sev-gfp-fz-expressing cell (green). (B-B”) Control cells. Aggregation is observed. Punctae (red) from internalization of Boss within the sev-gfp-expressing cell can be observed (green). (C-C”) Aggregation of hs-G4 boss-expressing cells with one of the experimental constructs, sev-gfp-fz (green) is shown.
Figure 50. S2 cells expressing Sev-chimera stained for acetylated Tubulin. (A-C) Individual cells fixed with Con A shows clear staining for microtubules. (A-A’) S2 cell expressing UAS-gfp-fz. (A’) Higher magnification. (B-B’) S2 cell expressing UAS-gfp-fz, counterstained with antibody against acetylated Tubulin to observe stable MTs. (B’) Higher magnification. (C-C’) Merge shows that Fz (green) and Tubulin (red) are clearly visible when the cells are fixed individually.
Figure 51. Aggregates stained for Tubulin. (A-D’) S2 cell aggregates expressing Boss and sev-gfp-fz. (A) hs > sev-gfp-fz expressing cells. (B) hs > boss expressing cells. (C) Staining for acetylated tubulin, which delineates stable MT. (D) Merge. When aggregates occur, it is difficult to make the entire aggregate adhere and flatten on to the slide, making staining of MT difficult. (D’) Magnification of (D). I could not observe nor determine any effects on the MT by the chimeras.
Figure 52. Ectopic expression of boss causes a mwh phenotype. (A) Adult wing of MS1096 > UAS-boss. Venation defects can be observed in both the anterior and posterior compartments. (A’) Multiple wing hairs can be observed. The hairs appear as punctae (arrows) due to the focal plane of the picture. Each puncta represents a hair extruding from the surface of the wing. There is an increase in mwh with an increase in boss overexpression. Here two copies were driven with MS1096-G4. More than 4-5 hairs can be observed with each mwh.
Table 39. Candidate genes that were screened

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indicates: did not have the stocks

RNAi controls: UAS-RNAi lines driven only with MS1096-G4 or en-G4 to ascertain whether knockdown of the transcripts alone in the wing gave a phenotype.
Chapter IV

General Discussion
“Ultimately, scientists will compile a wiring diagram of the plasma membrane in a myriad cell types of the human organism. For each, they will know how dozens of types of receptors, G proteins and effectors are connected. And they will be able to predict how the cells will operate in response to any combination of signals. As one wag has said, for those who would hope to develop therapies, such discoveries would be like giving a thief a wiring diagram to the alarm system at a bank.”

-Alfred Gilman, 1992

The fluid flow of information in cell communication occurs with the aid of key players, such as G-proteins, which coordinate cellular responses to an array of signals. In G-protein regulated signaling, there are a relatively modest number of components that can be tailored to achieve a level of complexity, due to convergence and divergence at every level. Multiple receptors may converge to activate a single type of G-protein, or a single specific receptor might interact with multiple G-protein subfamilies to trigger various signaling mechanisms. Likewise, a single G-protein may act on multiple effectors or different G-proteins may instruct a single effector and act on it either additively or synergistically. Where Sutherland and his successors laid the foundation for the study of G-protein signaling with the discovery of cAMP, others have followed suit over the ensuing decades and continued their efforts in different organisms to enrich our knowledge in identifying these components as well as the various interacting factors.

Indeed, the study of G-proteins has grown exponentially since the early investigations of Gilman and Rodman and the actual birth of the concept of G-proteins. Since then scientists have been intrigued to investigate the multitude of areas where G-proteins play a role, including their function in development. However, relatively little is known about the fifth class of G-alpha protein in the D. Melanogaster that was isolated and described almost two decades ago. Whereas four classes of G-alpha proteins are generally discussed: 1) Gαs, 2) Gαi/Gαo, 3) Gαq 4) Gα12/13, in the Drosophila five classes are recognized.

Here, in this thesis, I have provided evidence to show that Gαf does fit the requisite characteristics of a Gα protein (described in Chapter I), and that the original authors (Quan, et al., 1993) who made its discovery were justified in placing Gαf under a separate class (Figure 6). With the availability of RNAi lines targeted against this gene, as there are not any mutants currently present, I was able to commence preliminary characterization of this protein, which I described in Chapter II.
In Chapter III, I provided a cursory description of another project, involving a different G-protein, Gαo. There, I described my unsuccessful attempts to initiate multiple strategies (over the course of many years) to test whether subcellular localization of Gαo is critical in a specific developmental phenomenon known as planar cell polarity. Due to technical difficulties, that project failed to get off the ground. Thus, overall, my efforts as a graduate student were undertaken to better grasp the role of G-proteins in developmental functions in the context of the fly.

Heterotrimeric G-proteins play a key role in transducing signals from cell surface receptors (7-TM receptors) to effector proteins. Receptors that activate G-proteins are extensive and are widely utilized in nature to regulate processes ranging from perception to cell growth. However, whether heptahelical receptors (such as with Fz and Smo) that play a role in development are G-protein linked, to mediate developmental signals, has long been in dispute. With my efforts on Gαf, I was not able to obtain convincing data as to whether Gαf couples to any 7-TM receptors to transduce any specific signal.

Surprisingly, my studies in the fly wing uncovered a novel gene participating in the asymmetric cell division in the fly, which suggests that in other organisms additional G-proteins may play a similar role that have yet to be discovered. In addition, my studies show that this G alpha subunit, Gαf, appears to play redundant role to Gαi in this phenomenon.

Furthermore, the dramatic phenotypes seen with downregulation of Gαf in the wing, using the TRiP RNAi line against Gαf, underline the importance of this G-protein in controlling and regulation of patterning and development, as well as possibly any other unanticipated physiological processes. As study of this protein is still in its infancy, many outstanding questions remain to be addressed. In this thesis, I began to explore whether Gαf participates three specific signaling pathways (Wg, PCP/Fz and Hh). Aside from the Fz/PCP pathway, I was unable to conclusively rule out whether Gαf participates directly in any of these signaling pathways, and those questions still need to be resolved.
**Wnt/Wg pathway**

While I was able to obtain some compelling data for a role for Gαf in the Wg pathway, I was unsuccessful in determining definitively if Gαf participates in Wg transduction. Thus, to resolve this, I can employ a tissue culture assay designed to test TCF transcriptional activity and therefore analyze Wnt/Wg signaling activity (Molenaar et al., 1996). This assay uses the TOPFLASH (TCF optimal promoter) reporter, which harbors a minimal promoter bearing multimerized TCF-binding sites to drive the expression of a cDNA encoding the firefly luciferase gene. By using the standard HEK293 cells or *Drosophila* imaginal disc-derived clone 8 cells, transfected with constructs encoding Gαf, as well TOPFLASH reporter bearing eight consensus TCF binding sites, or FOPFLASH with mutant TCF binding sites, and an expression vector encoding Wg to stimulate the pathway in the presence or absence of Gαf, one can measure the activity of the Wg signaling pathway. I would expect that if Gαf is a positive regulator of the pathway, that luciferase expression would be low or undetectable in cells transfected with the reporter or plasmid alone (Gαf singly, for example). However, I would expect robust expression with the Wg-encoding plasmid when transfected together with the Gαf -encoding plasmid, which would be reduced in the presence of FOPFLASH (with defective TCF binding sites). Therefore, using this in vitro approach could provide clear molecular evidence as to whether Gαf levels can affect TCF activity.

**TGfβ/Dpp pathway**

Additionally, varying venation phenotypes were observed both with knockdown and overexpression of Gαf. With Gαf RNAi, I observed both loss and ectopic venation defects, while overexpressed Gαf had increased venation formation in the adult wing. To decipher the nature of this phenotype, I began by exploring the TGfβ/Dpp pathway, as mutations in this pathway result in distinct adult wing phenotypes such as vein patterning defects (Blair, 2007; de Celis, 1997; K. Yu et al., 1996). That is, mutants of the components of the Dpp signaling pathway at various levels from the ligand, receptor to the transcription factor have been shown to be necessary for vein formation and wing growth (de Celis, 1997; Eivers et al., 2009; Sander, et al., 2010). Conversely, overexpression of members of the pathway (such as the ligand Dpp or the degradation resistant Mad transcription factor) produces ectopic
vein formation (de Celis, 1997; Eivers, et al., 2009). Moreover, modulation of this signaling pathway in the wing has been shown to produce wg-like phenotypes (Eivers, et al., 2009; Zeng, et al., 2008). While I did observe downregulation of Wg targets, such as dll and vg with reduction of Gαf in the wing that is indicative of direct perturbations in Wg signaling, the possibility of a cross-talk between Wnt/Wg and TGFβ/Dpp signaling should not be disregarded, as this phenomenon has been described in various systems (Eivers, et al., 2009; X. Yu, et al., 1996). Ectopic Dpp signaling in the wing pouch does not affect wg expression, but it can cause phenotypes that are consistent with loss of Wg signaling phenotype, such as wing notching (Bennett & Alphey, 2002; Zeng, et al., 2008). Thus, overall it is conceivable that Gαf may serve a unique role that is receptor-independent (of a GCPR) to function in this particular signaling pathway. Further, given the observation of such prominent phenotypes, and as the Spalt staining with knockdown of Gαf were inconclusive, other approaches need to be evaluated in order to exclude a role for Gαf in the Dpp pathway. It will be key to perform immunostaining with other markers of the Dpp pathway on the wing discs of flies harboring two copies of UAS-Gαf (all variants), where conspicuous venation defects and narrowing of wings (possibly due to an increase in Dpp signaling) along the AP compartment were observed. In addition, experiments to show that mutants of the Dpp signaling pathway (such as dpp itself) can be compensated for, by overexpression of Gαf, would provide compelling data to implicate Gαf in Dpp signaling.

Test direct interaction of Gαf with 7-TM receptors

As the genetic experiments in Chapter II yielded less than definitive results, taking a biochemical approach to test whether the Gαf protein may couple to any of the mentioned 7-TM receptors (the two Frizzleds and Smo) might be more promising. Sites where heterotrimeric G-proteins have been shown to bind to GPCRs include the cytoplasmic third intracellular loop (I3) and carboxy-tail (CT) of the heptahelical receptors (Kostenis, Conklin et al., 1997; Hamm et al., 1988). To this end, I would generate recombinant fusion polypeptides of glutathione S-transferase with truncated forms of the 7-TM receptor (containing the I3 segment or of the segment from the CT), to determine which receptor segment (if any) bound to an epitope-tagged Gαf in vitro.
Role of $\text{G}_\alpha f$ into Asymmetric Cell Division

My results, as evidenced by the perturbations in bristle formation in the wing margin (both with overexpression of $\text{UAS-}G\alpha f$ as well as with TRiP $\text{UAS-}G\alpha f$ RNAi), suggest that $G\alpha f$ plays a role in determining daughter cell fates, in the external sense organ lineage. Interestingly, while I observed striking phenotypes in the adult wing with only expression of one copy of the TRiP $\text{UAS-}G\alpha f$ RNAi line, it took two copies of this line to observe any effects at the larval stage. These results imply that $G\alpha f$ may function after the larval stage, during pupariation, when metamorphosis takes place. Apart from the final divisions that take place in the wing, cell and tissue changes occur, allowing for the development of adult structures. These changes include the development of bristles and sockets from precursor cells. If $G\alpha f$ does function at this latter stage, it would be in agreement with the results related to the anterior margin defects observed with knockdown of the $Gaf$ transcript as well as ectopic $Gaf$ expression.

My in vivo genetic analyses and overexpression studies with $\text{UAS-}G\alpha f$ support a role for $G\alpha f$ in asymmetric cell division in SOP cells. (Figure 37; Appendix Figure 6) How this occurs will need to be explored in greater detail in the future. The genetic studies with $Gai$ also suggest that $Gaf$ plays a redundant role to $Gai$ in ACD in SOP cells. In contrast, the role of $Gao$ in relation to $Gaf$ has yet to be ascribed, since the RNAi lines I was working with did not appear to be potently downregulating $Gao$ transcript levels. Therefore, additional genetic studies (as described in Chapter II) will need to be undertaken to determine the role of $Gao$ relative to the expression of $Gaf$, and thereby establish if one inhibits the activity of the other or whether there is an additive effect with the expression of both.

Since the results of a role for $Gaf$ in ACD in SOPs cells are preliminary, further studies needs to be initiated to address the additional questions that have been raised by those results, including whether $Gaf$ plays a similar role in ACD in neuroblasts. To begin to address these relevant questions, performing cell biological assays to determine subcellular localization in this mechanism will be key.

In the two well-known manifestations of asymmetric cell divisions in Drosophila (NBs and SOPs) determinants of these cell types invariably localize over one of the two mitotic spindle poles. In NBs, this correlates with epithelial cells that delaminate and divide asymmetrically along the apical-basal axis; while in SOPs, cells divide along the anterior-posterior axis. Thus, the critical question here is whether $Gaf$
also localizes asymmetrically over the mitotic spindle pole? To address this, I would begin by analyzing the distribution of $G_\alpha f$ in SOP cells during pupal development. In interphase, one of the main determinants of ACD, Numb is homogenously distributed around the cell cortex (Frise et al, 1996). However, by metaphase, Numb and $G_\alpha i$ are found at the anterior cell cortex forming a crescent (Schaefer et al. 2001). If $G_\alpha f$ shares this similar function, and therefore feature, I would expect $G_\alpha f$ to colocalize in this area of the cortex; and ultimately, segregating into the same daughter cells as $G_\alpha i$.

Then, to directly test the requirement of $G_\alpha f$ in SOP cells, I would modulate levels of $G_\alpha f$ by using the TRiP UAS-$G_\alpha f$ RNAi line to disrupt $G_\alpha f$ function and observe any corresponding disruption to the determinants of this cell fate, such as Numb or $G_\alpha i$. If $G_\alpha f$ does indeed play a critical role in the cell fate transformation of the bristle lineage, as implicated by the defects of the stout bristles in the adult wing margin, I would expect misoriented spindles in the SOP cells that have reduced $G_\alpha f$, as well as loss of asymmetric localization of $G_\alpha f$ and that of other cell fate determinants.

I would also closely examine the cluster of cells in pupae that will give rise to the SOPs by using cell type markers to study this four-cell cluster: Senseless to delineate all four markers; Propero for sheath; Elav for neurons; and Su(H) for socket cells. (Figure 7) For example, since I frequently observed duplication of external cells at the expense of the internal cells when TRiP UAS-$G_\alpha f$ RNAi line was expressed in the wing, I would expect to see corresponding staining with Senseless of the four-cell cluster, which lack any of the internal cell markers when co-labeled with Elav or Prospero.

Following this, I would test the variants of $G_\alpha f$ to determine whether $G_\alpha f$ function involves the GTP-bound form of $G_\alpha f$ by ectopically expressing the three variants (wild type, GTP and GDP forms). I would subsequently repeat the same set of experiments to test if $G_\alpha f$ functions similarly to $G_\alpha i$ in neuroblasts by examining embryos (expressing either TRiP UAS-$Gf$-RNAi or UAS- $G_\alpha f$ transgenes with scabrous-G4). For example, $G_\alpha i$ mutant NBs generate two approximately equal size daughter cells (F. Yu et al., 2003). If $G_\alpha f$ truly plays a role redundant to that of $G_\alpha i$ in ACD, I would begin by assessing whether depletion of $G_\alpha f$ transcript in the embryo induces the same phenotype.
**Perspective on Gαf and Gαi**

In my studies Gαf appears to play a role in ACD and a possible, though undetermined, role in Hh signaling via an interaction with the Smo receptor. Interestingly, these are two mechanisms where the trimeric G-protein subunit, Gαi, has also been described (Schaefer et al. 2001; Ogden et al. 2008). In this thesis, I have implicated Gαf as playing a redundant role to Gαi, at least ACD of SOP cells. How it is that the proteins encoded by Gαf and Gαi might play redundant roles has yet to be examined.

While the sequence and phylogenetic analyses indicate Gαf protein to be a unique G alpha subunit, it bears two features strikingly similar to that of the Gαi protein. First, BLAST analysis using the CDD of NCBI indicates that it possesses a putative GoLoco Binding domain (Appendix Figure 5). In the neuroblast, activators of G-protein signaling, such as Pins, possess this domain and have been shown to bind to GDP-bound heterotrimeric Gαi/o-subunits that also contain this domain (Siderovski et al., 1999). GoLoco domain bearing proteins bind to Gα subunits and trigger the dissociation of βγ-subunits without requiring receptor activation or extracellular signals (Kimple et al., 2002), thereby acting as GDIs. In *Drosophila*, both Gαi and Gαo have been shown to bind Pins to mediate mitotic spindle orientation of NBs (F. Yu et al., 2002; Kopein and Katanaev, 2009). Curiously, if Gαf does possess this domain, it would underscore my preliminary finding and hypothesis for Gαf in ACD, as well as serving a redundant function to Gαi. However, ultimately, biochemical evidence will be needed to validate an interaction between Gαf and Pins.

Another feature where Gαf resembles Gαi is at its extreme C-terminus. (Appendix Figure 2, 3) Indeed, that Gαf appears to play a role in Hh signaling, albeit with perplexing results, might be attributed to this sequence, where the three terminal amino acids are identical. In addition, the two amino acids present on both proteins, preceeding the three identical amino acids at the extreme C-terminus, share the same properties. Importantly, one of the key regions of the G alpha subunit implicated in receptor interaction is the extreme C-terminus (Conklin, et al., 1993; Hamm et al., 1988; Sullivan et al., 1987; Masters et al., 1988). Taken together, it is perhaps not all that surprising that Gαf may also play a role in Hh signaling by interacting with the Smo receptor, as has been implicated with Gαi (Ogden et al., 2008).
Role of Gαf in Endocytosis

While it is well established that trimeric G-proteins function in signal transduction across the cell membrane, G-proteins have also been shown to localize to intracellular membranes and participate in the regulation of vesicle formation within the secretory and endocytic pathways (Colombo, et al., 1992; Leyte, et al., 1992; Nurnberg & Ahnert-Hilger, 1996). Indeed, evidence obtained with mammalian systems suggest that two heterotrimeric G-protein subunits, Gαs and Gαi, alter endocytosis and endosome trafficking by controlling coat assembly (Helms, 1995; Ktistakis, et al., 1992; H. C. Lin, et al., 1998; Van Dyke, 2000). Gαi has been shown in polarized cells to direct transport of newly synthesized proteins to the basolateral membrane, while Gαs to the apical plasma membrane (Pimplikar & Simons, 1993).

For many proteins endocytosis is a means of transport from one location to another. However, endocytosis is also a mechanism for cells to maintain transmembrane protein homeostasis, such as sequestering heptahelical receptors from the cell surface. This involves internalization of the portions of the plasma membrane into carrier vesicles. Multiple routes of internalization and sequestration of receptors exist in cells, which differ in their requirements of cellular machinery (the differences lie according to the size and composition of the proto-vesicle coat: clathrin, nonclathrin, caveolae and macropinosome) (Lamaze & Schmid, 1995). Most of these routes lead to fusion of vesicles to what is referred to as an early endosome that is mediated by a small GTPase, Rab 5 (Bucci et al., 1992; Gorvel, et al., 1991), which serves as a docking station where cargoes are sorted for recycling back to the membrane or sent for degradation by the lysosome.

My genetic studies with Smo, for instance, implicated a possible role for Gαf in Hh signaling. However, those experiments in Chapter II did not yield satisfactory and clear results, which led to the idea of a potential role for Gαf in a more basic signaling mechanism, such as endocytosis, whereby Gαf may play a role in sequestering the hepathelical receptor or bringing it to the membrane.

Thus, one means of probing whether Gαf is involved in endocytosis is via direct visualization of the protein by confocal microscopy. The subcellular localization of Gαf first needs to be determined, using flies that express (or cells over-expressing) an epitope tagged- Gαf (if an antibody against Gαf remains unavailable). Following that, the localization of Gαf should be compared with markers of the
endocytic pathway. If $G_{\alpha}f$ appears to be localized in the cytoplasm in addition to the plasma membrane, it would be prudent to double stain organelles such as Rab5 to see if $G_{\alpha}f$ colocalizes with the early endosome, for instance, or other with markers, such as Rab11 (recycling endosome marker) (Dollar, et al., 2002). Therefore, studies to illuminate whether $G_{\alpha}f$ associates with endosomes can be carried about via immunohistochemistry, which can be further corroborated biochemically through fractionation studies.

**Other 7-TM receptors**

*Lys-Asp-Glu-Leu (KDEL)*

The nature of putative regulators of $G_{\alpha}f$ is highly speculative at this juncture, and accordingly it can only be hypothesized what factors may be involved upstream in its regulation. If $G_{\alpha}f$ is indeed associated with an organelle outside of the plasma membrane, it might be regulated by cytosolic proteins or by membranous factors that bear resemblance to heptahelical cell surface receptors. The KDEL receptor, for instance, is a 7-TM protein that resides temporarily in the cis-Golgi spanning the lipid bilayer and chaperones back to the endoplasmic reticulum (ER) in transport vesicles, and may conceivably couple to heterotrimeric G-proteins (Townsley, et al., 1993). Its primary role is to bind proteins bearing the KDEL recognition sequence and mediate their return from the Golgi to the ER (Semenza, et al., 1990), where folding of newly synthesized secretory and membrane proteins occurs. If $G_{\alpha}f$, for instance, plays a more basic role in the fidelity of cellular functions, it may be associated with the secretory pathway and could couple to this putative GPCR.

*Bride of Sevenless*

Another heptahelical receptor that is worth consideration for investigation as a potential cognate receptor, which $G_{\alpha}f$ may couple to, is Bride of Sevenless. Boss is an atypical seven-transmembrane spanning protein that has been traditionally believed to function only as a ligand for the Sev protein in the Ras/MAPK pathway (Hart, et al., 1990; Reinke & Zipursky, 1988). However, more recent work by Kohyama-Koganei, et al. (2008) revealed Boss to be expressed in fat bodies, in addition to the known
Drosophila eye. This unexpected finding and results of their studies has implicated Boss as a potential receptor, serving a role as a glucose sensor in metabolism in the Insulin/TOR pathway.

In the lab, when boss was overexpressed ubiquitously in the fly, with either the tubulin-G4 or actin-G4 driver, the flies failed to emerge. They developed until the pupal stage but did not eclose. When closely examined, the carcasses exhibited many developmental problems, which included the eyes having a ‘burnt’ appearance (they failed to develop completely), and the remainder of the body appeared to have failed to develop entirely as well. Interestingly, flies failed to emerge when the tubulin driver was also used to drive the TRiP UAS-Gαf RNAi line to ubiquitously knockdown the Gαf transcript. These flies did not reach the pupal stage, as with boss. These results suggest that boss and Gαf may play a role in a critical process early in development, or, if speculating further, perhaps in modulation of life span. When I performed the candidate screen (Chapter III), utilizing the Boss overexpression (mwh) phenotype in the wing as my assay, I uncovered Gαf in the screen. In those conditions, I observed a decrease in mwh with a reduction of Gαf transcript. Preliminary overexpression studies where I over-expressed boss with Gαf appeared to show an increase in the observed mwh of boss in the wing. However, those studies would have to be repeated with greater numbers to confirm the observed phenotype. Therefore, whether Gαf plays a role in the Insulin/TOR pathway or whether Boss couples to Gαf and both are critically needed for other developmental processes, are questions that remain to be addressed.

Role of Cabeza (caz) (not a 7-TM receptor)

As I began work on characterizing Gαf, we serendipitously obtained caz mutants from a collaborating laboratory (J. W. Wang, et al., 2011). Caz encodes an RNA binding protein (Stolow & Haynes, 1995) and these mutant flies exhibit defective adult locomotion and decreased life span (J. W. Wang, et al., 2011). We noted that the wings of these mutant flies were nearly identical in phenotype to our flies over-expressing TRiP UAS-Gαf RNAi. The wings of these flies had similar polarity defects as well as mwh, for instance. Given the identical nature of the phenotypes, we suspected that Caz and Gαf proteins may work in the same signaling pathway, but I was unable to pursue this further. I did, however, overexpress caz with boss as well, and I observed what appeared to be robust enhancement of the mwh
phenotype. Therefore, in light of the observations, looking at any possible interaction between Caz and $G\alpha_f$ ought to be deliberated for future studies on $G\alpha_f$.

*Methuselah (Mth)*

A final heptahelical receptor worth deliberating is *methuselah*. *Mth* also has a putative 7-TM architecture, and the mutant fly is characterized by prolonged longevity (Y. J. Lin, et al., 1998). These flies have an extended life span that is 35% longer than that of wild type and are involved in the modulation of stress response (Y. J. Lin, et al., 1998). Whether $G\alpha_f$ has a role in ageing and life span is conjecture at this stage. However, given that the phenotypes of wings from *caz* mutants and $G\alpha_f$ RNAi wings appear to be nearly identical, one may suspect that $G\alpha_f$ may function in a similar mechanism and does so by coupling to a putative GPCR which plays a role in the life span of the *Drosophila*. 
Appendix
Figure 1. Predicted Palmitoylation site(s). CSS-Palm 3.0 was used to determine if Gaf possessed any potential palmitoylation sites, using default parameters (A). The cysteine at position 8 appears to be the most likely candidate for palmitoylation (B).
Figure 2. $G_{\alpha_f}$ is not predicted to be PTX sensitive. $G_{\alpha_f}$ does not possess a cysteine four amino acids from the C-terminus, which is present in $G_{\alpha_o}$. 
Figure 3. Protein sequence alignment of the C-termini of *Drosophila* Gαf, Gαi and Gαo. Shaded regions demonstrate identical residues. Similar, nonidentical residues are also indicated (+).
**Figure 4. Multiple Sequence Alignment of G proteins**

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**Legend:**
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- **Homo sapiens Gs**
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- **Homo sapiens G1f**
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- **C. elegans Gs**
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- **Homo sapiens KRas**
- **Mus musculus Rhab**
- **Homo sapiens Rhab-1**
- **Drosophila Rhab**

**Note:**
- The alignment includes amino acid sequences from various species, focusing on G proteins.
- The sequence data is presented in a multiple sequence alignment format, highlighting conserved and variant regions across the species.

**Additional Information:**
- The alignment is used to study evolutionary relationships and functional conserved regions among G proteins from different species.
- The coordinates and sequence data aligning different proteins are provided for further analysis and comparison.
Figure 4. Multiple Sequence Alignment of G-proteins
**Figure 4. Multiple Sequence Alignment of G-proteins**

Amino acid sequence alignment, with G-protein sequences from a range of vertebrates and invertebrates (*Homo sapiens, Mus musculus, Xenopus laevis, D. melanogaster, C. elegans*). Generated with Clustal 2.0.

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**Figure 5. Conserved Domains.** BLAST analysis using the Conserved Domain Database (Marchler-Bauer et al., 2004) of NCBI indicates the putative domains present in G\(\alpha_f\) protein, including the GoLoco binding site (boxed).
Figure 6. \( G_{af} \) and \( G_{ai} \) overexpression causes defects in the anterior wing margin. Female wings.

(A) Wild type. (B) \( MS1096-G4 > UAS-G_{af}-GDP \). Ectopic bristles are observed (arrow). (C) \( MS1096-G4/+ > UAS-G_{ai} \text{ WT}/+ \). Ectopic bristles emerging from one socket (arrow). (D) \( MS1096-G4/+ > UAS-G_{af} - GDP/+ \). Ectopic bristles emerging from a single socket (arrow), and a gap in the anterior wing margin (bracket) are visible.
Table 1
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Table 3
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References

Adler, P. N., Krasnow, R. E., & Liu, J. (1997). Tissue polarity points from cells that have higher Frizzled levels towards cells that have lower Frizzled levels. *Current biology: CB*, 7(12), 940-949.


