TWINS, THE B/PR55 REGULATORY SUBUNIT OF PROTEIN PHOSPHATASE IIA, IS NECESSARY FOR PHOTORECEPTOR FATE SPECIFICATION AND MORPHOGENETIC FURROW INITIATION IN THE *DROSOPHILA MELANOGASTER* EYE

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The Ras/MAPK pathway plays an essential role in the development and differentiation of eukaryotic organs. During *Drosophila* eye development, the cells of each unit, or ommatidium, of the eye are specified from a field of undifferentiated cells. As the morphogenetic furrow passes across the eye field, the Ras/MAPK pathway is reiteratively activated in cells posterior to the furrow to recruit first the eight photoreceptors, then the four cone cells, and finally the pigment cells. Understanding Ras/MAPK's regulation is essential to knowing how its activation can generate so many cellular outcomes.

The enzyme protein phosphatase 2A (PP2A) is critical for successfully transducing the Ras/MAPK signal. PP2A is composed of three subunits, the associated A and catalytic C subunits compose the core enzyme, and a variable B subunit which regulates the activity of the enzyme. The *Drosophila* gene *twins* encodes the B/PR55 regulatory subunit of PP2A. Mutations in *twins* have previously been shown to affect the development of peripheral sensory organs in the adult fly. Large patches of mechanosensory organs are missing from the thorax of the fly, while those that remain have duplicated bristles and sockets at the expense of the mechanosensors' neuron and sheath cells. Additionally,

notal tissue is sometimes transformed to wing at the posterior edges of the notum.

To better understand the role of *twins* in Ras/MAPK signaling during eye development, a genetic approach utilizing loss of function mutants and ectopic *twins* expression was used. Hypomorphic *twins* mutants were missing R7 photoreceptors, cone cells, and had disorganized pigment cells. Furthermore, these *twins* mutations were found to suppress the $egfr^{E1}$ mutation, a hypermorphic allele of the Ras/MAPK receptor EGFR. Ectopic expression of *twins* was able to rescue the morphogenetic furrow inhibition caused by loss of EGFR function, suggesting that *twins* functions downstream of EGFR in Ras/MAPK signalling. Additionally, *twins* suppressed furrow inhibition caused by ectopic *wg*, but not that caused by the loss of *Notch* activation, supporting *twins* role in regulating Ras/MAPK positively. These results suggest that *twins* is a positive regulator of Ras/MAPK, necessary for a subset of Ras/MAPK dependent ommatidial developmental events.

BIOGRAPHICAL SKETCH

Dennis Michael Bissonnette was born on February 10, 1976 in Framingham, Massachusetts. He spent his childhood watching baseball and frolicking in the back yard of his family's home in Bellingham, Massachusetts before moving to Ithaca, NY to attend college. Dennis graduated from Cornell University in 1998 with a B.S. in Biology from the College of Agriculture and Life Sciences. In 1998 Dennis entered the Doctoral Program in the Department of Neurobiology and Behavior at Cornell University and joined the laboratory of Professor Ronald Booker.

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CHAPTER ONE
INTRODUCTION

Protein Phosphatase 2A

The protein phosphatase 2A (PP2A) enzyme is included in one of four subgroups of the PPP (Protein Phosphatase P) family of serine / threonine protein phosphatases classified on the basis of substrate specificity and response to specific inhibitors and effectors (Barford et al., 1998). These enzymes are responsible for regulating a vast array of cellular events controlled by protein phosphorylation. PP2A is a heterotrimeric enzyme composed of a catalytic (C), associated (A), and a variable regulatory (B) subunit. The C and A subunits together form the core enzyme complex. The B subunit regulates the catalytic activity and substrate specificity of the core enzyme (Janssens and Goris, 2001; Mayer-Jaekel and Hemmings, 1994). The C and A subunits are ubiquitously expressed (Dougherty and Morrison, 2004), while the B subunits have a more restricted distribution in all eukaryotic animals. The B subunits can therefore confer a wide range of functionality to the PP2A enzyme depending on which subunit is expressed in the same tissue as the core enzyme subunits. The B subunits of PP2A are coded for by three multigene families known as B/PR55, B'/PR61 and B''/PR72 (Table 1-1). These families confer onto the PP2A holoenzyme distinct properties due to their restricted cellular locations (Millward et al., 1999).

PP2A has been found to function in a variety of cellular signaling cascades. Among these are the Bcl-2 apoptotic pathway (Hoof and Goris, 2003; Deng et al., 1998), the *wingless* signal transduction pathway

Table 1-1 Nomenclature and Distribution of Regulatory B-Type Subunits[1]

Regulatory Subunit	Isoform	Cell/Tissue Distribution	Subcellular Distribution
B family	Bα or B55 or PR55	Widespread, predominant PP2A regulatory subunit	Microtubules, neurofilaments, vimentin, cytoplasm, nucleus
	Bβ	Enriched in brain	Cytosol
	Bγ	Brain-specific	Enriched in cytoskeleton
	Bδ	Widely expressed	Cytosol
	Bα/Bβ	*Xenopus* Bα and Bβ homologues	
	CDC55	*Saccharomyces cerevisiae* Bα/Bβ homologues	
	B-homologue	*Dictyostelium discoideum*	Cytosol, enriched in centrosomes
	Pab1	Fission yeast Bα homologue	
	Twins/DPR55 -1, DPR-55-4	***Drosophila* Bα and Bβ homologues**	**Cytoplasm**
	AtBα, AtBβ	*Arabidopsis* Bα and Bβ homologues	Ubiquitous in all plant tissue
B' family	B56α	Highly expressed in heart and skeletal muscle	Cytoplasm
	B56β or B'α	Highly expressed in brain	Cytoplasm
	B56γ1, B'β1-4, or B'α3	Highly expressed in heart and skeletal muscle	Cytoplasm and nucleus
	B56γ2 or B'α2	Highly expressed in heart and skeletal muscle	Nucleus and cytoplasm
	B56δ or B'γ or 74-kDa/B"δ	Highly expressed in brain	Nucleus, cytosol, mitochondria, microsome
	B56ε or B'δ	Highly expressed in brain	Cytoplasm
	B56ζ1 or B56γ3	Highly expressed in heart and skeletal muscle	Nucleus and cytoplasm
	Par1p, Par2p	Fission yeast B' homologues	Cell cycle-dependent localization patterns
	Widerborst/DPR56	*Drosophila* B56 homologue	Cytoplasm
B' ' family	PR72 or Bδ/72	Enriched in heart and skeletal muscle	Cytosol
	PR130	Heart, brain, lung, muscle, kidney	Centrosome and Golgi apparatus
	PR59	Heart, kidney, lung, but not skeletal muscle	Nucleus
	PR48	Placenta	Nucleus

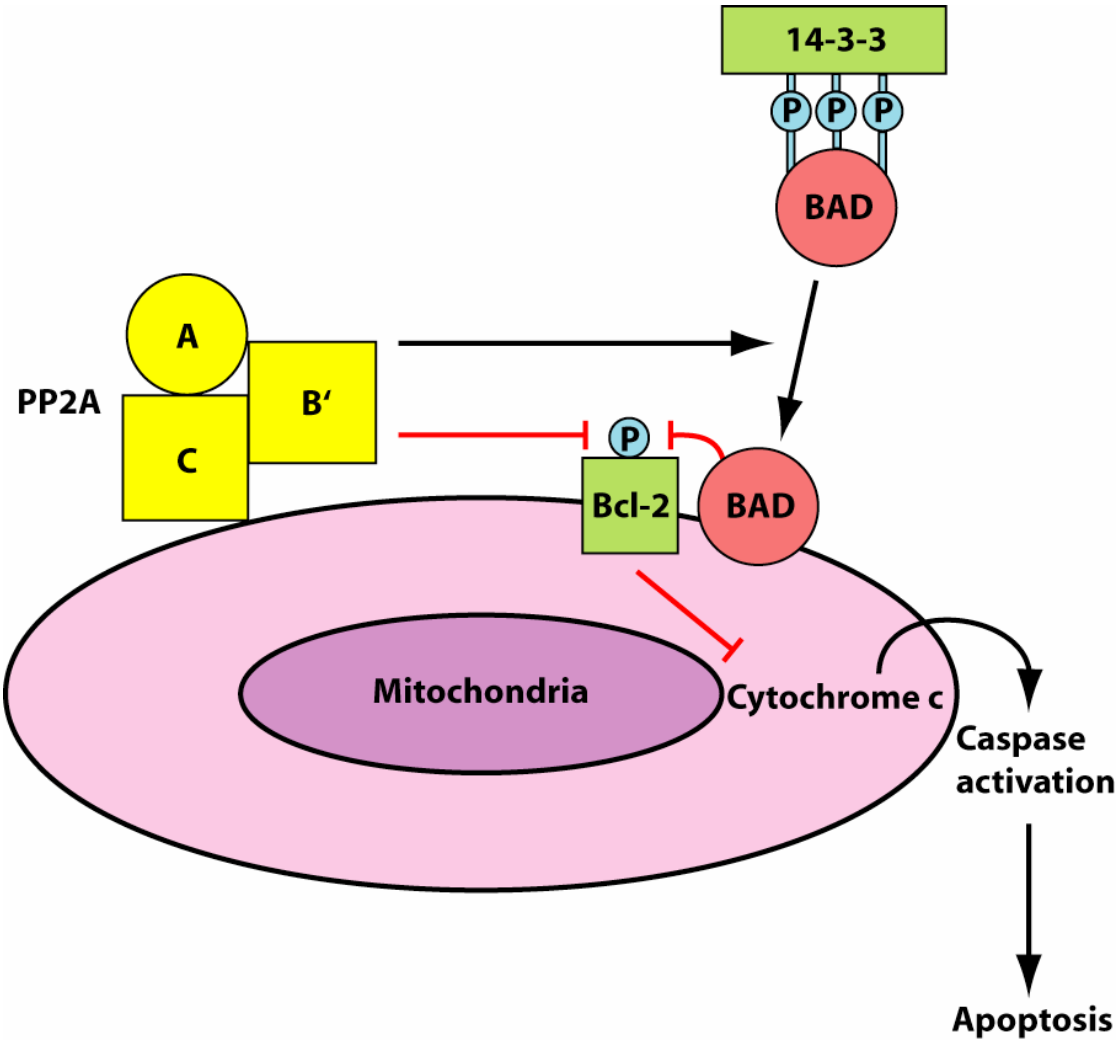
[1] After Sontag, 2001

(Bajpai et al., 2004), and the Ras/MAPK signaling pathway (Dougherty et al., 2005; Ory et al., 2003; Sieburth et al., 1999). In the Bcl-2 pathway PP2A inhibits anti-apoptotic factors such as Bcl-2, enhancing cytochrome c release from mitochondria which induces caspase activation (Figure 1-1). By associating with the B'/PR61 subunit, the A and C core enzyme dephosphorylates the protein BAD (Bcl-2-antagonist of cell death). This allows the dissociation of BAD from the 14-3-3 scaffolding complex, freeing BAD to inhibit Bcl-2 from its anti-apoptotic activities. Thus, PP2A functions to promote apoptosis when the A and C core complex associates with the B'/PR61 regulatory subunit.

PP2A and Wingless signaling

The *wingless* (*wg*) signal transduction pathway plays a key role in regulating many developmental events during *Drosophila* ontogeny (Fanto et al., 2004; Seto et al., 2004; Maves et al., 2003). During growth of the wing imaginal disc, *wingless* is necessary for specifying the presumptive wing pouch in the dorsal compartment of the disc. The pathway is then used again to establish the Dorsal-Ventral (DV) boundary of the wing pouch (Kim et al., 1996; Williams et al., 1994; Diaz-Benjumea et al., 1993). *wingless* is upregulated at the DV boundary in response to Notch and EGFR signaling. Wg acts at the DV boundary acts as a morphogen, upregulating genes necessary for proper development of the cells located on either side of the DV boundary (Figure 1-2). Short range targets such as *achaete* and *scabrous* require high concentrations of Wg, while long range targets like *vestigial* are upregulated by low levels of Wg.

Figure 1-1. Regulatory role of PP2A in the apoptotic signal transduction pathway upstream of the Bcl-2 family. Translocation of the B'/PR61 subunit to the PP2A core enzyme results in the assembly of a mitochondrial PP2A holoenzyme dephosphorylating and inactivating the anti-apoptotic Bcl-2. PP2A is also a BAD phosphatase, keeping this pro-apoptotic protein in a dephosphorylated and mitochondrial located state, where it is functional in inhibiting Bcl-2. The net result is the release of cytochrome c, required for the apoptotic response. (From Van Hoof and Goris, 2003)

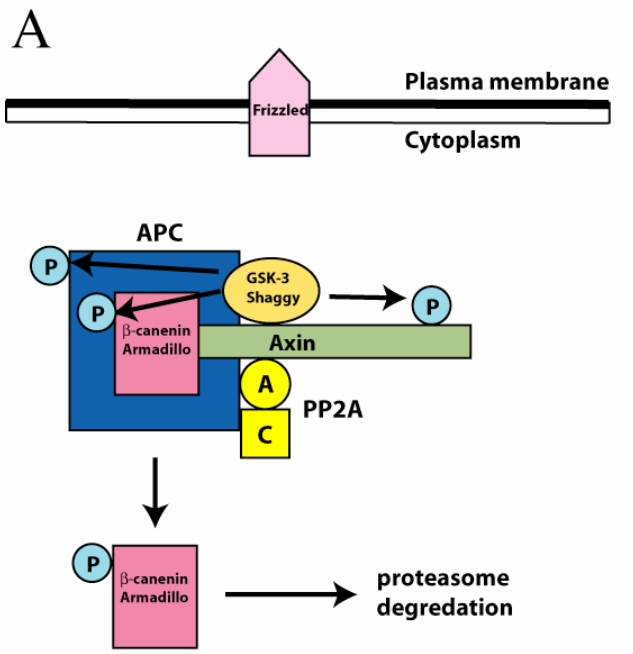

14-3-3
P
P
P
BAD
A
PP2A
B'
C
P
Bcl-2
BAD
Mitochondria
Cytochrome c
Caspase
activation
Apoptosis

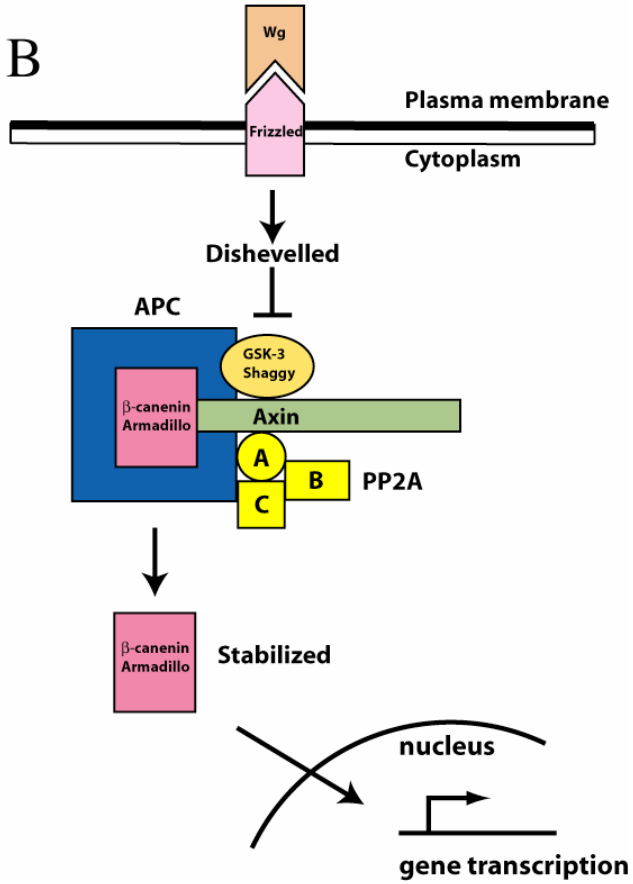
.Upregulation of these *wg* targets requires proper transduction of the *wg* signal. This process is dependent on the stabilization of the signaling factor *armadillo* in response to the binding of Wg to its receptor Frizzled (Neumann and Cohen, 1996; Zecca et al., 1996).

Figure 1-2 shows that during normal *wingless* signaling, Wg binds the membrane bound receptor Frizzled. This interaction causes the activation of cytoplasmic Dishevelled, which inhibits GSK-3 from properly phosphorylating Axin, APC, and β-catenin (*armadillo*). The end result is the release of Armadillo protein from the Axin-APC-GSK-3 complex in an unphosphorylated form that prevents it from proteasome degradation (Figure 1-2). Once released, and protected from degradation, Armadillo can initiate the upregulation of *wg* target genes (Greaves, 2003; Matinez Arias, 2003; Strutt 2003).

The successful transduction of Wg is dependent on the function of PP2A (see Figure 1-2 and Bajpai et al., 2004; Li et al., 2001; Gotz et al., 2000). PP2A can have a positive or negative influence on *wg* signaling depending on which regulatory subunit is associated with the core enzyme complex (Bajpai et al., 2004; Li et al., 2001; Gotz et al., 2000). Li et al (2001) have shown that overexpressing the B'/PR61 subunit inhibits the *wg* pathway, therefore reducing the level of cytoplasmic Armadillo. The opposite effect was observed by Bajpai et al. (2004). When the B/PR55 subunit (*twins*) was removed, the level of *wg* signaling was reduced, indicating a positive interaction of *twins* and PP2A with the *wg* signal transduction pathway.

Figure 1-2. Model for the regulation of the Wg signalling pathway by protein phosphatase 2A. (A) In unstimulated cells, GSK-3 (*shaggy*) phosphorylates the cytoplasmic scaffold protein complex of Axin, APC, and β-catenin (*armadillo*). Phosphorylated β-catenin is released from the complex and targeted for proteasome degradation. The inactive PP2A enzyme (A and catalytic C subunit) is bound to the scaffolding protein complex. (B) binding of the secreted ligand Wg to the membrane bound receptor Frizzled activates Disheveled protein that inhibits GSK-3 from phosphorylating its targets. When Wg binds Frizzled, B/PR55 (*twins*) becomes associated with PP2A, allowing it to work with Disheveled to inhibit GSK-3. This allows the release of unphosphorylated, stable β-catenin, which can translocate to the nucleus and affect gene transcription. (From Li et al., 2001)


A
Plasma membrane
Frizzled
Cytoplasm
APC
P
P
GSK-3
Shaggy
P
β-canenin
Armadillo
Axin
A
PP2A
C
P
β-canenin
Armadillo
proteasome
degredation


B
Wg
Plasma membrane
Frizzled
Cytoplasm
Dishevelled
APC
GSK-3
Shaggy
β-canenin
Armadillo
Axin
A
B
PP2A
C
β-canenin
Armadillo
Stabilized
nucleus
gene transcription

In *tws*^{p}/*tws*^{p} mutant wing discs, Wg is secreted at the DV boundary of the wing pouch, but fails to increase cytoplasmic Armadillo levels, indicating a failure of *wg* signaling. Examination of the epistatic relationship between *twins* and components of the *wingless* pathway suggests that *twins* functions downstream of *disheveled*, and upstream of GSK-3 (*shaggy*). Bajpai et al (2004) have proposed that *twins* functions in the pathway to work with Disheveled to promote the release of the unphosphorylated form of Armadillo (Figure 1-2). The functional role of PP2A in the *wingless* signal transduction pathway is highly dependent on the regulatory B subunit which becomes associated with the core enzyme complex. Through differences in the B subunits, PP2A can have a positive or negative influence on the success of the *wg* signal transduction pathway.

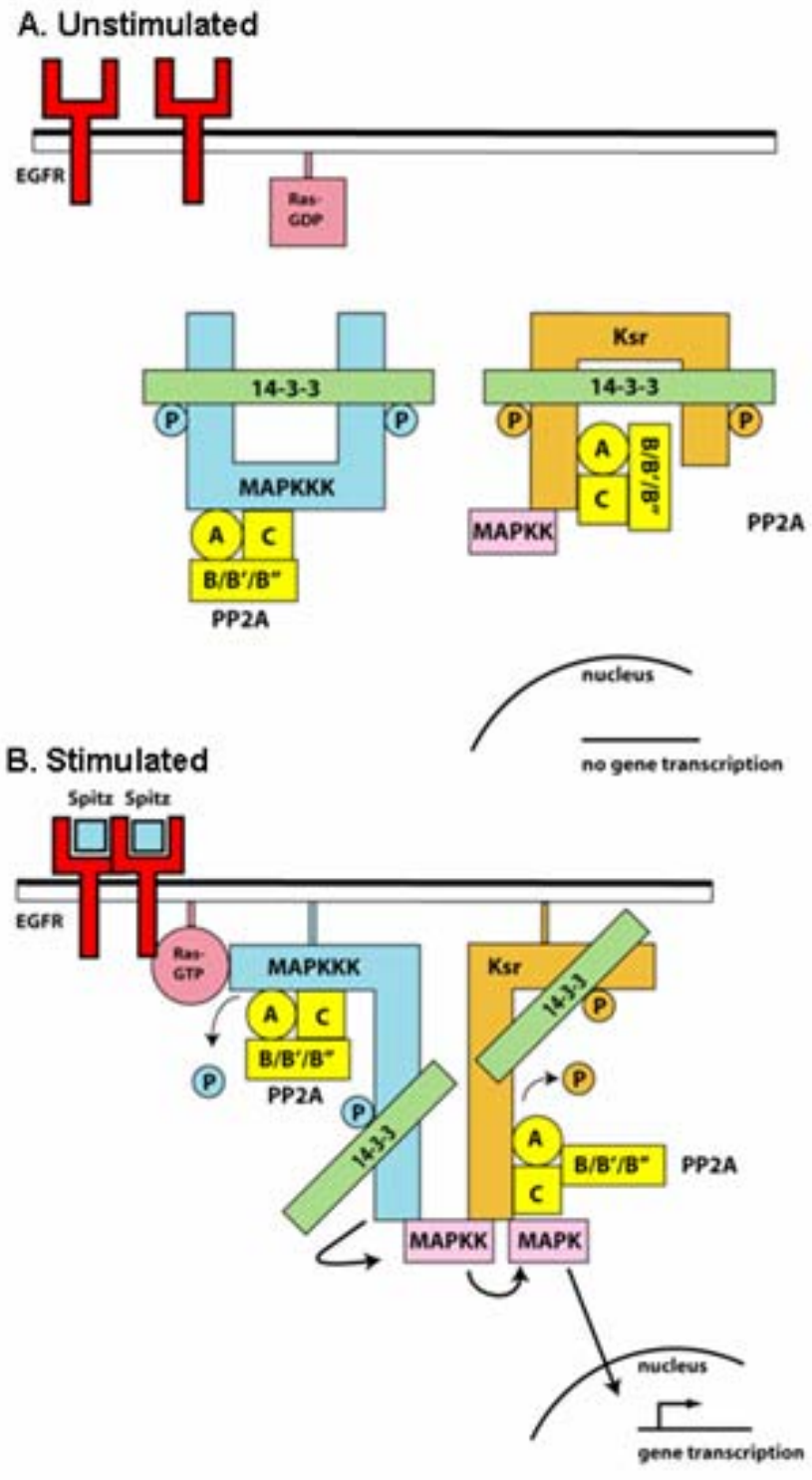
PP2A and Ras/Raf/MAPK Signaling

Receptor tyrosine kinases are responsible for many developmental decisions during the growth and specification of eukaryotic organs; among them the *Drosophila* eye has been useful in understanding their function (Freeman, 1996; Wassarman et al., 1995; van der Geer et al., 1994). During *Drosophila* eye development, the EGFR/MAPK pathway is necessary for establishing the eye field, as well as specifying the different cells of each unit, or ommatidium, of the compound eye. The MAPK signaling pathway is a means by which extracellular signals can be interpreted in a manner specific to different cell-types. As the morphogenetic furrow sweeps across the eye disc, the EGFR/MAPK pathway is reiteratively used to recruit the cellular components of each

ommatidia. By varying the intracellular proteins regulating the pathway, a variety of cellular outputs can be generated from a single pathway (Chang, et al., 2001). Each MAPK signal transduction pathway contains the three core units, MAPK kinase kinase (MAPKKK), MAPK kinase (MAPKK), and MAPK. Sequential activation of these enzymes provides signal amplification and transduction from the cell membrane to changes in gene activation. Modification of the scaffolding proteins that interact with the MAPK enzymes is one way to generate the specificity of the active complex (Chang et al., 2001; Morrison, 2001).

The Ras/Raf/MAPK pathway is a well-known example of the MAPK signal transduction pathway. This pathway utilizes Ras-GDP to transduce the extracellular signal to the nucleus through Raf (MAPKKK), MEK (MAPKK), and Erk (MAPK) (Chang and Karin, 2001) (Figure 1-3). Upon Ras activation, the scaffolding proteins Raf and Ksr must undergo a conformational change to become associated with the activated Ras and transduce the active Ras signal (Morrison, 2001). In an unstimulated state, both Raf and Ksr are inaccessible to the membrane bound Ras due to the conformation of the dimers each forms with the scaffolding protein 14-3-3 (Tzivion and Avruch, 2002). Ory, et al. (2003) have shown that dephosphorylation of serine residues on Raf and Ksr contributes to the stimulated conformation necessary for Raf/Ksr/Ras association at the cell membrane. Furthermore, this conformational change orients the Ksr-bound MEK (MAPKK) in a way that allows Raf to interact with it, initiating proper signal transduction. (Ory, et al., 2003, Raabe and Rapp, 2003). PP2A is the enzyme that facilitates this conformational change by

Figure 1-3. Model for the regulation of Raf and Ksr by protein phosphatase 2A. (A) In unstimulated cells, binding of dimeric 14-3-3 to phosphorylated Raf and Ksr retains both complexes in the cytoplasm. PP2A is bound to Raf and Ksr. In addition, inactive MEK is associated with Ksr. (B) Stimulation of cells by growth factors such as Spitz results in the assembly of an active signal transduction pathway. The PP2A holoenzyme dephosphorylates Raf and Ksr, freeing them from the scaffolding protein 14-3-3. Displacement of 14-3-3 facilitates the membrane recruitment of both proteins. As a result, an active signaling complex of Ras, Raf, MEK and Erk is formed at the membrane. (From Raabe and Rapp, 2003)


A. Unstimulated
EGFR
Ras-GDP
14-3-3
P
P
MAPKKK
A
C
B/B'/B"
PP2A
Ksr
14-3-3
P
P
A
C
B/B'/B"
PP2A
MAPKK
nucleus
no gene transcription
B. Stimulated
Spitz Spitz
EGFR
Ras-GTP
MAPKKK
A
C
B/B'/B"
PP2A
P
P
14-3-3
Ksr
14-3-3
P
P
A
C
B/B'/B"
PP2A
MAPKK
MAPK
nucleus
gene transcription

dephosphorylating Ksr and Raf serine residues. PP2A is bound to Ksr and Raf, and its level of activity is dependent on which regulatory subunit is associated with the core enzyme. When the B subunit encoded by the *Drosophila melanogaster* gene *twins* is associated with the core enzyme, the Ras/MAPK signal can be successfully transduced (Ory, et al., 2003).

The *twins* Gene

The *twins* gene is 12969 base pairs long and composed of seven exons located on the right arm of the third chromosome (Mayer-Jaekel et al., 1993). These seven exons can be alternatively spliced to produce thirteen different mRNAs, which in turn can be translated into one of two proteins (Mayer-Jaekel et al., 1994; Mayer-Jaekel et al., 1993). The Tws-P1 protein is 456 amino acids long, while the Tws-P2 is 443. These proteins are expressed during embryonic and larval development. They are first detected in the syncytial blastoderm, but later become restricted to the cental nervous system (CNS), imaginal discs, and gonadal tissue of the third instar larva (Mayer-Jaekel et al., 1994; Mayer-Jaekel et al., 1993; Uemura et al., 1993). Tws is present in the cytoplasm of interphase cells, and throughout the whole cell as it progresses through mitosis. The protein is expressed in dividing neuroblasts, and ubiquitously throughout imaginal disc tissue (Mayer-Jaekel et al., 1994; Mayer-Jaekel et al., 1993; Uemura et al., 1993).

Hypomorphic *twins* mutations affect several aspects of *Drosophila* development (Mayer-Jaekel et al., 1994; Mayer-Jaekel et al., 1993; Uemura et al., 1993). The most severe hypomorphic allele, tws^{p}, produces less than 1% of Tws protein compared to wild type (Mayer-Jaekel et al.,

1994; Shiomi et al., 1994; Mayer-Jaekel et al., 1993; Uemura et al., 1993) and is a recessive lethal mutation causing death as pharate adults. The weakest hypomorphic allele, *aar*^{2} (*abnormal anaphase resolution*) produces female sterility when homozygous. Although sterile, these flies survive (Mayer-Jaekel et al., 1994; Mayer-Jaekel et al., 1993; Uemura et al., 1993). The *aar*^{2} allele of *twins* was identified in a screen for mitotic mutants independently of the other *twins* alleles. During anaphase of mitosis, the chromatids fail to migrate to the poles of the mitotic spindles. As a result, the cells are unable to complete the division to produce two daughter cells (Gomes et al., 1993; Mayer-Jaekel et al., 1993). The lethality of the mutations are believed to be due to the inability of dividing cells to complete mitosis successfully

In addition to mitotic abnormalities, homozygous *tws*^{p} mutants display duplicated third instar wing pouches, and duplicated microchaete and macrochaete bristles. Bajpai et al., (2004) have shown that within the wing imaginal discs, *twins* is necessary for cells to respond to *wingless* signaling. *tws* functions to stabilize the Armadillo/beta-catenin structure that forms in response to Wg. Without this stabilization, the Wg signal will not be interpreted correctly.

The eyes of homozygous *twins* mutants are smaller and rougher than wild type eyes (Shiomi et al., 1994). These phenotypes could result from inappropriate establishment of the eye field, and misspecification of the ommatidial cells. In each of these processes, the EGFR/MAPK pathway is repeatedly used to induce eye development. Although the same pathway is utilized repetitively, different outcomes are achieved. One way to achieve multiple outcomes through a single pathway is to

utilize different pathway regulators downstream of the receptor. As has been observed in *wg* signalling, *twins* may regulate the EGFR/MAPK pathway during eye development to produce a variety of outcomes. It is the purpose of this thesis to understand the role of *tws* during *Drosophila melanogaster* eye development.

Drosophila melanogaster Eye Development

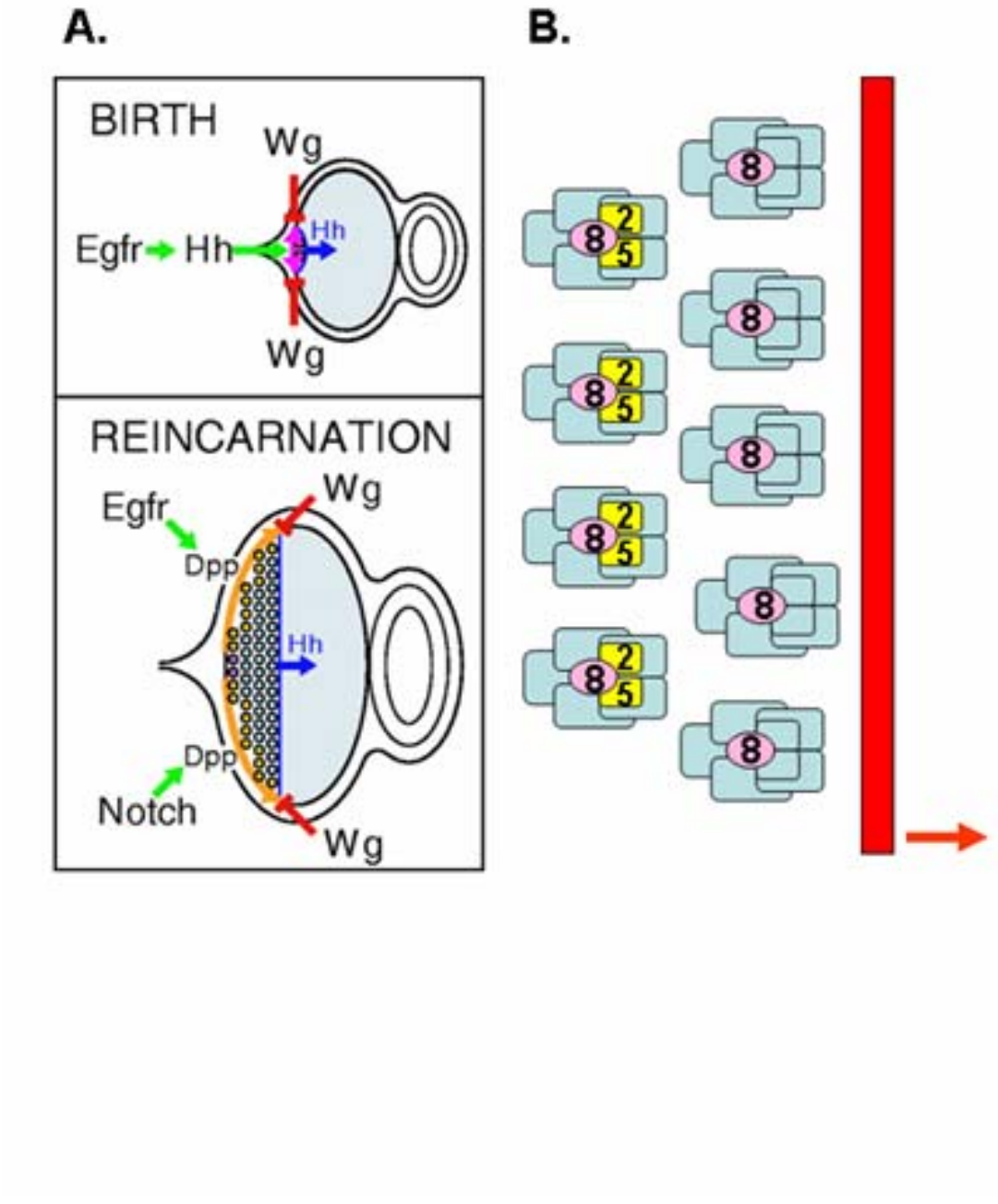
The adult *Drosophila* eye is a highly structured tissue composed of about 750 subunits called ommatidia arranged in a repetitive hexagonal pattern (Ready et al., 1976). Each ommatidium is composed of eight photoreceptor cells, four lens secreting cone cells, eleven pigment cells, and three bristle cells. The structured order of each ommatidium is so precise that it is often referred to as a neurocrystalline lattice (Baonza et al., 2001; Baker, 2000; Ready et al., 1976). Disruptions in assembly of the ommatidial cells, or the greater arrangement of ommatidia within the eye field, will produce an eye with a rough phenotype (Wolff and Ready, 1993; Ready et al., 1976).

The adult eye develops from a field of two bilateral groups of twenty cells that are generated late in embryogenesis (Baker, 2001; Garcia-Bellidio and Merriam, 1969). It is not until the second instar when the fate of these cells is restricted to that of antennal or eye tissue. During this period, activation of the Notch signaling pathway confers the eye fate, while activation of the Ras/RTK (Ras/Receptor Tyrosine Kinase) pathway, which antagonizes Notch signaling, promotes the antennal fate (Kumar and Moses, 2001a).

Cellular differentiation of the eye begins during the middle of the third instar. It begins at the posterior margin of the eye field, and slowly moves in the anterior direction. This differentiation can be visualized as a band, the morphogenetic furrow (MF), moving across the eye. The furrow is created through the apical constriction of eye epithelial cells, forcing a downward movement of cell bodies. The propagation of the furrow is linked to the neural differentiation posterior to its movement. The end result is a spatiotemporal recruitment of ommatidial cells within the eye field, with the youngest cells just posterior to the furrow (Wolff and Ready, 1993; Ready et al., 1976) (Figure 1-4).

The initiation of the MF is under the positive control of Hedgehog (Hh) and Decapentaplegic (Dpp), while negatively regulated by Wingless (Wg) (Chanut and Heberlein, 1997a; Chanut and Heberlein, 1997b; Dominguez and Hafen, 1997; Pignoni and Zipursky, 1997; Heberlein and Moses, 1995; Ma and Moses, 1995; Treisman and Rubin, 1995). Just prior to the first appearance of the MF, a stage Moses (2001) refers to as "birth," Hh is expressed at the posterior margin of the eye field (Figure 1-4). If Hh expression is eliminated, there is a total irreversible loss of pattern formation within the eye (Borod and Heberlein, 1998). Heberlein et al. (1995) showed that Hh is sufficient for MF initiation by expressing Hh ahead of the endogenous furrow, which induced ectopic MFs and retinal differentiation. Dpp is also necessary for MF initiation. Dpp has a broader range of expression than Hh at MF initiation. Dpp is expressed at the posterior and lateral margins of the eye disc. Like Hh, ectopic expression of Dpp can induce additional MFs (Chanut and Heberlein, 1997b; Pignoni and Zipursky, 1997). As new cells are recruited into the

Figure 1-4. Models for furrow birth and reincarnation in the eye-antennal imaginal dics and the recruitment of the ommatidial cells. A) Birth and reincarnation in the mid-third instar eye-antennal disc. Green arrows show inductive interactions, red symbols show inhibitory influences. Purple arrows and Hh in birth model indicates first ommatidial induction and purple ommatidia are the first ones produced. Blue furrows and arrows indicate Hh progression signal from the newly established ommatidia, and blue ommatidia are those produced by this mode of Hh induction. Orange arrows show progressive furrow re-initiation along the lateral margins and orange ommatidia are those induced by Dpp (From Kumar and Moses, 2001). B) Specification and recruitment of cells into the ommatidial clusters begins just posterior to the morphogenetic furrow. R8 is the first ommatidial cell specified form the undifferentiated cells (blue) posterior to the furrow. Once specified, R8 secretes Spitz, activating EGFR of its nearest undifferentiated neighbors, recruiting them into the cluster (yellow cells). Once specified, the yellow R2 and R5 cells begin secreting Spitz to recruit their nearest neighbors into the cluster. The process repeats until all ommatidial cells are specified. The red arrow indicates the direction of furrow progression.


A.
BIRTH
Wg
Egfr
Hh
Hh
Wg
REINCARNATION
Egfr
Wg
Dpp
Hh
Dpp
Notch
Wg
B.
8
2
5

ommatidia posterior to the furrow, they begin to express Hh to propagate the MF at the disc midline, and Dpp to promote propagation laterally (Kumar and Moses, 2001). Wg is expressed along the lateral margins of the disc, anterior to the advancing MF. If expressed ectopically within the MF, progression is halted. Similarly, the removal of Wg induces ectopic MFs (Ma and Moses, 1995; Treisman and Rubin, 1995). Kumar and Moses (2001) have shown that Ras activation, via the Epidermal Growth Factor Receptor (EGFR), and Notch receptor activation are also important players in MF initiation. They work upstream of Hh and Dpp to initiate the MF, and downstream of Wg to promote MF propagation, a phase Moses (2001) refers to as MF "reincarnation."

Within the MF high expression levels of the proneural gene *atonal* can be detected (Chanut and Heberlein, 1997a; Chanut and Heberlein, 1997b; Dominguez and Hafen, 1997; Pignoni and Zipursky, 1997; Heberlein and Moses, 1995; Ma and Moses, 1995; Treisman and Rubin, 1995). Atonal expression is induced by Notch within the furrow. As the MF progresses, directly posterior to it, Atonal is restricted to groups of approximately twelve cells through the process of "proneural enhancement" (Baker, 2000; Jarman et al., 1993). This is a process by which Notch causes the upregulation of Atonal within a cell cluster, and the protein Rough inhibits Atonal expression between the clusters. Following this "proneural enhancememnt," Notch-mediated lateral inhibition restricts Atonal expression to a single cell within each group. The end result is a row of evenly spaced Atonal expressing cells in a field of Rough expression (Baker, 2000).

The single cell left expressing Atonal becomes the R8 photoreceptor. This founder cell initiates the recruitment of the other photoreceptor and support cells of the ommatidium (Baker and Rubin, 1989; Tomlinson and Ready, 1987; Lebovitz and Ready, 1986). Unlike other sensory structures in *Drosophila melanogaster,* such as the macrochaete and microchaete mechanosensory organs whose fates are determined by their lineage (Baker, 2000; Artavanis-Tsakonas et al., 1999), most of the cells of the fly eye are present in an undifferentiated state prior to passage of the MF. After R8 establishment, R2 and R5 are quickly recruited, followed by R3 and R4. Recruitment of these four photoreceptors is accomplished through activation of the Ras/MAPK pathway (Figure 1-4). R8 secretes the EGFR ligand Spitz, which binds EGFR on Rough expressing cells surrounding R8 (Heberlein et al., 1991). These first cells to receive Spitz become the R2 and R5 cells. Once recruited, R2 and R5 begin secreting Spitz as well (Tomlinson et al., 1988). Their Spitz contribution, along with that of R8, recruits R3 and R4, which only weakly express Rough (Fanto and Mlodzik, 1999; Fanto et al., 1998).

After the establishment of this five cell pre-cluster, the remaining undifferentiated cells complete a coordinated S-phase and undergo mitosis in a process described as the “second mitotic wave.” Following the second mitotic wave, the undifferentiated cells surrounding the five cell pre-clusters begin to express the protein Lozenge (Lz), a protein necessary for establishing cell-type specific transcription programs (Flores et al., 1998, 2000). Lz expression, in combination with signaling through EGFR, upregulates BarH1 and BarH2 expression in two cells adjacent to

the five cell pre-cluster. These cells become photoreceptors R1 and R6 (Daga et al., 1996). After R1 and R6 are recruited into the pre-cluster, they inhibit BarH1 and BarH2 expression in adjacent cells via activation of the Notch pathway. This prevents additional cells from adopting the R1 and R6 fate (Parks et al., 1995; Cagan and Ready, 1989). R7, which resides next to R1 and R6, is the final photoreceptor to join the ommatidial cluster. Ras/MAPK pathway activation via the EGFR and Sevenless (Sev) receptor in R7 confers the R7 fate (Li et al., 2002; Dang et al., 1999; Li et al., 1997; Tang et al., 1997). Removing Notch signaling between R1/6 and R7 prevents the inhibition of BarH1 and BarH2, therefore allowing Ras activation to transform R7 into an ectopic R1/6.

The final cells recruited into the ommatidia are the four cone cells and the pigment cells. The cone cells' EGFR receptors are activated by Spitz secreted from R7. Because the cone cells do not have activated Sev receptors, the level of Ras activation is lower than in R7, conferring the cone cell fate, and not that of R7 (Lai et al, 1996). Ectopic activation of Ras in these cells converts the cone cells to an R7 fate. Conversely, a reduction of Ras signaling in R7 will convert it into a cone cell (Fortini et al., 1992). Like R7, the cone cells also require Notch activation to suppress BarH1 and 2 expression, preventing a transformation to the R1/6 phenotypes (Tsuda et al., 2002; Cagan and Ready, 1989). The pigment cells are the final cells to be recruited into the ommatidia. Although less is known about pigment cell specification, EGFR signaling, Lz, and BarH1 are necessary to maintain their cell fate (Flores et al., 1998; Miller and Cagan, 1998; Cagan and Ready, 1989).

The Ras/MAPK signaling pathway is used repeatedly during eye development in *Drosphila melanogaster*. It is first necessary for initiation of the morphogenetic furrow, and its subsequent propagation. It is then critical for recruitment of the photoreceptors and support cells of the ommatidia. Interestingly, EGFR mediated Ras/MAPK activation is not necessary for the establishment of the R8 founder photoreceptor. It is of great interest to understand the factors that regulate the spatio-temporal use of the Ras/MAPK signaling pathway. In this thesis I show that the gene *twins*, a regulator of the enzyme PP2A, is necessary for a subset of the EGFR mediated RAS/MAPK dependent processes in the development of the adult eye.

Chapter Two

twins is Necessary for R7 and Cone Cell Recruitment into the Ommatidia

Introduction:

The EGFR Ras/MAPK signaling pathway is used repeatedly to induce the cellular differentiation of the *Drosophila* ommatidial cells (Baker, 2001; Baonza et al., 2001). Ras activation in concert with other cell-type specific transcription factors is necessary to confer the distinct fates to each cell. As one cell type is established, it begins signaling adjacent cells via the EGFR receptor to initiate their differentiation (Baker 2000; Daga et al., 1996; Heberlein et al., 1991; Tomlinson et al., 1988; Higashijima et al., 1987). It is of interest to understand how this single pathway can be regulated to produce different outcomes depending on the spatio-temporal pattern of its activation.

The R7 photoreceptor is the last of the eight photoreceptors to be recruited into the developing ommatidial cluster. The undifferentiated R7 resides in a cluster of cells known as the R7 equivalence group (Chu-Lagraff et al., 1991). This collection includes the future cone cells as well as R7. The recruitment of R7 into the ommatidium is dependent on both Ras/MAPK and Notch pathway activation. Ras is activated in R7 via two separate RTK receptors, the EGFR and the Sevenless (Sev) receptor (Xu et al., 2000). Simultaneous activation of these receptors establishes a higher Ras activation than in the other photoreceptor cells. In addition to Ras signaling, Notch signaling from R1/6 is necessary to suppress BarH1 and 2 in the presumptive R7. Without Notch suppression of these

proteins, R7 would adopt an R1/6 cell fate (Tomlinson and Struhl, 2001; Cooper and Bray, 2000; Fortini et al., 1993). The high level of Ras signaling in R7 is necessary for the expression of the transcription factor *prospero* (*pros*). Without this Ras signaling, *pros* expression is blocked by the transcriptional repressors Yan and Tramtrack88 (Ttk88) (Xu et al., 2000). Similarly, cone cells require Ras and Notch signaling to suppress BarH1 and BarH2, therefore preventing an R1/6 fate. These cells also require Ras activation to adopt the cone cell fate. Because Ras is activated only through EGFR, and not Sev, cone cells have a lower level of Ras activation than does R7 (Kauffmann et al., 1996; Chu-Lagraff et al., 1991; Doe et al., 1991; Vaessin et al., 1991). This lower level of activated Ras is insufficient to fully remove the transcriptional repression of *pros*. Therefore, cone cells have a lower level of Pros expression than that of R7 (Xu et al., 2000).

During the specification of the *Drosophila* ommatidial cells, the Ras/MAPK pathway is used repeatedly to produce different cell fates. The mechanisms that regulate this pathway to allow the different outcomes are not completely understood. One way to achieve multiple outcomes through a single pathway is to utilize different pathway regulators downstream of the receptor. As has been observed in *wg* signalling, altering the B regulatory subunit of PP2A may regulate the Ras/MAPK pathway during eye development to induce one fate choice over another, thereby increasing the variety of fates that can be produced through the activation of a single pathway.

In an attempt to better understand how the Ras/MAPK signaling pathway is regulated during *Drosophila* eye development, we directed our

attention towards the B regulatory subunit of PP2A coded for by the gene *twins*. Because *twins* is expressed in all cells of the *Drosophila* imaginal discs during post-embryonic development, it was a good candidate for regulating the activity of PP2A in these tissues. Here, we show that the gene *twins*, a B/PR55 regulatory subunit of PP2A, is essential for the establishment of the R7 and cone cell fates. Loss of functional Tws protein has no effect on the Ras/MAPK dependent differentiation of the other photoreceptors, suggesting that Tws is specific for differentiation of the R7 equivalence group members.

Materials and Methods

Drosophila stocks

All flies were maintained at 25°C on standard yeast-glucose media. For all experiments, we used the background strain w^{1118} as a control line. The *tws* gene is located on the third chromosome (85F13-14). The P-insertional allele tws^{p} was a gift of D. Glover (Mayer-Jaekel et al., 1994; Mayer-Jaekel et al., 1993; Uemura et al., 1993). The *frt-*tws^{p} allele was a gift of T. Uemura. The hypermorphic EGFR allele $egfr^{E1}$ was a gift of N. Baker (Baker and Rubin, 1989). The *svp-lacZ* enhancer trap line, and the *y,w; ey-gal4, uas-flp; frt82b gmr-hid* used to make mitotic clones in the eye were obtained from the Bloomington Stock Center. All stocks were maintained over the balancer chromosomes *Tm6B* Tb^{1}, *Tm3* Sb^{1} Ser^{1}, or *CyO*.

Immunohistochemistry

For examination of photoreceptors and the morphogenetic furrow, wandering third instar larvae were dissected. Eye-antennal discs were dissected from larvae in cold PBS and fixed for 0.5 hours in PBS + 4% paraformaldehyde at room temperature. For examination of cone, pigment, and bristle cells, early pupae were dissected. Animals were removed from the pupal membrane and placed in cold PBS. The retina and optic lobes were dissected from the head of the animal and fixed in PBS + 4% paraformaldehyde. Antibody staining was performed as described in Tomlinson and Ready (1987). Fixed tissue was washed 4 x 10 minutes in PBS + 0.2% Triton X-100, and blocked for 0.5 hour in PBS + 0.2% Triton X-100 + 10% Normal Goat Serum (NGS). Primary antibody was added to fresh blocking solution, and tissue was incubated overnight at 4^0C with gentle shaking. Following incubation, discs were washed 6 x 10 minutes in PBS + 0.2% Triton X-100. Secondary antibody was added to PBS + 0.2% Triton X-100 + 5% NGS and incubated with the discs for 2 hours at room temperature with gentle shaking. Tissue was then washed 3 x 10 minutes in PBS and mounted in Vectashield. Discs were visualized on a Leica TCS SP2 confocal microscope.

Primary antibodies used were rabbit anti-Atonal (Jarman et al., 1995) 1:5000, rabbit anti-BarH1 (Higashijima et al., 1992a) 1:200, mouse anti-Boss (Kramer et al., 1991) 1:1000, and mouse anti-β-Galactosidase (Promega) 1:500. The following antibodies were obtained through the Developmental Studies Hybridoma Bank (developed under the auspices of the NICHD and maintained by The University of Iowa, Department of Biological Sciences, Iowa City, IA 52242), rat anti-Elav (O'Neill et al.,

1994) 1:1000, mouse anti-Cut (Blochlinger et al., 1993) 1:100, rat anti-DE-Cadherin (Oda et al., 1994) 1:20, and mouse anti-Prospero (Spana and Doe, 1995) 1:200. Secondary antibodies used were goat anti-mouse conjugated to Alexa Fluor 488 (Molecular Probes) 1:500, and goat anti-rat conjugated to Cy3 (Vector Laboratories) 1:500.

Generation of Mitotic Clones

Because *tws*^{p} is a recessive lethal mutation, mitotic clones were generated in the eye for examination of phenotypes in pupal and adult eyes. The result was an eye disc or adult eye composed entirely of cells with the *tws*^{p}*/tws*^{p} genotype. Females of the genotype *frt82b tws*^{p}*/ Tm6B, Tb* were crossed to males of the genotype *yw; ey-gal4, uas-flp; frt82b gmr-hid / Tm6B, Tb*. The *gmr-hid* construct expresses the pro-apoptosis gene *hid* in all cells of the eye due to the eye-specific promoter *gmr*. As a result, all clones homozygous for *gmr-hid* die. Therefore, all non-*Tb* animals had eyes of the genotype *tws*^{p}*/ tws*^{p}. These animals were selected for immunohistochemical examination. *Ey-gal4* is expressed in the presumptive eye field of the embryo. Therefore, the eye disc of all larval stages is composed of *tws*^{p}*/tws*^{p} cells.

Light Microscopy

Adult eyes were visualized with a Leica MZ FL III microscope and photographed with a Leica DC 500 camera. The following genotypes were examined. Wild type *w*^{1118} adults, *yw; ey-gal4, uas-flp; frt82b gmr-hid / frt82b tws*^{p} adults, *egfr*^{E1}*/ +* adults, and *egfr*^{E1}*/ +; tws*^{p}*/+* adults.

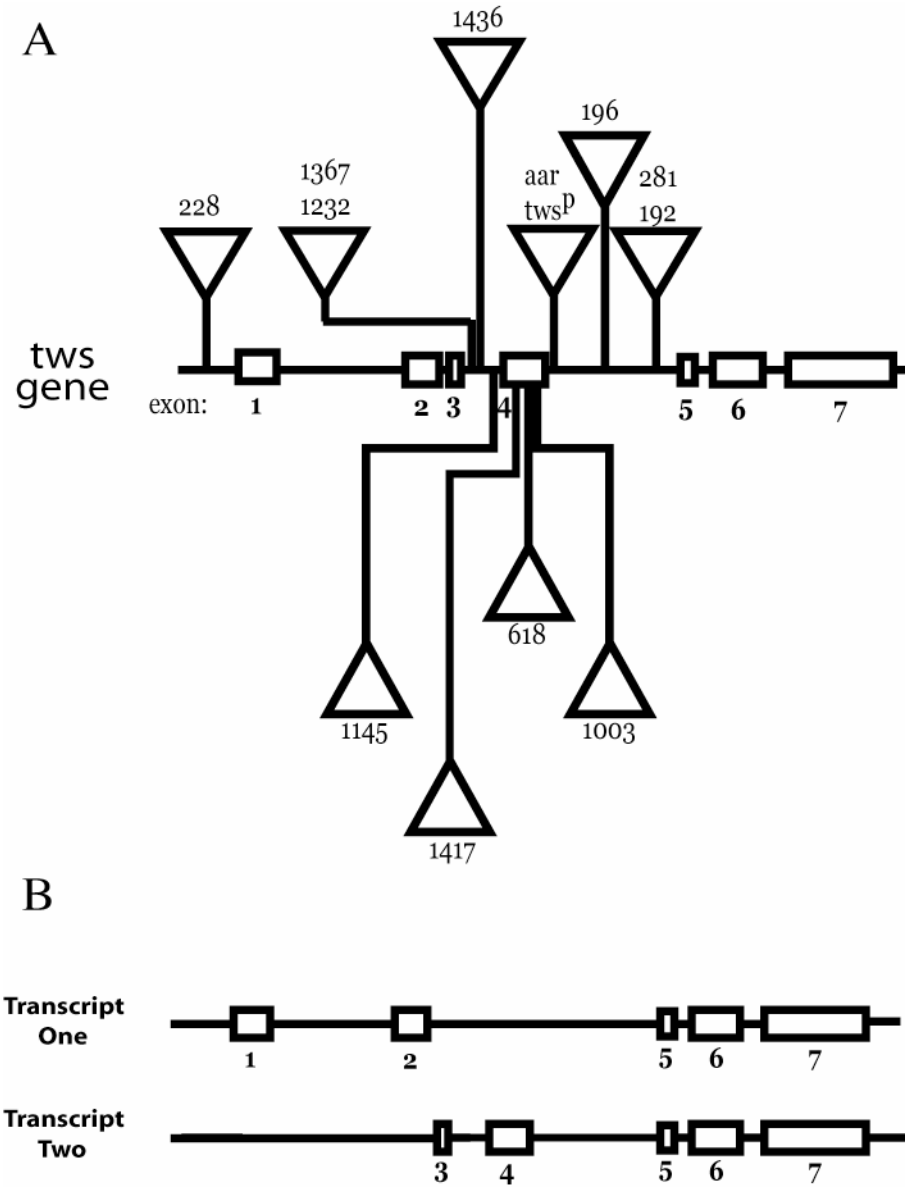
Results

Morphogenetic Furrow and R8

The hypomorphic *tws*^{p}mutation was originally identified as a *P-lacW* insertion into the *tws* locus, which is located at position 85F13-14 on the third chromosome (Mayer-Jaekel et al., 1993; Uemura et al., 1993). This insertion is between the fourth and fifth exons, and is not in the gene's coding region. *tws*^{p} is the most severe allele of a series of P-element insertions within the noncoding region of the *tws* locus (Figure 2-1). These insertions are recessive lethal mutations. Homozygous mutant animals die shortly after head eversion following pupation. Escapers can survive until pharate adulthood, with weaker alleles such as *tws*^{430} producing more escapers than the more severe alleles such as *tws*^{p}.

Examination of pharate adults demonstrates that *tws* mutants exhibit many defects in the development of adult sensory structures. Because all *tws* alleles and their allelic combinations are lethal, it was necessary to generate mitotic clones to visualize the phenotypes of the more severe *tws* alleles in the adult. Since mutations in the adult eye do not affect the survival of the animal, we examined the *tws*^{p}/*tws*^{p} phenotype in this structure. By using the *flp-frt* method (Chou and Perrimon, 1992), we were able to generate clones of *tws*^{p}/*tws*^{p} genotype in all cells of the adult eye. In our experiments, *flp* was under the control of the eye cell precursor specific promoter, *ey*. As a result, mitotic recombination was only induced in the eyes of the animals. The eyes of these flies were smaller and rougher than wild type animals (Figure 2-2), suggesting that *tws* is necessary for the development and specification of the adult eye.

Figure 2-1. Diagram of the *tws* gene (A) and its two major transcripts (B). The *tws* gene is composed of seven exons that are alternatively spliced into two major transcripts (B). One transcript is composed of exons 1,2,5,6,7. The second transcript is composed of exons 3,4,5,6,7. The proteins encoded by these transcripts are redundant in function. The triangles above and below the gene in (A) indicate P-element insertions.


A
1436
196
1367
1232
aar
tws^p
281
192
228
tws
gene
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1
2
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618
1145
1003
1417
B
Transcript
One
1
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Transcript
Two
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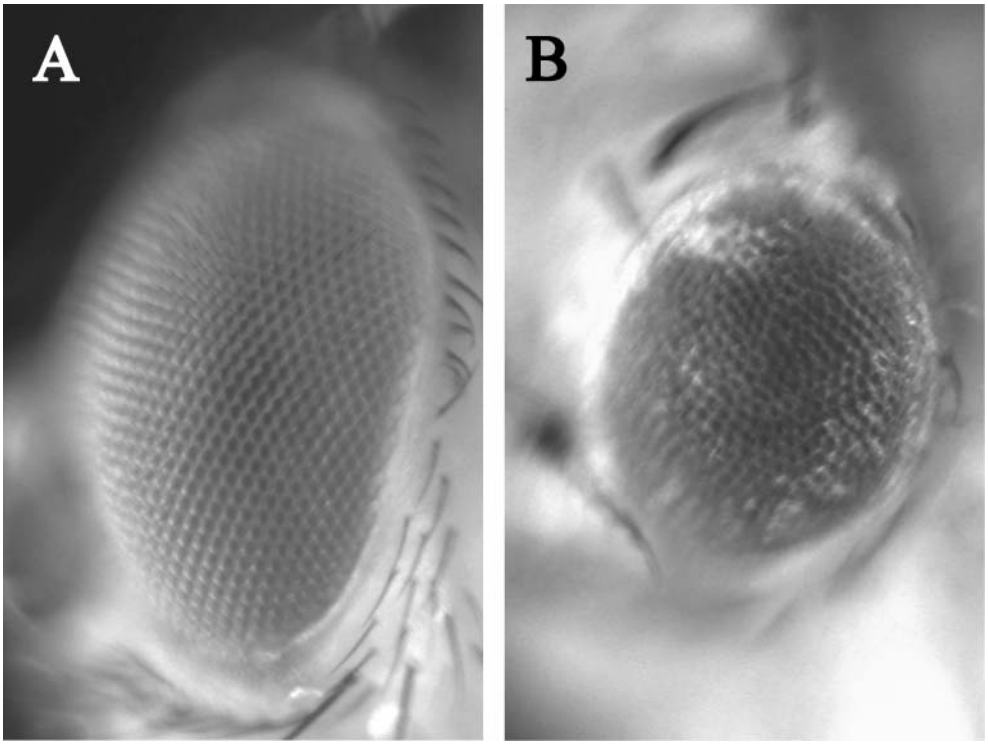
Figure 2-2. *tws^{p}/tws^{p}* adult eyes are small and rough. Light micrographs of A) wild type, and B) *tws^{p}/tws^{p}* mutant eyes generated as mitotic clones (*ey*-Gal4, *uas-flp; Frt82b tws^{p}/Frt82b gmr hid*) Magnification is the same in (A) and (B). Dorsal is towards the top, anterior is to the left. The adult eyes of the mutant are rough and small, lacking the neurocrystalline organization of the adult eye.

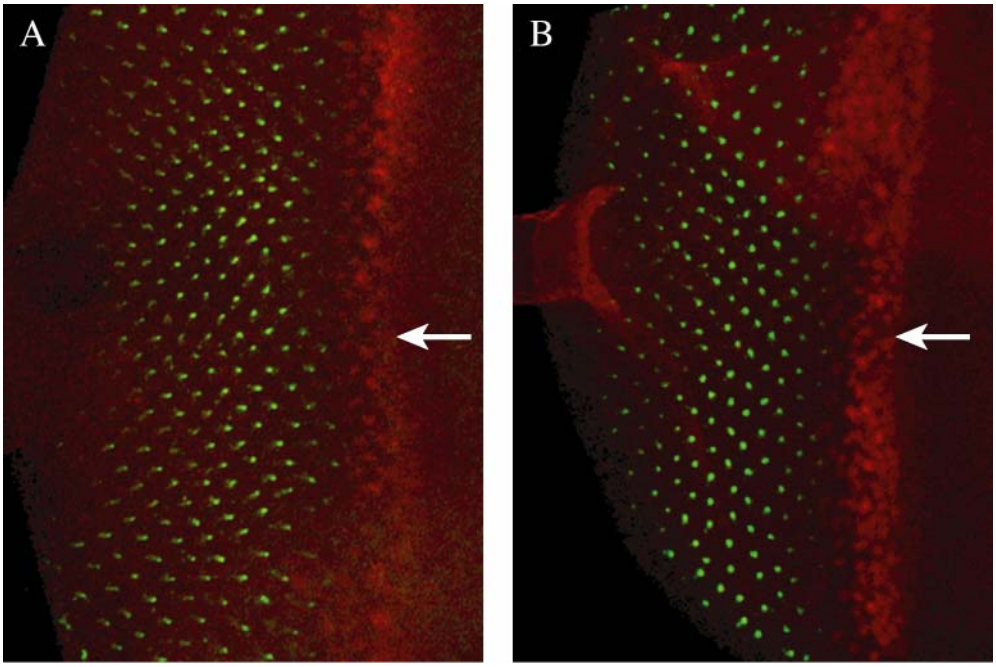

A
B

Because the *tws*^{p} mutation disrupted normal eye development I was interested in examining the initiation of the morphogenetic furrow, and ommatidial cell specification, the two processes which specify the adult eye

Movement of the morphogenetic furrow across the eye field is necessary to begin the process of photoreceptor specification. Within the furrow itself, the transcription factor *atonal* can be detected. *atonal* is necessary for specifying the proneural clusters from which the R8 cell will be specified (Baker, 2000). Although the Ras/MAPK pathway is activated within the furrow, its function there is unclear, as removal of the pathway in *egfr* null mutants does not prevent the furrow or R8 from being specified (Lesokhin et al., 1999). We examined whether or not *tws*^{p} has an effect on *atonal* expression. In these eye discs, Atonal was detected in a normal expression within the morphogenetic furrow (Figure 2-3). *tws* does not appear to be necessary for *atonal* expression in the MF.

R8 is the first photoreceptor specified, and is necessary to begin the recruitment of the other seven (Tomlinson and Ready, 1987; Lebovitz and Ready, 1986). Although Ras/MAPK signal transduction is not necessary to specify R8, overactivity of the pathway, as seen in the hypermorphic *egfr*^{E1} allele, can cause a failure in R8 specification and ommatidial spacing (Kumar et al., 1998; Freeman, 1996). If R8 can not be properly specified, it will be unable to present the *sev* ligand *boss* which is necessary for R7 specification. Without *boss* dependent

Figure 2-3. Expression of *atonal*, a bHLH transcription factor, is necessary for R8 formation in the eye-antennal discs. (A) wild type, and (B) tws^{p}/tws^{p} eye-antennal discs from late third instar larvae. Posterior is to the left. (A) Atonal expression (red) is patterned in the morphogenetic furrow (white arrow) and differentiating R8 cells in wild type. Terminally differentiated R8 cells express the protein Boss (green). (B) tws^{p}/tws^{p} eye-antennal disc shows normal Atonal expression (red) in the morphogenetic furrow. R8 cells are properly established and express the terminal R8 marker Boss (green).


A
B

Ras/MAPK activation, the presumptive R7 will fail to adopt the proper R7 fate (Xu et al., 2000; Chu-Lagraff et al., 1991; Doe et al., 1991; Vaessin et al., 1991).

To determine if R8 was being specified correctly, we looked to see if *boss* expression could be detected in the founding photoreceptor R8. tws^{p}/tws^{p} mutants displayed normal *boss* expression posterior to the morphogenetic furrow. Single, evenly spaced cells displayed *boss* expression, indicating that R8 was properly specified (Figure 2-3). Thus it is unlikely that *tws* has a role in the specification of the founding photoreceptor R8.

Recruitment of Early Photoreceptor Cells

The Ras/MAPK signaling pathway is essential not only for specifying the members of the R7 equivalence group, but also for proper specification of R1-R6. R8 does not require Ras/MAPK pathway activation for its specification, although it appears to be important for proper spacing between R8 photoreceptors (Dominguez et al., 1998; Xu and Rubin 1993). As each photoreceptor is specified, it expresses the neuronal marker *elav* as well as other receptor specific genes. In the case of R1 and 6, the transcription factor *BarH1* (*B-H1*) is upregulated in response to Ras/MAPK activation (Daga et al., 1996). For R3 and R4 (as well as R1 and R6), the transcription factor *seven up* (*svp*) is upregulated (Mlodzik et al., 1990).

In order to determine if tws^{p}/tws^{p} mitotic clones disrupted the specification of these photoreceptors, we examined *svp*, *B-H1*, and *elav* expression in the clones. In tws^{p}/tws^{p} mutant clones the expression of *svp*

and *B-H1* was normal. Two *elav* expressing photoreceptors exhibited *B-H1* expression, while four *elav* positive cells also expressed *svp* (Figure 2-4). Because the number and pattern of *svp* and *B-H1* expressing cells was found to be normal, it is unlikely that *tws* plays a role in regulating the transduction of the Ras/MAPK pathway during R1/6 and R3/4 specification.

Photoreceptor R7 is not Specified

The recruitment of R7 into the ommatidial cluster is dependent on the activation of the Ras/MAPK signaling pathway of the cells neighboring R1/6 and R8. The EGFR receptor of presumptive R7 is activated by Spitz secreted by R1/6, while its Sevenless receptor is activated by the neighboring R8 (Xu et al., 2000; Chu-Lagraff et al., 1991; Doe et al., 1991; Vaessin et al., 1991). When R7 is properly specified, the transcription factor *pros* is upregulated within the cell. In tws^{p}/tws^{p} adult eyes, there is no Pros expression (Figure 2-5). Although the photoreceptors of mutant ommatidia do express the neuron specific marker *elav*, none of the photoreceptor neurons express both Elav and Pros. It is therefore likely that the tws^{p} mitotic clones are not properly signaling through the Ras/MAPK signaling pathway to recruit R7.

Cone cells, Pigment cells, and Bristle cells

In addition to R7, the lens secreting cone cells are specified from the R7 equivalence group. The cone cells have the potential to become R7, but because they lack an activated Sevenless receptor, their level of Ras activation is insufficient to become an R7 (Xu et al., 2000; Doe et al.,

Figure 2-4. *tws*^{p} mutants properly specify photoreceptor cell (PRC) fates in eye-antennal discs from late third instar larvae as indicated by the R1,6 reporter BarH1 and the R3,4,1,6 reporter Svp-LacZ. (A,C) wild type, and (B,D) *tws*^{p}*/tws*^{p} third instar eye-antennal discs. Posterior is to the left. (A) Elav (red) is expressed in all PRC's . BarH1 (green) is expressed in differentiated R1 (arrowhead) and R6 (small arrow) PRCs. Colocalization of Elav and BarH1 in R1 and R6 is yellow. (B) *tws*^{p}*/tws*^{p} mutants display BarH1 expression (green) in R1 (arrowhead) and R6 (small arrow). (C) wild type Svp-LacZ expression (anti-ß-galactosidase, green) is observed in R3,4,1,6 (PRCs are numbered accordingly). Colocalization with Elav is yellow. (D) *tws*^{p}*/tws*^{p} displays normal Svp-LacZ expression in R3,4,1,6. The large arrows indicate the direction of morphogenetic furrow progression.

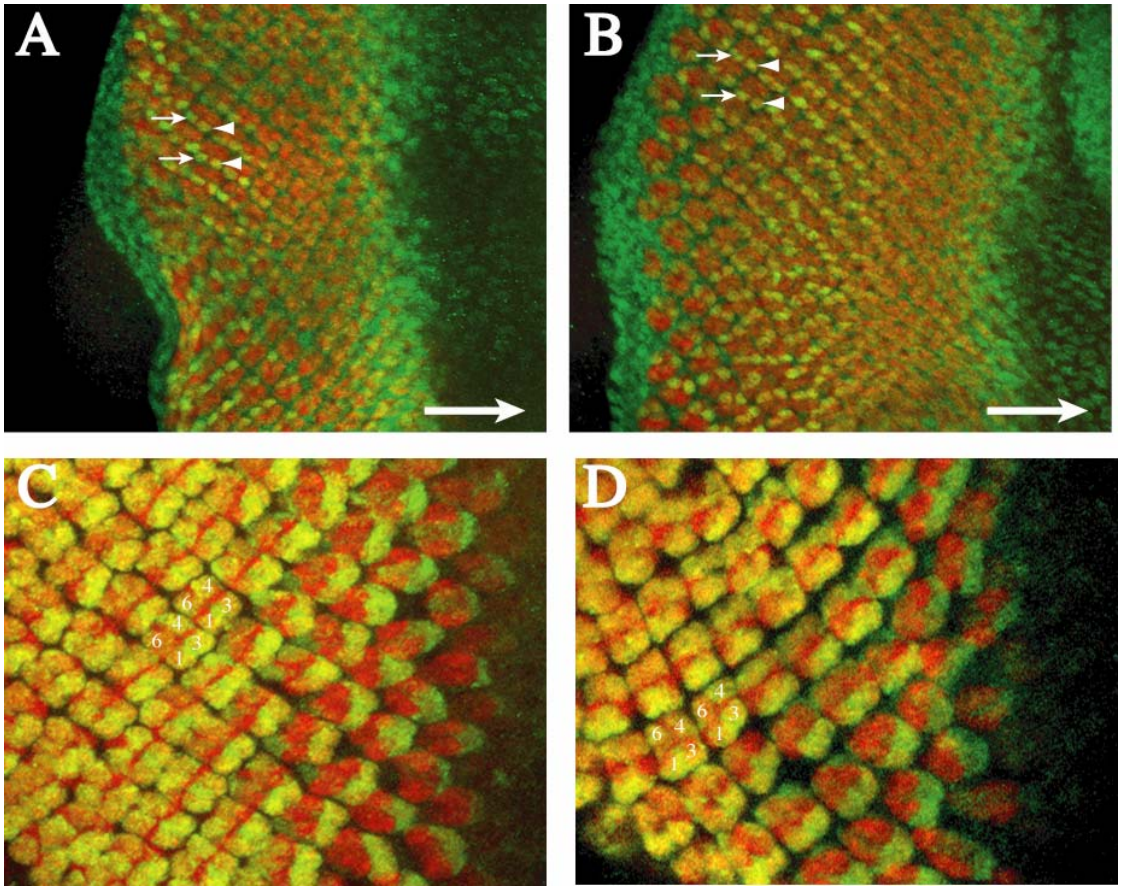
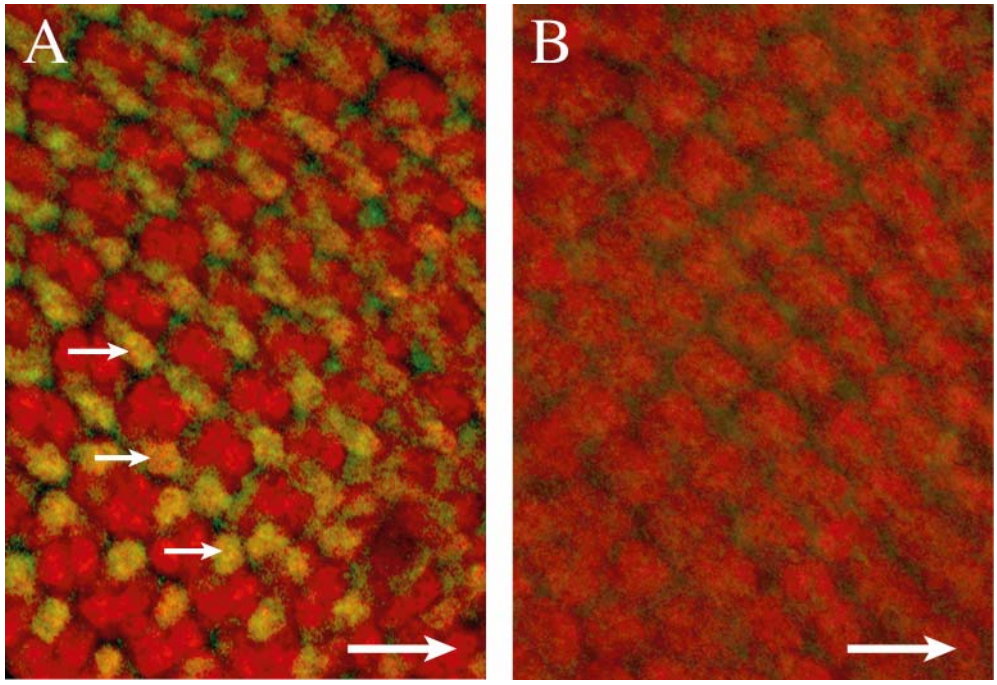

A
B
C
D

Figure 2-5. *tws*^{p} mutants fail to establish R7 as indicated by the loss of Prospero expression in eye-antennal discs from late third instar larvae. (A) wild type, and (B) *tws*^{p}*/tws*^{p}. (A) wild type eye disc stained with Elav (red) to identify PRCs. Prospero (yellow) is expressed in R7. (B) *tws*^{p}*/tws*^{p} mutants have no Prospero expression (yellow), indicating a loss of R7.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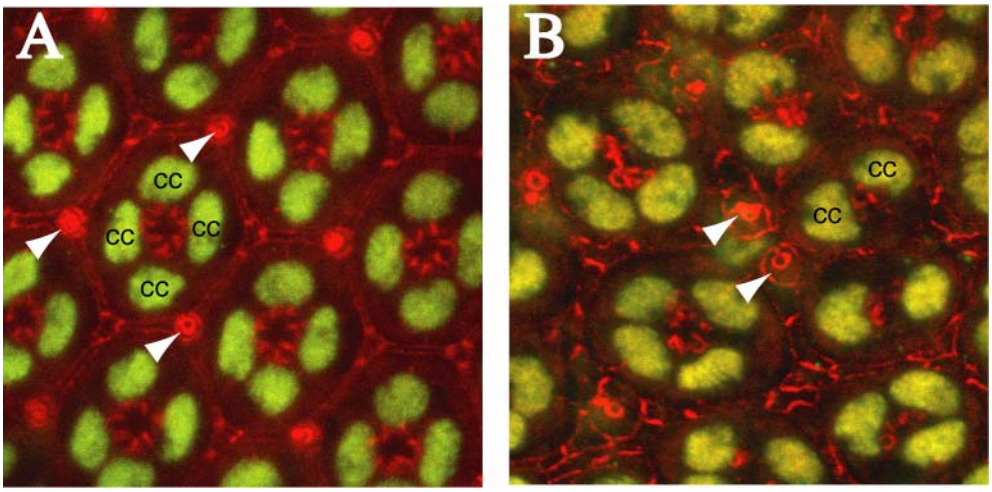
1991). The presumptive cone cells receive stimulation via the R1/6 secreted Spitz. This Spitz activation of the EGFR receptor is sufficient to induce low levels of Pros expression, which in turn contributes to the upregulation of the cone cell specific transcription factor *cut* (Xu et al., 2000). We examined the level of cut expression in the tws^{p}/tws^{p} mitotic clones. The mutant clones had reduced numbers of Cut positive cells (Figure 2-6). Wild type ommatidia have four evenly spaced cone cells. Although some tws^{p}/tws^{p} mutant clones had a full complement of cone cells, many ommatidia had one to three cone cells missing. Along with the loss of Pros expression, tws^{p}/tws^{p} mutant clones fail to specify the proper cell fates from the R7 equivalence groups.

Following the specification of the cone cells, the pigment and bristle cells that make up the hexagonal perimeter of each ommatidium are specified. Although the factors necessary for pigment cell specification are not completely understood, it is known that Ras signaling is necessary for their proper specification (Freeman, 1996; Cagan and Ready, 1989). The pigment cells of tws^{p}/tws^{p} mitotic clones were examined using anti-DE-Cadherin. This protein helps maintain the hexagonal form of the ommatidia by promoting cell-cell adhesion between the ommatidial cells. The pigment and bristle cells of the mutant clones were unorganized and improperly spaced (Figure 2-6). Wild type ommatidia have each of the six corners occupied by a bristle or pigment cell. These two cell types alternate each corner they occupy. Mutant ommatidia often had bristle cells residing next to each other, or extra pigment cells lying between the bristle cells (Figure 2-6). It is unclear if

Figure 2-6. Cone, bristle, and pigment cell establishment is disrupted in *tws*^{p} mutants. (A) wild type, and (B) *tws*^{p}/*tws*^{p} retinas consisting entirely of *tws*^{p}/*tws*^{p} clones (*ey*-Gal4, *uas-flp; Frt82b tws*^{p}/*Frt82b gmr hid*) dissected from young pupae (148 hours after egg deposition). (A) Cut expression (green) is observed in the four cone cells (cc) of each ommatidium. Phalloidin (red) identifies the bristle cells (arrowheads) and pigment cells of the ommatidia. Three bristle cells surround each ommatidium. (B) *tws*^{p}/*tws*^{p} retinas have a reduced number of Cut expressing (green) cone cells (cc). Mutants may have between one and four cone cells. *tws*^{p} mutants also exhibit missing and misplaced bristle cells (arrowheads) and pigment cells.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twins is necessary for the specification of pigment and bristle cells, the spacing of these cells, or both.

EGFR interaction

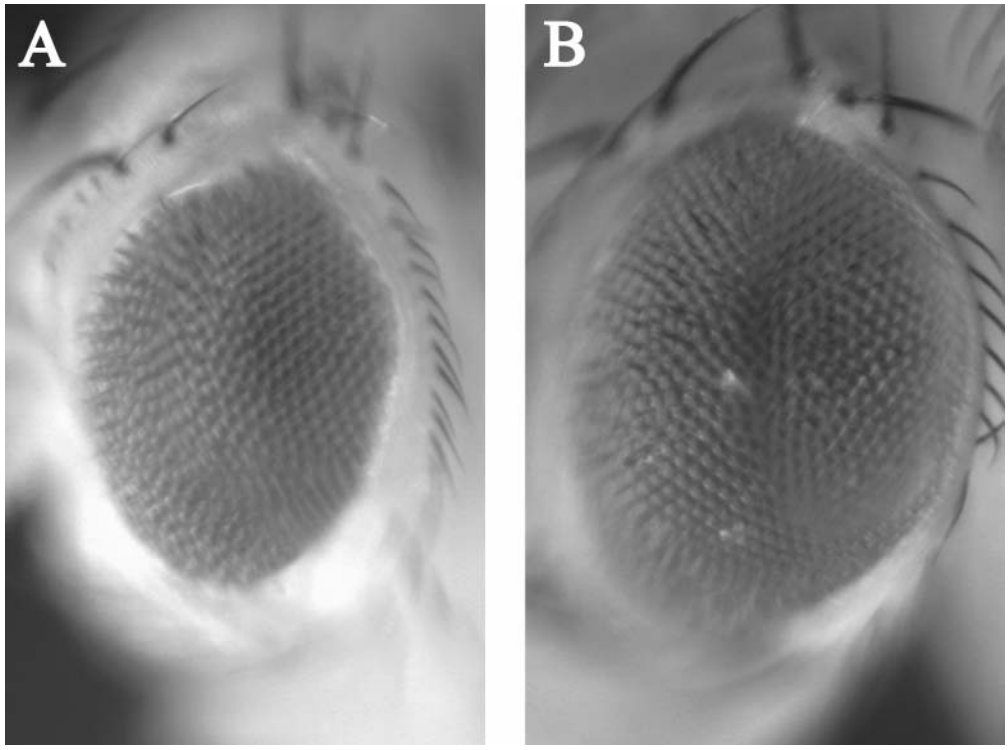
The *egfr*^{E1} (*Ellipse*) mutation is a Dominant hypermorphic allele of EGFR. A single amino acid substitution in the kinase domain of the receptor constitutively activates its signal transduction pathway (Lesokhin et al., 1999). As a result of this hyperactivity of the Ras/MAPK pathway, the R8 founder photoreceptor is not properly specified in heterozygotes. These mutants have fewer R8's, and they are not evenly spaced. Because these flies have fewer R8's at irregular intervals, the other photoreceotors are not recruited properly, and the eyes of the adult are small and rough (Lesokhin et al., 1999).

PP2A is necessary for proper transduction of the Ras/MAPK pathway (Ory et al., 2003). Because *tws* regulates the activity of PP2A, a reduction in *tws* may also effect the transduction of the Ras signal. We therefore examined the adult eyes of *egfr*^{E1}/+ ; *tws*^{p}/+ transheterozygotes to determine if there was a genetic interaction between the two mutations. The transheterozgotes had eyes that were larger and less rough than the *egfr*^{E1}/+ flies (Figure 2-7). It is therefore likely that *tws* is necessary for proper transduction of the Ras/MAPK signal.

Discussion

The Ras/MAPK signal transduction pathway is necessary for the morphogenesis of the adult eye of *Drosophila melanogaster*. This pathway is utilized repeatedly to specify the fates of the different cells of

Figure 2-7. *egfr*^{E1}*/+; tws*^{p}*/ +* adult eyes show a decrease in the small, rough eye phenotype of *egfr*^{E1} animals. Light micrographs of A) *egfr*^{E1}*/ +* adult eye and B) *egfr*^{E1}*/+; tws*^{p}*/ +* adult eye at the same magnification. Dorsal is towards the top, and anterior is to the left. The dominant hyperactive Ellipse allele of EGFR causes a small eye by disrupting proper morphogenetic furrow migration. One copy of the mutant *tws*^{p} allele is able to reduce the Ellipse phenotype, increasing the size of the eye.


A
B

the ommatidia (Kumar and Moses, 2001; Baker 2000; Raabe, 2000; Flores et al., 1998; Miller and Cagan, 1998; Cagan and Ready, 1989). The spatial and temporal activation of the pathway is critical for establishing the correct cell fates of the eye. Ectopic expression can lead to precocious ommatidial development, or improper fate adoption (Lesokhin et al., 1999). In addition to when and where the pathway is turned on, the level of its cellular activation is also important for determining what fate a cell will adopt (Xu et al., 2000).

It is of great interest to understand the mechanisms regulating the consequences of Ras/MAPK activation at different time points and locations. An understanding of the pathway's regulation may reveal how repeated use of the same system is able to generate the different cell fates of the adult eye. Here we show that *twins*, the B/PR55 regulatory subunit of the enzyme PP2A, is necessary for establishing the R7, cone, and pigment cell fates of the *Drosophila* adult eye. These fates represent a subset of Ras/MAPK dependent ommatidial cell fate specification.

The Specification of R8

The movement of the morphogenetic furrow across the eye field is necessary to begin the process of ommatidial development. As the furrow passes, cells just posterior to it become specified as the R8 founding photoreceptor. This process requires the progressive restriction of the transcription factor *atonal* from a band within the furrow to the single R8 cells evenly spaced just posterior to the furrow (Baker, 2000; Jarman et al., 1993; Baker and Rubin, 1989; Lebovitz and Ready, 1986). While it has been shown that Ras/MAPK activation occurs within the furrow, it

does not appear necessary for proper R8 specification. Removal of Ras/MAPK signaling from the furrow does not prevent R8 from being specified, or properly spaced (Lesokhin et al., 1999). Similarly, we have shown that in *tws*^{p} mutant eye discs, loss of Tws does not affect the expression of *atonal*. R8 is properly specified and expresses the terminal R8 marker *boss*. *Tws* may be necessary for Ras/MAPK signal transduction, but its removal has no consequences on *atonal* expression and R8 specification, as Ras/MAPK is not necessary in this process.

Recruitment of Early Photoreceptor Cells

Signaling from R8 is required for the recruitment of photoreceptors R1 through R6. This process is also dependent on proper Ras/MAPK signaling (Jones and Moses, 2004). Loss of this signaling results in a decreased number of photoreceptor cells or their adoption of improper fates. After the specification of R8, R2/5 and R3/4 are recruited to create the five cell pre-cluster through activation of the Ras/MAPK pathway. These cells then recruit R1/6 to the cluster by utilizing this pathway once again. Each time a cell is recruited, the cluster secretes Spitz, which binds EGFR on the precursor to initiate Ras/MAPK activation and fate specification. Once a cell fate is adopted by a precursor, it begins to express transcription factors specific for that cell type.

We have shown that *tws*^{p}/*tws*^{p} mutant eye discs do not affect the recruitment of photoreceptors R1-6 and R8 into the ommatidial cluster. The mutant eye discs contained the proper number of cells expressing *BarH1*, a transcription factor specific for R1/6, and *svp*, a transcription factor specific for R1/6 and R3/4 in each ommatidia. Although activation

of the Ras/MAPK pathway is essential for recruitment of photoreceptors R1-6, it is not necessary to have functional Tws for this process to occur properly.

tws interacts with Ras/MAPK

The model proposed for the Ras/MAPK pathway suggests that PP2A is essential for successful transduction of the Ras signal (Figure 1-3). If this model is valid, *twins* would be expected to interact with the different downstream elements of the pathway. PP2A has been proposed to interact with both Ras and Raf (MAPKKK) downstream of EGFR (Ory et al., 2003; Raabe and Rapp, 2003; Wassarman et al., 1996). Through genetic interaction studies, Wassarman et al. (1996) have shown that PP2A can have an inhibitory effect on Ras activation, but a positive effect on Raf activation. The B regulatory subunit controlling the core enzyme in these studies was not identified, and altering it is likely responsible for this differential effect (Wassarman et al., 1996).

By dephosphorylating serine residues on Ksr and Raf, PP2A promotes the displacement of the scaffolding protein 14-3-3 from these proteins. The displacement of 14-3-3 permits Ksr and Raf to adopt a conformational change, bringing them from the cytosol to the membrane of the cell (Ory et al., 2003; Raabe and Rapp, 2003). Once these proteins are at the membrane, Raf can become associated with the activated Ras, as well as the Ksr bound MEK (Chang and Karin, 2001; Morrison, 2001). Raf activates MEK, which can then in turn activate Erk (MAPK) (Ory et al., 2003; Marshall, 1995).

tws is a positive regulator of PP2A. When it is recruited into the core complex composed of the A and C subunits, it activates the enzyme (Ory et al., 2003; Mayer-Jakel and Hemmings, 1994). We have shown that tws^{p}, a hypomorphic allele of *tws*, reduces the small, rough eye phenotype seen in the EGFR hyperactive mutant $EGFR^{E1}$. Without functional Tws, PP2A would remain inactive, and unable to dephosphorylate Ksr and Raf. This would result in a failure of signal transduction, as the two proteins would be unable to associate with the cell membrane and each other. By decreasing the level of Tws, the level of Ras/MAPK activation is also reduced. While *tws* has an apparent positive influence on Ras/MAPK signalling, another B regulatory subunit, such as B'/PR61 or B''/PR72, may be responsible for the inhibitory effect that Wassarman et al. (1996) observed between PP2A and Raf.

tws^{p} Disrupts R7 Equivalence Group Fates

The photoreceptor R7 is the final photoreceptor to be recruited into the ommatidial cluster. R7 is recruited from a cluster of cells known as the R7 equivalence group (Matsuo et al., 1997; Ready et al., 1976). Each of the cells of this group has the potential to become R7 depending on its level of Ras/MAPK activation. The cell that has the highest level of activation will adopt the R7 fate. Cells with a lower level of activation will adopt the fate of the lens secreting cone cells. High levels of Ras/MAPK activation are achieved in the precursor to R7 through activation of the EGFR and Sev receptors, while only EGFR is activated in the cone cell precursor. This level of activation is reflected by the expression of the transcription factor *prospero*. High levels of Pros are

present in R7 while significantly lower levels are seen in the cone cells (Xu et al., 2002; Kauffmann et al., 1996; Doe et al., 1991).

We have shown that in *tws*^{p} mutant clones no cell of the R7 equivalence group properly adopts the fate of the final photoreceptor. Furthermore, the cone cells that originate from the R7 equivalence group are not properly established. It is plausible that loss of functional Tws protein reduces the ability of PP2A to dephosphorylate Ksr and Raf, therefore preventing their conformational change and association with the cell membrane. Ligand dependent activation of EGFR and Sev would produce activated Ras, but it would be unable to interact with Ksr and Raf to transduce the signal. This would prevent the removal of the *pros* transcriptional inhibitors Yan and Ttk88, resulting in a loss of *pros* expression. We have also shown that *tws*^{p} clones exhibit a loss of the terminal cone cell marker *cut*. Ommatidia normally have four *cut* expressing cone cells, but in *tws*^{p} mutants many ommatidia display a loss of one to three cone cells. The loss of Tws appears to inhibit the level of *pros* expression below the threshold necessary to initiate R7 and cone cell fate specification.

When R7 and the cone cells are specified from the R7 equivalence group, Notch signaling is required in addition to Ras/MAPK activation in the precursor cells to specify their proper fates (Parks et al., 1995; Cagan and Ready, 1989). The activation of the Notch pathway is necessary to suppress the transcription factor *BarH1*. Without Notch activation to suppress this gene, the R7 and cone cell precursors will adopt the R1/6 cell fate upon activation of their Ras/MAPK pathway (Tomlinson and Struhl, 2001). We did not observe any ectopic BarH1 expressing cells in

the tws^{p}/tws^{p} mitotic clones. It is thus unlikely that the loss of R7 and cone cells is due to their adoption of an alternative photoreceptor fate. It is possible that they adopt the pigment cell fate or remain undifferentiated, slated to be removed during the apoptotic wave that occurs during late pupal development.

Tws codes for the B/PR55 regulatory subunit of PP2A. Our data suggest that *tws* is necessary to regulate PP2A positively during the recruitment and differentiation of R7, cone, and pigment cells. It is possible that PP2A is positively regulated by another subunit such as the B'/PR61 or B''/PR72 during the recruitment of the other photoreceptors. The B'/PR61 subunit is coded for by the gene *widerborst* (*wdb*), and like *twins*, it is expressed in the *Drosophila* eye. This subunit has been shown to positively regulate PP2A to promote apoptosis (Van Hoof and Goris, 2003; Deng, et al., 1998), and to properly orient hairs on the cuticle of *Drosophila* wings (Hannus et al., 2002). It is also possible that additional, unidentified genes coding for B regulatory subunits may be differentially expressed in ommatidial precursors. By altering the regulation of Ras/MAPK signaling, the different ommatidial cells can be properly specified and recruited.

tws is a positive B/PR55 regulatory subunit of PP2A activity in the Ras/MAPK signal transduction pathway. Our results demonstrate that *tws* is essential for cell fate specification of the R7 equivalence group members. Without functional Tws, the level of Ras/MAPK activation may be insufficient to achieve the necessary *prospero* transcription that restricts the cells to their fates.

Chapter Three

twins is Necessary for the Progression of the Morphogenetic Furrow

Introduction

The movement of the morphogenetic furrow (MF) across the *Drosophila* eye is essential for ommatidial development. Passage of the MF is necessary for founding the R8 photoreceptor, and to begin the recruitment of the other cells that make up the ommatidial cluster (Heberlein and Treisman, 2000; Ready et al., 1976). The initiation of the morphogenetic furrow can be divided into two phases known as "birth" and "reincarnation" (Kumar and Moses, 2001). The Ras/MAPK pathway plays a major role in controlling these two genetically separable processes.

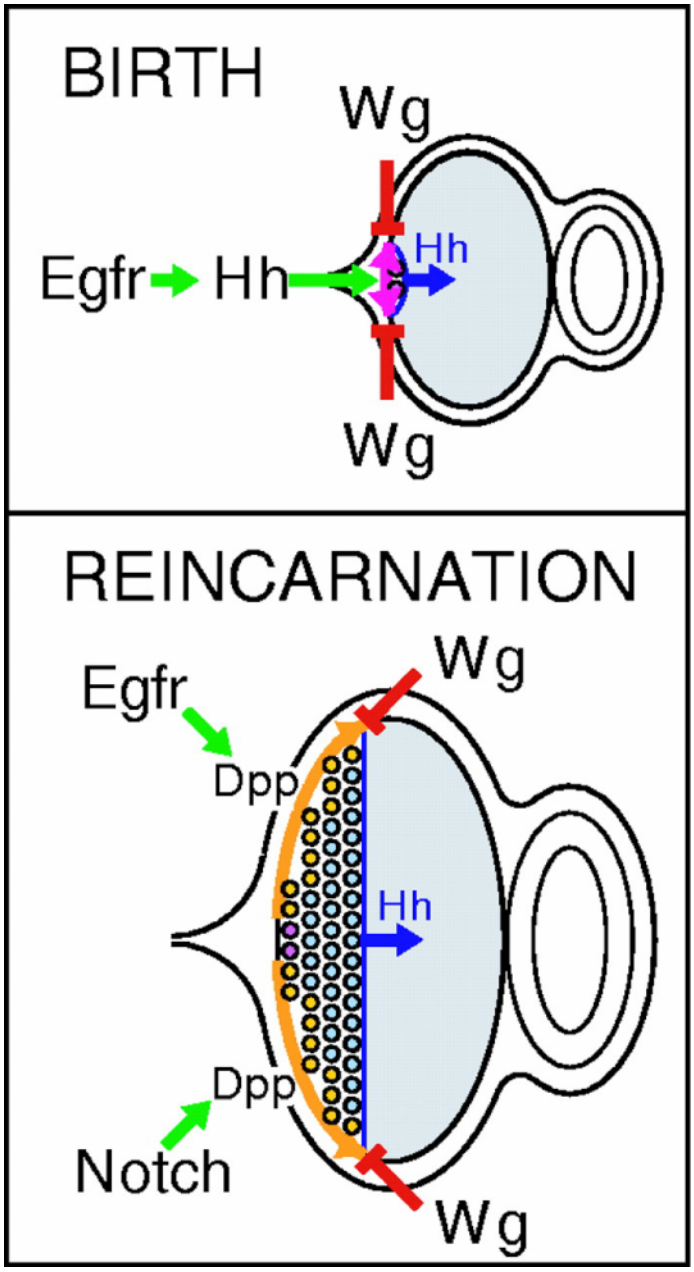
The birth phase of MF initiation occurs midway through the third instar larval stage at the posterior margin of the eye disc. During this process, Ras/MAPK signaling through EGFR functions to regulate positively Hedgehog signaling for the birth. If Hedgehog signaling is blocked, the furrow will not be born regardless if Ras/MAPK is ectopically expressed at the posterior margin (Kumar and Moses, 2001; Borod and Heberlein, 1998; Dominguez and Hafen, 1997). While these factors are promoting the birth, Wingless signaling opposes them by inhibiting the birth of the morphogenetic furrow (Chanut and Heberlein, 1997a; Chanut and Heberlein, 1997b; Ma and Moses, 1995). Kumar and Moses (2001) have shown that the positive birth factors operate downstream of Wingless. Expression of their activated forms will rescue the birth inhibition conferred by ectopic expression of activated Wingless

protein at the posterior eye disc margin. After the birth of the furrow, the factors inducing it will drive the progression of the furrow in the anterior direction for approximately eight ommatidial rows, after which additional factors are necessary for its continued progression during the reincarnation phase. (Kumar and Moses, 2001).

The reincarnation phase of MF initiation again utilizes the EGFR mediated Ras/MAPK pathway. Removal of this pathway after the birth phase will cause the furrow's progression to stop after only eight ommatidial rows are established (Kumar and Moses, 2001). During the reincarnation phase of MF initiation, activation through EGFR as well as activation of the Notch pathway is required to induce the reincarnation of the furrow. EGFR functions upstream of Notch activation, and together the two pathways upregulate Dpp expression. *dpp*, though unnecessary for furrow birth, is essential for inducing MF reincarnation along the lateral margins of the eye field (Mlodzik, 2000; Burke and Basler, 1996; Curtiss and Ma et al., 1993). Along the medial region of the furrow, Hedgehog expressed from the newly produced ommatidia drive furrow reincarnation medially (Figure 3-1).

Because tws^{p}/tws^{p} *Drosophila* mutants exhibit eyes that are smaller than wild type, and PP2A is known to regulate Ras/MAPK signal transduction positively (Dougherty, et al., 2005; Ory, et al., 2003; Sieburth, et al., 1999), we examined if *tws* has a role in either the birth or reincarnation phases of MF initiation. tws^{p}/tws^{p} mutants successfully complete the birth phase of MF initiation, but are unable to achieve reincarnation of the furrow. In tws^{p}/tws^{p} mutants the furrow is inhibited after it has established eight ommatidial rows, consistent with the

Figure 3-1. Models for furrow birth and reincarnation in the eye-antennal imaginal dics. Green arrows show inductive interactions, red symbols show inhibitory influences. Purple arrows and Hh in birth model indicates first ommatidial induction and purple ommatidia are the first ones produced. Blue furrows and arrows indicate Hh progression signal from the newly established ommatidia, and blue ommatidia are those produced by this mode of Hh induction. Orange arrows show progressive furrow re-initiation along the lateral margins and orange ommatidia are those induced by Dpp. (From Kumar and Moses, 2001)


BIRTH
Wg
Egfr
Hh
Hh
Wg
REINCARNATION
Egfr
Wg
Dpp
Hh
Dpp
Notch
Wg

phenotype observed when the Ras/MAPK pathway is inhibited after birth. Furthermore, ectopic expression of *tws* along the lateral margins of the eye-antennal disc was able to induce ectopic furrows, a phenotype also observed when Ras/MAPK is ectopically activated in this location. The function of *tws* to regulate Ras/MAPK positively is further supported by its interaction with inhibitors and activators of MF initiation. As seen with Ras/MAPK activation, ectopic expression of *tws* was able to rescue MF inhibition conferred by *wg* expression and a dominant negative form of EGFR, but not that caused by a loss of functional Notch activation. These observations suggest that *tws* positively regulates the Ras/MAPK pathway during initiation of the morphogenetic furrow.

Materials and Methods

Drosophila stocks

All flies were maintained at 25°C on standard yeast-glucose media. For all experiments, we used the background strain, w^{1118}, as a control line. The following stocks were obtained from Bloomington Stock Center. *w; wg^{sp-1}/ CyO; dpp^{blk}-gal4 / Tm6b Tb*, which expressed Gal-4 protein along the posterior and lateral margins of the third instar eye-antennal disc. *w; uas-wg*, which was used to express ectopically *wg*. *w; Dr^1 e^1/ Tm3 Sb^1 e^1 uas-dl.DN*, which was used to express ectopically dominant negative *delta*. *y w; uas-EGFR.DN; uas-EGFR.DN*, which was used to express ectopically dominant negative *egfr*. The *w; uas-tws^1* allele used to express ectopically *twins* was generated as described below. All stocks were maintained over the balancer chromosomes *Tm6B Tb^1*, *Tm3 Sb^1 Ser^1*, or *CyO*.

Females of the genotype *uas-tws*^{1} were crossed to males of each of the other *uas* lines to generate male offspring carrying the *uas-tws*^{1} and either *uas-wg*, *uas-EGFR.DN*, or *uas-Dl.DN*. Each of these males were then crossed to females of the genotype *dpp*^{blk}*-gal4 / Tm6B Tb*. *Tb*$^{+}$ to express ectopically the genes of interest along the posterior and lateral margins of the third instar eye disc.

Generation of uas-tws^{1}

In order to ectopically express *tws*, it was necessary to generate a fly carrying a *uas-tws* construct. LD12394, a full length cDNA clone of *tws*, was obtained from BACPAC (Oakland, CA; Stapleton *et al.*, 2002a, b). The *tws* cDNA ORF was excised by an EcoRV/Bsu36I digest and directionally cloned into a *pUAST* plasmid (Brand and Perrimon, 1993). The *pUAST* plasmid was modified with an adapter at the EcoRI restriction site. The adapter had restriction sites for SwaI and Bsu36I, making the opened plasmid compatible with the excised *tws* ORF. Embryos were injected with the above construct along with *pπ25.7wc* helper plasmid (Karess and Rubin, 1984) and transformants were selected using standard procedures (Santamaria, 1986; Rubin and Spradling, 1982). A line was obtained with an insertion on the second chromosome and designated as *uas-tws*^{1}.

Immunohistochemistry

For examination of photoreceptors and the morphogenetic furrow, wandering third instar larvae were dissected. Eye-antennal discs were dissected from larvae in cold PBS and fixed for 0.5 hours in PBS + 4% paraformaldehyde at room temperature. Antibody staining was performed as described in Tomlinson and Ready (1987). Fixed tissue was washed 4 x 10 minutes in PBS + 0.2% Triton X-100, and blocked for 0.5 hour in PBS + 0.2% Triton X-100 + 10% Normal Goat Serum (NGS). Primary antibody was added to fresh blocking solution, and tissue was incubated overnight at 4^0C with gentle shaking. Following incubation, discs were washed 6 x 10 minutes in PBS + 0.2% Triton X-100. Secondary antibody was added to PBS + 0.2% Triton X-100 + 5% NGS and incubated with the discs for 2 hours at room temperature with gentle shaking. Tissue was then washed 3 x 10 minutes in PBS and mounted in Vectashield. Discs were visualized on a Leica TCS SP2 confocal microscope.

Primary antibodies used were rabbit anti-GFP conjugated to Alexa Fluor 488 (Molecular Probes) 1:500 and rat anti-Elav (O'Neill et al., 1994) 1:1000 obtained through the Developmental Studies Hybridoma Bank (developed under the auspices of the NICHD and maintained by The University of Iowa, Department of Biological Sciences, Iowa City, IA 52242). The secondary antibody used was a goat anti-rat conjugated to Alexa Fluor 488 (Molecular Probes) 1:500. Phalloidin conjugated to Alexa Fluor 546 (Molecular Probes) 1:50 was used to stain cell membranes.

Results

Inhibition of Morphogenetic Furrow Progression

In order to investigate the effect of the *tws*^{p} mutation on the morphology of the adult eye, we generated *tws*^{p}/*tws*^{p} mitotic clones in every cell of the developing eye disc. This was accomplished using the *flp-frt* method. The eyes of these animals were smaller and rougher than wild type eyes (see Figure 2-2). The movement of the morphogenetic furrow across the presumptive eye field is necessary to initiate ommatidial development. If this process is inhibited, ommatidia will not be specified, and the adult *Drosophila* eye will be smaller than wild type. Because the Ras/MAPK pathway is essential for the initiation of the MF, we were interested in understanding the role of *tws* in MF progression.

Using antibodies against Elav, we were able to monitor the number of ommatidial rows generated in the *tws*^{p}/*tws*^{p} mutants. At 106 hours after egg deposition (AED) in a constant 25^{0}C environment, the MF in wild type animals has progressed about halfway across the eye field, generating sixteen ommatidial rows (Figure 3-2a). New ommatidial rows are added to the eye field at the rate of one row every 1-1.5 hours. The eye field of *tws*^{p}/*tws*^{p} mutant animals have a reduced number of ommatidial rows at 102 hours AED. At this time in development, the animals have only eight ommatidial rows (Figure 3-2b). This suggests that in the mutant clones, birth of the MF occurs, but reincarnation is retarded.

tws can induce ectopic furrows

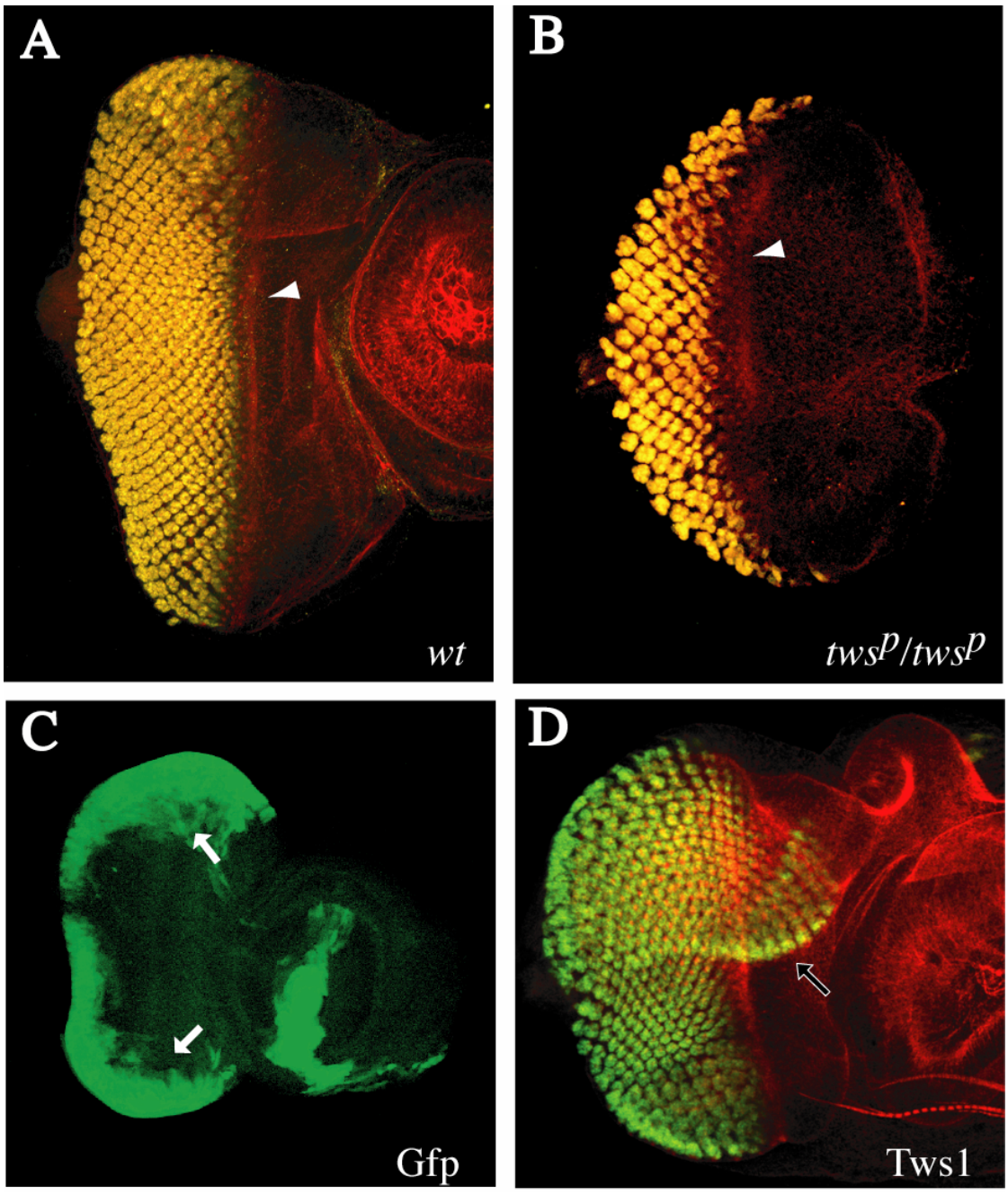
Ectopic activation of the Ras/MAPK pathway has been shown to induce ectopic furrows (Kumar and Moses, 2001). Since tws^{p}/tws^{p} animals have a phenotype in which MF progression has been halted shortly after birth, we were interested in learning if ectopic *tws* could induce additional furrows. To answer this question, we used the dpp^{blk}-*Gal4* driver in combination with our *uas-tws* construct to ectopically express *tws* in a wild type background. The dpp^{blk}-*Gal4* construct expresses Gal4 along the posterior and lateral margins of the eye disc (Figure 3-2c). This differs from the endogenous *dpp* pattern of expression which is also extends along the MF (Curtiss and Mlodzik, 2000; Ma et al., 1993).

When *tws* was ectopically expressed along the posterior and lateral margins of the eye field, initiation of ectopic furrows occurred in the lateral region of the eye. This was observed as an overgrowth of the lateral margins of the normal MF (Figure 3-2d). The induction of ectopic furrows was previously observed when Ras/MAPK was ectopically expressed along the lateral margins of the developing eye disc (Kumar and Moses, 2001). Similar to what was seen with Ras/MAPK activation, overexpression of *tws* appears to induce the initiation of ectopic morphogenetic furrows.

tws interaction with wingless and Notch

Because ectopic *tws* is able to induce ectopic furrows, we were interested in determining epistatic relationships with the other factors controlling MF birth and reincarnation. During birth, and again at

Figure 3-2. Reincarnation of the morphogenetic furrow fails in *tws*^{p} mutants. (A) wild type, and (B) *tws*^{p}/*tws*^{p} eye-antennal disc dissected 106hrs. after egg deposition. Discs are stained with anti-Elav (yellow) labeling photoreceptor cells (PRCs), and Phalloidin (red) which marks F-actin. White arrowheads indicates morphogenetic furrow. (A) wild type disc with sixteen ommatidial rows. (B) *tws*^{p}/*tws*^{p} disc with eight ommatidial rows. *tws* signaling is sufficient to induce furrow initiation. Expression of *tws* is controlled by *dpp*^{blk}-Gal4. Hollow arrows show ectopic furrows, and solid arrows show Gal4 expression. (C) *dpp*^{blk}-Gal4 is expressed along the posterior and lateral margins of the third instar eye-antennal eye disc as indicated by *uas-GFP* expression (green). (D) Ectopic furrow along the lateral margin is induced by *uas-tws* expression. Elav is green, F-actin is red.


A
wt
B
twsP/twsP
C
Gfp
D
Tws1

reincarnation, *wingless* functions to inhibit the initiation of the furrow (Chanut and Heberlein, 1997a; Chanut and Heberlein 1997b; Ma and Moses, 1995). When we ectopically expressed *wg* using *dpp^{blk}-Gal4*, MF birth was prevented (Figure 3-3a). When *wg* was co-expressed with *tws*, we observed the induction of an ectopic furrow at the lateral margins of the eye disc (Figure 3-3b). *tws* was able to induce a furrow in the presence of *wg*, suggesting that *tws* function is downstream of *wg* to initiate the furrow.

Notch functions during the reincarnation of the furrow. Unlike *wg*, *Notch* is a positive regulator of the MF, and its expression induces the formation of ectopic furrows (Kumar and Moses, 2001). Since *tws* also functions to induce furrow initiation, the epistatic relationship of these two genes was investigated. We removed *Notch* expression by using a Dominant Negative form of the *Notch* ligand *Delta* (*uas-Dl.DN*). When expressed under the control of *dpp^{blk}-Gal4*, the reincarnation of the furrow was blocked (Figure 3-4a). Since the birth of the furrow was unaffected, only six to seven ommatidial rows were produced. Co-expression of *Dl.DN* with *tws* did not rescue the reincarnation phenotype (Figure 3-4b). This suggests that *tws'*, like EGFR (Kumar and Moses, 2001), function is upstream of *Notch* during furrow reincarnation, and downstream of *wg* during both birth and reincarnation.

Interaction with Ras/MAPK to Initiate Furrow

We have shown that *tws* is necessary for furrow reincarnation during MF initiation, and capable of inducing furrows when ectopically expressed. *tws* function is upstream of *Notch* and downstream of *wg*

Figure 3-3. *tws* expression rescues inhibition of morphogenetic furrow birth caused by Wg. Ectopic expression of proteins named in each panel along the posterior and lateral margins of late third instar eye-antennal discs is controlled by dpp^{blk}-Gal4. Arrowheads indicate furrow inhibition, hollow arrows show ectopic furrows. Elav is yellow, F-actin is red. (A) Birth of furrow is inhibited when *wg* is ectopically expressed. (B) Ectopic expression of *tws* rescues *wg* inhibition, initiating ectopic furrows at the lateral margins.

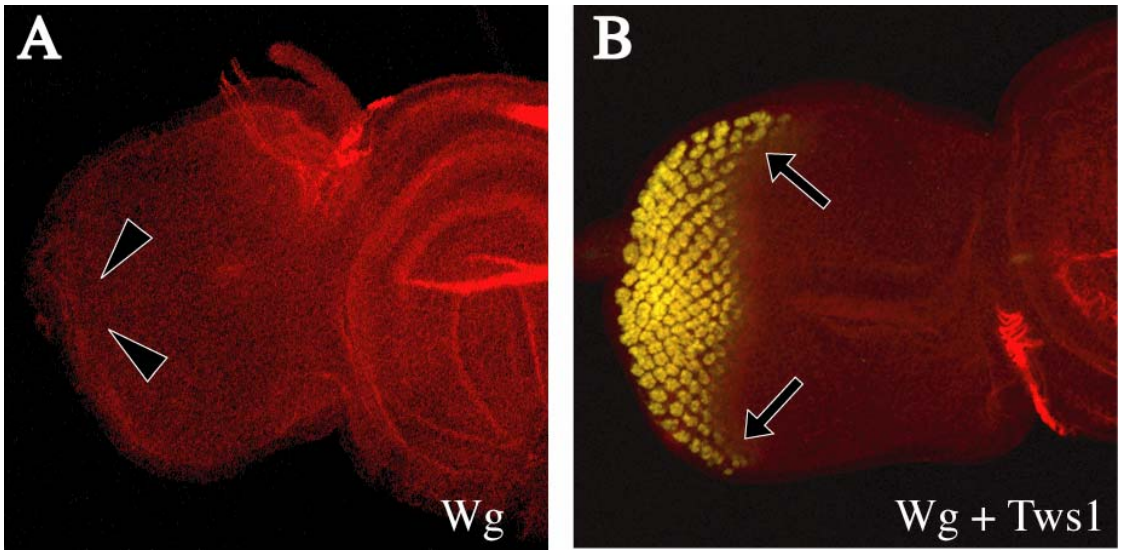
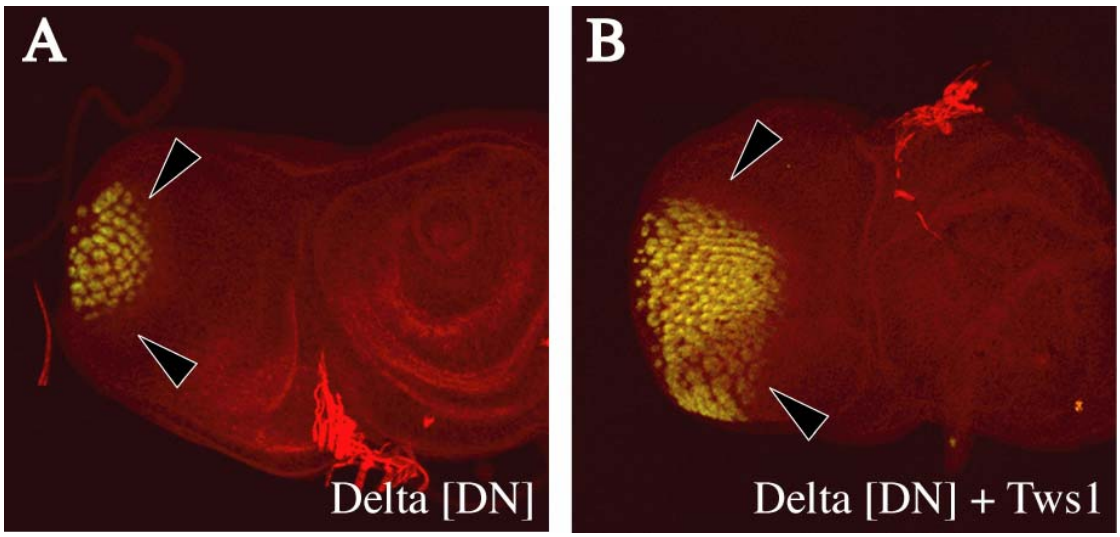
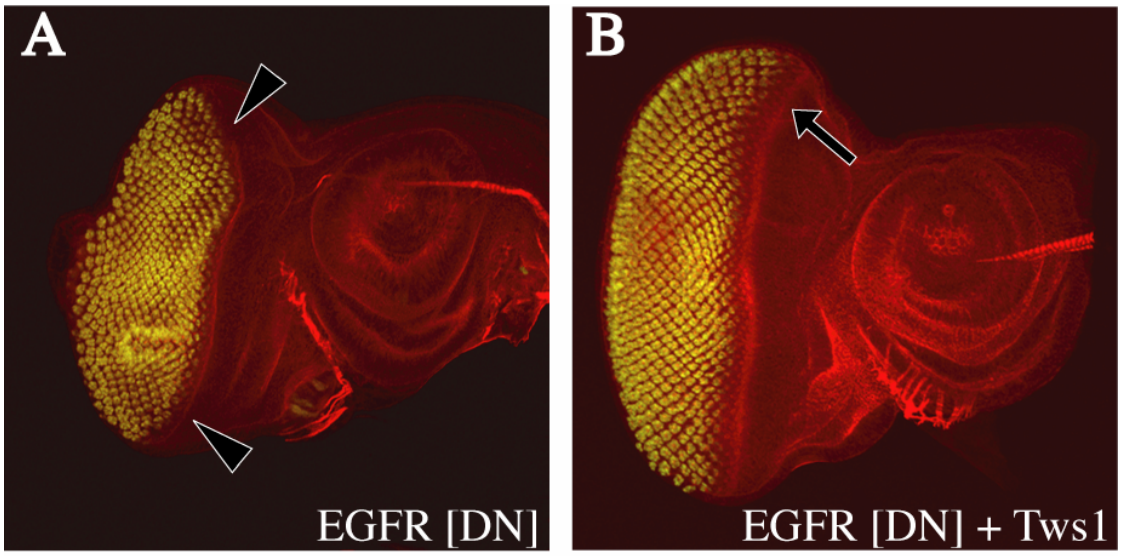

A
Wg
B
Wg + Tws1

Figure 3-4. *tws* expression fails to rescue reincarnation inhibition caused by loss of Notch signaling (*uas-Dl.DN*) along the lateral margins of the eye disc. Ectopic expression of proteins named in each panel along the posterior and lateral margins of late third instar eye-antennal discs is controlled by dpp^{blk}-Gal4. Arrowheads indicate furrow inhibition. Elav is yellow, F-actin is red. (A) Loss of Notch signaling inhibits reincarnation of the furrow, but not birth. (B) Ectopic expression of *tws* does not rescue reincarnation inhibition caused by *uas-Dl.DN*.


A
Delta [DN]
B
Delta [DN] + Tws1

during reincarnation. Because *tws* regulates the interaction of PP2A with the Ras/MAPK pathway, we examined the nature of *tws'* epistatic relationship with Ras/MAPK. To investigate this, we expressed a Dominant Negative form of EGFR (*uas-EGFR.DN*) under the control of dpp^{blk}-*Gal4* (Figure 3-5a). This caused an inhibition of furrow reincarnation, but not birth since endogenous EGFR activation necessary for birth occurs before dpp^{blk}-*Gal4* is expressed. Co-expression of *EGFR.DN* with *tws* caused an induction of ectopic furrows at the lateral margins (Figure 3-5b). These results suggest that *tws* is functioning downstream of EGFR. This is consistent with *tws* function to regulate Ras/MAPK activity positively downstream of EGFR through its interaction with PP2A.

Figure 3-5. *tws* expression rescues reincarnation inhibition caused by loss of Ras/MAPK pathway activation (*uas-EGFR.DN*). Ectopic expression of proteins named in each panel along the posterior and lateral margins of late third instar eye-antennal discs is controlled by dpp^{blk}-Gal4. Arrowheads indicate furrow inhibition, hollow arrow shows ectopic furrows. Elav is yellow, F-actin is red. (A) Reincarnation of furrow is inhibited along the lateral margins when Dominant Negative EGFR is ectopically expressed there. (B) Ectopic expression of *tws* rescues EGFR.DN reincarnation inhibition, initiating ectopic furrows.


A
EGFR [DN]
B
EGFR [DN] + Tws1

Discussion

tws^{p} inhibits reincarnation

We have shown that in *tws^{p}/tws^{p}* mutant eye discs, the reincarnation phase of morphogenetic furrow initiation, a Ras/MAPK dependent event, is delayed. These mutants were successful during the birth phase of initiation to generate a furrow, but were unable to reincarnate the furrow after it has progressed eight ommatidial rows. This is consistent with *tws* function to positively regulate the Ras/MAPK signaling pathway, which is necessary for both the birth and reincarnation of MF initiation (Kumar and Moses, 2001).

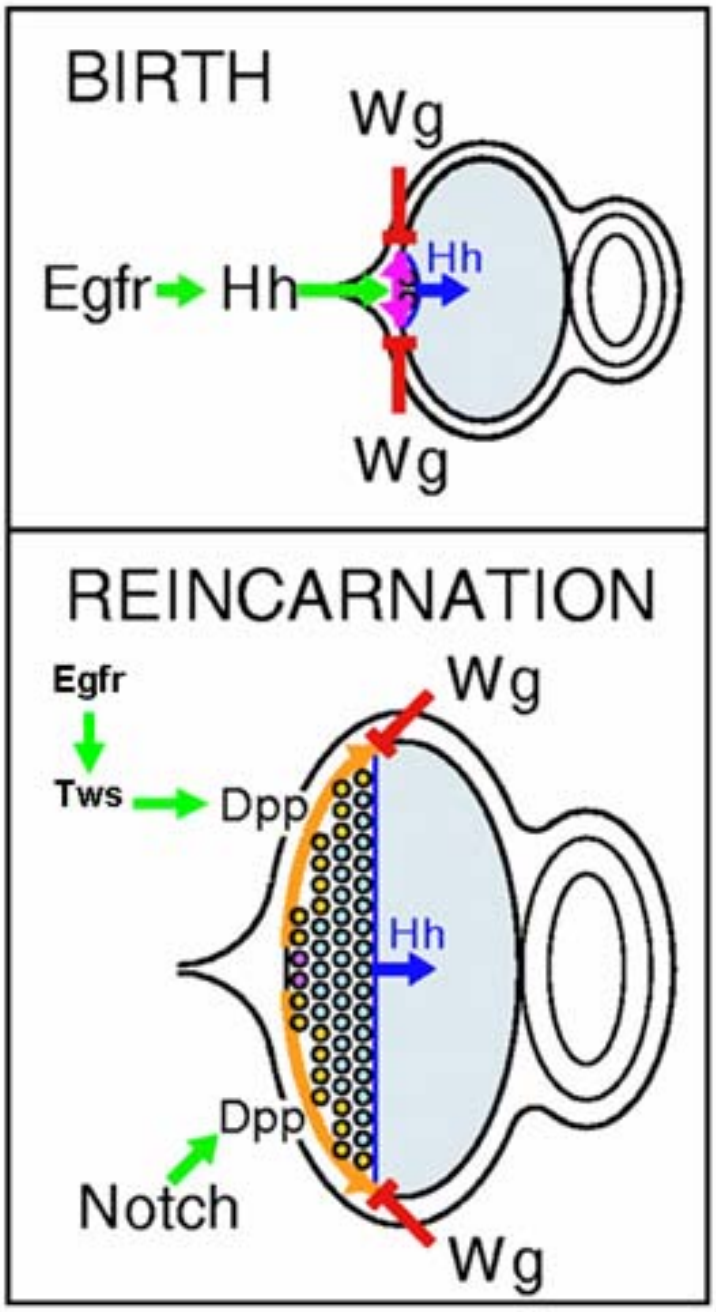
The Ras/MAPK pathway must be activated during two time intervals that Kumar and Moses have termed temperature-sensitive points one and two (TSP1 and TSP2). At 25^{0}C TSP1 occurs 84-90 hours after egg deposition (AED) to initiate the birth of the MF, while TSP2 occurs 96-102 hours AED to induce the reincarnation of the furrow. Removal of Ras/MAPK at any time interval other than the two TSPs will have no effect on the initiation of the furrow. However, if Ras/MAPK signaling is removed during TSP1, birth will be inhibited, while removal during TSP2 will inhibit reincarnation. This results in no eye, and reduced eye size phenotypes, respectively (Kumar and Moses, 2001).

Our results show that *tws^{p}* has the effect of only inhibiting the reincarnation of the furrow, while birth appears to occur normally. Although Ras/MAPK is required for both processes, *tws* appears only necessary for one. It is possible that the *tws* B/PR55 regulatory subunit is only used for reincarnation purposes, while another B regulatory subunit may be utilized during the birth phase. It would be of interest to study the

effects of other B regulatory mutants on MF birth. Since ectopic expression of *tws* was able to induce MF initiation, *tws* appears to be sufficient for MF birth. It may be possible that, like Ras/MAPK, *tws* is necessary for MF birth, but tws^{p} does not reduce the level of functional protein enough to inhibit birth. This would be true if reincarnation is more sensitive to Ras/MAPK activation levels than birth, and requires a higher level of pathway activation to successfully reincarnate the furrow. This could be tested by developing a complete *tws* null allele and examining furrow birth in this mutant.

Our results support the hypothesis that *tws* functions to promote the reincarnation of the MF during the TSP2 period of Ras/MAPK activation (Figure 3-5). Ectopic expression of *tws* was able to rescue the EGFR.DN phenotype of reincarnation inhibition. Like Ras/MAPK, *tws*' function appears to act downstream of the MF inhibitor *wingless*, but upstream of the positive MF factor *Notch*. These results are consistent with the theory that *tws* functions to regulate Ras/MAPK signal transduction positively through its interaction with the enzyme PP2A.

Figure 3-6. Models for furrow birth and reincarnation in the eye-antennal imaginal dics. Green arrows show inductive interactions, red symbols show inhibitory influences. Purple arrows and Hh in birth model indicates first ommatidial induction and purple ommatidia are the first ones produced. Blue furrows and arrows indicate Hh progression signal from the newly established ommatidia, and blue ommatidia are those produced by this mode of Hh induction. Orange arrows show progressive furrow re-initiation along the lateral margins and orange ommatidia are those induced by Dpp. In this model, Tws function is downstream of EGFR to regulate the Ras/MAPK signal transduction pathway positively upon activation of EGFR. (From Kumar and Moses, 2001)


BIRTH
Wg
Egfr
Hh
Hh
Wg
REINCARNATION
Egfr
Wg
Tws
Dpp
Hh
Dpp
Notch
Wg

Chapter Four

twins is Necessary for Mechanosensory Bristle Development

Introduction

twins, the B/PR55 regulatory subunit of the enzyme PP2A has many effects on *Drosophila* post-embryonic development. We have shown that *twins* is necessary for specifying the fates of the R7 equivalence group. Without proper *twins* expression, R7 and the cone cells fail to be specified, and the pigment and bristle cells surrounding the photoreceptors are disorganized. Furthermore, we have shown that *twins* is necessary for the reincarnation phase of morphogenetic furrow initiation. When *twins* is not properly expressed, the birth of the furrow occurs normally, but it can not reincarnate during the second Ras/MAPK dependent phase. Both of these phenotypes, ommatidial fate specification and morphogenetic furrow reincarnation, are consistent with *twins* role in positively regulating the Ras/MAPK signal pathway.

In addition to its involvement in adult eye development, there is evidence that *twins* plays a role in the development of other sensory structures in *Drosophila*. The specification of mechanosensory machrochaete and microchaete bristles, and wing disc compartment specification are all dependent on proper *twins* expression (Bajpai et al., 2004; Shiomi et al., 1994; Uemura et al., 1993). *tws* mutants exhibit a duplicated mechanosensory bristle phenotype that lacks the associated neuron and sheath cell. In wild type animals, the four cells of the mechanosensory structure (bristle, socket, neuron, sheath) arise from a single progenitor. Because the bristle and socket are duplicated in the

twins mutants, it would be of interest to understand what happens to the neuron/sheath progenitor in these mutants.

In conjunction with the sensory organ phenotype, the wing discs of *twins* mutants exhibits a duplicated wing pouch phenotype (Uemura et al., 1993). In wild type animals, the ventral half of the wing disc is restricted to a wing pouch fate, from which the wing will develop. The dorsal end is restricted to the notal fate that produces the dorsal thorax of the fly. *twins* mutants display excessive wing pouch tissue at the expense of notal tissue in the third instar wing disc (Bajpai et al., 2004; Uemura et al., 1993). Activation of the Ras/MAPK pathway is necessary for specifying notal fate, while *wingless* is responsible for restricting the ventral disc to wing pouch phenotype (Baonza et al., 2000; Wang et al., 2000; reviewed in Klein, 2001). Because *twins* interacts with both of these pathways in adult eye development, it is likely to be involved in wing disc fate specification.

Further characterization of *twins* role in adult eye specification is also needed to better understand the role of PP2A in regulating the Ras/MAPK pathway. If *twins* is not involved during the specification of photoreceptors R1-6 and the birth phase of morphogenetic furrow initiation, it may be possible that another B regulatory subunit is modulating the activity of PP2A to permit Ras/MAPK activation.

Materials and Methods

Drosophila stocks

All flies were maintained at 25°C on standard yeast-glucose media. For all experiments, we used the background strain w^{1118} as a control line. The *tws* gene is located on the third chromosome (85F13-14). The P-insertional alleles tws^{196} and tws^{1003} were obtained from the Szeged Stock Center (Rorth et al., 1998; Rorth, 1996). The P-insertional allele tws^{p} was a gift of D. Glover (Mayer-Jaekel et al., 1994; Mayer-Jaekel et al., 1993; Uemura et al., 1993), and the tws^{430} allele was a gift of C. Zuker as part of a third chromosome mutagenesis collection. The *hh-lacZ* (Ma et al., 1993), and *neur-lacZ* (Huang et al., 1991) enhancer trap lines were obtained from the Bloomington Stock Center. All stocks were maintained over the balancer chromosomes *Tm6B* Tb^{1}, *Tm3* Sb^{1} Ser^{1}, or *CyO*.

Immunohistochemistry

For examination of wing discs, wandering third instar larvae were dissected. Wing discs were dissected from larvae in cold PBS and fixed for 0.5 hours in PBS + 4% paraformaldehyde at room temperature. For examination of early pupal thoraxes, young pupae (26 hours after puparium formation) were removed from their pupal membranes and cut open along the ventral midline to fillet the animal open. Antibody staining was performed as described in Tomlinson and Ready (1987). Fixed tissue was washed 4 x 10 minutes in PBS + 0.2% Triton X-100, and blocked for 0.5 hour in PBS + 0.2% Triton X-100 + 10% Normal Goat Serum (NGS). Primary antibody was added to fresh blocking solution, and tissue was incubated overnight at 4^{0}C with gentle shaking. Following

incubation, discs were washed 6 x 10 minutes in PBS + 0.2% Triton X-100. Secondary antibody was added to PBS + 0.2% Triton X-100 + 5% NGS and incubated with the discs for 2 hours at room temperature with gentle shaking. Tissue was then washed 3 x 10 minutes in PBS and mounted in Vectashield. Discs were visualized on a Leica TCS SP2 confocal microscope.

Primary antibodies used were mouse anti-Prospero (Spana and Doe, 1995) 1:200 obtained through the Developmental Studies Hybridoma Bank (developed under the auspices of the NICHD and maintained by The University of Iowa, Department of Biological Sciences, Iowa City, IA 52242). The secondary antibody used was goat anti-mouse conjugated to Alexa Fluor 488 (Molecular Probes) 1:500. β-Galactosidase expression was visualized through an x-gal enzymatic reaction (Ashburner, 1989).

Light Microscopy

Pharate adult whole mount thoraxes were visualized with a Leica MZ FL III microscope and photographed with a Leica DC 500 camera. Animals were dissected from their pupal membrane, mounted in a 1:1 glycerol/water solution and photographed.

Results

Mechanosensory cell fate specification

tws^{p} mutants experience a plethora of phenotypes in the development of their sensory structures. The mechanosensory macrochaetes and microchaetes covering the dorsal thorax of the fly each consist of four types of cells. These four components of each

mechanosensor are the bristle, socket, neuron, and sheath cell which arise from a single sensory organ precursor (SOP) (Figure 4-1) (Okabe et al., 2001; reviewed in Jan and Jan, 1994; Roegiers et al., 2004; Ghysen et al., 1993; Ghysen and Dambly-Chaudière, 1989). Examination of tws^{p}/tws^{p} pharate adults has shown that *tws* is necessary for the neuron and sheath cell to develop properly (Shiomoi et al., 1994). In tws^{p} mutants, the sensory hairs and sockets are duplicated at the expense of the neuron and socket cells (Figure 4-2) and (Shiomoi et al., 1994). In addition to the duplication of sensory bristles and sockets, tws^{p}/tws^{p} animals displayed a cuticle balding phenotype in which large patches of sensory bristles were missing from the thorax (Figure 4-2). This loss of bristles is due to a failure of SOP specification at the DV boundary of the wing pouch (Bajpai et al., 2004).

The restriction of the SOP daughter cells to their proper fates involves *Notch* mediated lateral inhibition (Figure 4-1). In a process similar to that seen during proneural cluster restriction to specify the founding photoreceptor R8, one of the two SOP progeny, the pIIa cell, inhibits the other daughter, the pIIb cell. During this process, pIIa prevents pIIb from adopting the pIIa fate, and thus forces the adoption of the pIIb fate (Bellaiche et al., 2001; reviewed in Roegiers et al., 2004). As a result, pIIa will divide to produce the hair and socket cells while pIIb gives rise to the neuron and sheath cells. The transcription factor *prospero* (*pros*) is transiently expressed in the presumptive neuron and sheath cells, and is necessary for the adoption of their respective cell fates (Manning and Doe, 1999).

Figure 4-1. Mechanosensory organ specification. (a) Adult mechanosensory bristles consist of a single hair shaft (Sf) and socket (So) on the external surface of the animal's cuticle. One neuron (N) and sheath (Sh) cell are associated with each organ on the internal surface of the cuticle. A glial (G) cell is also associated with the organ, but often migrates away following specification of a complete sensory organ. (b) The specification of each sensory organ cell type is lineage dependent. Precursor cells asymmetrically allocate proteins into daughter cells conferring specific cellular fates. In this example Prospero (green) is shown which confers the neuronal fate. As a cell divides, Notch mediated lateral inhibition (N) between the new daughter cell further restricts the fates a cell can adopt. Here the pIIa cell inhibits the pIIb cell from adopting the pIIa fate.

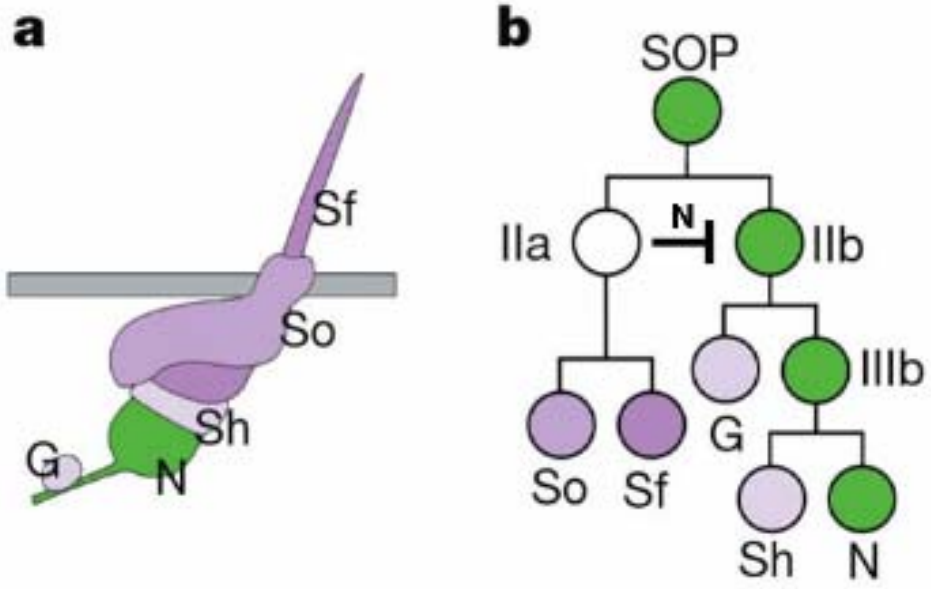
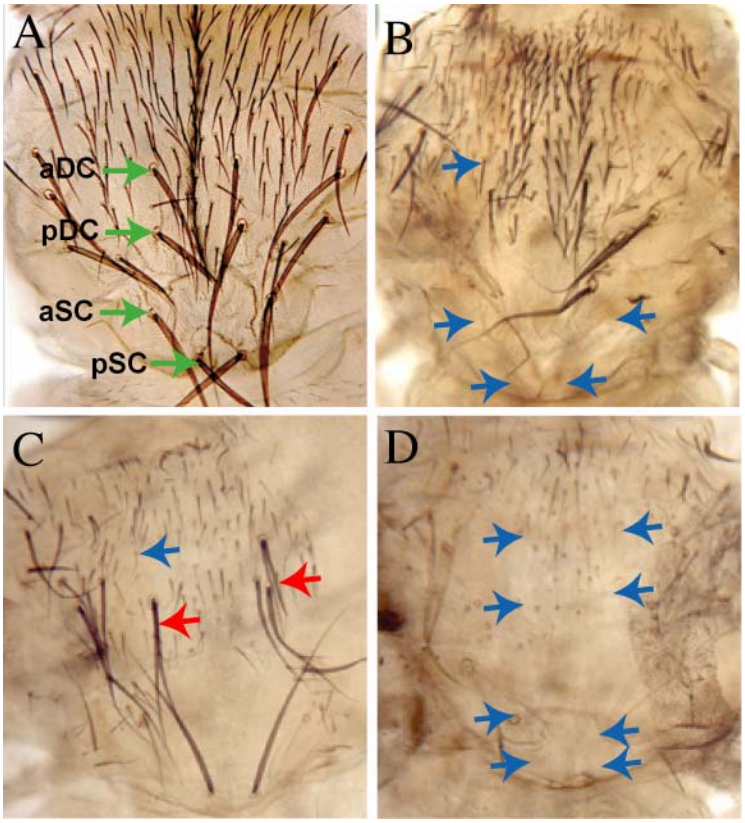

a
Sf
So
Sh
G
N
b
SOP
IIa
N
IIb
IIIb
G
So
Sf
Sh
N

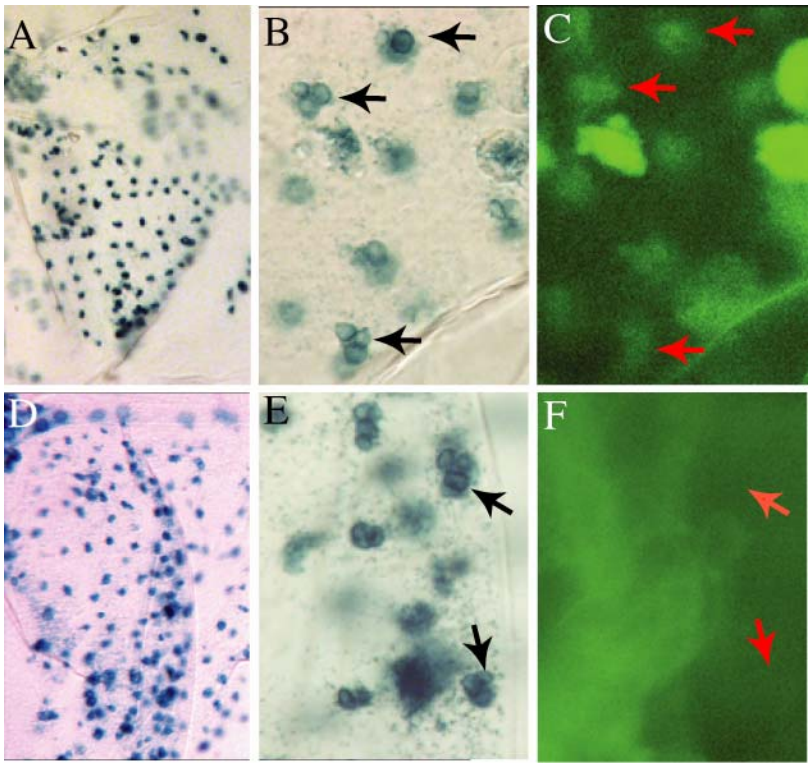
Figure 4-2. *tws* bristle phenotype in the *Drosophila* notum. All images are of pharate adults dissected from their pupal case. Anterior is towards the top. (A) Wild-type. Large macrochaete bristles labeled are anterior dorsocentral (aDC), posterior dorsocentral (pDC), anterior scutellar (aSC), and posterior scutellar (pSC) bristles. Shorter hairs are microchaetes. (B) *tws430/tws430* mutants exhibit a loss of sensory bristle phenotype. Loss of five macrochaetes are shown (blue arrows). (C) tws^{1003}/tws^{430} and (D) tws^{196}/tws^{430} exhibit even more severe microchaete and macrochaete loss (blue arrows). (C) tws^{10033}/tws^{430} displayes the duplicated sensory bristle phenotype (red arrow).


A
aDC
pDC
aSC
pSC
B
C
D

tws^{430}/*tws*^{430} mutants do not express *pros* during neuronal/sheath cell development (Figure 4-3a). This loss of Pros expression is not due to the loss of the neuron/sheath progenitor. When a sensory precursor divides, it is able to properly produce two daughter cells. The gene *neuralized* is expressed in the precursors of all the macro/microchaete mechanosensors. When Prospero is absent from *tws*^{430}/*tws*^{430} mutants, these animals still contain the correct number of progenitor cells (Figure 4-3b).

The pIIa cell is unresponsive to the *Notch* inhibitory signal pIIb reciprocates on it. This *Notch* signal is blocked by the membrane associated protein *numb* present in pIIa, but not pIIb (Roegiers et al., 2004; Bellaiche et al., 2001). When the SOP divides, *numb* is asymmetrically localized into one of the daughter cells, pIIa. It would be of interest to determine the expression pattern of *numb* in a *tws* mutant background. Because *tws* appears to possess two pIIa cells, it is possible that *numb* is be localized equally into the daughter cells of the SOP. *tws* may play a role in establishing proper orientation of the SOP to successfully segregate *numb*. Playing a role in SOP orientation would not be the first time *tws* has been identified in contributing to cellular orientation during mitosis. It is known that in *tws* mutants, cells of the developing central nervous system fail to establish proper mitotic spindle arrangements. This failure to orient the spindles creates problems for the migration of chromatids during mitotic anaphase (Gomes et al., 1993; Mayer-Jaekel et al., 1993). The chromatids become disorganized, and the cells do not complete mitosis. If *tws* has a similar role in establishing the

Figure 4-3. *tws* mutants have no Prospero expression in neural and glial cell precursors. (A,B,C) Wild-type and (D,E,F) tws^{430}/tws^{430} notum of flies dissected at 26 hours after puparium formation. (A,B,D,E) X-gal staining of *neuralized-lacZ* (blue), a marker of the sensory organ precursor (SOP) and its progeny. (C,F) Magnified view of the same animals as in B and E respectively. Neural and glial cell precursors stained with Prospero antibody (green). (A,D) Microchaete SOPs are properly established in both wild type and tws^{430}/tws^{430} mutants. (B,E). Magnified view of the nota shows various SOP progeny at the 3-cell stage (arrows), which includes pIIa (socket and bristle precursor), a neuron, and a glial cell. (C) Prospero (green) is expressed in the developing neuron and glial cell progeny of the SOP. (F) No Prospero expression is detected in tws^{430}/tws^{430} associated with the X-gal stained cells in E (arrows).


A
B
C
D
E
F

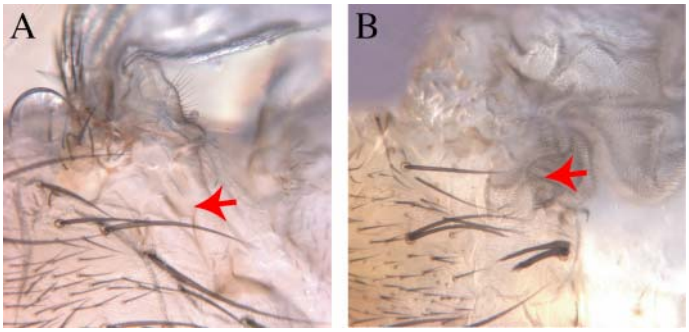
proper orientation of protein trafficking scaffolding, the *tws* mutants may fail to chauffeur *numb* properly into only pIIa.

Compartmentalization of the wing disc

One of the many phenotypes observed in *tws* mutants is the "twinning" of the third instar wing pouch. In these animals there is ectopic pouch tissue along the posterior dorsal zone of the wing disc at the expense of presumptive notal tissue (Bajpai et al., 2004; Mayer-Jaekel et al., 1993; Uemura et al., 2003). This fate transformation of notum to wing can also be seen in tws^{p}/tws^{p} pharate adults (Figure 4-4). In these animals there is ectopic wing tissue located at the posterior region of the notum.

During the second instar of the larval life cycle, the wing disc is subdivided into ventral and dorsal regions. The dorsal zone gives rise to the notum, while the ventral is the presumptive wing of the adult. In wild type animals, *wingless* is the first gene to be expressed initiating this subdivision. *wg* is expressed in a wedge shaped region located at the ventral anterior end of the early second instar wing disc (Klein, 2001; Baonza et al., 2000; Wang et al., 2000). Following the expression of *wg*, EGFR is activated by its ligand Vein, which is upregulated along the dorsal posterior zone of the second instar wing disc. These two proteins, Wg and activated EGFR, function to inhibit each other's expression while also specifying presumptive wing and notum, respectively (Klein, 2001; Baonza et al., 2000; Wang et al., 2000).

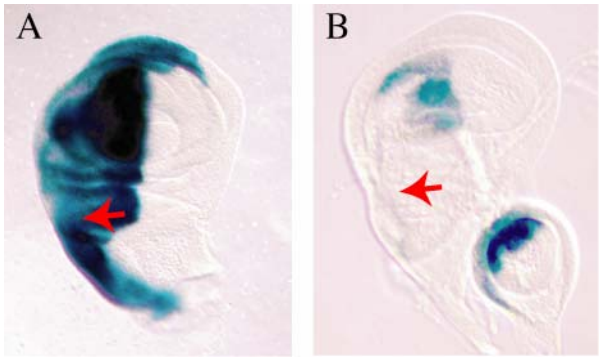
Figure 4-4. *tws^{p}/tws^{p}* mutants fail to establish proper wing hinge fate. *tws^{p}/tws^{p}* mutants exhibit a notum to wing tissue transformation. Anterior is to the left, and animals are viewed dorsally. Red arrows point to the posterior hinge region which in wild type is composed of cuticular tissue. (A) wild type, and (B) *tws^{p}/tws^{p}* pharate adults.


A
B

The cause of the ectopic wing pouch tissue may be due to a breakdown of the *wg* EGFR mutual inhibitory process. If EGFR fails to become activated by *vein*, then *wg* will be free to extend its pattern of expression outside of the ventral anterior zone, and into the dorsal notal tissue (Baonza et al., 2000; Wang et al., 2000). We have examined this possibility by determining the expression pattern of the posterior wing disc marker *hedgehog*. *hedgehog* is a target gene of activated EGFR. Upon its activation, EGFR induces the upregulation of *engrailed*, which in turn upregulates *hedgehog* expression (Baonza et al., 2000). In addition to the encroachment of *wg* into the notal zone of the wing disc, the failure of EGFR to become activated will result in the loss of the posterior marker *hedgehog*. In the wing discs of tws^{p}/tws^{430} mutants, there is a loss of Hedgehog expression from the posterior region of the wing disc (Figure 4-5). It is likely that the lowered level of *tws* expression is blocking PP2A from functioning properly in the Ras/MAPK signaling pathway, therefore inhibiting the successful transduction of the EGFR signal and upregulation of *hedgehog* expression. It would be of interest to see if ectopic expression of *tws* or activated EGFR in the ventral region of the wing disc would be sufficient to inhibit *wg* expression and transform wing tissue to a notal fate.

Bajpai et al., 2004, have shown that *tws* is necessary for proper signal reception during specification of the wing margin mechanosensory and chemosensory hairs of the adult. Without proper *wg* reception, factors such as *armadillo* that are necessary for specifying the sensory cells, are not properly regulated, inhibiting the sensory cells from being established. We have observed that in addition to the wing margin

Figure 4-5. *tws^{p}/tws^{430}* mutants fail to establish posterior notum as indicated by the loss of Hedgehog-LacZ expression in third instar wing discs. Posterior is to the left, and ventral is up. (A) wild type, and (B) *tws^{p}/tws^{430}* wing discs dissected from late third instar larvae. (A) wild type wing disc with x-gal staining (blue) showing Hh expression in the posterior region of the wing disc. (B) *tws^{p}/tws^{430}* mutants have a reduced level of Hh-LacZ staining indicating a failure in *hh* expression.

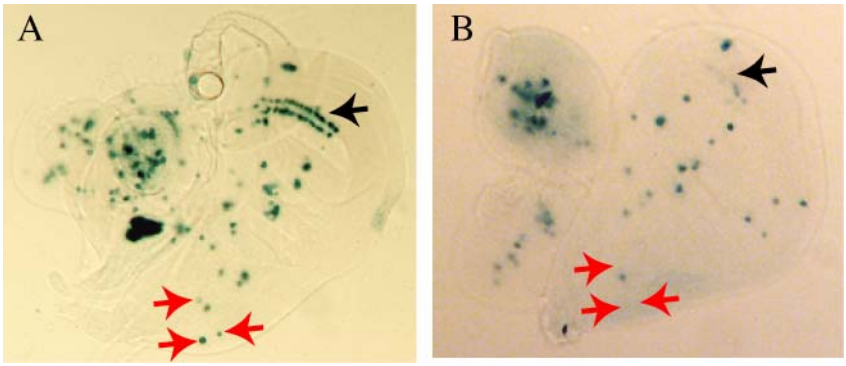

A
B

sensory cells, mechanosensory SOPs are missing throughout the third instar wing disc (Figure 4-6). It would be of interest to examine the cause of the missing SOPs. Because *wg* is known to be responsible for establishing only a subset of SOPs (Bajpai et al., 2004; Tomoyasu et al., 1998) it is possible that *tws* is regulating other genes necessary to specify mechansensory SOPs. By the third instar stage, the wing disc is subdivided into many compartmentalized zones. Each of these zones can be identified by various genetic markers (reviewed in Hidalgo, 1998). Because the establishment of SOPs is dependent on proper reception of different morphogen concentrations, any disruption in gene expression may alter the morphogen levels, preventing SOP specification. If there is a decrease in EGFR activation, leading to ectopic *wg* expression in the notal zone, the expression of compartmental genes may be perturbed, resulting in the loss of SOPs.

Regulation of Ras/MAPK

We have shown that *tws* is necessary for fate specification of R7 equivalence group cells, and reincarnation of the morphogentic furrow during adult eye development. Both of these events represent a subset of Ras/MAPK dependent processes that occur in adult eye development. It would be of interest to determine which additional B subunits are necessary for regulating the other Ras/MAPK dependent processes. Since it is known that PP2A is essential for dephosphorylating Ksr and Raf to allow their association with Ras, and activation of MEK and Erk (Ory, et al., 2003, Raabe and Rapp, 2003; Tzivion and Avruch, 2002; Morrison, 2001), it is likely that other B subunits are necessary for functionality of

Figure 4-6. *tws^{p}/tws^{p}* mutants fail to establish SOP fates indicated by the loss of *neuralized-lacZ* expression, an SOP marker, in third instar wing discs. Posterior is to the right, and ventral is up. (A) wild type, and (B) *tws^{p}/tws^{p}* wing discs dissected from late third instar larvae. (A) wild type wing disc with x-gal staining (blue) showing *neuralized-lacZ* expression in the wing margin, black arrow, and three macrochaete precursors, red arrows. (B) *tws^{p}/tws^{p}* mutants have a reduced number of *neuralized-lacZ* staining SOPs in the wing margin, black arrow, and are missing the three macrochaete precursors shown in A.


A
B

PP2A during photoreceptor R1-6 specification and birth of the morphogenetic furrow. Understanding the role these subunits play in Ras/MAPK signaling will help to better understand how regulating a single pathway can produce different developmental outcomes.

In addition to examining the role of other B subunits in Ras/MAPK signal transduction, development of a *tws* null allele may prove to be of great value. It is possible that the lack of a mutant phenotype during furrow birth and photoreceptor R1-6 establishment may be due to a requirement for different levels of *tws* expression. If these processes are less sensitive to *tws* levels, and do not require as much functional protein as reincarnation and R7 equivalence group soecification, then the mutant phenotype may be masked by the low level of functional Tws in tws^{p}/tws^{p} mutants.

Conclusion

In this thesis we have shown that *twins*, the B/PR55 regulatory subunit of the phosphatase PP2A is essential for the development of the adult *Drosophila melanogaster* eye. *twins* is necessary for proper specification of R7, the final photoreceptor specified in each ommatidium. *twins* is also necessary for the specification of the four cone cells, non-neural elements of the eye that secrete the lens of each ommatidium. Without proper *twins* activity, these cells fail to be specified. Furthermore, *twins* appears to be necessary for proper spacing of the pigment and bristle cells which isolate each ommatidium from their neighbor. All of these cells are specified from the R7 equivalence group, a collection of undifferentiated cells with the potential to adopt either the

R7, cone, or pigment cell fate. *Tws* is likely acting in these processes through its regulation of PP2A, an enzyme necessary for the successful transduction of the Ras/MAPK signal by dephosphorylating Raf and Ksr.

In addition to its function in ommatidial cell fate specification, *tws* is necessary for the reincarnation phase of morphogentic furrow initiation, but not its birth. Both of these events are dependent upon Ras/MAPK activation. Without functional *twins*, the furrow is born but progresses only until the second Ras/MAPK dependent checkpoint. Without functional Ras/MAPK pathway activation, the furrow's progression is halted. It is now evident that *tws* is a gene necessary for at least two distinct Ras/MAPK dependent eye development phenomena; morphogenetic furrow initiation, and ommatidial cell specification.

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