THE UNIVERSITY OF CHICAGO

INTERACTIONS BETWEEN EYES ABSENT AND JAK/STAT SIGNALING DURING *DROSOPHILA* DEVELOPMENT

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LIST OF ABBREVIATIONS

Abl	Abelson
BC	Border cells
Cdc	Cell division cycle
ChIP	Chromatin immunoprecipitation
CoIP	Co-immunoprecipitation
Dac	Dachshund
Dome	Domeless
Dpp	Decapentaplegic
Drk	Downstream of receptor kinase
DV	Dorsal-ventral
ED	Eya domain
EGFR	Epidermal growth factor receptor
Elav	Embryonic lethal abnormal vision
En	Engrailed
Et	Eye transformer
Ey	Eyeless
Eya	Eyes absent
Fj	Four-jointed
FLP	Flippase
FnIII	Fibronectin type-III domain
FRT	Flippase recognition target
Gal4	Galactose-responsive transcription factor
GFP	Green fluorescent protein
GMR	Glass-multimerized reporter
Hh	Hedgehog
Hth	Homothorax

Hop	Hopscotch
Jak	Janus kinase
Lat	Latran
LMEE	Lozenge minimal enhancer element
MF	Morphogenetic furrow
Myr	Myristoylation
N	Notch
NLS	Nuclear localization sequence
Omb	Optomotor blind
Pak	p21-activated kinase
PIAS	Protein inhibitors of Stat
PPN	Preproneuronal region
PTB	Phosphotyrosine binding
Ptc	Patched
PTP	Protein tyrosine phosphatase
pY	Phospho-tyrosine
R	Photoreceptor cell
Rac	Ras-related C3 botulinum toxin substrate
RD	Retinal determination
Ro	Rough
RT-PCR	Reverse transcriptase polymerase chain reaction
RTK	Receptor tyrosine kinase
RTL	Rough-LacZ^{tau}
S2	Schneider 2 Cells
Sev	Sevenless
SH2	Src homology 2
Slbo	Slow border cells

So	Sine oculis
SOCS	Suppressor of cytokine signaling
Stat	Signal transducers and activators of transcription
Stg	String
TGF	Transforming growth factor
Toy	Twin of eyeless
UAS	Upstream activating sequence
Upd	Unpaired
Wg	Wingless
WT	Wild-type

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ABSTRACT

Development of an adult organism from a fertilized embryo requires coordinated cell proliferation, specification, differentiation and morphogenesis. These processes are controlled by a limited collection of conserved signaling pathways that integrate with tissue specific gene regulatory networks. The best understood mechanism for integration involves transcriptional interactions, in which combinatorial control of gene expression by signaling effectors together with transcription factors dictates the appropriate developmental program. Additional opportunities for interaction present when one considers factors that can act both in the nucleus and cytoplasm. The Drosophila retinal determination gene *eyes absent* (*eya*) provides an excellent model for studying how such a protein might interact with signaling pathways in multiple locations within a cell. In this dissertation, I explore Eya's interactions with the Jak/Stat pathway in the nucleus and the cytoplasm, and how these interactions differ between developmental contexts.

Eya, though initially discovered in Drosophila, is conserved across metazoans and encodes a dual-function transcriptional cofactor and phosphatase that shuttles between the nucleus and cytoplasm. Eya's nuclear role in transactivation has been extensively studied, but understanding of its functions in the cytoplasm remain limited. In Drosophila, tyrosine phosphorylation of Eya promotes its cytoplasmic accumulation, leading to the hypothesis that interactions with phosphotyrosine-based signaling networks will direct its cytoplasmic functions. In this study, I show that *eya* synergizes with components of the Jak/Stat pathway in photoreceptor axon targeting. This discovery both identifies a phosphotyrosine-based signaling circuit with which Eya may interact and defines a novel developmental context for Jak/Stat

signaling. Further characterization of the *eya*-Jak/Stat genetic synergy using subcellularly restricted *eya* transgenes suggests that the interactions involve cytoplasmic Eya. Expanding on this, biochemical analyses in cell culture show that Eya can form protein complexes with three Jak/Stat pathway members, Hop, Socs36E and Socs44A. Structure-function experiments conclude that the Eya-Socs44A complex is mediated by direct interaction between the Src Homology 2 domain of Socs44A and tyrosine phosphorylated Eya. Subcellularly, Eya becomes redistributed into cytoplasmic punctate structures, suggesting interactions with Socs44A contribute to the dynamics of Eya localization and function within a cell.

To investigate whether Eya interacts with Jak/Stat members in contexts outside the eye and if the interactions have effects on pathway signaling, genetic interactions were explored in two additional tissues, the egg chamber and wing imaginal disc. I find that *eya* has a novel role in regulating border cell migration during egg chamber maturation, a process which also requires Jak/Stat signaling. Interestingly, *eya* and effectors of the pathway no longer genetically synergize as they do in axon targeting, suggesting context-specific interactions. In the wing, I find that ectopic *eya* increases expression of a Jak/Stat pathway reporter, indicating that Eya can provide positive input to signaling. Characterization of this effect with subcellularly restricted *eya* transgenes reveals involvement of Eya's role in the nucleus as a transcriptional coactivator. This suggests that Eya, together with its transcriptional cofactor Sine oculis (So), may regulate activity of the pathway by altering gene expression, for example members of the Jak/Stat signaling pathway. Transcriptional interactions appear mutual in which the Jak/Stat pathway may also augment Eya-So transcriptional output, as effectors of the pathway synergize with ectopic *eya* to induce expression of the Eya-So target *dachshund*. Taken all together, my work identifies

novel interactions between Eya and the Jak/Stat signaling pathway, and suggests that the mechanism and consequence of these interactions differ depending on the tissue. This work sets forth a model in which gene networks integrate with signaling pathways both during cytoplasmic signal transduction and downstream transcriptional regulation to generate context-dependent cellular behaviors.

CHAPTER I

Introduction

Development is a complex coordination of cellular decisions occurring in the right sequence, place and time. Within a tissue, this process is known as organogenesis, and merges survival cues with signals influencing specification, differentiation and morphogenesis. One particularly well-suited model for studying the balance and interplay between these processes is the developing Drosophila eye. Fly eye development begins with a five-day period of cell proliferation that spans the early first instar to mid-third instar larval stage. During this time, regional specification demarcates antennal versus eye fates along the anterior-posterior axis of the imaginal disc and establishes the dorsal-ventral boundary. A sharp transition occurs in the mid/late third instar eye disc, when cells stop proliferation and then differentiate into the photoreceptors and non-neuronal cell types that will make up the adult eye. The onset of differentiation is triggered by a progressive wave called the morphogenetic furrow (MF) that starts from the posterior and moves anteriorly across the entire eye field over a two-day period that spans mid-third instar to early pupa. Once photoreceptor cells have differentiated, they undergo elaborate morphogenetic changes necessary for transducing information to the brain. In a late third instar imaginal disc, when the MF has traveled about half-way across the tissue, the entire progression from specification to morphogenesis can be appreciated within different regions of the tissue relative to this wave of differentiation (Figure I-1). This feature of capturing temporal progression in the physical organization of the tissue, combined with the power of

Drosophila genetics, has made the fly eye a useful tool for elucidating mechanisms of developmental regulation.

Because the molecular mechanisms that drive fundamental developmental processes are extraordinarily conserved across metazoans, the developing fly eye has also proven to be an ideal system for investigating the interplay between signal transduction pathways and tissue-specific gene regulatory networks. Signal transduction pathways are the primary source of external information received by a cell, and gene regulatory networks provide the cell-intrinsic effector mechanisms that govern context-appropriate responses to the signaling information. These two circuits must be deeply intertwined for proper execution of development, and understanding of how such integration is achieved is critical not only for understanding normal development but also to achieving success with stem cell based regenerative medicine and to identifying and correcting the signaling imbalances that underlie diseases such as cancer.

In this thesis, I present my discovery of a novel interaction between *eyes absent* (*eya*), a transcription factor that operates within an eye-specific gene regulatory network, and the Jak/Stat signal transduction pathway, during retinal cell morphogenesis. Extending the analysis beyond the eye revealed that *eya* and the Jak/Stat pathway intersect in other developmental events, but with the nature of the interaction and ensuing output changing based on the context. As a framework for considering my work, this introduction summarizes current understanding of the individual roles for Eya and the Jak/Stat signaling pathway in Drosophila eye development. The chapter begins with a summary of Eya and the Jak/Stat pathway, followed by description of the adult fly visual system and its cell types, and ends with discussion of current understanding of

how Eya and Jak/Stat signaling independently contribute to proliferation, regional specification, differentiation and morphogenesis during eye development.

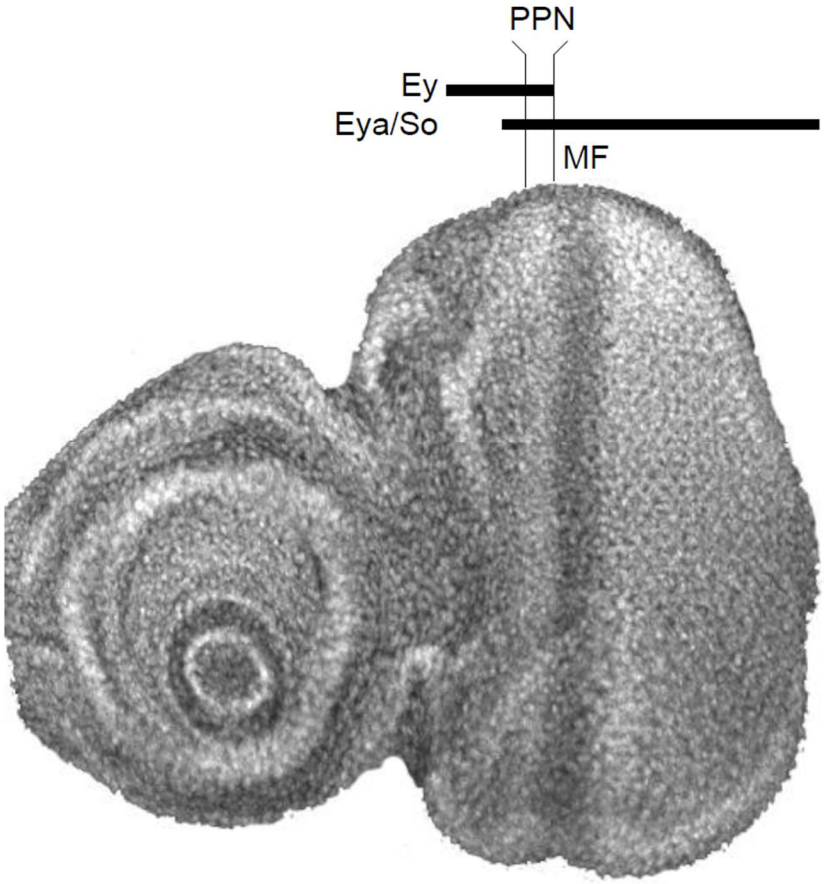

Figure I-1 | Late third instar antennal-eye imaginal disc
Third instar antennal-eye disc stained with the nuclear marker, DAPI oriented anterior to the right, posterior to the left, dorsal up and ventral down. The morphogenetic furrow (MF) can be seen as a depression in the disc. The domain of *eyeless* (*ey*) expression is anterior to the MF while the *eyes absent* (*eya*) and *sine oculis* (*so*) expression domain initiates just ahead of the furrow and continues in and posterior to the furrow. A portion of the overlapping domain between Ey and Eya-So just ahead of the MF is known as the preproneuronal region (PPN). Cells anterior to the MF are proliferating and specifying their eye fate while cells posterior to the MF are differentiating into photoreceptors and undergoing morphogenesis.

I.1 – Overview of Eya and the Jak/Stat signaling pathway

I.1.1 – Eya is a core member of the retinal determination gene network

Eye development is regulated by a collection of "master regulator" transcription factors collectively known as the retinal determination (RD) gene network. In addition to *eya*, other core members of the RD network include the Pax6 protein, *eyeless* (*ey*), the SIX family homeodomain protein, *sine oculis* (*so*) and the DACH box family protein, *dachshund* (*dac*) (Figure I-2A). These genes have earned their place in the RD network by being both necessary and sufficient for eye formation. As such, their loss within the presumptive eye field results in loss of eye tissue, while their ectopic expression outside of the normal eye field can induce formation of ectopic eye tissue (Bonini *et al.* 1993, 1997; Cheyette *et al.* 1994; Quiring *et al.* 1994; Mardon *et al.* 1994; Serikaku and O'Tousa 1994; Halder *et al.* 1995; Shen and Mardon 1997; Pignoni *et al.* 1997).

ey is the earliest of these RD genes to be expressed, with its expression onset coinciding with embryonic specification of the eye anlagen (Quiring *et al.* 1994). *ey* expression persists throughout the eye portion of the antennal-eye imaginal disc until the third instar when it is turned off within the MF and posterior to it in differentiating cells (Halder *et al.* 1998). Anterior to the MF, Ey induces expression of downstream RD genes, *eya* and *so* (Niimi *et al.* 1999; Zimmerman *et al.* 2000). A subdomain of the region of Ey/Eya/So overlap immediately abutting the MF is known as the preproneuronal region (PPN) and is determined by long-range Dpp signaling that primes cells for imminent differentiation (Figure I-1 and (Greenwood and Struhl 1999; Baonza and Freeman 2001)).

eya was first discovered in Drosophila and has amassed broad interest due to its evolutionary conservation, its expression and function in the development of multiple organs in addition to the eye, its unusual molecular characteristics, and its relevance to human disease (Bonini *et al.* 1993, 1998; Abdelhak *et al.* 1997; Duncan *et al.* 1997; Xu *et al.* 1999; Söker *et al.* 2008). Eya family proteins are defined by a highly conserved C-terminal Eya Domain (ED) that enables binding to So/Six and Dac/Dach proteins and also carries tyrosine phosphatase activity (Figure I-2B and (Xu *et al.* 1997; Zimmerman *et al.* 1997; Chen *et al.* 1997; Bui *et al.* 2000; Li *et al.* 2003; Rayapureddi *et al.* 2003; Tootle *et al.* 2003)). The N-terminal half of Eya shows less conservation except for a tyrosine-rich Eya Domain 2 (ED2) that encodes a novel threonine phosphatase activity and is embedded within a proline/serine/threonine-rich transactivation domain (Figure I-2B and (Xu *et al.* 1997; Zimmerman *et al.* 1997; Silver *et al.* 2003; Okabe *et al.* 2009; Liu *et al.* 2012)). In Drosophila eye development, *eya* expression begins in the second instar disc in the eye field and persists in third instar discs in regions anterior to, within and posterior to the MF (Figure I-1 and (Bonini *et al.* 1993; Kenyon *et al.* 2003)). This expression pattern positions Eya to contribute to the complete sequence of events that drive eye development.

First described as a novel nuclear factor, Eya was subsequently shown to be a transcription cofactor that provides transactivation to its binding partner, *sine oculis* (*so*) (Pignoni *et al.* 1997). Together, the Eya-So transcription factor regulates the expression of numerous target genes including cell cycle regulators, other RD genes and proneuronal genes, thus contributing to proliferation, specification and differentiation of the eye (Yan *et al.* 2003; Pappu *et al.* 2005; Pauli *et al.* 2005; Jemc and Rebay 2007). Eya was later shown to shuttle

dynamically between the nucleus and cytoplasm (Xiong *et al.* 2009), raising the possibility of transcription-independent roles. Several recent studies suggest that cytoplasmic Eya function is important for morphogenesis. These include Eya function in the context of photoreceptor axon targeting in Drosophila where it interacts with the non-receptor tyrosine kinase, Abelson (Abl), and in lung epithelial polarity in mammals where it interacts with apical junction complexes (Xiong *et al.* 2009; El-Hashash *et al.* 2011, 2012). Understanding of how Eya functions in the cytoplasm is limited, and this dissertation describes the discovery and exploration of interactions between Eya and cytoplasmic components of the Jak/Stat pathway.

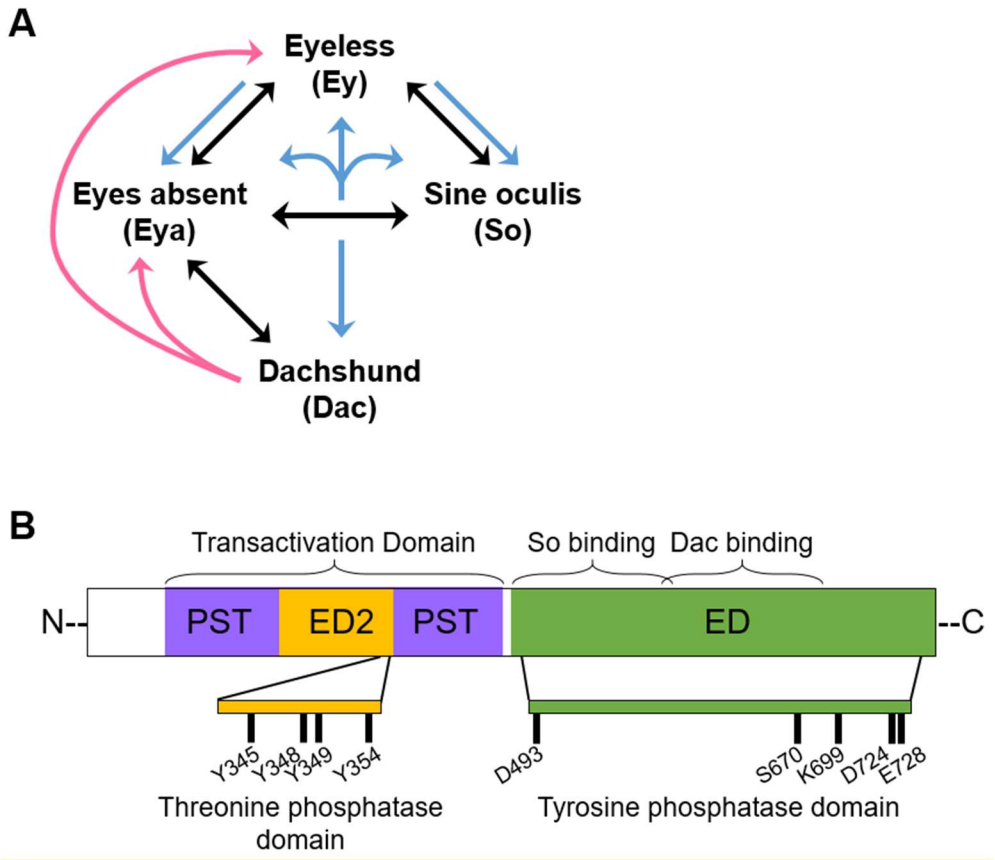

Figure I-2 | Genetic hierarchy of the RD network and domain structure of Eya

Figure I-2 | Genetic hierarchy of the RD network and domain structure of Eya (continued) (A) RD network hierarchy where blue arrows depict direct transcriptional regulation, pink arrows depict genetic interactions with unknown molecular mechanism and black arrows depict protein-protein interactions. (B) Protein structure of Drosophila Eya. The Eya domain (ED) is a highly conserved C-terminal region which contains Eya's So- and Dac-binding domains and tyrosine phosphatase domain. The N-terminal transactivation domain contains a secondary Eya domain (ED2) which is less conserved, flanked by regions enriched for proline, serine and threonine amino acids. Embedded within the ED2 is a region encoding a conserved threonine phosphatase; nothing is known about the structural fold or catalytic mechanism of the threonine phosphatase and the precise boundaries that define it have not been clearly mapped. The amino acids shown for Eya's two phosphatases are those verified by in vitro phosphatase assays as being required for optimal enzymatic activity.

I.1.2 – The Jak/Stat signal transduction pathway

The Janus Kinase (Jak)/Signal Transducers and Activators of Transcription (Stat) pathway is an evolutionally conserved mechanism for transducing signals from cytokines, growth factors and interferons into gene expression changes within a cell (reviewed in (Darnell *et al.* 1994; Li 2008)). In mammals, its primary relevance is in hematopoiesis and immune response where misregulation causes a range of malignancies including hematopoietically derived cancers (reviewed in (Bowman *et al.* 2000 and Jatiani *et al.* 2010)). Since vertebrate Jak/Stat signaling contains 4 JAKs and 7 STATs while Drosophila has just one of each, research in the fly has been instrumental in revealing pertinent functions for this pathway. Monumental examples include discovery and characterization of Jak/Stat's contribution to the stem cell niche (reviewed in (Bausek 2013)) and heterochromatin landscape (reviewed in (Silver-Morse and Li 2013)). Thus, Drosophila provide a genetically tractable system with a simplified but evolutionally conserved version of the pathway that regulates many of the same developmental processes that it regulates in vertebrates.

In Drosophila, signaling begins when Unpaired (Upd) ligand (Upd 1-3) binds to a transmembrane receptor, domeless (dome), which causes activation of the JAK, hopscotch (Hop) (Harrison *et al.* 1998). Hop then phosphorylates Stat, which dimerizes to form an active transcription factor (Yan *et al.* 1996a). In addition to activating expression of target genes that promote survival and growth, p-Stat dimers also activate transcription of Suppressor of Cytokine Signaling (SOCS) encoding genes, which provide critical negative feedback on the pathway (Figure I-2 and (Karsten *et al.* 2002)). In addition to SOCS, other negative regulators include tyrosine phosphatases and protein inhibitors of Stat (PIAS) (Figure I-3). The following discussion will center on discovery and function of these genes in the fly.

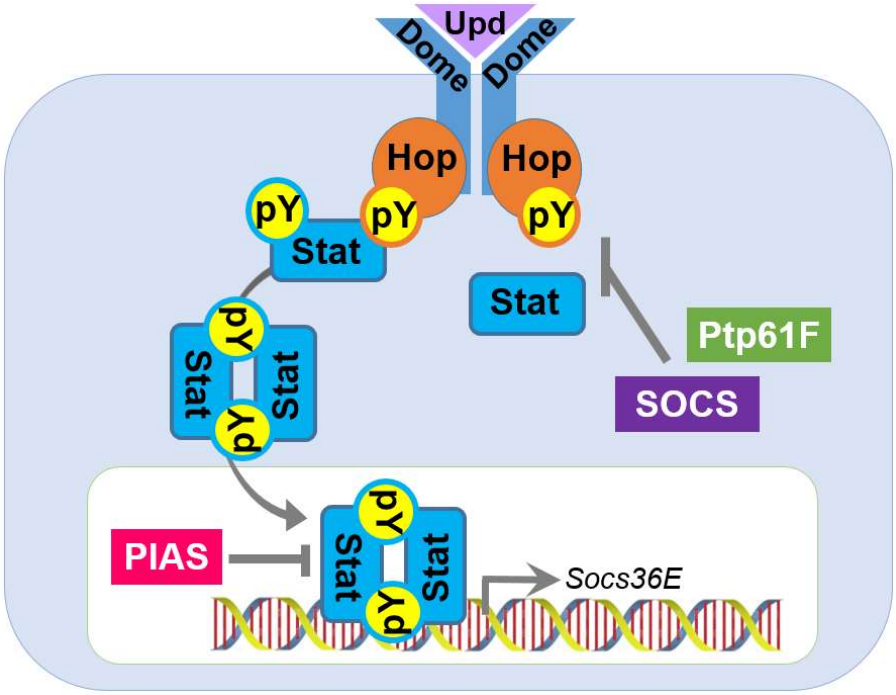

Figure I-3 | Schematic of the Jak/Stat signaling pathway in Drosophila
Upd binding to Dome induces receptor dimerization and transactivation of the receptor-associated Jak, Hop. Activated Hop then phosphorylates Stat, which will itself dimerize to create an active transcription factor. Stat dimers bind to tandem Stat binding sites in the DNA to regulate transcription of target genes such as *Socs36E*. SOCS and other negative regulators such at Ptp61F attenuate signaling by interfering with the Dome-Hop-Stat complex in multiple ways while PIAS prevents Stat transcription factor activity.

upd-1 was the first mutant to be described in the pathway and was characterized as the *outstretched* group of mutations yielding small eye and held-out wing phenotypes (Muller 1930). Cloning of the *upd-1* locus revealed that Upd-1 is a secreted, glycosylated protein that, when expressed in S2 cells, leads to tyrosine phosphorylation of Hop (Harrison *et al.* 1998). Subsequent whole genome sequencing revealed that there are two additional *upd*-like genes located adjacent to *upd-1*; however only *upd-1* appears relevant in the eye, and so from here-on-out, will simply be referred to as *upd* (Agaisse *et al.* 2003; Gilbert *et al.* 2005; Hombría *et al.* 2005). *upd* is expressed within the ventral domain of the first instar imaginal disc, then at the dorsal-ventral (DV) midline at the posterior of the second instar disc, but turns off after MF initiation (Figure I-4 and (Tsai and Sun 2004; Reynolds-Kenneally and Mlodzik 2005; Bach *et al.* 2007; Gutierrez-Aviño *et al.* 2009)). Distribution of Upd across the eye field is regulated by heparin sulfate proteoglycans, Dally and Dally-like-protein (Dlp). Loss of these glypicans either in null clones or by RNAi reduces Jak/Stat signaling activity while in transcription assays, expression of *dally* enhances Jak/Stat signaling activity (Zhang *et al.* 2013). Heparin sulfate proteoglycans are macromolecules within the extracellular matrix to which heparin sulfate glycosaminoglycan chains attach (reviewed in (Lin 2004)). Due to these interactions, heparin sulfate proteoglycans have been shown to regulate distribution of other morphogens such as Wg, Hh and Dpp (reviewed in (Yan and Lin 2009)), and now potentially cytokines such as Upd.

dome encodes a transmembrane protein most similar to the IL-6 receptor family, with an extracellular cytokine-binding module (CBM), three extracellular fibronectin-type-III domains (FnIII) and a necessary intracellular domain (Brown *et al.* 2001). In their work, Brown *et al*. show that *dome* maternal and zygotic mutant embryos produce segmentation defects similar to

those observed in *upd*, *hop* and *Stat92E* mutants. Additionally, they find that *dome* loss dominantly suppresses the abnormal head formation induced by ectopic expression of *upd* (Brown *et al.* 2001). Taken all together, this suggests that Dome transduces Upd signal to Hop and Stat.

A second Dome-like gene located adjacent to the *dome* locus in the genome is *eye transformer* (*et*) also known as *latran* (*lat*) (Hombría and Brown 2002; Makki *et al.* 2010; Kallio *et al.* 2010). Et/Lat contains CBMs similar to those in Dome but lacks the FnIII repeats. Recently, Et/Lat has been shown to disrupt Dome homodimers to create signaling-incompetent Dome:Et/Lat heterodimers (Fisher *et al.* 2016). Studies have shown that both Dome and Et/Lat can be trafficked through endocytic machinery, but Et/Lat vesicles are targeted for lysosomal degradation while clathrin-mediated Dome vesicles are important for proper Jak/Stat signaling (Devergne *et al.* 2007; Fisher *et al.* 2016). An observation from Devergne *et al*. which will become important for discussion in Chapter IV is that Dome vesicles form visible puncta in both S2 cells and follicle cells of the egg chamber (Devergne *et al.* 2007). While the regulation and functions of vesicular versus non-vesicular Jak/Stat signaling are still not known, their downstream effects are reliant on Hop's kinase activity on Stat.

hop was first discovered based on its segmentation phenotypes (Perrimon and Mahowald 1986) and subsequent cloning showed that it encodes a non-receptor tyrosine kinase of the JAK family (Binari and Perrimon 1994; Chen *et al.* 2002). Hop contains a conserved FERM domain, an SH2 domain and two JAK kinase domains, one functional and the other a regulatory pseudokinase (reviewed in (Yamaoka *et al.* 2004)). Two independent screens, one for P-element induced mutations that produce similar segmentation defects as *hop* mutants and another for

cDNA clones carrying SH2 domains similar to that found in human STATs resulted in identification of *marrelle*, now known as *Stat92E* (Hou *et al.* 1996; Yan *et al.* 1996a). *hop* and *Stat92E* are the sole Jak and Stat in Drosophila, and *Stat92E* is likely the sole downstream effector of *hop*, as it is a strong dominant suppressor of the hyperactivating hop^{Tum} allele (Hanratty and Dearolf 1993; Yan *et al.* 1996b).

Sequence homology has identified three SOCS-like genes in Drosophila: *Socs36E*, *Socs44A* and *Socs16D.* SOCS proteins are potent negative regulators of cytokine and growth factor signaling (reviewed in (Linossi *et al.* 2013)). Mammalian studies reveal that SOCS inhibit Jak/Stat signaling by SH2-mediated interactions that occlude Stat binding to Jak-phosphorylated receptor complexes (Endo *et al.* 1997; Naka *et al.* 1997; Nicholson *et al.* 1999; reviewed in (Piessevaux *et al.* 2008)). There is also evidence that SOCS can target activated receptor complexes for degradation through a conserved motif that recruits elongins B and C which facilitate ubiquitination of bound substrates (Kamura *et al.* 1998). In addition to antagonizing Jak/Stat signaling, SOCS factors also regulate signal transduction from receptor tyrosine kinases (RTKs) (reviewed in (Trengove and Ward 2013)). *Socs36E* is the canonical pathway repressor in that it is a transcriptional target of Stat which provides negative feedback on Jak/Stat and RTK signaling (Callus and Mathey-Prevot 2002; Karsten *et al.* 2002; Rawlings *et al.* 2004; Baeg *et al.* 2005; Almudi *et al.* 2009). *Socs44A* presents interesting modes of pathway regulation as it can inhibit the Jak/Stat pathway, but does not appear to be a target of Stat (Rawlings *et al.* 2004) and is the sole example of a SOCS protein that synergizes with EGFR signaling (Rawlings *et al.* 2004). Finally, *Socs16D* has no demonstrated roles in development.

In addition to SOCS proteins, Jak-mediated phospho-tyrosine potentiation is limited by the action of tyrosine phosphatases and protein inhibitors of Stat (PIAS). Two independent RNAi screens found that the tyrosine phosphatase *Ptp61F* named for its chromosomal location antagonizes Jak/Stat activity (Müller *et al.* 2005; Baeg *et al.* 2005). The mechanism of Ptp61F is unclear since RNAi-mediated knockdown resulted in increased phosphorylation of both Hop and Stat (Baeg *et al.* 2005). Given that Hop phosphorylates Stat, loss of Ptp61F activity on just Hop could cause an increase in phosphorylation of both, but this does not rule out that Stat is an independent substrate of Ptp61F as well. PIAS inhibits Stat signaling by interfering with Stat binding to DNA and SUMOylation (Chung *et al.* 1997; Liu *et al.* 1998; Ungureanu *et al.* 2003). Drosophila PIAS are genetic antagonists of Jak/Stat signaling, as overexpression of *dPIAS* decreases eye size which is suppressed by overexpression of Stat while knockdown suppresses the small-eye phenotype of *upd* loss-of-function mutants (Betz *et al.* 2001). *In vitro* pull-down assays show that dPIAS specifically interacts with pY-Stat (Betz *et al.* 2001), however whether this interferes with DNA binding or leads to SUMOylation of Stat is not known.

The Jak/Stat pathway is deployed in many tissues and developmental processes (reviewed in (Arbouzova and Zeidler 2006)), but owing to the fact that eye formation is dispensable for survival, studies in this tissue have arguably been the most comprehensive for gaining insight to this pathway's pleiotropic roles for multiple steps in organogenesis. In Drosophila eye development, Jak/Stat signaling regulates early growth, stimulates cell survival by cell competition, promotes eye identity by repressing eye antagonists and is needed post-differentiation for morphogenesis (Zeidler *et al.* 1999a; b; Tsai and Sun 2004; Chao *et al.* 2004; Ekas *et al.* 2006; Rodrigues *et al.* 2012). These requirements are further evidenced by expression

of a pathway activity reporter at the appropriate developmental times and places predicted by the loss-of-function phenotypes. This reporter, known as 10xStat-eGFP (Bach *et al.* 2007), contains 5 tandem pairs of Stat binding sites upstream of a GFP and thus provides a sensitive readout of transcriptional output from the pathway (Figure I-4). In this dissertation, I discover an additional role for Jak/Stat signaling in photoreceptor axon targeting and show that it synergizes with Eya in that process.

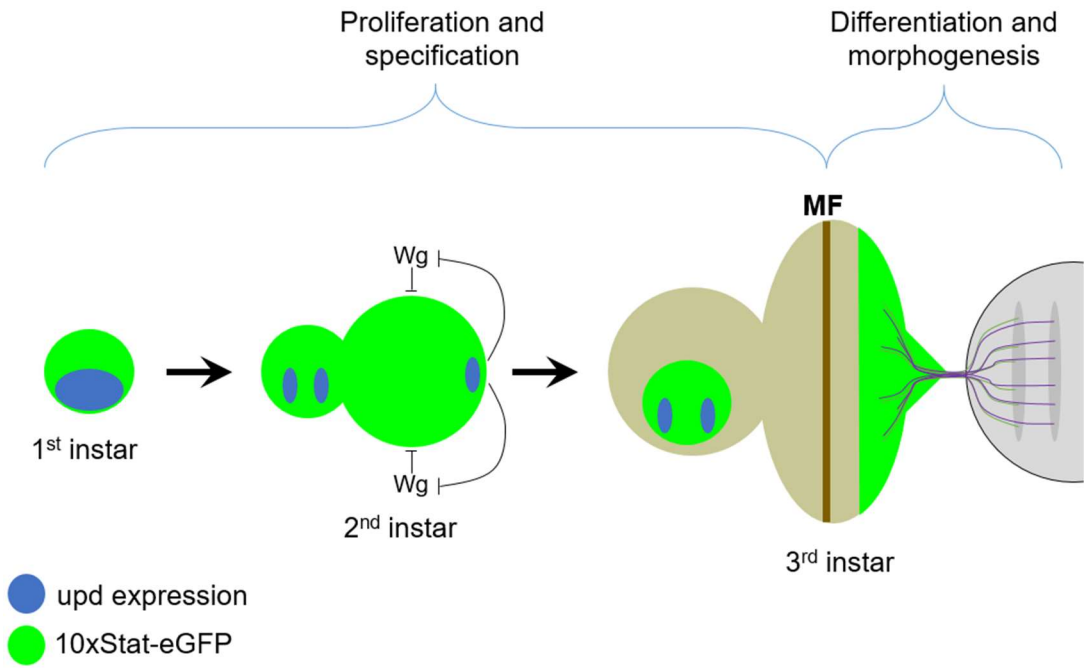

Figure I-4 | Activity of the Jak/Stat pathway during Drosophila eye development
The 10xStat-eGFP reporter is a widely-used transcriptional readout of pathway activity. Its expression within the eye reflects activity of the Jak/Stat pathway during multiple stages of eye development. Expression throughout the first and second instar discs reflects requirements for this pathway in proliferation of the eye field as well as specification, while expression in the posterior of the third instar eye disc reflects known requirements in ommatidial rotation, and based on the findings from this dissertation, additional requirements for photoreceptor axon targeting.

I.2 – Adult Drosophila eye structure

The adult Drosophila visual system is comprised of a pair of compound eyes that connect to the optic lobes of the brain. Each compound eye contains approximately 750 repeating units, individually known as an ommatidium (Ready *et al.* 1976; Tomlinson and Ready 1987). Within an ommatidium, there are eight photoreceptor cells (R1-R8) surrounded by pigment, bristle and cone cells. These cell types have fixed organization with respect to each other due to the stereotyped order in which their fates are specified, but will orient with opposite chirality according to their position relative to the DV axis. Thus while any ommatidium in isolation is indistinguishable from another ommatidium, the dorsal and ventral halves of the eye are rotationally symmetric across the DV boundary. The regularity of spatial organization within the eye disc is then mapped onto the optic lobe of the brain via innervation by the photoreceptor axons. Photoreceptor axons target to one of two neuropiles: either the lamina, which is important for motion detection, or the medulla which processes color (Morante and Desplan 2004). Outer photoreceptors, R1-R6, target to the lamina, while the two inner photoreceptors, R7 and R8, target to the medulla.

I.3 – Drosophila eye development

The compound eye derives from a small epithelial sac in the head of the embryo that, during larval life, undergoes a defined sequence of events in which precursor cells expand in number by proliferation and assume eye identity, differentiate into the various cell types necessary in the retina, and finally undergo morphogenetic changes that specialize cell shape and organization to allow the adult animal to visually interface with its surroundings. Below, I

discuss Eya and Jak/Stat pathway contributions to proliferation, specification, differentiation and morphogenesis of the fly eye.

I.3.1 – Proliferation

The Drosophila eye derives from ~15 Ey-expressing cells that are set aside as a flat epithelial sac during embryogenesis (Garcia-Bellido and Merriam 1969). These cells proliferate during larval development as the disc grows in size and regionally specifies eye versus antennal/head fates. Proliferation occurs in two waves (reviewed in (Kumar 2011)), termed the first and second mitotic waves. The first mitotic wave describes a continuous, multi-day period of asynchronous proliferation beginning in the first instar larva that continues into third instar, expanding the embryonic retinal precursors into the 44,000 cells that ultimately make up the antennal-eye imaginal disc. Early in the 3rd instar, the MF initiates at the posterior and marks the cessation of the first mitotic wave as it progresses anteriorly, arresting cells within the PPN in G1 as it goes (Ready *et al.* 1976; Firth *et al.* 2010). Regulating this transition from asynchronous proliferation to G1-arrest and differentiation is therefore crucial for proper eye development. The roles of Eya and Jak/Stat signaling in modulating this transition will be discussed below.

The second mitotic wave describes one final round of synchronous cell division that occurs posterior to the MF. Progenitors derived from the second mitotic wave contribute to the last three photoreceptors recruited to the ommatidia, R1, R6 and R7, as well as the cone, pigment and interommatidial bristle cells. Disruptions to the second mitotic wave result in an insufficient progenitor pool, and stochastic loss of late-specified fates (de Nooij and Hariharan 1995; Freeman 1996). Jak/Stat signaling does not appear relevant to the second mitotic wave (Tsai and

Sun 2004) and roles for Eya have not been explored; thus the second mitotic wave will not be discussed further.

I.3.1.1 – Eya-So regulate proliferation ahead of the MF by mediating cell cycle progression

Eya-So regulates transcription of the cdc25 homolog, *string* (*stg*), and therefore contribute to gene expression changes that accompany the transition from proliferation to differentiation. Stg is expressed at low levels throughout the antennal-eye imaginal disc except within the MF where it is absent and immediately anterior the MF where it is highly expressed (Thomas *et al.* 1994). This pattern of expression plus prior observations that retinal cells arrest in G1 just prior to differentiation fueled the model that high Stg synchronizes cells prior to passage through the MF (Ready *et al.* 1976; Firth *et al.* 2010). Since Eya is high in the PPN, one hypothesis is that it regulates *stg* levels just anterior to the furrow. Support for this idea came when microarray and qRT-PCR studies identified *stg* as an Eya-So transcriptional target (Figure I-5 and (Jemc and Rebay 2007)). While this model has not been proven, evidence that Eya-So can regulate *string* includes loss of *stg* expression in eya^2 tissue and sufficiency of Eya overexpression to induce *stg* in both the eye and the wing imaginal discs (Jemc and Rebay 2007).

I.3.1.2 – Jak/Stat signaling is required for early eye proliferation

The first mitotic wave is driven by activation of the Notch pathway at the DV midline which locally activates *upd* expression in the posterior of the eye disc (Figure I-4 and (Chao *et al.* 2004)). Long-range diffusion of Upd activates Jak/Stat signaling across the eye field, promoting cell proliferation and growth. Loss of Notch signaling by clonal expression of a dominant negative, N^{DN}, blocks eye development; co-expression of *upd* rescues eye formation in the N^{DN}, consistent with Jak/Stat signaling operating downstream of Notch. *upd* appears to be a

direct target of Notch signaling, as N^{ts} mutants in the restrictive temperature show both reduced eye sizes and undetectable *upd* mRNA by in situ hybridization while overexpression of active Notch in N^{act} clones induces *upd-LacZ* (Chao *et al.* 2004). Thus, Notch mediated proliferation in the first mitotic wave of eye development is propagated through Jak/Stat signaling.

Jak/Stat signaling impacts proliferation by regulating CyclinD, a key growth-promoting factor in the eye (Datar *et al.* 2000; Tsai and Sun 2004). However it is unclear whether this regulation is via Stat-mediated transcriptional activation of *cyclinD* or via Hop-mediated phosphorylation of CyclinD or its regulators. Several groups have shown that activation of Jak/Stat signaling by overexpression of *upd* generates enlarged eyes. Intriguingly, driving *upd* expression with multiple Gal4 drivers can produce this phenotype: *eyeless-Gal4*, which expresses prior to the 3rd instar disc and anterior to the furrow; *dpp-Gal4*, which expresses in the MF; and *elav-Gal4* and *GMR-Gal4*, which express posterior to the MF (Chen *et al.* 2002; Bach *et al.* 2003; Tsai and Sun 2004). The Hou group show that the *upd* overproliferation phenotypes can be enhanced by co-expression of *cyclinD* (Chen *et al.* 2003). Expanding on this result, Tsai and Sun show by quantification of phospho-Histone 3 staining and *in situ* hybridization of *cyclinD* that *GMR>upd* increases *cyclinD* transcription ahead of the furrow (Tsai and Sun 2004). Together, these data lead to the conclusion that proliferation within the first mitotic wave mediated by CyclinD is driven by long-range effects of Upd (Figure I-5).

In addition to having a role in proliferation, Jak/Stat signaling is also required cell autonomously for cell competition, a process in which cells in a genetically heterogeneous population compare their fitness relative to that of their neighbors and then make survival versus death choices (reviewed in (Amoyel and Bach 2014)). Both *hop* and *Stat92E* null eye clones are

significantly smaller than their twin spots, suggesting compromised survival (Zeidler *et al.* 1999a; Luo *et al.* 1999; Ekas *et al.* 2006; Rodrigues *et al.* 2012). Rodrigues et al. show that the size of *Stat92E* clones can be restored to normal by co-expressing the caspase inhibitor, P35, or by generating clones in a *minute* background, which are themselves compromised in growth. Conversely, clonal cells with increased Jak/Stat signaling by overexpression of Hop will outcompete wild-type neighbors. These observations suggest that active Jak/Stat signaling is important for maintaining the proliferative advantage required for cell survival.

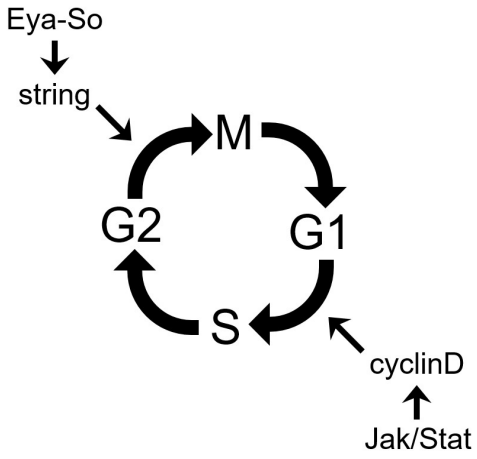

Figure I-5 | Eya and the Jak/Stat pathway promote cell cycle progression
Genetic interactions show that Eya and the Jak/Stat pathway lead to expression of cell cycle regulators, String and CyclinD, respectively, to regulate cell cycle progression and thus proliferation.

I.3.2 – Regional specification

One of the earliest developmental decisions that a cell makes is acquisition of tissue identity, a process referred to here as regional specification. Two mutually antagonistic interactions between signaling pathways determine antennal versus eye fate and eye versus head

cuticle fate; the former employs antagonistic EGFR-Notch interactions (Kumar and Moses 2001) while the latter involves opposing Jak/Stat-Wingless signaling (Ekas *et al.* 2006). During the second larval instar, Notch signaling becomes localized to the DV midline by dorsal and ventral restriction of Notch ligands, Delta and Serrate, respectively (Papayannopoulos 1998; Cho and Choi 1998; Domínguez and de Celis 1998; reviewed in (Singh *et al.* 2012)). This localized Notch activity induces expression of *upd* and promotes eye development in two ways: by Jak/Stat-mediated promotion of growth, which was discussed in the previous section, and by Jak/Stat-mediated repression of head cuticle fate, which will be discussed below. The reginal specification decisions that result from these antagonistic interactions are then maintained by positive feedback within the RD network in which Ey induces downstream RD members, *eya* and *so* (Niimi *et al.* 1999; Zimmerman *et al.* 2000), which cooperatively induce expression of fellow RD members, *ey* and *dac*. Thus, Eya-So affirm eye identity initiated by Ey expression early in the tissue.

I.3.2.1 – Jak/Stat antagonism of Wg signaling directs MF initiation and eye field formation

Wingless (Wg) signaling antagonizes eye fate and MF initiation and progression. *wg* opposes eye fate by promoting expression of the head and antennal marker, *homothorax* (*hth*) (Pichaud and Casares 2000). This is evidenced by loss of *wg* using a temperature sensitive allele resulting in expansion of the eye field into lateral regions of the head (Royet and Finkelstein 1996; Lee and Treisman 2001). Conversely, removal of negative regulators increases Wg signaling and causes replacement of eye tissue with Hth-positive, head-like cuticle (Royet and Finkelstein 1996; Baonza and Freeman 2002).

Jak/Stat signaling emanating from the posterior midline helps restrict Wg signaling to the lateral margins of the eye field, thereby allowing MF initiation at the posterior (Figure I-4). The first hint that Jak/Stat might antagonize Wg signaling came from the observation that the Jak/Stat pathway reporter, 10xStat-eGFP, had complementary expression with the wg-lacZ enhancer trap (Ekas *et al.* 2006). As previously mentioned, *upd* is locally induced at the DV boundary by Notch signaling (Chao *et al.* 2004; Reynolds-Kenneally and Mlodzik 2005), but how *upd* is restricted along the anterior-posterior axis to just the posterior is not known. Since Upd is a diffusible ligand, this local signal must act from long-range to restrict Wg activity to the lateral margins. Indeed, Ekas *et al*. find that *wg* was de-repressed cell autonomously in both *hop* and *Stat92E* clones while ectopic Jak/Stat activity by Flp-out mediated expression of *hop* in dorsal and ventral regions was sufficient to repress *wg*. Tsai *et al*. elaborate on the developmental relevance of this antagonism by showing that loss of Jak/Stat signaling in a temperature sensitive *Stat92E* background blocks MF initiation due to ectopic *wg* (Tsai *et al.* 2007). Exploration into mechanism reveals that Jak/Stat signaling represses Wg activity by opposing an effector of dorsal Wg signaling, the Iroquois complex (Iro-C) (Gutierrez-Aviño *et al.* 2009).

This mechanism does not explain regulation of ventral-lateral Wg activity. One possibility is that Jak/Stat activates expression of the TGF-β morphogen, *decapentaplegic* (*dpp*), as it does in the gonad (Wang *et al.* 2008), and that Dpp signaling then represses Wg signaling in the ventral region to allow MF initiation (Hazelett *et al.* 1998). This model could be tested by attempting to suppress ectopic *wg* activation in loss-of-function Jak/Stat clones by overexpression of *dpp*. Taken together, although details of the molecular mechanism remain to

be discovered, Jak/Stat-mediated repression of *wg* is crucial for eye specification and initiation of the MF (Figure I-4).

I.3.2.2 – Eya maintains eye fate by promoting expression of other RD genes

The decisions that result from the actions of the signaling pathways discussed above are maintained within cells by members of the RD network. Because Eya is not at the top of the RD hierarchy, its role in regional identity is most pertinent to maintenance of eye specification by feedback onto other RD members (Figure I-2A). As precursor cells transition into the PPN just anterior to the encroaching MF, Ey initiates expression of downstream RD members, *eya* and *so*, by directly binding enhancer elements for both genes (Halder *et al.* 1998; Niimi *et al.* 1999; Zimmerman *et al.* 2000; Pappu *et al.* 2005). Within the PPN, the handoff from Ey to Eya-So is reinforced by a positive feedback loop in which Eya-So activate *ey* expression through So-binding sites in the *eyeless* enhancer (Pauli *et al.* 2005; Atkins *et al.* 2013). Through a yet-to-be discovered mechanism, Eya-So then switch from activating *ey* to repressing it, as evidenced by loss-of-function *eya* and *so* clones anterior to the MF displaying loss of *ey*, but showing de-repression of *ey* in clones posterior to the MF (Atkins *et al.* 2013). Taken together, Ey is responsible for the earliest steps in regional specification but then targets *eya* and *so* anterior to the MF, initiating a positive feedback loop that locks in eye fate.

Eya and So then cooperate with Ey to activate expression of fellow RD gene, *dac*. In the eye, Dac is expressed at the posterior margin prior to MF initiation, and within the migrating MF during differentiation (Mardon *et al.* 1994). Ey and Eya-So regulate *dac* expression at two redundant enhancer regions, one in a non-coding region and a second within an intron (Pappu et al. 2005). There are no known transcriptional targets for *dac*, meaning that mechanistic

understanding of its role in development remain limited. Despite this, *dac* is a bona fide RD member as evidenced by the fact that loss-of-function mutants develop with dramatically reduced or absent eyes (Mardon *et al.* 1994) and ectopic expression is sufficient to induce eye formation (Shen and Mardon 1997).

Eya's role in maintaining eye fate is most clearly demonstrated in *eya* mosaic clones which cause loss of *dac* (Pignoni *et al.* 1997) and de-repression of antennal markers, Cut and Hth (Bessa *et al.* 2002; Salzer and Kumar 2009). Consistent with these observations, *eya* null tissue fails to generate retinal tissue and is instead replaced with head cuticle (Pignoni *et al.* 1997). In conclusion, early *ey* expression initiates that of *eya* and *so.* Eya-So then ensure eye fate specification by positive feedback on *ey* and expression of the final RD gene, *dac* (Figure I-2A).

I.3.3 – Differentiation

Following specification and proliferation of the eye field, differentiation occurs in a progressive sweep across the eye disc as the MF advances anteriorly, leaving rows of ommatidia in its wake. MF progression is mediated by Hedgehog (Hh) protein secreted by maturing photoreceptors which induces long-range activation of a serine-threonine kinase, Raf, in the PPN. Raf in this region downregulates the proneuronal repressor, *hairy*, which then allows expression of the proneuronal gene, *atonal* (*ato*) (Greenwood and Struhl 1999). The earliest cells to differentiate are the five photoreceptors, R8, R2, R5, R3 and R4 that form a precluster. R8 is defined molecularly by *ato* expression, R2/R5 by *rough* (*ro*) expression and R3/R4 by *ro* and *seven-up (svp)* expression (Figure I-6, zones 1-2). The remaining precursor cells surrounding the preclusters undergo one final synchronized round of division in the second mitotic wave. Following that, recruitment of R1/R6 (defined by *svp* and *lozenge* (*lz*) expression) and R7

(defined by *prospero* (*pros*) expression) completes photoreceptor differentiation (Figure I-6, zones 3-4). Following this, four cone cells join the ommatidium. Finally, recruitment of the interommatidial pigment and bristle cells during pupal stages completes ommatidial differentiation. Except for R8, which requires *ato* as an inductive cue for differentiation (Suzuki and Saigo 2000), R7, which require additional RTK signal from R8 (Reinke and Zipursky 1988) and the bristle cells, which require the proneuronal achaete-schute complex (Furman and Bukharina 2012), differentiation of all subsequent cell types occurs by reiterative deployment of EGFR signaling (reviewed in (Freeman 1997)).

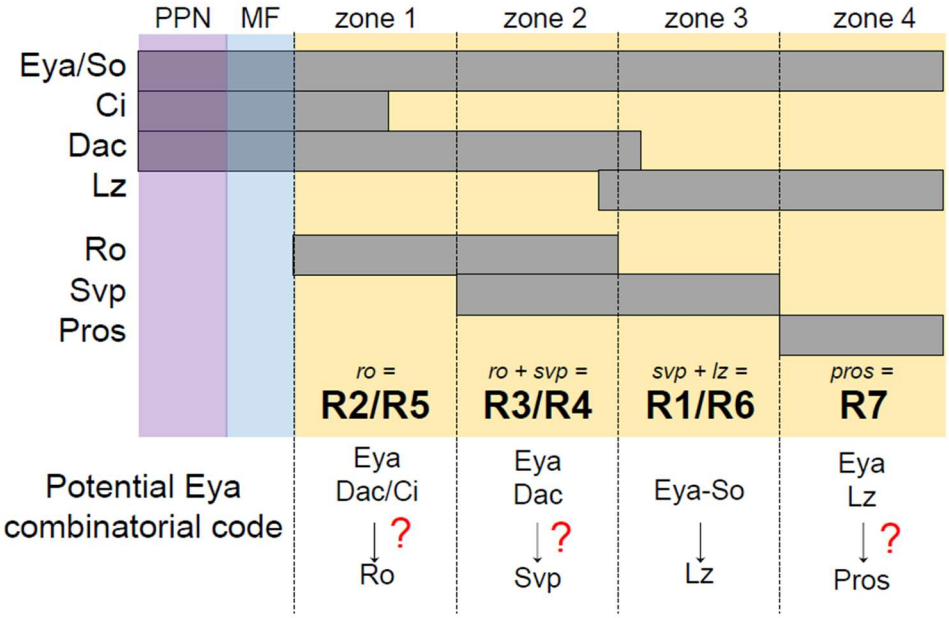

Figure I-6 | Combinatorial code of Eya regulating photoreceptor (R) cell fates
Eya is hypothesized to operate in a combinatorial code with Cubitus interruptus (Ci), Dac and Lozenge to regulate determination of different photoreceptor (R) cells.

I.3.3.1 – Eya may participate in the combinatorial codes that specify photoreceptor fates

Eya is hypothesized to act in combination with other transcription factors to mediate cell fate decisions. Supporting this, Eya is broadly expressed posterior to the MF (Figure I-1 and I-6) such that GMR-FLP induction of *eya* or *so* clones in the second mitotic wave results in random loss of R1, R6 or R7 cells (Pignoni *et al.* 1997) and GMR-driven Eya RNAi knockdown produces flies with roughened eyes and loss of cone cells, as evidenced by reduction in *cut* expression (Karandikar *et al.* 2014). Initially, EGFR signaling, which acts reiteratively to specify photoreceptor cells (Freeman 1996), was proposed to interface directly with Eya by mediating post-translational phosphorylation at two MAPK consensus sites that, based on genetic rescue assays with overexpressed cDNA constructs, appeared critical for Eya function (Hsiao *et al.* 2001). Arguing against this, recent experiments with genomic rescue constructs suggest that phosphorylation at these sites is not required (Jusiak *et al.* 2012). An alternative model for Eya's role in differentiation is combinatorial interactions with other transcription factors to define appropriate photoreceptor fate. Indeed, work by Hayashi and Saigo in which they carefully dissect the expression patterns of *ato*, *ro*, *svp*, *lz*, *pros*, *eya*, *dac* and *cubitus interruptus* (*ci*) lead them to propose that combinations of Eya with Ci, Dac and Lz dictate expression of *ro*, *svp* and *pros* and thus photoreceptor cell fate choice (Figure I-6 and (Hayashi *et al.* 2008)).

Although the combinatorial model is supported by overlapping expression domains between Eya with Ci, Dac and Lz that correlate with *ro*, *svp* and *pros* expression patterns, molecular mechanisms for Eya's regulation of *ro*, *svp* or *pros* are lacking. The only event with experimental evidence of direct Eya contribution is determination of R1/R6 which requires *lz*

expression (Figure I-6, zone 3). *lz* contains functional So binding sites which, when mutated, will cause loss of reporter gene activity (Yan *et al.* 2003).

Much weaker evidence supports the possibility of Eya-Dac regulation of *svp* expression in R3/R4 and R1/R6 (Figure I-6, zone 2). First, double dac^{-}/eya^{-} null clones show decreased levels of *svp* (Hayashi *et al.* 2008). Second, *in vitro* pull-down assays indicate interactions between Dac's C-terminal Dachbox and Eya's ED (Chen *et al.* 1997), although rescue assays suggest that this domain is dispensable for normal Dac function in the eye (Tavsanli *et al.* 2004). Third, Dac has an N-terminal Dachbox which shows sequence similarity with Ski/Sno co-repressors and contains a helix-turn-helix that may allow Dac to directly bind DNA (Hammond *et al.* 1998; Kim *et al.* 2002). Taken together, Eya-Dac may form a transcriptional complex that potentially work to activate *svp* expression which promotes specification of R3/R4 and R1/R6 (Figure I-6, zone 2).

Mechanisms by which Eya might influence specification of other photoreceptor fates are purely speculative. As previously mentioned, Hh is expressed in maturing photoreceptors and can have long-range effects in the PPN. Ci is the downstream effector of Hh signaling and its expression pattern is tightly regulated in posterior eye tissue by Cullin3 (Ou *et al.* 2002). Whether and how Eya, Dac and Ci cooperate to regulate *ro* is not known (Figure I-6, zone 1). Analogously, it is interesting to speculate that cooperative regulation by Eya and Lz might regulate *pros* expression. Lz negatively regulates *svp* and positively regulates *pros* where *lz* mutant clones show extra photoreceptor cells with inappropriate *svp* expression (Daga *et al.* 1996) while overexpression of Lz induces ectopic *pros* (Behan *et al.* 2002). Given that Eya-So regulate *lz*, perhaps the three then cooperate to activate *pros* expression (Figure I-6, zone 4). A

great deal of work identifying appropriate enhancers and dissecting the regulatory contributions of the relevant transcription factors will be needed to test these models.

I.3.3.2 – Eya enhancers demonstrate restricted expression of *eya* in pigment and cone cells

More recently, Eya has been shown to be required for differentiation of cone and pigment cells (Karandikar *et al.* 2014). Until work by Karandikar and colleagues, examination of Eya's roles posterior to the MF was limited to Gal4-UAS techniques that bypassed Eya's early requirements (Xiong *et al.* 2009; Karandikar *et al.* 2014; Yu *et al.* 2015). Karandikar et al. offer a new tool which leverages their finding of two unique *eya* enhancers that are sufficient to drive expression either just anterior to the MF or just within ommatidial clusters (Karandikar *et al.* 2014). They generated genomic rescue constructs in which a 45.5 kb region of genomic DNA surrounding the *eya* locus was recombineered into a bacterial artificial chromosome and then integrated into an alternative site on the 3rd chromosome (Jin *et al.* 2013). By using genomic rescue constructs lacking one enhancer or the other, they decoupled Eya's early requirements in proliferation and specification from its later requirement in differentiation and morphogenesis. Using this approach, they demonstrate that loss of Eya expression in ommatidial clusters results in a loss in cone fate, as detected by the cone cell marker, Cut, but an increase in pigment cells due to higher levels of Ci (Karandikar *et al.* 2014). Ci is normally degraded in cells posterior to the MF by Cullin3 ligase and ectopic Hh signaling causes an increase in pigment cells (Ou *et al.* 2002, 2007). Thus, Karandikar et al. propose that Eya may play a role in degrading Ci in regions outside of the MF. An alternative model would be combinatorial transcriptional regulation, analogous to that proposed for photoreceptor cell determination. There could also be additional Eya enhancers that further refine expression to photoreceptors versus cone versus pigment cells.

In support of that, a previously uncharacterized pigment cell specific enhancer was found to map near the *eya* locus (N. Sanchez and I. Rebay, unpublished).

I.3.3.3 – Socs36E competes with Drk to dampen Sevenless RTK activity

RTK signaling is critical for photoreceptor cell determination. R1-R6 fates are induced by reiterative EGFR signaling while R7 also requires activation of Sevenless (Sev) RTK (reviewed in (Kumar 2012)). *sev* loss-of-function will result in transformation of R7 into an equatorial cone cell (Tomlinson and Ready 1986) while *sev* gain-of-function induces supernumerary R7 cells (Basler *et al.* 1991), demonstrating Sev is necessary and sufficient for R7 fate. However, *sev* expression is not restricted to R7 (Tomlinson *et al.* 2011); instead, the activity of *sev* expressed in R3/R4 and R1/R6 photoreceptors is attenuated to prevent those cells from improperly assuming R7 fate. Work by Almundi et al. showed that Socs36E competitively interacts with Sev to prevent Drk transduction of this RTK signal in non-R7 cells (Almudi *et al.* 2009, 2010). They found that *Socs36E* expresses highly in R3/R4 and at intermediate levels in R1/R6, but is absent from R2/R5 and R7, poising Socs36E to be a negative regulator of Sev in non-R7 fated cells. Consistent with this idea, *Socs36E* loss-of-function mutants produce supernumerary R7 cells, similar to *sev* gain-of-function phenotypes, while *Socs36E* overexpression suppresses *sev* gain-of-function phenotypes (Almudi *et al.* 2009). Mechanistically, they show by co-immunoprecipitation and Förster resonance energy transfer (FRET) assays in S2 cells that Socs36E competes for binding to Sev to prevent binding of Drk, which transduces Sev activity (Almudi *et al.* 2010). Thus, although it remains to be tested whether Socs36E-Sev interactions result in degradation of Sev in R3/R4 and R1/R6 or if

Socs44A is also involved in Sev regulation, *Socs36E* does genetically antagonize Sev signaling in these photoreceptors to prevent improper R7 determination.

I.3.4 – Morphogenesis

After differentiation, cells must acquire specific morphological features and/or organize into morphological structures that allow them to perform their specialized functions. In the case of eye organogenesis (reviewed in (Pichaud 2014)), the most relevant morphogenetic events that involve the Jak/Stat pathway and Eya are ommatidial rotation and photoreceptor axon targeting (Figure I-7). After precluster determination, nascent ommatidia begin to rotate according to their location along the DV axis. Ommatidial clusters in the dorsal half rotate 90° clockwise while those in the ventral half rotate 90° counterclockwise. Another morphogenetic event for photoreceptor neurons is outgrowth of their axons from the retina to the brain. Photoreceptor axon targeting begins shortly after differentiation when R8s provide the pioneer axon that the other seven photoreceptors then fasciculate with and follow. Once they reach the brain, the R1-R6 axons terminate at the lamina, while R7 and R8 travel deeper to target the medulla (Figure I-7). The regularity of these projection patterns provides a sensitive and readily quantifiable phenotype that has been used to identify genes whose loss or overexpression causes mistargeting.

I.3.4.1 – The Jak/Stat pathway regulates *four-jointed* to mediate ommatidial rotation

Graded Jak/Stat signaling from the DV midline activates a gradient of a secreted molecule, *four-jointed* (*fj*), which is then interpreted by cells to determine their position relative to the DV boundary. Fj is a Golgi-localized kinase that phosphorylates cadherin domains of two proteins involved in planar cell polarity: Fat (Ft) and Dachsous (Ds) (Ishikawa *et al.* 2008; Brittle *et al.* 2010; Simon *et al.* 2010). A recent study shows that Fj has a bias towards phosphorylating

Ft, and because phosphorylated Ft most strongly associates with unphosphorylated Ds, this leads to Fj levels correlating with strength of cell-cell interactions (Hale *et al.* 2015). As a result, *fj* gain- or loss-of-function clones produce ommatidial inversions along the edges of clone. Since Fj differentials likely establish DV polarity (i.e. high -> low *fj* :: medial -> lateral DV position, Figure I-7), it follows that the inversion occurs on the margin facing away from the DV boundary for loss-of-function clones but on the equatorial side for gain-of-function clones (Zeidler *et al.* 1999b).

Notch and Jak/Stat signaling positively regulate *fj* while Wg represses it at lateral margins, as demonstrated by loss of *hop* or ectopic *wg* leading to a reduction of *fj* and ectopic *upd* causing an increase in *fj* (Zeidler *et al.* 1999b). In support of the Jak/Stat pathway being an upstream effector of the DV patterning signal that drives ommatidial rotation, two separate groups show that perturbations to Jak/Stat signaling components produced misoriented ommatidial clusters (Zeidler *et al.* 1999a; Luo *et al.* 1999). Both studies found that while clones lacking either *hop* or *Stat92E* are smaller than wild-type twin spots due to defects in proliferation as discussed in section I.3.1.2, they do survive and show normal differentiation of photoreceptors. However, ommatidia near clone boundaries show inverted orientation similar to those observed in *fj* clones. Thus, localized Jak/Stat signaling from the DV midline contributes to a gradient of *fj* which then establishes planar cell polarity that ommatidial clusters interpret for appropriate rotation relative to the DV axis.

I.3.4.2 – Eya cooperates with Abl to control photoreceptor axon targeting

A second morphogenetic event in the eye is projection of photoreceptor axons to the brain. Although discussion of Eya until now has focused on its role as a nuclear transcription co-

factor, recent studies have shown that interactions with the non-receptor tyrosine kinase, Abelson (Abl), cause cytoplasmic retention of Eya and that the two synergize to regulate photoreceptor axon targeting (Figure I-7 and (Xiong *et al.* 2009)). Abl has previously been shown to affect embryonic axonal connections of the central nervous system (Gertler *et al.* 1989), and thus when genetic interactions revealed that *abl* heterozygotes dominantly suppress Eya's ability to induce ectopic eyes, Xiong et al. explored photoreceptor axonogenesis as a potential context for synergy. They found that *eya* and *abl* are both required cell autonomously for proper R1-R6 axon targeting as *eya* hypomorphs and *abl* mutant clones both fail to fasciculate evenly or terminate at the lamina. Furthermore, *eya* and *abl* synergize in this process, as *eya* heterozygosity dominantly enhances *abl* mutant mistargeting phenotypes (Xiong *et al.* 2009). In vitro work suggests that this synergy results from Eya being tyrosine phosphorylated by Abl. Intriguingly, Abl-mediated phosphorylation of Eya causes Eya to be sequestered in the cytoplasm, and genetic rescue assays indicate that cytoplasmic localization of Eya is required for full function.

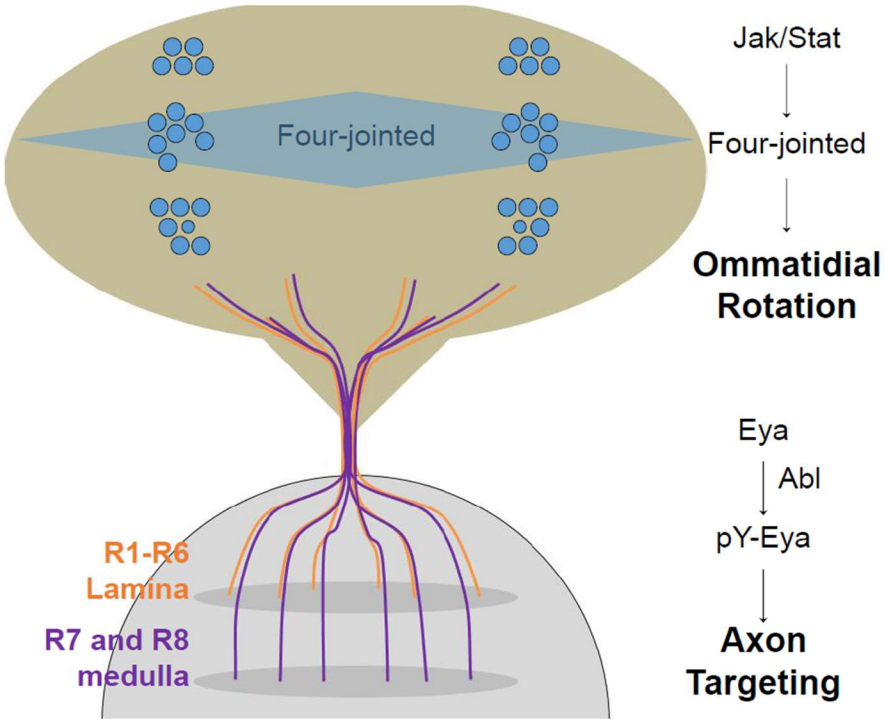

Figure I-7 | Roles of Eya and the Jak/Stat pathway in morphogenesis
Cartoon of a third instar eye disc connected to the brain oriented anterior up, posterior down, dorsal to the right and ventral to the left. Shown within the eye disc are blown-up representations of R cell clusters rotating according to the DV boundary as they differentiate. Ommatidial clusters in the dorsal half will rotate clockwise while those in the ventral half will rotate counterclockwise. Differentiated photoreceptors also send axonal projections to the brain where the outer R1-R6 innervate the lamina while inner R7 and R8 innervate the medulla. The Jak/Stat pathway has demonstrated roles in ommatidial rotation while Eya affects axon targeting.

At the time that these experiments were done, the hypothesis was that Eya's protein tyrosine phosphatase (PTP) activity would be the mechanism by which cytoplasmic Eya directs proper axon targeting. This was based on the finding that genetic rescue assays with cytoplasmic Eya failed when amino acid mutations were made to the PTP (Xiong *et al.* 2009) and on prior knowledge of other PTPs playing important roles in embryonic and/or photoreceptor axon targeting. These PTPs, which all also happen to be integral membrane proteins, include Ptp69D,

LAR, Ptp10D and Ptp99A (Desai *et al.* 1997; Garrity *et al.* 1999; Sun *et al.* 2001). Although there are no known ligands or receptors for these four PTPs, they all appear to act by relaying information to the actin cytoskeleton through the adapter protein, Dreadlock (Dock), to recruit the Pak kinase which then activates Cdc42/Rac at growth cones (reviewed in (Li *et al.* 2001)). However, since Eya's PTP has recently been shown to be dispensable for all aspects of Drosophila development, including axon targeting (Jin *et al.* 2013), Eya may interact with cytoplasmic complexes relevant to axonal outgrowth and targeting in other ways. This dissertation describes how efforts to identify the relevant cytoplasmic signaling centers that interface with Eya during photoreceptor axon targeting led to the discovery of genetic interactions with components of the Jak/Stat signaling pathway.

I.4 – Scope of the thesis

When Eya was first being characterized in Drosophila eye development, its nuclear localization and function within the RD network led to the assumption that it works as a transcription factor to regulate complex developmental outcomes by modulating gene expression. While this has proven to be correct, more recently, additional non-transcriptional functions for Eya have been discovered in mammalian systems (reviewed in (Rebay 2015)). In this light, the idea that Drosophila Eya might similarly partition its functions between nuclear and cytoplasmic compartments stimulated a genetic screen designed to elucidate cytoplasmic interactions relevant to Eya function during photoreceptor axon targeting.

This thesis addresses that frontier by elucidating interactions between Eya and cytoplasmic components of the Jak/Stat signaling pathway. Although the primary contexts

addressed in this thesis are morphogenetic ones, future explorations may choose to focus on interactions between Eya and Jak/Stat signaling during proliferation and specification, and to explore whether interactions also occur transcriptionally.

Chapter II presents the results of a screen that identifies both genetic and biochemical interactions between Eya and components of the Jak/Stat signaling pathway and reveals a novel role for the Jak/Stat pathway in regulating axon targeting.

Chapter III explores whether the interactions described in Chapter II support the model that Eya is a novel positive regulator of Jak/Stat signaling and whether Eya and Jak/Stat pathway components genetically interact in other tissues.

Chapter IV summarizes the conclusions of the work presented in this dissertation and discusses future perspectives.

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CHAPTER II

Retinal axon guidance requires integration of Eya and the Jak/Stat pathway into phosphotyrosine-based signaling circuits in Drosophila

Charlene Hoi, Wenjun Xiong, Fangfang Jiang and Ilaria Rebay

WX performed most of the screen whose results are shown in Supplemental Table II-1; CH's contribution to these data are Src64B genetic interactions, csw genetic interactions with NLS- and Myr-Eya, additional numbers to Csk and Dab genetic interactions and all statistical analysis. FJ contributed to some of the quantification in Figure II-2A and performed the experiments shown in Figure II-5 E and F and Supplemental Figure II-3. CH performed all other experiments and wrote the paper. This manuscript is in press in Genetics.

II.1 – Abstract

The transcriptional coactivator and phosphatase eyes absent (Eya) is dynamically compartmentalized between the nucleus and cytoplasm. Although the nuclear transcriptional circuits within which Eya operates have been extensively characterized, understanding of its cytoplasmic functions and interactions remains limited. Our previous work showed that phosphorylation of *Drosophila* Eya by the Abelson tyrosine kinase can recruit Eya to the cytoplasm, and that *eya-abelson* interactions are required for photoreceptor axons to project to correct layers in the brain. Based on these observations, we postulated that photoreceptor axon targeting might provide a suitable context for identifying the cytoplasmic signaling cascades with which Eya interacts. Using a dose-sensitive *eya* misexpression background, we performed an

RNAi-based genetic screen to identify suppressors. Included among the top 10 hits were non-receptor tyrosine kinases and multiple members of the Jak/Stat signaling network (*hop*, *Stat92E*, *Socs36E*, and *Socs44A*), a pathway not previously implicated in axon targeting. Individual loss-of-function phenotypes combined with analysis of axonal projections in *Stat92E* null clones confirmed the importance of photoreceptor autonomous Jak/Stat signaling. Experiments in cultured cells detected cytoplasmic complexes between Eya and Hop, Socs36E and Socs44A; the latter interaction required both the Src Homology 2 motif in Socs44A and tyrosine phosphorylated Eya, suggesting direct binding and validating the premise of the screen. Taken together, our data provide new insight into the cytoplasmic phosphotyrosine signaling networks that operate during photoreceptor axon guidance and suggest specific points of interaction with Eya.

II.2 – **Introduction**

A remarkable feature of multicellular animal development is that the complexity and diversity in tissue type and patterning is achieved using fewer than a dozen signaling pathways, including Notch, Receptor Tyrosine Kinase, Wnt, Hedgehog, TGFß, Jak/Stat and Hippo. These cascades are repeatedly deployed in different contexts to regulate the majority of the critical proliferation, survival, specification and differentiation decisions (reviewed in Voas and Rebay 2004; Housden and Perrimon 2014). One strategy to achieve context-specific developmental regulation is for proteins and pathways to function together in interconnected networks. Further, individual proteins within these networks may encode multiple independent functions that can be executed in distinct parts of the cell, cell types, or stages of development. In this way, even a

modest number of individual proteins and core pathways can create vast combinatorial possibilities.

Eyes absent (Eya), a protein conserved from plants to humans, presents an ideal model to study integration because its multi-functionality and dynamic subcellular localization provide opportunities for interaction with many signaling pathways (reviewed in Jemc and Rebay 2007; Tadjuidje and Hegde 2013). In metazoans, Eya has been studied extensively as a transcriptional coactivator and core member of the retinal determination gene network (reviewed in Silver and Rebay 2005; Kumar 2009). In this context, Eya participates in nuclear transcriptional complexes to regulate programs of gene expression that direct cell proliferation, differentiation and survival in a variety of tissues and organs, including the Drosophila eye (Bonini *et al.* 1993; Bonini *et al.* 1998; Pignoni *et al.* 1997; Ohto *et al.* 1999; Heanue *et al.* 1999; Zou *et al.* 2004; Nica *et al.* 2006; Hirose *et al.* 2010). Eya also carries two different phosphatase domains, one with specificity for phosphotyrosine and a second directed toward phosphothreonine (Li *et al.* 2003; Rayapureddi *et al.* 2003; Tootle *et al.* 2003; Okabe *et al.* 2009). Although Eya's tyrosine phosphatase is not required for normal development in *Drosophila* (Jin *et al.* 2013), in mammals it dephosphorylates H2AX to promote repair and survival in response to DNA damage (Cook *et al.* 2009; Krishnan *et al.* 2009) and aPKCζ to direct lung epithelial polarity and morphogenesis (El-Hashash *et al.* 2012). Eya's threonine phosphatase is less well characterized, but appears to act both cytoplasmically to regulate innate immunity and nuclearly to provide transactivation and regulate the activity of other transcription factors (Okabe *et al.* 2009; Liu *et al.* 2012; Xu *et al.* 2014; Jin and Mardon 2016).

Despite increased awareness from mammalian studies of the requirement for Eya function in the cytoplasm, understanding of the specific signaling cascades with which it interacts remains limited. To increase knowledge of the signaling networks with which Eya interfaces, we took advantage of our observation that Drosophila Eya can be sequestered in the cytoplasm upon tyrosine phosphorylation (pY) by the Abelson (Abl) kinase (Xiong *et al.* 2009) by performing a genetic modifier screen designed to uncover interactions with genes encoding phosphotyrosine binding proteins. The photoreceptor axon mistargeting phenotypes associated with Eya misexpression provided sensitized backgrounds, and secondary genetic tests allowed us to distinguish interactions likely to impact Eya's cytoplasmic versus nuclear functions. Prominent among the final list of candidate cytoplasmic interactors were several non-receptor tyrosine kinases and multiple members of the Jak/Stat pathway. Follow-up genetic experiments, including MARCM analysis of *Stat92E* clones, confirmed the pathway is required in the differentiating photoreceptors for axon targeting. To our knowledge, these results provide the first demonstration, either in invertebrates or vertebrates, of a requirement for the Jak/Stat pathway in axon guidance. Focusing on the Jak/Stat pathway factors, we validated the premise of our screen by showing that Eya can be recruited to cytoplasmic complexes via pY-SH2 mediated interactions. Together our results uncover novel requirements for phosphotyrosine-based signaling networks in photoreceptor axon targeting and provide a strong impetus for future mechanistic explorations of how cytoplasmic Eya interfaces with these pathways in both *Drosophila* and mammals.

II.3 – Results and discussion

II.3.1 – A genetic screen for SH2/PTB family genes that synergize with *eya* during photoreceptor axon targeting

We previously showed that Eya shuttles between the nucleus and cytoplasm and that Abl-mediated tyrosine phosphorylation (pY) can sequester it in the cytoplasm (Xiong *et al.* 2009). These observations led us to hypothesize that pY-Eya might be recruited to cytoplasmic signaling complexes through interactions with Src Homology 2 (SH2) or Phospho-Tyrosine Binding (PTB) domain-containing proteins. To test this idea, we leveraged our prior demonstration of genetic synergy between *eya* and *abl* during axon guidance (Xiong *et al.* 2009) to design an RNAi-based genetic interaction screen aimed at identifying SH2/PTB factors relevant to Eya function in differentiating photoreceptors.

Our prior work showed that in viable hypomorphic *eya* mutants, photoreceptor axons fasciculate aberrantly and frequently overshoot the lamina (Xiong *et al.* 2009). MARCM clones ruled out the possibility that these phenotypes stemmed from defects in the target layers of the brain, and *GMR-Gal4* driven *eya* knockdown further confirmed its requirement in the photoreceptors (Xiong *et al.* 2009). Although the $GMR{>}Eya^{RNAi}$ mistargeting phenotype was modifiable, as evidenced by its enhancement upon *abl* knockdown (Xiong *et al.* 2009), we opted against using it as the primary background for our screen. Briefly, because we predicted positive genetic interactions between *eya* and relevant SH2/PTB genes, and therefore enhancement of the $GMR{>}Eya^{RNAi}$ phenotype, we were concerned that the difficulty in distinguishing a relevant enhancement from a non-specific additive interaction would lead to a high false positive rate.

As an alternative, we explored the suitability of an *eya* overexpression phenotype for the screen. Using the membrane marker Ro-lacZ^{tau} (RTL) to label photoreceptors R2-R5 (Garrity *et al.* 1999), we quantified the average number of axon bundles that mistarget past the lamina to the medulla in *GMR-Gal4>UAS-Eya^{WT}* animals as 25.7 ± 6.1 (mean ± standard deviation), a significant increase over the driver alone baseline of 8.1 ± 2.2 (Figure II-1B vs. II-1A, and II-1D; and Xiong *et al.* 2009). Reducing endogenous *eya* dosage with heterozygosity strongly suppressed the mistargeting (9.2 ± 4.4), confirming that the *GMR>Eya^{WT}* phenotype resulted from increased Eya activity rather than from a dominant negative effect of *eya* overexpression (Figure II-1C-D).

Although the similarity between *eya* loss- and gain-of-function phenotypes may seem surprising, there is precedent for biological processes, including photoreceptor axon guidance, being sensitive to precisely balanced gene dosage such that both loss and gain produce similar phenotypes. For example, analogous to our observations with *eya*, either increased or decreased activity of the p21-activated kinase *Pak* (Hing *et al.* 1999) or of the transcription factor *hindsight* (Oliva and Sierralta 2010) causes photoreceptor axon mistargeting. It is also important to note that our system of counting the number of RTL-labeled axons that overshoot their laminar target, although highly effective as a first-pass quantification of a phenotype or interaction, ignores qualitative differences. For example, *eya* knockdown tends to produce thicker fascicles and greater disruptions to the lamina plexus than *eya* overexpression (Figure II-1B, Supplemental Figure II-1 and Xiong *et al.* 2009). Future detailed mechanistic studies will be required to understand the requirement for balanced Eya levels in photoreceptor axon guidance, and the *eya* interactors identified in our screen should provide useful tools in that endeavor.

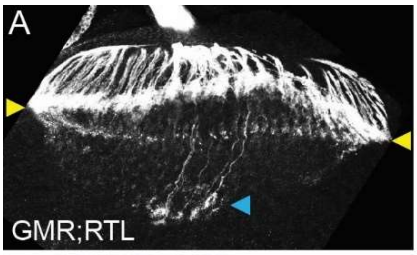
Given the general caveats associated with overexpression, it was critical to confirm the *GMR>Eya*^{WT} mistargeting phenotype could be appropriately modified by a verified *eya* interacting gene. Building on our prior work (Xiong *et al.* 2009), we showed that reduced *abl* dose, either by heterozygosity or by expression of a weak *UAS-Abl*^{RNAi} transgene that on its own did not produce targeting defects, dominantly suppressed *GMR>Eya*^{WT} (13.4 ± 3.5 and 13.5 ± 5.4, respectively; Figure II-1D shows data for *Abl*^{RNAi}). Thus reduced *abl* dominantly enhances *eya* loss-of-function and dominantly suppresses *eya* gain-of-function induced axon mistargeting. We conclude that despite both overexpression and knockdown of *eya* causing axons to overshoot the lamina, these genetic backgrounds produce genetically distinct and biologically informative phenotypes and interactions.

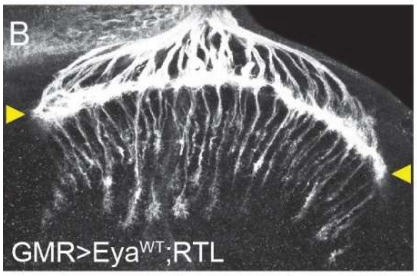
We also tested sensitivity of the *GMR>Eya*^{WT} background to enhancement by co-expressing a second *UAS-Eya*^{WT} transgene that had a comparably strong phenotype as the first (24.0 ± 5.4), but found only a modest increase to 30.3 ± 4.1 mistargeted axon bundles per brain. Thus the overexpression phenotype was dose-sensitive toward suppressive interactions, but was not easily enhanced (Figure II-1D). Because we predicted that reducing the dosage of an SH2/PTB containing factor relevant to cytoplasmic Eya functions would suppress, the *GMR>Eya*^{WT} background seemed ideal for the screen.

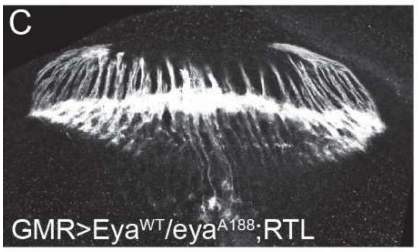
We screened a collection of 63 RNAi transgenes that targeted 36 SH2/PTB family genes for their ability to suppress the *GMR>Eya*^{WT} axon targeting phenotype (Figure II-1E). This panel included two independent RNAi transgenes per gene whenever possible to minimize the likelihood of false-positives from off-target effects and false-negatives from insufficient knockdown. Our use of *GMR-Gal4* restricted knockdown to retinal cells posterior to the

morphogenetic furrow, thereby bypassing any earlier requirements for the SH2/PTB genes to be tested. The screen was performed blind as to SH2/PTB gene identity, and ~10-20 3rd larval instar eye-brain complexes were dissected, stained and scored for each interaction test.

To prioritize further study, suppressors were binned into strong, moderate or mild interest groups based on p-value. 11 strong ($p<0.001$), six moderate ($p<.01$) and ten mild ($p<0.05$) hits were recovered, identifying 21 potential *eya* interacting genes (Table II-1 shows genes with one strong or moderate hit; full screen results are in Supplemental Table II-1). The rather high hit rate from the primary screen may reflect extensive connectivity between phosphotyrosine signaling modules (Koytiger *et al.* 2013). Inclusion of *dab*, a positive factor in the Abl signaling network (Song *et al.* 2010), in this initial list provided proof of principle for the screen by highlighting the importance of *eya-abl* interactions to axon guidance and thereby further validating the biological relevance of the *eya* overexpression phenotype. To narrow the scope of the follow-up experiments to a technically manageable level, we focused only on the strong and moderate suppressors, with one exception, *Stat92E*.







D

	w^{1118}	*eya*/+	abl^{RNAi}	Eya^{WT} Line 2
GMR-Gal4	8.0 ± 2.3 (50)	not tested	6.5 ± 2.8 (12)	24.0 ± 5.4 (21)
GMR>Eya^{WT} Line 1	25.7 ± 6.1 (11)	9.2 ± 4.4 (10)	13.5 ± 5.4 (22)	30.3 ± 4.1 (17)
GMR>Myr-Eya	18.9 ± 5.0 (65)	11.1 ± 4.3 (34)	8.7 ± 4.7 (31)	not tested
GMR>NLS-Eya	26.6 ± 4.9 (64)	11.6 ± 4.7 (7)	22.9 ± 4.3 (20)	not tested

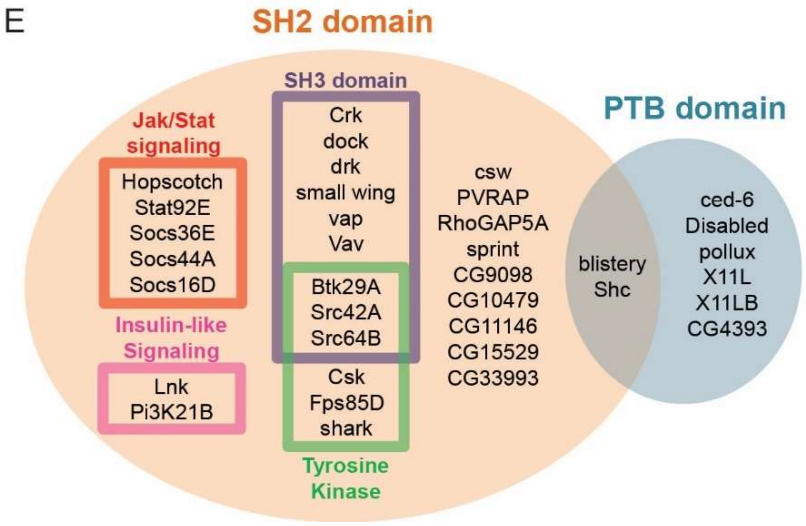


Figure II-1 | Eya overexpression axon targeting phenotypes provide dose-sensitive, suppressible genetic backgrounds
(A-C) The Ro-lacZ^{tau} (RTL) marker labels R2-R5 and is visualized by staining for β-galactosidase of 3rd instar larval brains. (A) Wild type RTL pattern shows most R2-R5 axons terminate correctly at the lamina, whose edges are marked by yellow arrowheads; a few axon bundles overshoot to the medulla, blue arrowhead. (B) *eya* overexpression causes many photoreceptor axons bundles to overshoot the lamina, marked by yellow arrowheads. (C) *eya* heterozygosity suppresses *eya* overexpression defects. (D) Quantification of genetic interaction tests demonstrating *eya* overexpression phenotypes are dose-sensitive and suppressible. N values are shown in parentheses. (E) 36 SH2/PTB-domain containing genes in *Drosophila*, grouped into known categories of signaling, that were targeted in our RNAi-based genetic screen.

II.3.2 – Genetic triage highlights potential links between Eya and both the Jak/Stat signaling pathway and non-receptor tyrosine kinases

Three secondary genetic tests were used to verify these hits as bona fide genetic interactors and to determine which genes may be relevant to cytoplasmic Eya function. First, we asked whether lines that suppressed *GMR>Eya^{WT}* conversely enhanced *eya* loss-of-function axon

targeting phenotypes (Table II-1). All but three genes (*CG15529, plx* and *vap)* enhanced *GMR>Eya*^{RNAi}, while *shark* yielded mixed results as one *shark* RNAi line enhanced and the other suppressed. Next, we used membrane-tethered myristoylated Eya (Myr-Eya) and nuclearly-localized Eya (NLS-Eya) (Xiong *et al.* 2009) to distinguish interactions specific to Eya's cytoplasmic and nuclear functions, respectively. *GMR-Gal4*-driven expression of both *Myr-Eya* and *NLS-Eya* produce axon guidance defects that can be suppressed by *eya* heterozygosity (Figure II-1D), indicating gain-of-function effects. However, we hypothesized that *Myr-Eya* and *NLS-Eya* perturb axon targeting via distinct mechanisms given their restricted subcellular localizations. Consistent with this idea, *abl* knockdown suppressed the axon mistargeting phenotypes associated with *Myr-Eya* but not *NLS-Eya* (Figure II-1D). Of the candidate lines tested, all except *csw* and *X11Lβ* significantly suppressed *GMR>Myr-Eya* mistargeting defects (Table II-1). When tested against *GMR>NLS-Eya*, only *Src64B* and *csw* suppressed, while the remaining ten lines enhanced. Because relevant interactions with *eya* gain-of-function phenotypes should manifest as suppression, we suspected the enhancement of *GMR>NLS-Eya* reflected additive effects rather than real interactions. Consistent with this prediction, *GMR-Gal4* knockdown of these SH2/PTB genes produced significant targeting defects (Figure II-2A). In sum, the results of our secondary screens whittled the number of candidates to ten genes: *Csk, hop, Lnk, shark, Socs36E*, *Socs44A, Src42A, Src64B, Stat92E*, and *X11L*. Among these, *Src64B* was unique in its potential to influence both cytoplasmic and nuclear Eya functions.

	RNAi element	GMR>Eya^{WT};RTL	GMR>Eya^{RNAi};RTL	GMR>Myr-Eya^{WT};RTL	GMR>NLS-Eya^{WT};RTL
	none (w^{1118})	25.7 ± 6.1	18.8 ± 2.5	18.9 ± 5.0	26.6 ± 4.9
Jak/Stat-signaling	Stat92E^{43866}	19.8 ± 2.6* ⬇	21.1 ± 3.3* ⇧	13.6 ± 4.5** ⬇	31.6 ± 4.9** ⇧
	Stat92E^{106980}	19.3 ± 6.1* ⬇	25.0 ± 3.7*** ⇧	12.9 ± 4.8*** ⬇	42.3 ± 7.3*** ⇧
	Socs36E^{51821}	22.3 ± 3.8			
	Socs36E^{52182}	17.7 ± 4.4** ⬇	22.0 ± 4.4* ⇧	11.2 ± 4.1*** ⬇	27.5 ± 7.6
	Socs44A^{33489}	25.4 ± 3.8			
	Socs44A^{102764}	14.3 ± 5.3*** ⬇	21.0 ± 4.0* ⇧	12.1 ± 7.3** ⬇	40.7 ± 3.5*** ⇧
Tyrosine Kinase	hop^{40037}	22.4 ± 4.8			
	hop^{102830}	16.6 ± 6.8*** ⬇	23.2 ± 5.1* ⇧	14.3 ± 6.2* ⬇	39.8 ± 6.6*** ⇧
	Src42A^{100708}	12.7 ± 4.3*** ⬇	21.3 ± 4.0* ⇧	10.6 ± 5.1*** ⬇	36.1 ± 5.4*** ⇧
	Src64B^{35252}	15.5 ± 2.9*** ⬇	22.8 ± 3.8*** ⇧	9.8 ± 3.2*** ⬇	20.2 ± 4.9*** ⬇
	Csk^{32877}	16.7 ± 4.0*** ⬇	24.5 ± 4.7*** ⇧	12.1 ± 5.2*** ⬇	41.0 ± 6.5** ⇧
	shark^{25304}	17.2 ± 4.7*** ⬇	23.3 ± 2.3*** ⇧	10.3 ± 3.8*** ⬇	36.8 ± 4.5*** ⇧
	shark^{105706}	18.4 ± 3.1** ⬇	16.1 ± 3.4** ⬇		
	Lnk^{32892}	22.6 ± 3.3			
	Lnk^{103646}	13.4 ± 6.5*** ⬇	23.3 ± 4.6* ⇧	12.6 ± 5.3*** ⬇	45.3 ± 6.0*** ⇧
	vap^{44638}	20.2 ± 5.7* ⬇			
	vap^{107341}	17.9 ± 5.9** ⬇	17.1 ± 2.9* ⬇		
	csw^{21756}	18.6 ± 4.7** ⬇	24.5 ± 3.4*** ⇧	19.9 ± 6.2	20.9 ± 4.4*** ⬇
	csw^{21757}	22.7 ± 2.9			
	CG15529^{50228}	21.8 ± 7.7			
	CG15529^{100438}	17.9 ± 2.8** ⬇	18.8 ± 2.6		
	Dab^{13005}	18.1 ± 6.7** ⬇	23.6 ± 2.9*** ⇧	14.8 ± 3.5** ⬇	13.7 ± 3.6*** ⬇
	Dab^{14008}	13.0 ± 4.3*** ⬇	24.0 ± 2.7*** ⇧	11.8 ± 2.7*** ⬇	25.7 ± 5.0
	plx^{27335}	6.54 ± 3.1*** ⬇	19.8 ± 3.3		
	plx^{106969}	26.6 ± 4.5			
	x11L^{27479}	13.5 ± 4.4*** ⬇	24.7 ± 5.1* ⇧	11.1 ± 4.4*** ⬇	34.8 ± 4.6*** ⇧
	X11Lβ^{8309}	10.4 ± 5.1*** ⬇	21.8 ± 3.9* ⇧	19.8 ± 6.0	33.4 ± 4.2*** ⇧
	X11Lβ^{14872}	27.4 ± 5.8			

Table II-1 | Short-list of candidate Eya-interacting SH2/PTB genes after primary and secondary genetic interaction tests

A short-list of RNAi lines identified in the primary screen as suppressors were crossed to *GMR>Eya^{RNAi};RTL*, *GMR>Myr-Eya^{WT};RTL* and *GMR>NLS-Eya^{WT};RTL* backgrounds as secondary tests. Numbers shown are averages of overshooting axons per brain with standard deviation. * p-value <0.05, ** p-value < 0.01, *** p-value < 0.001. Superscripted numbers for each RNAi element indicate the VDRC stock line used. Solid black down arrows mark significant suppressive interactions; open up arrows mark enhancement. Full table of results for all RNAi lines screened along with N values can be found in Supplemental Table II-1.

Two groups of genes were notably enriched in this short-list: the Jak/Stat pathway (*hop, Stat92E, Socs36E and Socs44A*) and non-receptor tyrosine kinases (*Csk, hop, shark, Src42A and Src64B*). In the follow-up studies described below, we focused on uncovering the mechanistic links that may underlie the Jak/Stat signature. Two reasons drove this decision. First, because the

Jak/Stat pathway had not been previously implicated in axon guidance in any system, discovery of a novel context for Jak/Stat signaling in neuronal development had the potential for broadest impact. Second, with the exception of *Stat92E*, the interactions that produced our ten gene short-list were based on a single RNAi transgene, either because only one line was available (*Csk, Src42A, Src64B and X11L*) or because only one of the two lines tested showed interaction in the primary (*hop, Lnk, Socs36E* and *Socs44A*) or secondary (*shark*) screens (Table II-1 and Supplemental Table II-1). Independent identification of four different Jak/Stat pathway members provided collective validation, and so mitigated the risk of pursuing spurious off-target effects of single hits. In the future, comparison of target gene mRNA levels between pairs of lines and addition of Dicer2 to the line that did not interact could be performed to determine whether such discrepancies reflect false-positives due to off-target effects or false-negatives due to either poor RNAi efficiency or additive interactions. We consider the latter to be the most likely explanation given that our screen required suppression of *GMR>Eya*^{WT} by RNAi knockdowns that on their own often produced strong mistargeting defects (Figure II-2A). Thus a "hit" in the primary screen often reflects not only suppression of *eya* overexpression phenotypes by SH2/PTB knockdown, but also mutual suppression of SH2/PTB knockdown phenotypes by increased *eya* dose. In cases where the additive effects were sufficiently strong as to mask such mutual suppression, inconsistent results between independent lines would be expected.

A

RNAi element	GMR;RTL	n
none (w^{1118})	8.0 ± 2.3	50
hop^{40037}	15.3 ± 2.1***	12
hop^{102830}	22.3 ± 3.6***	18
$Stat^{43866}$	19.7 ± 4.5***	15
$Stat^{106980}$	18.8 ± 3.0***	7
$Socs36E^{51821}$	17.1 ± 2.8***	18
$Socs36E^{52182}$	16.7 ± 3.1***	9
$Socs44A^{33489}$	9.5 ± 3.6	13
$Socs44A^{102764}$	11.9 ± 2.8**	16
$Src42A^{100708}$	18.0 ± 1.6***	8
$Src64B^{35252}$	18.1 ± 3.7***	17
Csk^{32877}	17.4 ± 3.5***	8
$shark^{25304}$	19.0 ± 1.9***	6
$shark^{105706}$	21.5 ± 1.1***	8
Lnk^{103646}	23.3 ± 1.3***	7
$x11L^{27479}$	19.7 ± 2.5***	7

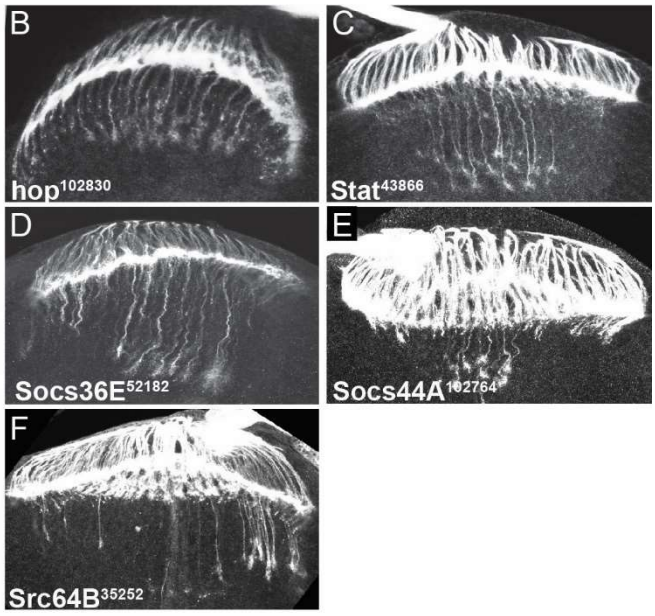


Figure II-2 | Eya-interacting SH2/PTB genes are required for photoreceptor axon guidance (A) Quantification of axon mistargeting phenotypes resulting from RNAi knockdown of ten SH2/PTB genes from Table II-1. The RNAi lines indicated were crossed to *GMR;RTL*. Superscripted numbers indicate the VDRC stock used. Numbers shown are averages of overshooting axons per brain with standard deviation. * p-value <0.05, ** p-value < 0.01, *** p-value < 0.001. N values are shown on the right. (B-F) Representative brains of the indicated genotype stained with β-galactosidase.

The second category of interactors, the non-receptor tyrosine kinases, underscores the importance of phosphotyrosine-based signaling for photoreceptor axon targeting. Previous work hinted at this, as a number of tyrosine phosphatases were found to be critical for regulating axon targeting (Garrity *et al.* 1999; Schindelholz *et al.* 2001; Jeon *et al.* 2008). Our results identify a set of candidate tyrosine kinases that could provide the opposing catalytic activity. The remaining two genes on the short-list, *Lnk* and *X11L*, encode adapter proteins. X11L has been implicated in amyloid precursor protein (APP) regulation in both *Drosophila* and mammalian cells (Hase *et al.* 2002; Gross *et al.* 2008), with no reported connections to either Jak/Stat or tyrosine kinase signaling. *Lnk* negatively feeds back on Jak/Stat signaling in mammalian cells,

although in *Drosophila* it has been studied as a positive regulator of insulin/insulin-like growth factor signaling (Werz *et al.* 2009; Slack *et al.* 2010; Oh *et al.* 2010; Almudi *et al.* 2013). The Jak/Stat ligands Unpaired (Upd) 2 and 3 influence insulin signaling in *Drosophila* (Rajan and Perrimon 2012; Woodcock *et al.* 2015), thus potentially implicating Lnk in Jak/Stat signaling in flies.

II.3.3 – Jak/Stat signaling is necessary for photoreceptor axon guidance

Jak/Stat signaling in *Drosophila* is transduced via three ligands, Upd1-3, one receptor, Dome, one Janus kinase (JAK), Hopscotch (Hop), and one signal transducer and activator of transcription (STAT), Stat92E (Stat). Signaling can be attenuated by a variety of negative regulators, including the suppressor of cytokine signaling (SOCS) proteins encoded by *Socs36E* and *Socs44A* (Rawlings *et al.* 2004a; Rawlings *et al.* 2004b; Arbouzova and Zeidler 2006). Jak/Stat signaling directs a diverse spectrum of developmental events including cell migration, proliferation, patterning and morphogenesis (reviewed in Arbouzova & Zeidler 2006 and Li 2008). During eye development, the pathway promotes growth of the early eye field, contributes to initiation and movement of the morphogenetic furrow and to regional eye specification, transduces information from the dorsal-ventral axis to direct ommatidial rotation and regulates specification and maintenance of the optic lobe neuroepithelium (Zeidler *et al.* 1999; Luo *et al.* 1999; Tsai and Sun 2004; Chao *et al.* 2004; Ekas *et al.* 2006; Gutierrez-Aviño *et al.* 2009; Ngo *et al.* 2010; Wang *et al.* 2011).

Identification of *hop*, *Stat92E*, *Socs36E* and *Socs44A* in our genetic screen (Table II-1) and finding that *GMR*-driven knockdown of these genes produces axon mistargeting phenotypes (Figure II-2 A-E) suggested that the Jak/Stat pathway is also active in post-mitotic

photoreceptors. In support of this, expression of the 10xStat-eGFP pathway activity reporter suggests that early expression of the ligand, *upd*, which is absent by 3rd instar, enables sustained Jak/Stat signaling in the photoreceptors at the time they are sending out their axons (Bach *et al.* 2007). As predicted, *GMR-Gal4* driven knockdown of the receptor *dome*, but not of the *upd* ligands, produced significant mistargeting defects (Figure II-3 A-C).

To confirm further the requirement for Jak/Stat signaling in photoreceptor axon guidance, we generated MARCM clones of a *Stat92E* null allele, $stat^{85c9}$. Axons originating in photoreceptors that lacked *Stat92E* overshot the lamina into wild-type brain tissue (Figure II-3D), while clones within the brain did not cause mistargeting of wild-type photoreceptors (data not shown). This demonstrates that correct axon targeting requires *Stat92E* in the photoreceptors. The result also eliminates the possibility that RNAi-induced phenotypes resulted from either leaky *GMR-Gal4* expression in the brain (Li *et al.* 2012) or off-target effects. Together, our results indicate a cell autonomous role for Jak/Stat signaling in post-mitotic photoreceptors that directs axon projections to the correct layers of the brain.

One apparent inconsistency between this conclusion and our genetic screen results is that SOCS factors typically antagonize Jak/Stat signaling (Chen *et al.* 2000; Callus and Mathey-Prevot 2002; Rawlings *et al.* 2004b) yet knockdown of *Socs*, *hop* or *Stat92E* all suppressed $GMR{>}Eya^{WT}$. Since axon targeting is a novel context for Jak/Stat signaling, one possibility is that the SOCS factors do not antagonize the pathway in post-mitotic photoreceptors as they do in other situations (Callus and Mathey-Prevot 2002; Rawlings *et al.* 2004b). Arguing against this, knockdown of either *Socs36E* or *Socs44A* suppressed the $GMR{>}hop^{RNAi}$ mistargeting phenotypes (Figure II-3 E-F). In addition to their roles as negative regulators of Jak/Stat signaling, SOCS

factors also influence a variety of growth factor signaling cascades (De Sepulveda *et al.* 1999; Chen *et al.* 2000; Bayle *et al.* 2004; Rawlings *et al.* 2004b; Kazi *et al.* 2012; Trengove and Ward 2013). Thus the *eya-Socs* synergy observed in our screen could reflect interactions relevant to those other pathways. Mechanistically, although SOCS proteins belong to the family of Cullin-Ring E3 ubiquitin ligases (reviewed in (Piessevaux *et al.* 2008)), a model in which they target Eya for degradation seemed unlikely because the predicted increase in Eya levels upon *Socs* knockdown should enhance, rather than suppress, *GMR>Eya*^{WT} phenotypes. Consistent with such reasoning, *Socs* knockdown did not alter Eya protein levels in the *GMR>Eya*^{WT} background (data not shown).

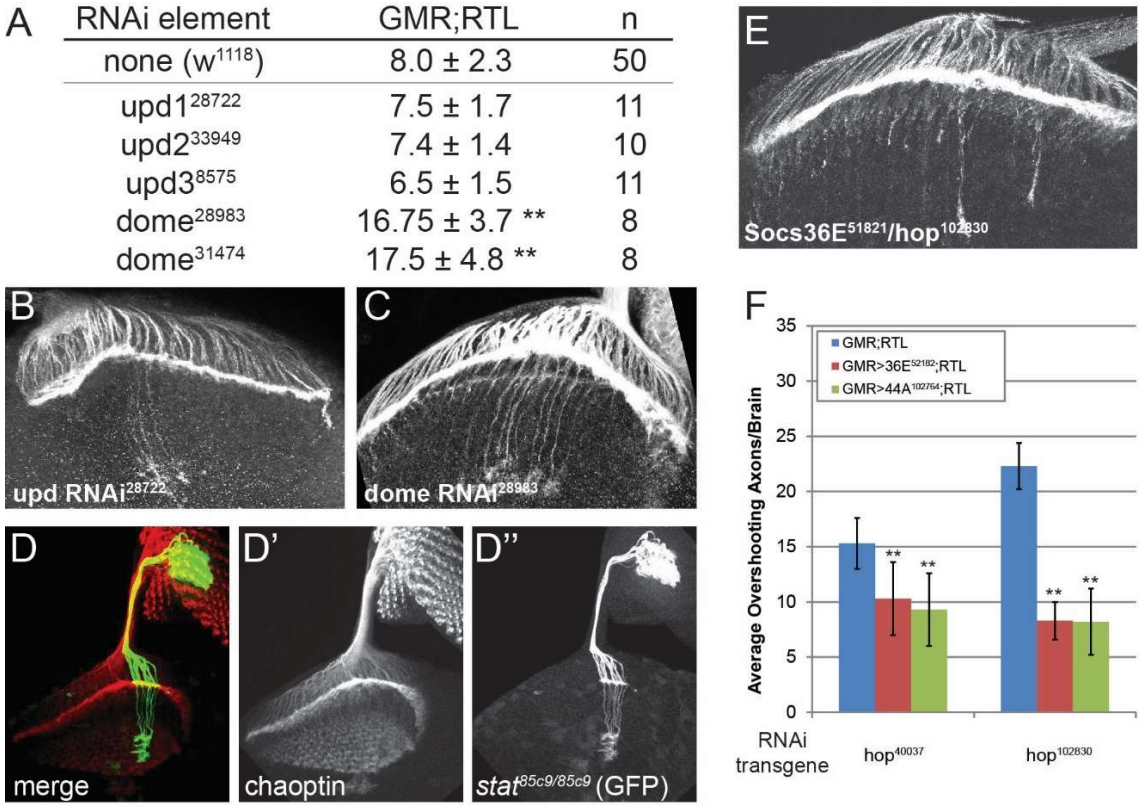


Figure II-3 | Jak/Stat signaling is required cell autonomously in photoreceptors for axon targeting

Figure II-3 | Jak/Stat signaling is required cell autonomously in photoreceptors for axon targeting (continued)
(A) Quantification of axon mistargeting phenotypes resulting from RNAi knockdown of *upd* and *dome*. The RNAi lines indicated were crossed to *GMR;RTL*. Superscripted numbers indicate the Bloomington stock used. Numbers shown are averages of overshooting axons per brain with standard deviation. * p-value <0.05, ** p-value < 0.01, *** p-value < 0.001. N values are shown to the right. (B-C) Representative brains of the indicated genotype stained with β-galactosidase. (B) *upd* knockdown does not perturb axon targeting. (C) Knockdown of *dome* leads to significant overshooting of the lamina. (D) GFP+ (green) MARCM clone showing that the axons of *Stat92E* null photoreceptors overshoot the lamina. Chaoptin staining (red) marks all photoreceptors in the eye disc (top right) and highlights their axonal projections through the optic stalk and into the brain. (E) Representative brain stained with β-galactosidase showing that *Socs36E* knockdown suppresses the axon mistargeting phenotypes of *GMR>hop*^{RNAi} (compare to Figure II-2B) (F) Quantification and statistics of axon overshooting phenotypes in double knockdown interaction assays show that *Socs* genes function as Jak/Stat pathway antagonists. *GMR;RTL* (blue), *GMR>Socs36E*^{RNAi}*;RTL* (red) and *GMR>Socs44A*^{RNAi}*;RTL* (green) were crossed to the *hop*^{RNAi} line indicated on the X axis. N values per experiment from left to right are 10, 7, 10, 13, 13 and 14. P-values were calculated by performing student's T-test between the experimental cross and driver alone control (i.e. red/green vs blue). * p-value <0.05, ** p-value < 0.01, *** p-value < 0.001. Superscripted numbers refer to VDRC RNAi line.

Given the sensitivity of photoreceptor axon targeting to both loss and overexpression of *eya* (Figure II-1, Table II-1 and Supplemental Figure II-1), we asked whether the same might hold true for *hop, Stat92E, Socs36E* and *Socs44A. GMR-Gal4* driven overexpression of *hop* was lethal prior to 3rd instar, and *Stat92E* overexpression did not produce targeting defects (data not shown and Figure II-4 A and C). *Stat92E* overexpression also did not modify *GMR>Eya*^{RNAi} (Figure II-4 B and F), suggesting endogenous signaling is limited and insufficient to activate the extra Stat protein. Consistent with this interpretation, previous reports have shown that although *UAS-Stat* can rescue *Stat92E* null phenotypes, its overexpression in an otherwise wild type background does not perturb eye development (Ekas *et al.* 2006).

In contrast, *GMR-Gal4* driven overexpression of either *Socs36E* or *Socs44A* significantly perturbed axon targeting (Figure II-4 A, D-E). We took advantage of this result to validate

independently the *Socs-eya* synergy predicted by our genetic screen interactions. Just as *Socs* knockdown suppressed *eya* overexpression phenotypes (Table II-1), *Socs* overexpression suppressed *eya* knockdown phenotypes (Figure II-4 B, G-H). Thus cooperative *eya-Socs* interactions appear integral to photoreceptor axon targeting.

A

UAS element	GMR;RTL	N
none (w^{1118})	8.0 ± 2.3	50
$Stat^{WT}$	8.1 ± 2.0	9
$Socs36E^{WT}$	30.8 ± 5.9***	9
$Socs44A^{WT}$	23.7 ± 4.0***	7

B

UAS element	GMR;RTL	N
Eya^{RNAi}	18.8 ± 2.5	33
Eya^{RNAi}, $Stat^{WT}$	19.3 ± 2.9	9
Eya^{RNAi}, $Socs36E^{WT}$	9.7 ± 2.4***,***	7
Eya^{RNAi}, $Socs44A^{WT}$	12.5 ± 3.5**,***	14

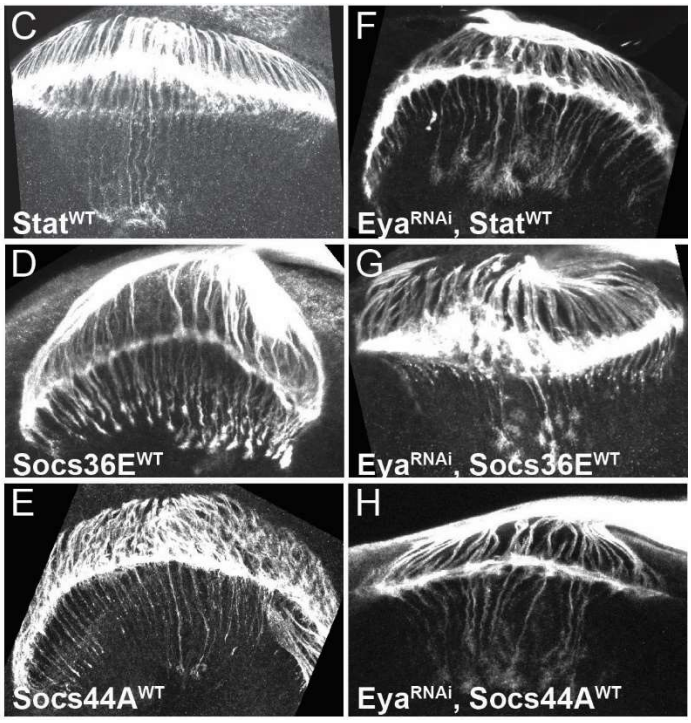


Figure II-4 | Eya and SOCS synergize in axon targeting
(A) Quantification of axon phenotypes resulting from overexpression of *Stat92E*, *Socs36E* and *Socs44A*. (B) Quantification of axon phenotypes from genotypes in (A) in a $GMR>Eya^{RNAi}$ background. (C-H) Representative images of the indicated genotypes stained with anti-β-galactosidase. P-values were calculated by performing student's T-test between the experimental cross and the control of that group. In B, the first is relative to $GMR>Eya^{RNAi}$ and the second is relative to $GMR>SOCS^{WT}$, revealing mutual suppression. * p-value <0.05, ** p-value <0.01, *** p-value < 0.001.

II.3.4 – Eya co-immunoprecipitates and co-localizes with Socs44A via an SH2-pY mediated interaction

Given that the impetus for our genetic screen was the possibility that SH2/PTB domains interact with pY-Eya, we next asked whether Eya could co-immunoprecipitate (CoIP) Jak/Stat proteins from lysates of Drosophila S2 cultured cells transiently transfected with epitope-tagged expression constructs. CoIPs were performed both with and without co-transfection of Abl. Abl stimulates Eya tyrosine phosphorylation and cytoplasmic accumulation (Xiong *et al.* 2009 and Figure II-6 A vs. B, quantified in N) and thus should enhance SH2/PTB-pY-Eya interactions. We found that Flag-tagged Eya can CoIP HA-tagged Hop, Socs36E and Socs44A, but not Stat, and that Abl improved Eya's CoIP of Socs36E and Socs44A, but not that of Hop or Stat (Figure II-5 A-D). Abl is expressed endogenously in S2 cells (Cherbas *et al.* 2011), and some tyrosine phosphorylation of Eya can be detected in cells transfected with Eya alone (Tootle *et al.* 2003), perhaps explaining the low levels of Socs36E and Socs44A CoIP detected when Abl was not supplied by co-transfection (Figure II-5 C-D). The Abl-independence of the Eya-Hop CoIP (Figure II-5A) suggests that either Hop phosphorylates Eya at different tyrosine residues, or that pY-SH2 interactions do not drive the CoIP.

Because Abl increased Eya's CoIP with Socs36E and Socs44A, we focused on them as the top candidates for forming SH2-pY-Eya complexes. To test this, we generated three deletion mutants that removed either the N-terminus (ΔNT), the SH2 domain (ΔSH2), or the Socs Box (ΔSB) and a point mutant (SH2*, Socs36E^{R500K} or Socs44A^{R214K}) that compromises SH2 function in homologous mammalian SOCS proteins (Nicholson *et al.* 1999). Consistent with expectations, the Socs44A SH2 domain was required for CoIP while the SH2* point mutant

weakened the interaction (Figure II-5F and Supplemental Figure II-2). Similar experiments with Socs36E did not identify any single domain as being required for CoIP (Figure II-5E), suggesting that the Eya-Socs36E interaction is more complicated, either requiring multiple Socs36E domains to assemble the complex and/or additional proteins to bridge the interactions. Socs44A and Socs36E are both endogenously expressed in S2 cells (Callus and Mathey-Prevot 2002; Zhu *et al.* 2013) and can CoIP each other (Supplemental Figure II-3). Thus, Socs44A is an appealing candidate to bridge the Eya-Socs36E interaction, as its association with both proteins would confer the sensitivity to Abl seen in the Eya-Socs36E CoIPs (Figure II-5 C-D).

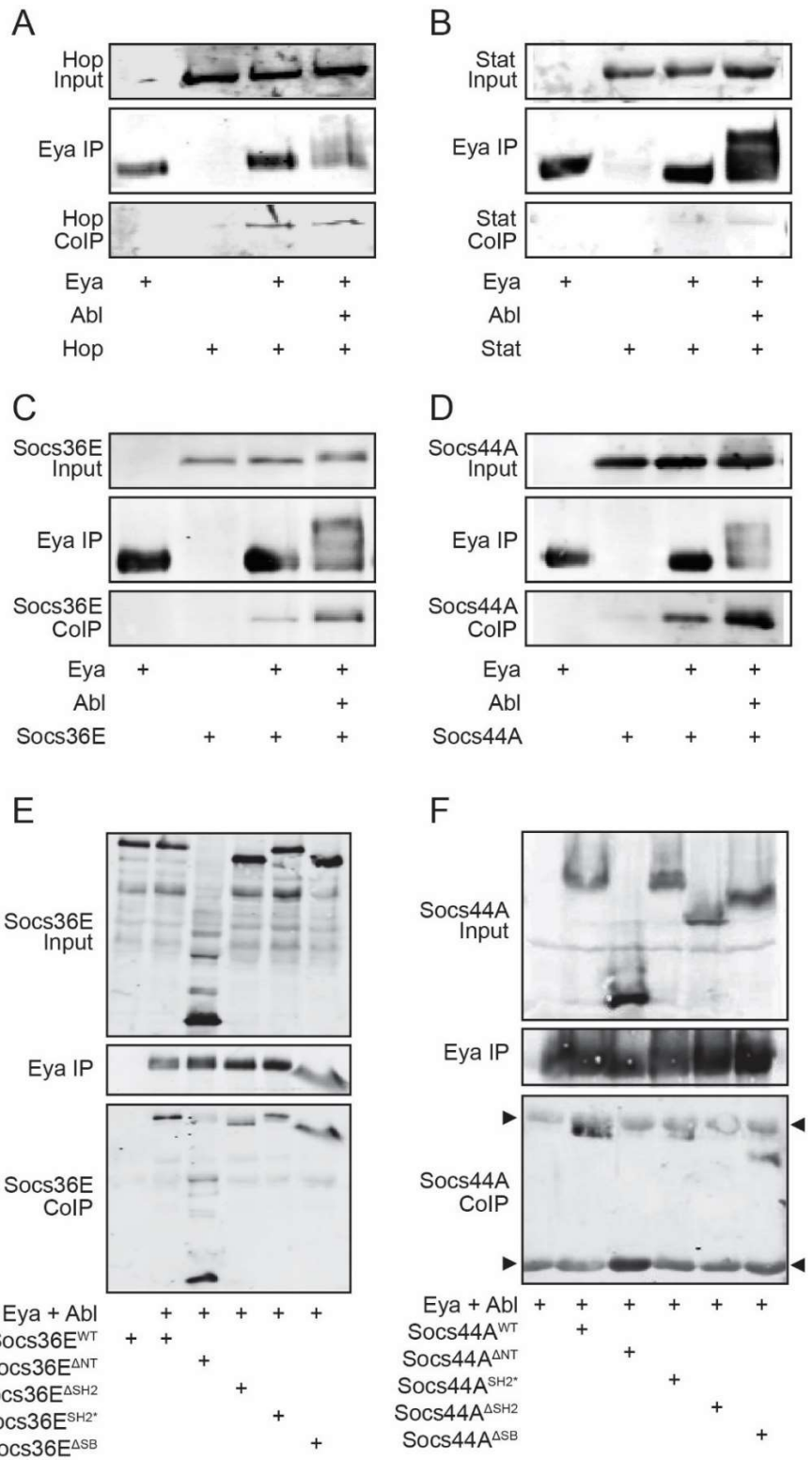


Figure II-5 | pY-SH2 dependence of Eya co-immunoprecipitation with Hop, Socs36E and Socs44A

Figure II-5 | pY-SH2 dependence of Eya co-immunoprecipitation with Hop, Socs36E and Socs44A (continued)
(A-F) Western blots showing CoIP results from transfected Drosophila S2 cells. Top panels, blotted with anti-HA, show input levels for the HA-tagged Jak/Stat pathway factor. Middle panels, blotted with anti-Flag, show IP of Flag-Eya. Bottom panels show CoIP of the HA-tagged Jak/Stat pathway protein. Key below indicates factors transfected in each experiment. (A-D) Eya CoIPs full length Hop, Socs36E and Socs44A but not Stat. Broad smeary band in lane 4, middle panels, reflects the extensive tyrosine phosphorylation of Eya that occurs in the presence of Abl (Xiong *et al.* 2009). (A) Flag-Eya CoIPs HA-Hop in the presence or absence of Abl. (B) Flag-Eya does not CoIP HA-Stat. (C & D) Flag-Eya CoIP of Socs36E (C) and Socs44A (D) is increased in the presence of Abl. (E-F) Eya-Socs44A but not Eya-Socs36E requires the SH2 domain of that SOCS factor. (E) Flag-Eya CoIPs all HA-Socs36E mutant constructs. (F) Flag-Eya CoIPs all HA-Socs44A deletion constructs except that lacking the SH2 domain. Black triangles mark IgG bands. Full-length Socs44A (lanes 2 and 4) runs below IgG heavy chain while Socs44A$^{\Delta NT}$ runs coincident with IgG light chain (lane 3).

The pY-SH2-mediated CoIP of Eya and Socs44A predicted cytoplasmic co-localization. Our previous work has shown that Eya is predominantly nuclear but can be relocalized to the cytoplasm upon phosphorylation by Abl (Xiong *et al.* 2009 and Figure II-6 A-B). Socs44A accumulated in the cytoplasm, with strong association to the plasma membrane (Figure II-6 C and F-I; localization of Hop, Stat and Socs36E is shown in Supplemental Figure II-4). In Eya-Socs44A co-transfected cells, we observed a subtle increase in the proportion of cells with cytoplasmic Eya while Socs44A localization was not changed (Figure II-6 F-G and N). Occasionally, membrane-associated Eya puncta were observed and these tended to co-localize with Socs44A-positive spots (Figure II-6G). The frequency of cells displaying these punctate structures dramatically increased when Abl was co-transfected (Figure II-6 H-I and O). Confirming the SH2 requirement for Eya recruitment, we did not observe Eya-containing puncta when Socs44A$^{\Delta SH2}$ was expressed (Figure II-6 J-K and O). To separate Abl's role in phosphorylating Eya from its role in recruiting Eya to the cytoplasm, we examined the response

of Myr-Eya which is constitutively targeted to the cytoplasmic membrane (Figure II-6 D-E). Only when Abl was co-transfected did Socs44A promote Eya punctate localization (Figure II-6 L-M and O). We speculate that these puncta represent Eya-Socs44A complexes and that they are mediated by pY-SH2 interactions.

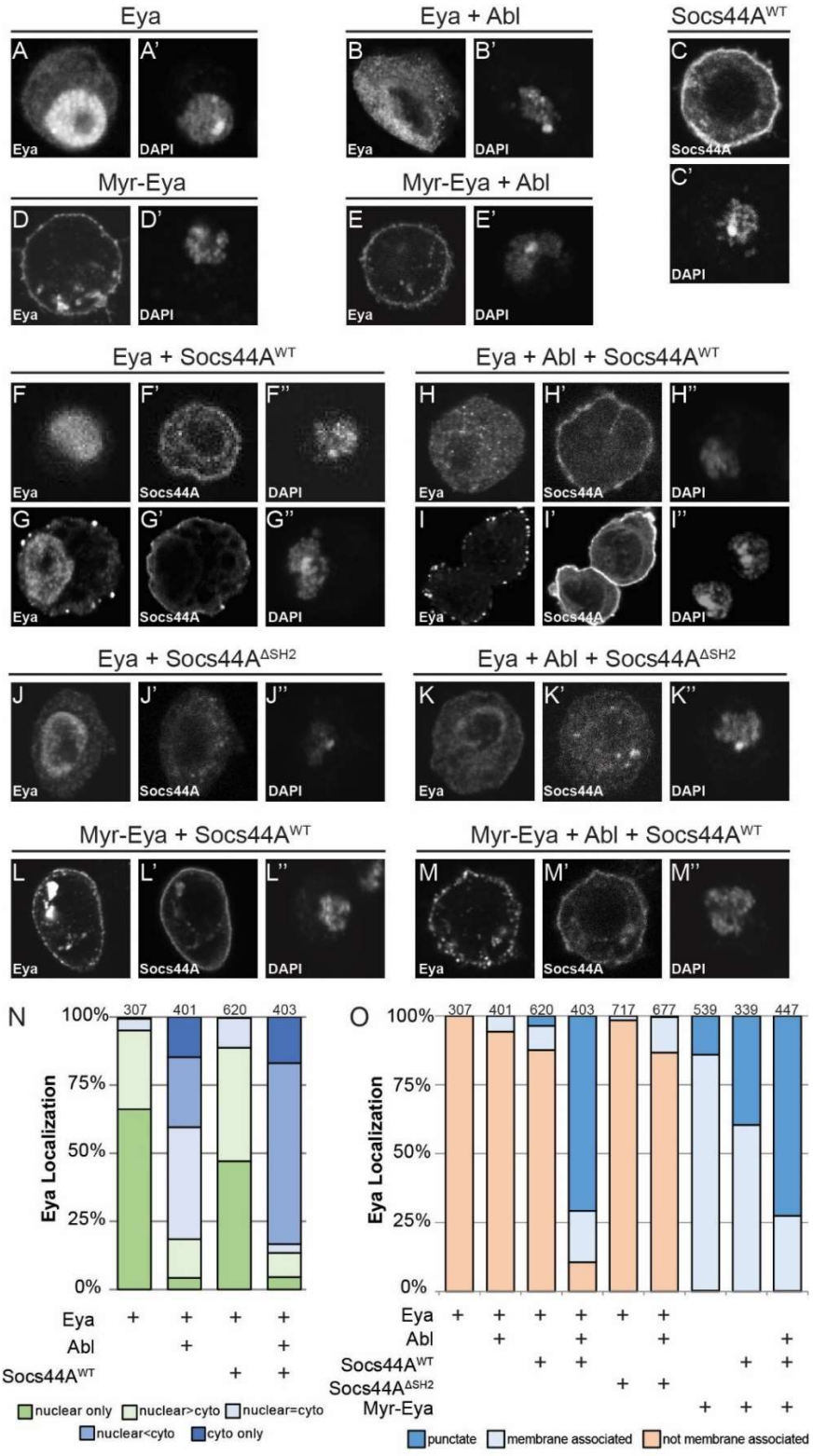


Figure II-6 | Eya localizes to cytoplasmic punctate structures when co-transfected with Abl and Socs44A

Figure II-6 | Eya localizes to cytoplasmic punctate structures when co-transfected with Abl and Socs44A (continued)
(A-M) Individual transfected S2 cells showing representative subcellular distribution of Flag-Eya and HA-Socs44A. Nuclei are marked with DAPI. (A) Flag-Eya is predominantly nuclear. (B) Co-transfection of Abl causes cytoplasmic redistribution of Flag-Eya. (C) Full-length HA-Socs44A appears cytoplasmic and membrane associated. (D) Myr-Flag-Eya is tightly membrane-associated, with some punctate structures apparent. (E) Co-transfection of Abl does not alter Myr-Flag-Eya distribution. (F, G) Two representative cells showing that co-transfection of Socs44A does not induce a major redistribution of Eya (F), although in a small fraction of cells, bright membrane proximal punctate structures are seen (G). Socs44A appears more tightly membrane associated in cells in which Eya puncta form (G'), with reduced general cytoplasmic accumulation (compare to F'). (H-I) Two representative cells showing Eya cytoplasmic (H) and punctate (I) localization increases in response to co-transfection of Abl and Socs44A. In cells with Eya puncta, Socs44A tends to be strongly membrane-associated (I'). (J-K) Redistribution of Eya to membrane-associated puncta requires the SH2 domain of Socs44A. Socs44A$^{\Delta SH2}$ localizes diffusely throughout the cytoplasm and is not strongly membrane-associated (Supplemental Figure II-3L), and localization does not change with co-transfection of Eya (J) or Eya and Abl (K). (L) Socs44A only slightly increases the punctate distribution of Myr-Eya. (M) Co-transfection of Abl and Socs44A results in punctate Myr-Eya distribution in 75% of cells. (N) Quantification of nuclear versus cytoplasmic distribution of Flag-Eya in experiments A, B and F-I. N values are shown across the top of the graph. (O) Quantification of cytoplasmic Eya distribution into punctate structures in experiments A, B, and D-M. Punctate classification is a subset of membrane associated (i.e. some cells categorized as having punctate staining also have membrane associated staining). N values are shown across the top of the graph.

II.4 – Concluding remarks

Our genetic screen was initially motivated by the idea that the cytoplasm was a likely site for Eya's phosphotyrosine phosphatase activity (Xiong et al. 2009), a model which was subsequently validated in mammals (El-Hashash *et al.* 2012). Recent work has shown that this catalytic function of Eya is dispensable for *Drosophila* development (Jin et al. 2013). What then might be the role of cytoplasmic Eya? One possibility is that Eya might act as a scaffolding factor that nucleates the formation of specific protein complexes in response to different upstream signals. Because our biochemical experiments relied on interactions between overexpressed proteins in cultured cells, it will be important to confirm the results using

endogenous proteins from Drosophila tissues. Another possibility is that Eya might modulate the composition or function of such complexes via its phosphothreonine phosphatase activity. Eya's threonine phosphatase activity has been implicated in innate immunity in both mammals and *Drosophila*, and although substrates have not been identified, cytoplasmic activity was implied by the identified interactions (Okabe *et al.* 2009; Liu *et al.* 2012). Whether threonine phosphatase function also contributes to normal developmental processes such as axonal outgrowth and targeting is not known. Intriguingly, the Jak/Stat pathway is relevant to innate immunity in both *Drosophila* and mammals (reviewed in Agaisse and Perrimon 2004; O'Shea and Plenge 2012). In *Drosophila*, Jak/Stat signaling regulates hemocyte (blood cell) development and release of Upd cytokines in response to infection (Agaisse *et al.* 2003; Minakhina *et al.* 2011; Copf *et al.* 2011; Woodcock *et al.* 2015). The tyrosine kinases from our short-list of candidates also have potential links to immunity. *shark* and *Src42A* are important for Draper-mediated glial phagocytosis of axonal debris (Ziegenfuss et al. 2008) which, if left uncleared, stimulates an immune response, and Src64B overexpression has been shown to be sufficient for inducing an immune response in larva (Williams 2009). Even if Eya's threonine phosphatase activity is not involved, extending exploration of the interactions we have uncovered in the context of axon guidance to other biological contexts in which the candidate interacting partner is known to function may provide new insight into the cytoplasmic signaling networks in which Eya participates during development and disease.

II.5 – Materials and methods

II.5.1 – Fly strains

The screen was carried out by crossing RNAi lines acquired from the Vienna Drosophila RNAi Center (VDRC) to 5 different recombinant stocks containing GMR-Gal4 and Ro-LacZ^{tau}: 1) *GMR-Gal4/CyO-actGFP;Ro-lacZ^{tau}*, 2) *GMR>Eya^{WT}/CyO-actGFP;Ro-lacZ^{tau}* , 3) *GMR>Eya^{RNAi}/CyO-actGFP;Ro-lacZ^{tau}* , 4) *GMR-Gal4/CyO-actGFP;Uas-NLS-Eya^{WT},Ro-lacZ^{tau}/TM6*, and 5) *GMR-Gal4/CyO-actGFP;Uas-Myr-Eya^{WT},Ro-lacZ^{tau}/TM6* (Xiong *et al.* 2009).

UAS-Stat92E and *stat^{85c9}* flies were generously provided by E. Bach. MARCM clones were generated by heat shocking *hsFLP, Elav-Gal4, UAS-mCD8::GFP;;FRT82B, stat^{85c9}/FRT82B, tub-Gal80* flies 48 hours after egg lay for 2 hours at 37°C.

Socs36E and *Socs44A* RNAi recombinants were confirmed by wing vein phenotypes using the engrailed driver (Rawlings *et al.* 2004b). *upd* and *dome* RNAi lines were acquired from the Bloomington Stock Center. *UAS-Socs36E* flies were a gift from B. Mathey-Prevot and *UAS-Socs44A* flies were shared by D. Harrison.

II.5.2 – Co-immunoprecipitation, immunostaining and antibodies

Drosophila S2 cells were cultured at 25°C in Schneider's medium (Sigma-Aldrich) supplemented with 10% Insect Medium Supplement (Sigma), penicillin and streptomycin (Invitrogen). Cells were transfected with 1.0ug of each plasmid using dimethyldioctadecyl-ammonium bromide (DDAB, Sigma) transfection and induced with $CuSO_4$. Expression constructs included pMT-Myc-Abl (Xiong *et al.* 2009), Eya B isoform cDNA cloned into the gateway pAFW vector, Hop, Stat92E, Socs36E and Socs44A cDNA cloned into the gateway

pAHW vector, and SOCS mutants quikchanged from pAHW-SOCS^{WT} plasmids. Oligos used were:

1) Socs36E$^{\Delta NT}$: 5'-GGTACCAACACCATGAGCAGCTTCTACTGGGGC-3' and

5'-GCCCCAGTAGAAGCTGCTCATGGTGTTGGTACC-3'

2) Socs36E$^{\Delta SH2}$: 5'-CTCGAGAAGATCACGAACAGCGGTCACAAGTTCAG-3' and

5'-CTGAACTTGTGACCGCTGTTCGTGATCTTCTCGAG-3'

3) Socs36E$^{SH2*\ (R500K)}$: 5'-GGCACGTTCCTGCTGAAAGACTCCGCCCAGGAGG-3' and

5'-CCTCCTGGGCGGAGTCTTTCAGCAGGAACGTGCC-3'

4) Socs36E$^{\Delta SB}$: 5'-CTGCACAGAAGGCAGACCTAGGAATTCGCGGCCGCA-3' and

5'- TGCGGCCGCGAATTCCTAGGTCTGCCTTCTGTGCAG-3'

5) Socs44A$^{\Delta NT}$: 5'-AACACCATGTACTGGGGTGAG-3' and

5'- CTCACCCCAGTACATGGTGTT-3'

6) Socs44A$^{\Delta SH2}$: 5'-AACCAAGTGTGGATCTTGCACCGC -3' and

5'-G CGGTGCAAGATCCACACTTGGTT-3'

7) Socs44A$^{SH2*\ (R214K)}$: 5'-TTTCTAGTC AAAGACTCGGAA-3' and

5'-TTCCGAGTCTTTGACTAGAAA-3'

8) Socs44A$^{\Delta SB}$: 5'CGGTACTCCAACTAGCTCGAG-3' and

5'-CTCGAGCTAGTTGGAGTACCG-3'

For transfections containing Abl or Hop, cells were treated with 100μM pervanadate and 200μM H_2O_2 prior to fixation or lysis. Co-immunoprecipitation assays were performed by lysing cells in lysis buffer (50mM Hepes, 150mM NaCl, 1mM EDTA, 0.5mM EGTA, 0.9M glycerol) supplemented with protease inhibitors (Roche), 0.5mM DTT and 0.1% Triton, incubating the cell

lysate with anti-Flag agarose beads (Sigma) overnight at 4°C, washing three times with lysis buffer, and resolving the proteins on 8% SDS-PAGE gels. Proteins were visualized by immunoblotting using either mouse or rabbit anti-Flag (1:1000, Sigma) and rabbit or mouse anti-HA (1:1000, Rockland or 12CA5 UChicago Frank W. Fitch Monoclonal Antibody Facility, respectively) and secondary antibodies (1:2000, Li-COR Biosciences).

Eye imaginal discs and brains were dissected from pre-pupal third instar larvae and stained as previously described (Xiong *et al.* 2009) with rabbit anti-β-gal (1:20,000, Promega). Transfected S2 cells were settled onto poly-L-lysine treated slides, fixed with 4% paraformaldehyde for 10 minutes and stained with anti-Flag and anti-HA as described above, followed by secondary antibodies (1:2000, Jackson ImmunoResearch). Samples were mounted with N-propyl-gallate solution in 90% glycerol.

Images were taken using a Zeiss LSM 510 confocal microscope. All axon images were taken at 0.5-1.5μm steps for the entire depth of the brain and then projected maximally in ImageJ. All single cell images are single scans through the middle of the cell. Unless otherwise stated, statistical p-values were calculated by performing student's T-test between control crosses with no RNAi transgene and the cross of interest. * p-value <0.05, ** p-value < 0.01, *** p-value < 0.001.

II.6 – Acknowledgements

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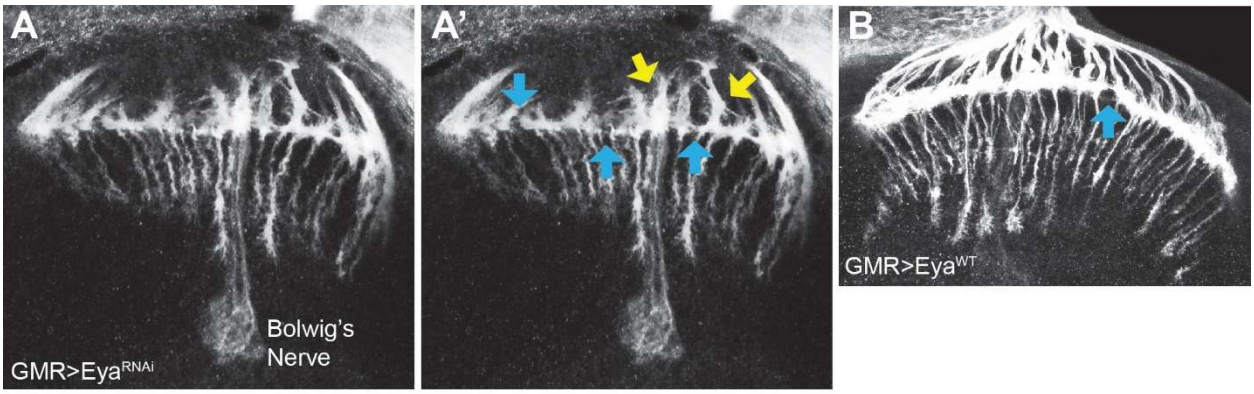
II.8 Supplemental table and figures

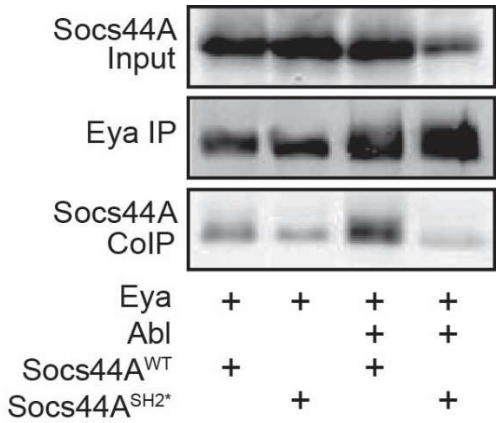
		RNAi element	GMR>Eya^{WT}	n	GMR>Eya^{RNAi}	n	GMR>Myr-Eya^{WT}	n	GMR>NLS-Eya^{WT}	n
		none (w1118)	25.7 ± 6.1	11	18.8 ± 2.5	33	18.9 ± 5.0	65	26.6 ± 4.9	64
SH2 domain	Jak/Stat-signaling	hop^{40037}	22.4 ± 4.8	26						
		hop^{102830}	16.6 ± 6.8*** ⬇	42	23.2 ± 5.1* ⇧	11	14.3 ± 6.2* ⬇	17	39.8 ± 6.6*** ⇧	9
		Stat92E^{43866}	19.8 ± 2.6* ⬇	16	21.1 ± 3.3* ⇧	24	13.6 ± 4.5** ⬇	5	31.6 ± 4.9** ⇧	5
		Stat92E^{106980}	19.3 ± 6.1* ⬇	12	25.0 ± 3.7*** ⇧	16	12.9 ± 4.8*** ⬇	19	42.3 ± 7.3*** ⇧	21
		Socs36E^{51821}	22.3 ± 3.8	19						
		Socs36E^{52182}	17.7 ± 4.4** ⬇	9	22.0 ± 4.4* ⇧	16	11.2 ± 4.1*** ⬇	14	27.5 ± 7.6	11
		Socs44A^{33489}	25.4 ± 3.8	16						
		Socs44A^{102764}	14.3 ± 5.3*** ⬇	32	21.0 ± 4.0* ⇧	61	12.1 ± 7.3** ⬇	14	40.7 ± 3.5*** ⇧	20
		Socs16D^{48210}	22.4 ± 3.3	25						
		Socs16D^{100568}	21.4 ± 4.4	27						
	Insulin signaling	Lnk^{32892}	22.6 ± 3.3	21						
		Lnk^{103646}	13.4 ± 6.5*** ⬇	33	23.3 ± 4.6* ⇧	11	12.6 ± 5.3*** ⬇	34	45.3 ± 6.0*** ⇧	20
		Pi3K21B^{33556}	22.1 ± 5.2	19						
		Pi3K21B^{104179}	23.0 ± 4.8	8						
	SH3 domain	Crk^{19061}	27.1 ± 8.3	14						
		Crk^{106498}	20.9 ± 3.7* ⬇	15						
		dock^{37524}	26.1 ± 2.8	16						
		dock^{107064}	24.9 ± 4.1	12						
		drk^{105498}	22.5 ± 5.2	8						
		sl^{7173}	21.9 ± 5.3	10						
		sl^{7174}	21.5 ± 6.4	15						
		vap^{44638}	20.2 ± 5.7* ⬇	18						
		vap^{107341}	17.9 ± 5.9** ⬇	30	17.1 ± 2.9* ⬇	15				
		Vav^{6241}	21.1 ± 3.4* ⬇	10						
		Vav^{103820}	23.3 ± 3.4	12						
	Tyrosine kinase	Btk29A^{22675}	23.6 ± 3.1	4						
		Btk29A^{106962}	22.5 ± 2.7	11						
		Src42A^{100708}	12.7 ± 4.3*** ⬇	25	21.3 ± 4.0* ⇧	25	10.6 ± 5.1*** ⬇	25	36.1 ± 5.4*** ⇧	19
		Src64B^{35252}	15.5 ± 2.9*** ⬇	22	22.8 ± 3.8*** ⇧	19	9.8 ± 3.2*** ⬇	22	20.2 ± 4.9*** ⬇	18
		Csk^{32877}	16.7 ± 4.0*** ⬇	16	24.5 ± 4.7*** ⇧	31	12.1 ± 5.2*** ⬇	36	41.0 ± 6.5** ⇧	20
		Fps85D^{36053}	23.0 ± 2.4	6						
		Fps85D^{107266}	22.0 ± 3.5	11						
		shark^{25304}	17.2 ± 4.7*** ⬇	22	23.3 ± 2.3*** ⇧	18	10.3 ± 3.8*** ⬇	23	36.8 ± 4.5*** ⇧	12
		shark^{105706}	18.4 ± 3.1** ⬇	12	16.1 ± 3.4** ⬇	21				
		csw^{21756}	18.6 ± 4.7** ⬇	12	24.5 ± 3.4*** ⇧	10	19.9 ± 6.2	19	20.9 ± 4.4*** ⬇	20
		csw^{21757}	22.7 ± 2.9	13						
		PVRAP^{26046}	24.2 ± 4.8	17						
		RhoGAP5A^{41779}	23.9 ± 4.6	9						
		RhoGAP5A^{41780}	23.5 ± 4.2	10						
		RhoGAP5A^{102000}	23.2 ± 3.8	9						
		spri^{25140}	21.9 ± 3.2	3						
		spri^{101164}	23.2 ± 4.3	11						
		CG9098^{27001}	23.5 ± 4.5	15						
		CG10479^{45098}	20.7 ± 4.5* ⬇	27						
		CG10479^{106226}	21.3 ± 4.1* ⬇	21						
		CG11146^{47795}	23.7 ± 6.0	22						
		CG11146^{101324}	20.9 ± 3.6* ⬇	8						
		CG15529^{50228}	21.8 ± 7.7	24						
		CG15529^{100438}	17.9 ± 2.8** ⬇	14	18.8 ± 2.6	12				
		CG33993^{40702}	22.1 ± 6.6	23						
		CG33993^{102754}	21.0 ± 7.1	8						
	PTB domain	by^{22823}	28.7 ± 3.9	19						
		Shc^{40464}	20.5 ± 6.8* ⬇	29						
		Shc^{103906}	20.3 ± 3.5* ⬇	19						
		ced-6^{16313}	21.8 ± 3.6	13						
		Dab^{13005}	18.1 ± 6.7** ⬇	13	23.6 ± 2.9*** ⇧	21	14.8 ± 3.5** ⬇	11	13.7 ± 3.6*** ⬇	12
		Dab^{14008}	13.0 ± 4.3*** ⬇	36	24.0 ± 2.7*** ⇧	16	11.8 ± 2.7*** ⬇	9	25.7 ± 5.0	12
		plx^{27335}	6.54 ± 3.1*** ⬇	13	19.8 ± 3.3	21				
		plx^{106969}	26.6 ± 4.5	15						
		x11L^{27479}	13.5 ± 4.4*** ⬇	23	24.7 ± 5.1* ⇧	9	11.1 ± 4.4*** ⬇	28	34.8 ± 4.6*** ⇧	22
		X11Lβ^{8309}	10.4 ± 5.1*** ⬇	23	21.8 ± 3.9* ⇧	12	19.8 ± 6.0	12	33.4 ± 4.2*** ⇧	18
		X11Lβ^{14872}	27.4 ± 5.8	9						
		CG4393^{105381}	23.6 ± 4.1	10						

Supplemental Table II-1 | Complete genetic screen results

Supplemental Table II-1 | Complete genetic screen results (continued)
Complete results from the primary and secondary genetic screens. SH2/PTB genes are grouped into known categories of signaling and superscripted numbers indicate the VDRC line. All lines were crossed to *GMR>Eya^{WT};RTL* for the primary screen, and hits were subsequently crossed to *GMR>Eya^{RNAi};RTL*, *GMR>Myr-Eya^{WT};RTL*, and *GMR>NLS-Eya^{WT};RTL* for secondary tests. Data shown in each column are averages of overshooting axons per brain with standard deviation, followed by p-value (* p-value <0.05, ** p-value < 0.01, *** p-value < 0.001), followed by an arrow indicating the direction of the genetic interaction (solid black down arrows mark significant suppression; open up arrows mark enhancement), followed by the number (N) of brains scored.

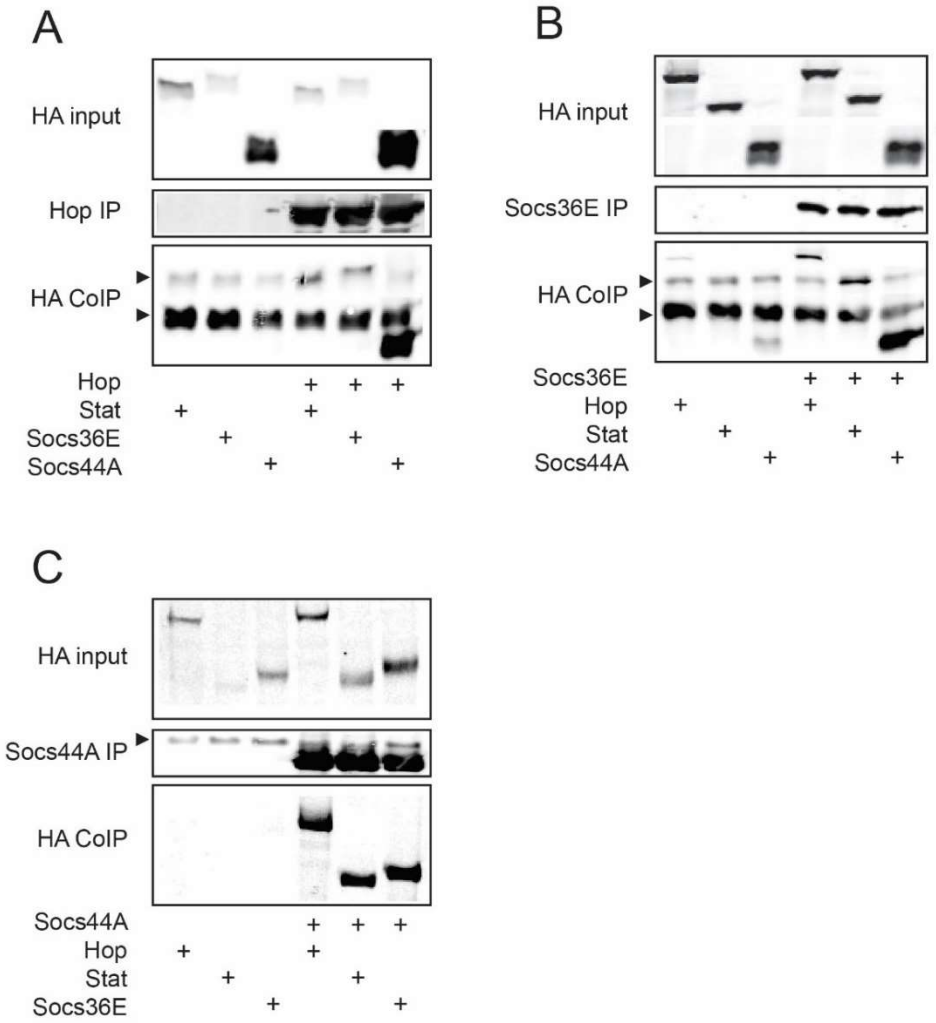


Supplemental Figure II-1 | Comparison of *eya* knockdown versus overexpression axon guidance phenotype
(A) *GMR>Eya^{RNAi}* animals have brains with thick axon fascicles (highlighted in A' by yellow arrows) and an uneven lamina plexus with frequent gaps highlighted in A' by blue arrows. (B) In contrast, *GMR>Eya^{WT}* animals only occasionally have disruptions to the lamina plexus (blue arrow).

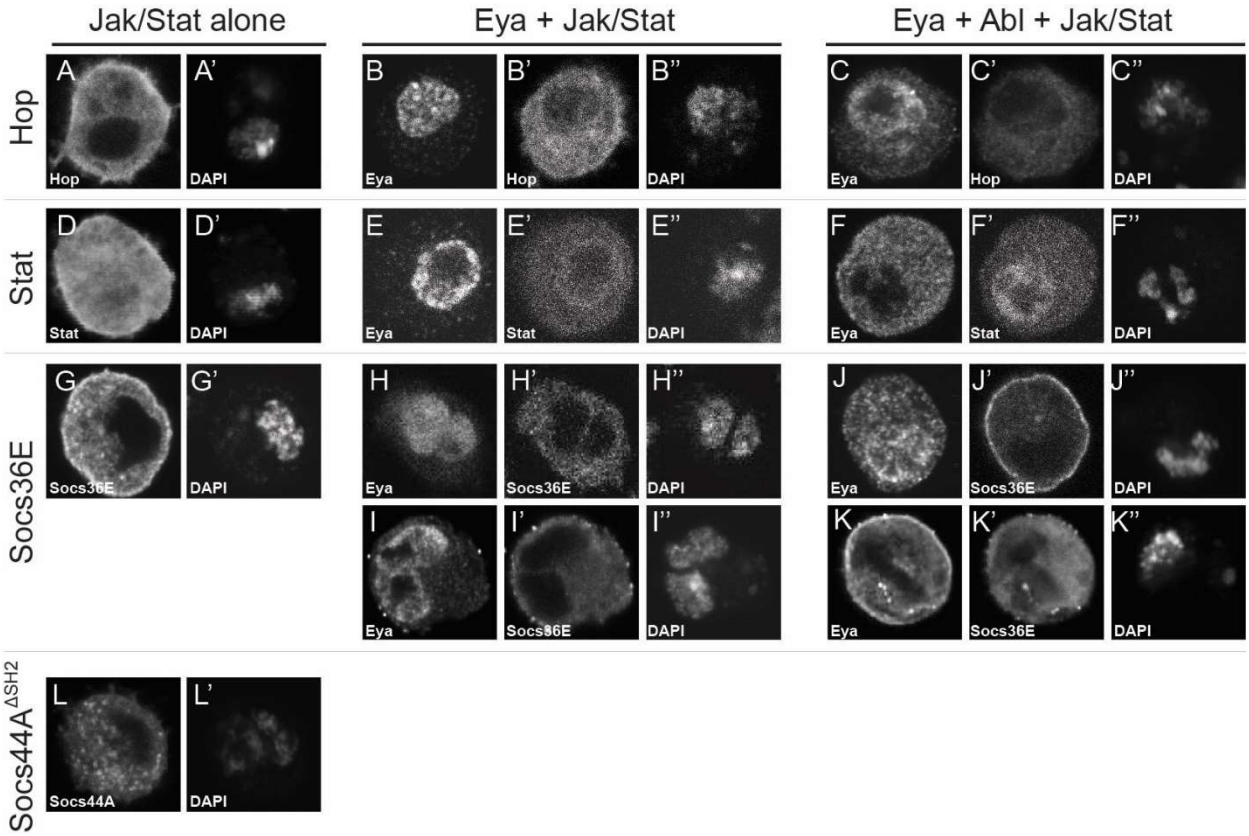


Supplemental Figure II-2 | A missense mutation in the Socs44A SH2 domain disrupts CoIP with pY-Eya

Supplemental Figure II-2 | A missense mutation in the Socs44A SH2 domain disrupts CoIP with pY-Eya (continued)
Western blot showing that Flag-Eya co-transfected with Abl only weakly CoIPs HA-Socs44A^{SH2*}(Socs44A^{R214K}). Top panel, blotted with anti-HA, shows input levels for Socs44A constructs. Middle panel, blotted with anti-Flag, shows IP of Flag-tagged Eya. Bottom panel shows CoIP of WT, but not SH2 mutated, Socs44A with Eya when Abl is cotransfected.



Supplemental Figure II-3 | Jak/Stat components CoIP each other
(A-C) Western blots showing CoIP results from transfected Drosophila S2 cells. Top panels, blotted with anti-HA, show input levels for the HA-tagged Jak/Stat genes. Middle panels, blotted with anti-Flag, show IP of Flag-tagged Jak/Stat genes. Bottom panels show CoIP of HA-tagged Jak/Stat genes. Key below indicates factors transfected in each experiment. Black triangles mark IgG bands. Stat and Socs36E both run very close to IgG heavy chain while Socs44A runs below IgG light chain. (A) Flag-Hop CoIPs HA-Stat, HA-Socs36E and HA-Socs44A. (B) Flag-Socs36E CoIPs HA-Hop, HA-Stat and HA-Socs44A. (C) Flag-Socs44A CoIPs HA-Hop, HA-Stat and HA-Socs36E.



Supplemental Figure II-4 | Subcellular localization of Hop, Stat or Socs36E in S2 cells co-transfected with Eya or with Eya and Abl, and Socs44A$^{\Delta SH2}$

(A-N) Individual transfected S2 cells showing representative subcellular distribution of HA-tagged Jak/Stat genes and Flag-Eya. Nuclei are marked with DAPI. (A-C) Hop localization is predominantly cytoplasmic and becomes subtly nuclear upon co-transfection of Eya. (A) HA-Hop is exclusively cytoplasmic. Co-transfection of Eya (B) or Eya and Abl (C) enables detection of HA-Hop in the nucleus. Hop's mammalian orthologs, Jak1 and Jak2, do localize to the nucleus (Lobie *et al.* 1996; Ram and Waxman 1997). Eya localization is unaffected by co-transfection of Hop (B) and still relocalizes to the cytoplasm with co-transfection of Abl and Hop (C). (D-H) Stat localizes throughout the cell and becomes more nuclear with co-transfection of either Eya or Eya and Abl. (D) HA-Stat localizes to both the nucleus and cytoplasm. (E-F) Two representative cells showing that co-transfection of Eya will cause HA-Stat to become more nuclear while Flag-Eya localization is unchanged. (G-H) Two representative cells showing that co-transfection of Eya, Abl and Stat results in HA-Stat becoming more nuclear but Flag-Eya still relocalizes to the cytoplasm. (I-M) HA-Socs36E is exclusively cytoplasmic with some membrane-association. (J-K) Two representative cells showing that co-transfection of Socs36E does not induce dramatic redistribution of Flag-Eya (J), although some instances of cytoplasmic Eya accumulating into puncta are observed (K). (L) HA-Socs44A$^{\Delta SH2}$ is heterogeneously distributed throughout the cytoplasm. This localization does not change with co-transfection of Eya (Figure II-6J) or Eya and Abl (Figure II-6K).

CHAPTER III

Interactions between Eya and the Jak/Stat pathway are context-dependent

Charlene Hoi and Ilaria Rebay

III.1 – Abstract

Like most signaling circuits, both the retinal determination (RD) gene network and the Jak/Stat signaling pathway are used repeatedly in a variety of tissues and at multiple stages throughout Drosophila development. Our previous findings of genetic synergy between the RD gene, *eyes absent* (*eya*), and members of the Jak/Stat pathway during photoreceptor axon targeting (Chapter II), prompted us to ask whether the underlying mechanism might involve regulation of Jak/Stat pathway transcriptional output, and more broadly, whether *eya* also interacts with Jak/Stat pathway genes in other tissues. Investigations into the first question concluded that *eya* does not regulate Jak/Stat pathway activity during photoreceptor morphogenesis. However, experiments based on ectopic expression of Eya in the wing found that Eya-So can increase Jak/Stat pathway output, suggesting the mechanism might be relevant in contexts outside the eye. Investigations into the second question uncovered genetic antagonism rather than synergy as observed in the eye between *eya* and Jak/Stat effectors during border cell migration, a morphogenetic event associated with egg chamber maturation. Together, this work highlights the idea that Eya-Jak/Stat interactions may be recurrent across tissues, but suggests that both the outcome from the interaction and the molecular mechanism underlying it may differ depending on context.

III.2 – Introduction

Regulatory gene networks and signal transduction pathways integrate to regulate tissue-specific developmental processes. One of the earliest models for studying this was the Drosophila eye, in which links between the retinal determination (RD) gene network and multiple signaling pathways including Notch, RTK, Hedgehog, Dpp, and Wingless have been uncovered (reviewed in (Voas and Rebay 2004; Silver and Rebay 2005)). The work described in Chapter II adds the Jak/Stat pathway to this list. Although functions for Eya and other RD genes have been identified outside the eye (Mardon *et al.* 1994; Bai 2002; Fabrizio *et al.* 2003), whether and how they interface with the same signaling pathways is not well understood.

The RD network is a highly conserved collection of transcription factors and co-factors that work together to regulate transcription of target genes important for specification, proliferation and differentiation in gonad, muscle and, of course, the eye (Boyle *et al.* 1997; Pignoni *et al.* 1997; Bonini *et al.* 1998; Heanue *et al.* 1999; Bai 2002; Fabrizio *et al.* 2003). In addition to necessity in these contexts, ectopic expression of RD genes is also sufficient to induce ectopic eye formation in legs and wings (dashed lines of Figure III-1 and (Mardon *et al.* 1994; Halder *et al.* 1995; Bonini *et al.* 1997; Pignoni *et al.* 1997)). At the top of the network is *eyeless* (Ey), which initiates expression of *eyes absent* (Eya) and *sine oculis* (So) (Niimi *et al.* 1999; Zimmerman *et al.* 2000) whose protein products can bind together to form a bi-partite transcription factor (Pignoni *et al.* 1997). Eya and So then regulate target gene expression including *dachshund* (Dac) (Pappu *et al.* 2005) and *ey* (Pauli *et al.* 2005), with the latter interaction providing positive feedback on the network. In addition to their functions in eye development, the RD genes are redeployed in other tissues (Mardon *et al.* 1994; Bonini *et al.*

1998; Bai 2002; Fabrizio *et al.* 2003). For example, *ey*, *so* and *eya* are expressed in the egg chamber, but Eya is the only RD gene to have a characterized function in which it represses polar cell fate in somatic follicle cells (Bai 2002). Additionally, *dac* is the only RD gene to be expressed in the wing (Mardon *et al.* 1994), although its function there is not known.

The Jak/Stat pathway is a conserved signal transduction pathway broadly important for cell proliferation, differentiation, survival and apoptosis in multiple tissues. In the fly, compromised Jak/Stat pathway activity has pleiotropic effects, revealing roles in early proliferation (Tsai and Sun 2004; Chao *et al.* 2004), specification (Ekas *et al.* 2006), cell competition (Rodrigues *et al.* 2012), ommatidial rotation (Zeidler *et al.* 1999a; b), muscle development (Liu *et al.* 2009), wing hinge development (Johnstone *et al.* 2013; Ayala-Camargo *et al.* 2013), proximal-distal patterning (Ayala-Camargo *et al.* 2007), hematopoiesis (Minakhina *et al.* 2011), germ cell maintenance (Gregory *et al.* 2008), collective cell migration in the egg chamber (Ghiglione 2002; Silver *et al.* 2005; Starz-Gaiano *et al.* 2009) and innate immunity (reviewed in Agaisse and Perrimon 2004) Jak/Stat signaling in these various contexts employs activation of the single fly Janus kinase, *hopscotch* (*hop*), and transduction of this activity through the single Signal transducer and activator of transcription, *Stat92E*.

Given that our recent findings highlight synergistic interactions between Eya and members of the Jak/Stat pathway (Chapter II), this plethora of developmental contexts in which both Eya and Jak/Stat signaling are relevant highlight additional tissues in which the two potentially integrate (Figure III-1). In this chapter, we explore potential interactions in 3rd instar eye development, the migrating border cell cluster of developing egg chambers, and the wing. We find that Eya is neither necessary nor sufficient for Jak/Stat pathway activity in the eye, that

Eya and Jak/Stat effectors potentially antagonize in border cell migration, that Eya is able to induce ectopic Jak/Stat signaling in regions of the wing, and that Jak/Stat effectors are important for Eya-mediated phenotypes in the wing. Taken all together, these findings underscore the complexity of Eya-Jak/Stat interactions from one context to another and suggest that each tissue may possess unique conditions that dictate the nature of the interactions.

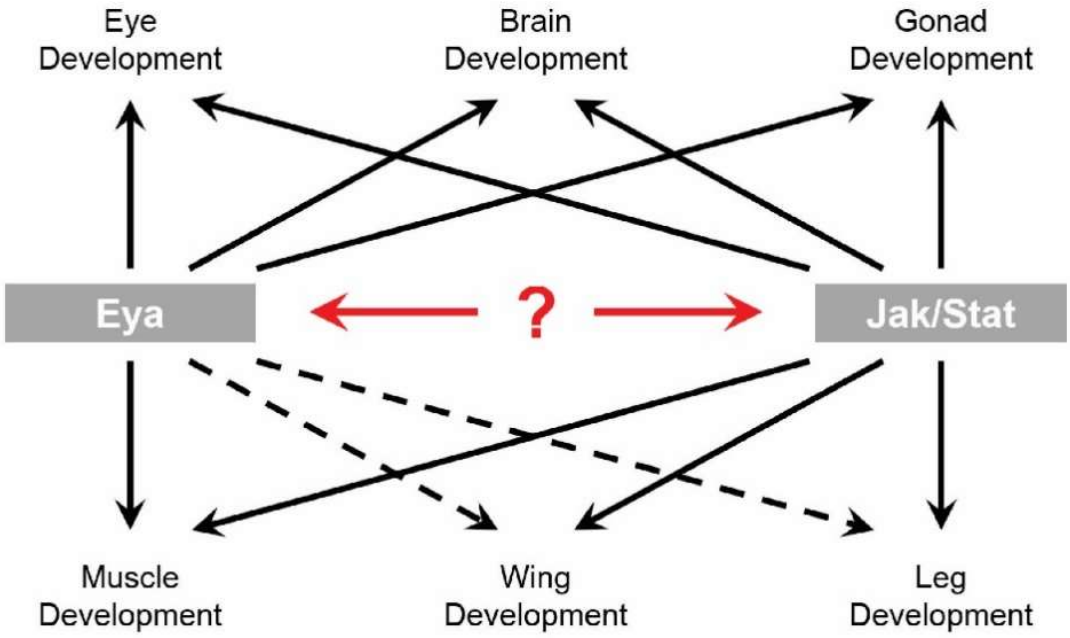


Figure III-1 | Eya and the Jak/Stat pathway are required for the development of multiple contexts
Eya and Jak/Stat signaling have both been implicated in a number of overlapping developmental contexts. Solid lines indicate demonstrated requirements while dashed lines for Eya indicate no endogenous requirement, but sufficiency for Eya in these contexts to induce ectopic eyes. The central question addressed in this chapter represented by the red arrow heads and question mark is whether Eya and the Jak/Stat pathway work together or independently in these contexts.

III.3 – Results

III.3.1 – *eya* does not affect Jak/Stat pathway activity in differentiated photoreceptor cells of 3rd instar retinal tissue

eya synergizes genetically with multiple Jak/Stat-related genes: *hop*, *Stat92E*, *Socs36E* and *Socs44A* to regulate photoreceptor axon targeting (Chapter II), but it is unclear whether this synergy affects output from the Jak/Stat pathway. Biochemical experiments show that Eya can interact with both Hop and SOCS factors (Figure II-5), raising the possibility that these interactions result in positive input to the pathway, perhaps by Eya interfering with SOCS' normal antagonism of Hop (Figure III-8) (Rawlings *et al.* 2004; Stec *et al.* 2013).

To test whether Eya positively regulates the pathway, we asked whether altering Eya levels changes the expression of a synthetic reporter known as 10xStat-eGFP (Bach *et al.* 2007). 10xStat-eGFP contains 5 tandem pairs of Stat binding sites upstream of a GFP and is widely accepted as a faithful transcriptional readout of pathway activity. In the third instar eye disc, this reporter is observed in the posterior of the eye disc (Figure III.2 A'), overlapping with a subset of the *eya* expression domain (Figure III.2 A''). I generated both *eya* overexpressing (Figure III-2 B) and null clones (Figure III-2 C) in a background containing this reporter to ask whether altered Eya levels can affect the intensity of the 10xStat-eGFP reporter. No ectopic induction of the reporter was observed in Eya overexpression clones (Figure III-2 B), indicating that either the level of pathway activity is already at maximum, or that Eya is insufficient for activating the pathway. Reporter expression was also unchanged in moderate and small sized *eya* null clones (Figure III-2 C), demonstrating that Eya is not necessary for pathway activity. Larger *eya* clones did seem to increase 10xStat-eGFP reporter levels, however these effects were likely from

concurrent compensatory proliferation and fate changes that occur as head tissue replaces the dying retinal tissue (Pignoni *et al.* 1997; Weasner and Kumar 2013).

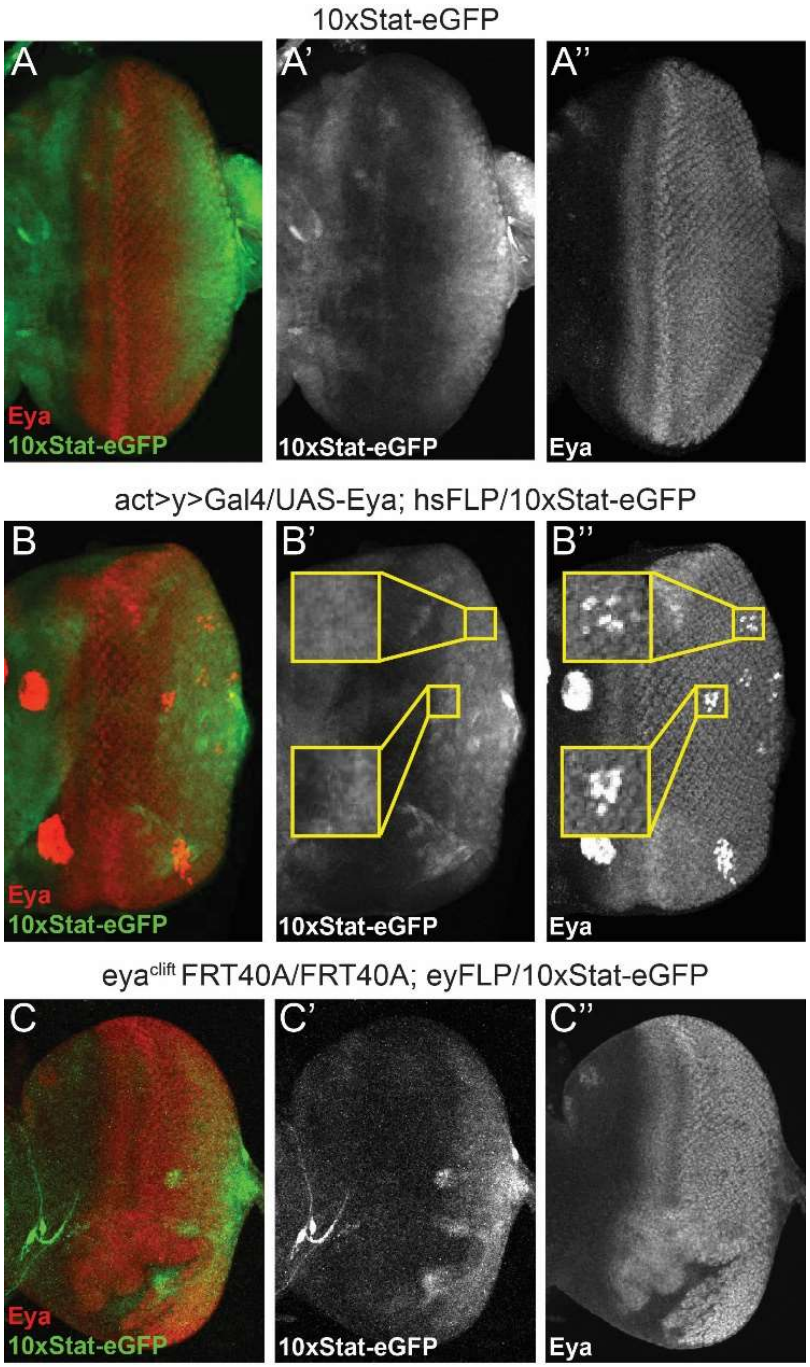


Figure III-2 | Eya is neither necessary nor sufficient to affect Jak/Stat pathway activity in the eye

Figure III-2 | Eya is neither necessary nor sufficient to affect Jak/Stat pathway activity in the eye (continued)
(A) Wild-type 3rd instar eye disc. Eya (red) and Jak/Stat pathway activity (green) are overlapping in the posterior of the eye. (B) Overexpression clones of *eya* do not affect the 10xStat-eGFP reporter. (C) Smaller *eya* null clones do not affect the 10xStat-eGFP reporter.

III.3.2 – Antagonistic interactions between *eya* and *hop* in border cell migration of the egg chamber

Gonad development is another context in which Eya and the Jak/Stat pathway may integrate. In both male and female gonads, *eya* is present in the somatic cells that support the germarium (Bai 2002; Fabrizio *et al.* 2003) and Jak/Stat signaling plays roles in the germ stem cells (Gilbert *et al.* 2005; López-Onieva *et al.* 2008; Wang *et al.* 2008). In the case of testes, these two cell types never overlap, while in the ovaries, both Eya and Jak/Stat signaling coincide in a cluster of cells known as the border cells. Border cells originate from somatic follicle cells that delaminate with polar cells and migrate from the anterior end of the egg to meet the anterior of the yolk sack during egg chamber maturation (Figure III-3 A). In brief, egg chambers within the ovary are germline cells surrounded by somatic follicle cells which are important in making the eggshell and patterning the embryo. While the follicle cells begin as a uniform monolayer surrounding the germ cells, follicle cells at the anterior are displaced towards the posterior during stage 9 (Figure III-3 A and B), a process which completes by stage 10 (Figure III-3 A and C). At the anterior and posterior ends of the egg chamber are polar cells which, during stage 9, express *unpaired* to recruit a few nearby anterior follicle cells into a cluster of motile cells known as the border cells (BC) (Xi *et al.* 2003; Starz-Gaiano *et al.* 2009). Jak/Stat signaling within the BCs is

required for their migration towards the posterior until reaching the edge of the oocyte during stage 9 (Beccari *et al.* 2002)

Eya is expressed in follicle cells and functions downstream of Hedgehog signaling to repress polar cell fate (Bai 2002), though the specific mechanism for this is not well characterized. Bai and Montell show that Eya overexpression in polar cells using *upd-Gal4* prevents BC migration, leading us to wonder whether endogenous *eya* in the follicle cells that delaminate with polar cells can affect BC migration. To test if Eya levels in follicle cells affect BC migration, I overexpressed or knocked down *eya* using the *slbo* and *c306* drivers. *Slbo-Gal4* expresses in the BCs but not the polar cells while *c306-Gal4* turns on earlier and expresses broadly in follicle cells but then becomes restricted to BCs by stage 9 (Aranjuez *et al.* 2012). Migrating BCs were visualized by staining with FasIII antibody which localizes to the cell-cell boundary of polar cells. *slbo*-driven overexpression of *eya* in the BC's causes severe BC delay (Figure III-3 D and red line in F) and knockdown of *eya* also causes delays (green line in Figure III-3 F), but not as great as *eya* overexpression, indicating that Eya levels do affect proper BC migration. The results using *c306-Gal4* (Figure III-3 G) were much weaker, despite this driver having a broader expression profile.

Eya's previously described requirement in repression of polar cell fate (Bai 2002) and this newly demonstrated role in BC migration likely occur So-independently. Bai and Montell show with *so-LacZ* that So is expressed in the germarium and stalk cells. They report that Ey is found in the nuclei of all follicle cells and could not detect any Toy or Dac expression. To confirm their *so-LacZ* reporter observations, I stained for So using a polyclonal antibody and found high levels in the the germarium (Figure III-3 E) with no evidence of coexpression in

follicle cells with Eya and occasional low levels in stalk cells (data not shown). Thus, the egg chamber is a unique context for Eya activity since it likely functions independently from So, a topic that will be discussed further below.

I next asked whether *eya* genetically interact with genes of the Jak/Stat pathway in BC migration. *upd* signal from polar cells triggers active Jak/Stat signaling within BCs, in which activated Stat promotes migration by transcriptionally activating *slbo*, a pro-migratory gene (Silver and Montell 2001). Thus, Jak/Stat activity within anterior follicle cells is important for collective cell migration of the BCs during maturation of the egg chamber (Beccari *et al.* 2002; Ghiglione 2002; Silver *et al.* 2005; Monahan and Starz-Gaiano 2013). Consistent with these previous reports, *slbo*-mediated knockdown of *hop* and *Stat92E* resulted in BC migration defects (Figure III-3 F). To explore the possibility of genetic interactions between *eya* and Jak/Stat genes, I asked whether *hop* or *Stat92E* knockdown could modify *slbo>Eya*^{WT} BC migration defects but failed to see suppression, suggesting that unlike their relationship in axon targeting (Chapter II), *eya* does not synergize with *hop* or *Stat92E* during BC migration. Raising the possibility of genetic antagonism, *hop* knockdown enhanced the *slbo>Eya*^{WT} BC migration defect (Figure III-3 F, orange line). Because similar enhancement was not observed with *Stat*^{RNAi}, the enhancement with *hop*^{RNAi} may reflect additivity rather than a true genetic interaction.

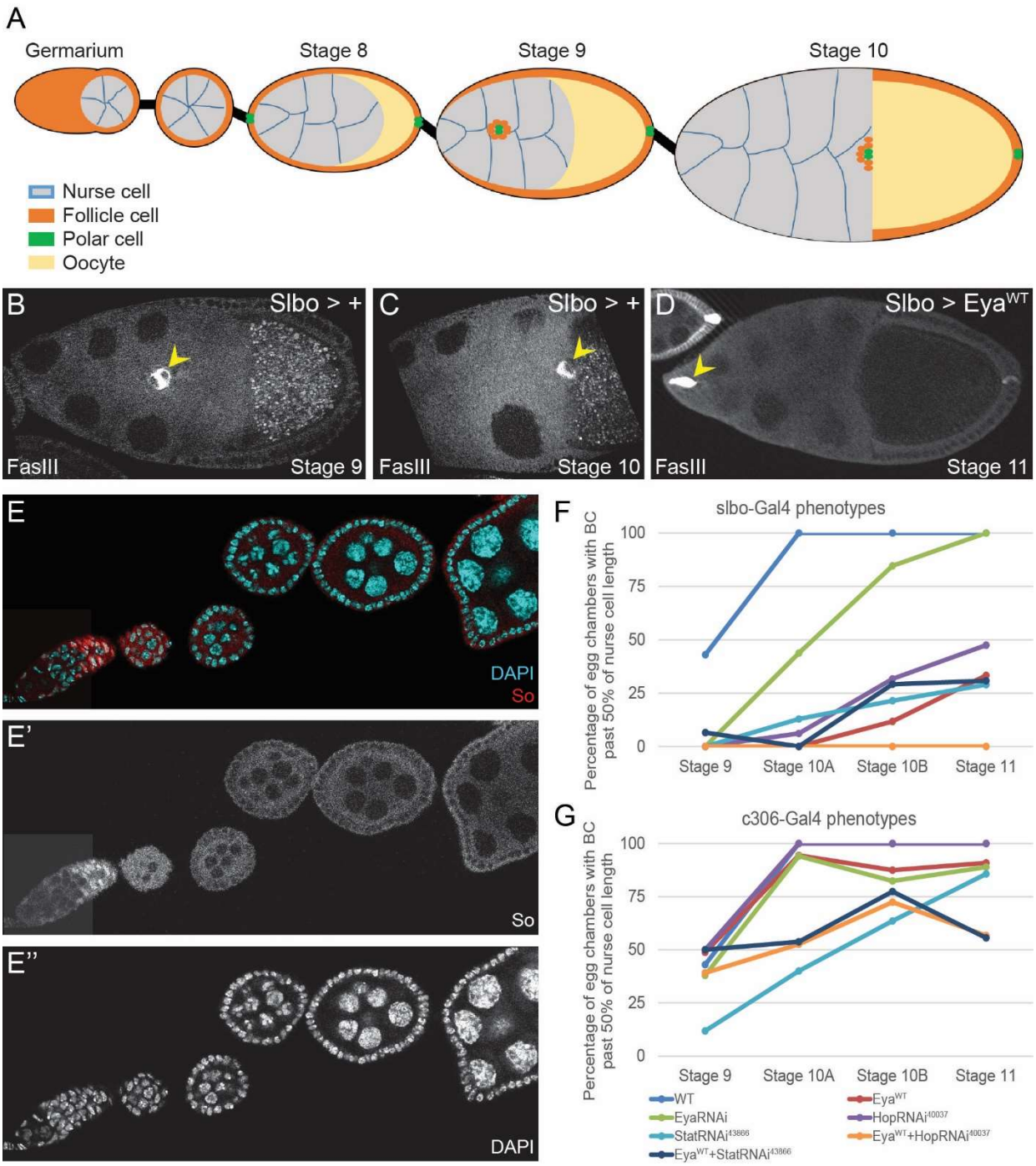


Figure III-3 | Eya plays a role in border cell migration

Figure III-3 | Eya plays a role in border cell migration (continued)
Border cell (BC) migration is a normal part of oocyte development and is affected by Eya as well as Jak/Stat signaling. (A-C) Wild-type BC migration occurs in Stage 9 egg chambers and completes by Stage 10. (A) Schematic of wild-type egg chamber development. BCs can be visualized by staining for FasIII. (B-C) Stage 9 egg chamber (B) showing FasIII-stained polar cells marked by the yellow arrowhead as migrating through the nurse cells, which completes by Stage 10 (C). (D) Follicle cells overexpressing *eya* show very delayed BC migration. Even at Stage 11, ~75% will have BC clusters in the first half of the nurse cell length. (E) So staining in egg chambers reveals that it is mostly in the germarium and does not colocalize with Eya in follicle cells. (F) Quantification of BC migration defects from misexpression of *eya* or knockdown of *hop* or *Stat92E* using *slbo-Gal4*. *slbo>Eya*^{RNAi} causes modest BC defects. *slbo*-driven overexpression of *eya* or knockdown of *hop* and *Stat92E* cause severe BC defects. Double knockdown of *eya* and *hop* causes even more severe migration defects. (G) Quantification of BC migration defects from misexpression of *eya* or knockdown of *hop* or *Stat92E* using *c306-Gal4*. These phenotypes were not as severe as those acquired with the *slbo*-driver and double UAS genotypes did not yield any evidence of interactions.

III.3.3 – Ectopic *eya* transcriptionally regulates activity of the Jak/Stat pathway in the wing

Although Eya is not endogenously expressed in the wing disc, ectopic overexpression studies in this tissue have identified relationships within the RD network and with other pathways that have proven relevant for other developmental contexts (Shen and Mardon 1997; Pignoni *et al.* 1997; Kango-Singh *et al.* 2003; Pappu *et al.* 2005; Xiong *et al.* 2009; Morillo *et al.* 2012). Thus I next asked whether the wing could be used to reveal Eya's intrinsic potential for regulating Jak/Stat signaling activity. The Jak/Stat pathway is required for hinge growth and patterning in the wing (Ayala-Camargo *et al.* 2013), and accordingly, the 10xStat-eGFP reporter is present in regions surrounding the pouch region of the 3rd instar wing disc (Figure III-4 A and (Bach *et al.* 2007)). *engrailed* (*en*)-*Gal4* expresses in the posterior of the wing disc and using to knockdown *Stat92E* produced loss of 10xStat-eGFP signal (Figure III-4 B) only in the *en-Gal4* domain, confirming its appropriateness as a driver.

To test whether Eya can affect activity of the Jak/Stat pathway, I used *en-Gal4* to overexpress *eya*. Ectopic activation of the 10xStat-eGFP reporter occurred within, but not throughout, the domain covered by the driver (Figure III-4 C). I quantified *en>Eya*^{WT} changes in 10xStat-eGFP levels by taking the ratio of fluorescence in the posterior half of the wing pouch versus the same area in a region outside of the driver's domain in the anterior wing pouch (Figure III-4 C'', yellow boxes). By these methods, I found that *en>Eya*^{WT} produced a 1.5-fold increase in 10xStat-eGFP levels (Figure III-4 F).

To separate Eya's nuclear and cytoplasmic functions, I repeated the experiment with *UAS-Flag-NLS-Eya* (Figure III-4 D) and *UAS-Flag-Myr-Eya* (Figure III-4 E) that were inserted into the same genomic region and thus have equivalent levels of expression. *en>NLS-Eya* produced a nearly 2-fold increase in the 10xStat-eGFP reporter whereas *en>Myr-Eya* 10xStat-eGFP levels were equivalent to control animals (Figure III-4 F). These results suggest that the induction of 10xStat-eGFP reporter in response to *eya* overexpression results from Eya's role as a nuclear transcription co-factor for So. Validating this conclusion further, *eya* overexpression is no longer able to induce 10xStat-eGFP expression if *so* is knocked down (Figure III-4 F). Thus, ectopic *eya* can induce Jak/Stat pathway activity in the wing by Eya-So-mediated transcriptional regulation. Because the 10xStat element does not include So binding sites, such regulation likely occurs at one or more upstream genes in the Jak/Stat pathway.

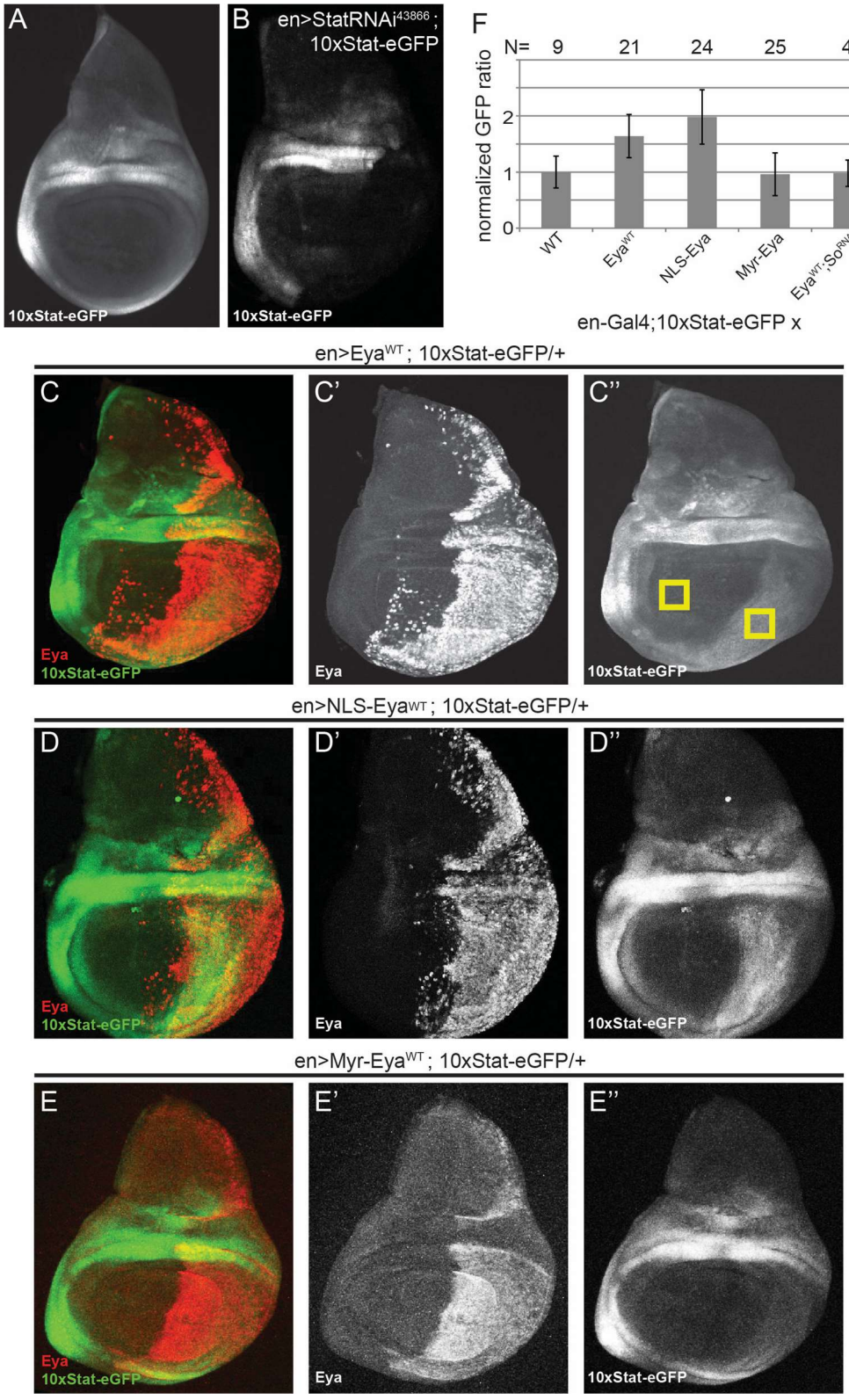


Figure III-4 | Ectopic *eya* can stimulate Jak/Stat pathway activity in the wing

Figure III-4 | Ectopic *eya* can stimulate Jak/Stat pathway activity in the wing (continued) (A-E) 10xStat-eGFP is an endogenous GFP reporter for Jak/Stat pathway activity and its responsiveness to *eya* identifies Eya as a potential positive regulator of the pathway. (A) Wild-type wing discs with 10xStat-eGFP in the wing hinge and areas surrounding the pouch. (B) The 10xStat-eGFP reporter in the engrailed domain is sensitive to pathway modulation, as *Stat92E* knockdown is sufficient for loss of the reporter. (C) *engrailed*-driven *eya* (red, C') induced ectopic activation of the 10xStat-eGFP reporter in the posterior wing pouch (green, C'', right hand yellow box). Yellow boxes indicate areas measured for quantification. (D-E) Eya-mediated induction of the 10xStat-eGFP occurs by the activity of nuclear Eya (D) and not cytoplasmic Eya (E). (F) Quantification of fluorescence ratios in the posterior wing pouch versus the anterior (yellow boxes depicted in C'') show that Eya stimulates activity of the Jak/Stat reporter transcriptionally. *NLS-Eya* but not *Myr-Eya* overexpression results in reporter activation, and this is compromised when *so* is knocked down.

III.3.4 – *hop* and *Stat92E* synergize with *eya* in ectopic Eya-mediated phenotypes in the wing

Since Eya can transcriptionally promote Jak/Stat signaling, we wondered whether Jak/Stat signaling can in turn promote Eya-So transcriptional activity. Ectopic studies of Eya and So in the wing have been useful in the past for studying factors that may affect Eya-So transcriptional regulation (Pignoni *et al.* 1997; Kango-Singh *et al.* 2003; Pappu *et al.* 2005; Xiong *et al.* 2009; Morillo *et al.* 2012). Specifically, ectopic expression of *eya* will induce expression of its partner *so* which then together induce ectopic *dac* expression. Thus, factors that synergize with or antagonize Eya-So function will respectively increase or repress ectopic *dac* induction. Given that *eya* synergizes with *hop* and *Stat92E* in photoreceptor axon targeting, I predicted *hop* and *Stat92E* might collaborate with Eya in ectopic *dac* induction. To test this, I asked whether knockdown of these genes suppressed Eya's ability to induce ectopic Dac patches. Endogenous *dac* is expressed in a defined backwards 'S'-like stripe that begins at the posterior

half at the dorsal side of the wing and ends on the anterior side of the pouch on the ventral side of the wing (Figure III-5 D). Overexpression of *eya* with the *dpp*-driver induces patches of ectopic Dac in the presumptive notum as well as the wing pouch within the dpp strip (Figure III-5 A, yellow arrowheads marks pouch). The number of ectopic patches that appears decreases when *hop* or *Stat92E* are knocked down by RNAi (Figure III-5 B and C), thus indicating that *hop* and *Stat92E* synergize with *eya* in *dac* induction.

The RD network regulates biological processes important for growth and survival, including proliferation. This effect can be modeled in the wing by ectopically co-expressing *eya* and *so* which produces a much stronger activation of the RD network than just ectopic expression of *eya* such that the induced *dac* stripe is more pronounced and cells in the wing disc overproliferate (Figure III-5 E). To extend my previous findings of *hop* and *Stat92E* synergizing with *eya* in ectopic *dac* induction, I asked whether *hop* and *Stat92E* knockdown also suppress *dpp>Eya, So* overproliferation phenotypes. I measured the total area of wing discs and found that while *hop* and *Stat92E* knockdown with *dpp-Gal4* do not affect wing size (Figure III-5 G, blue bars), they do reduce the amount of overproliferation from *dpp>Eya, So* (Figure III-5 G, orange bars). Taken all together, *hop* and *Stat92E* synergize with Eya-So to activate Eya-So target genes such as *dac* and to promote proliferation.

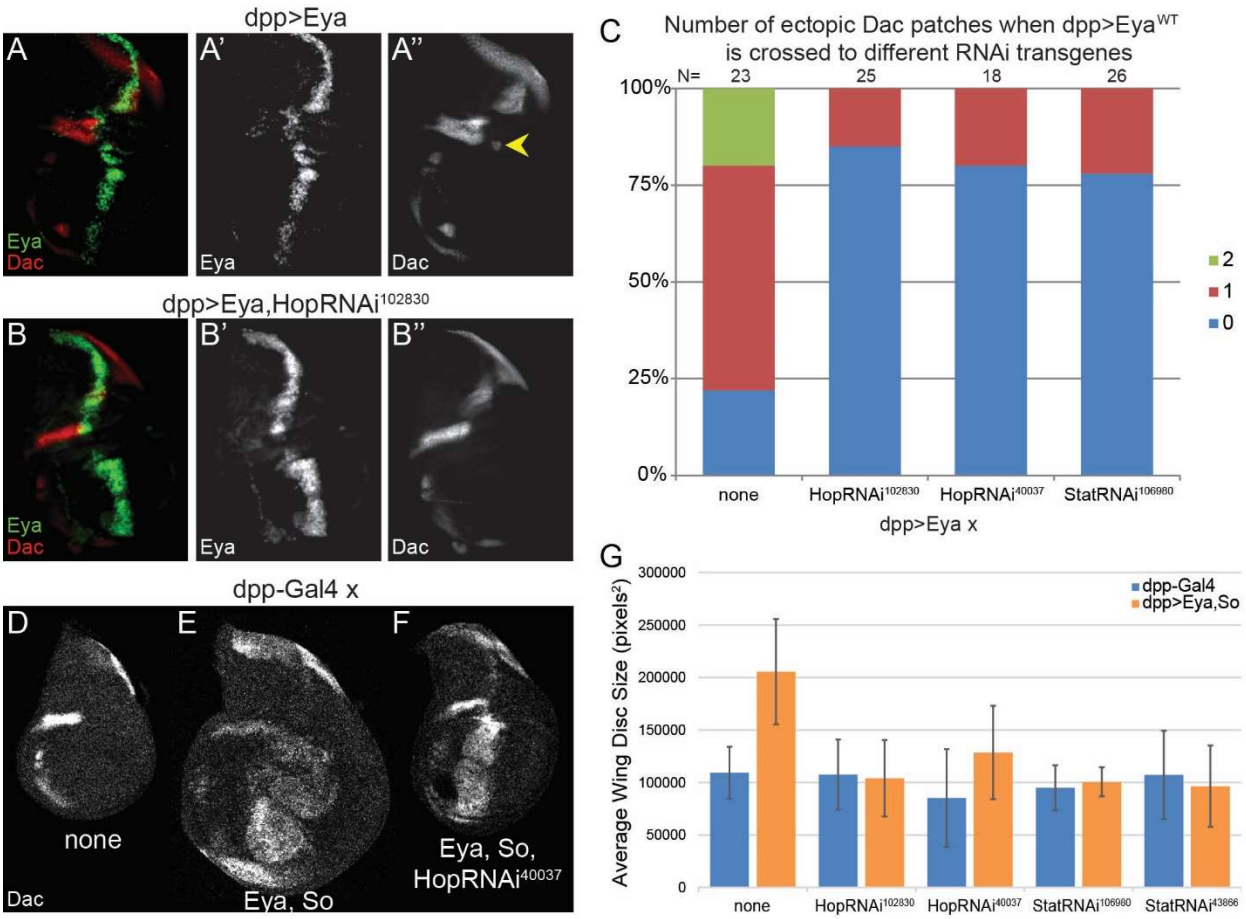


Figure III-5 | *hop* and *Stat92E* synergize with *eya* in Eya-mediated phenotypes in the wing
(A-C) *hop* and *Stat92E* synergize with *eya* to induce ectopic Dac patches. (A) *dpp>Eya* causes ectopic *dac* within the dpp strip (yellow arrow shows ectopic patch in the wing pouch). (B) Knockdown of *hop* suppresses Eya's ability to induce ectopic Dac. (C) Quantification of the number of ectopic Dac patches occurring in the wing pouch shows that *hop* and *Stat92E* knockdown suppresses Eya's ability to ectopically activate *dac*. (D-G) *hop* and *Stat92E* synergize with Eya-So in overproliferation of the wing. (D) Wild-type wing disc with Dac staining. (E) *dpp>Eya, So* causes a much stronger band than *dpp>Eya* of ectopic Dac as well as overproliferation of the entire wing disc.(F) Knockdown of *hop* suppresses Eya-So mediated overproliferation of the wing. (G) Quantification of wing size by measuring area shows that knockdown of *hop* and *Stat92E* on their own does not affect wing size (blue bars), but in combination with Eya-So overexpression will suppress overproliferation (orange bars).

III.4 – Discussion

The studies described in this chapter explore interactions between Eya and the Jak/Stat pathway in multiple developmental contexts: eye development, BC migration, and wing development. The results suggest that mechanisms and output from interactions between Eya and Jak/Stat signaling are context-dependent. Since interactions between gene networks and signal transduction pathways are a common means of integrating information during development, these findings provide an basis for understanding how regulatory diversity can be achieved by reuse of different configurations of the same signaling modules.

III.4.1 – Negative regulators potentially balance Eya's positive regulation of the Jak/Stat pathway

Our experiments in the eye and wing sought to explore whether the genetic synergy observed between *eya* and genes of the Jak/Stat pathway in axon guidance resulted from Eya modulating Jak/Stat pathway output. Although initial experiments in the eye yielded negative results, assuming the 10xStat-eGFP reporter is not already at maximum level, it is possible that the presence of negative regulators limits the effects of *eya* overexpression. There are several known negative regulators of Jak/Stat signaling such as SOCS proteins, PIAS, PTP61F and Omb (Betz *et al.* 2001; Rawlings *et al.* 2004; Müller *et al.* 2005; Baeg *et al.* 2005; Tsai *et al.* 2015). Of these, *omb* is present at low levels throughout the eye and high levels at lateral margins (Poeck *et al.* 1993; Tsai *et al.* 2015). In the wing, *omb* is targeted by dpp and expressed in a region spanning the anterior/posterior margin (Nellen *et al.* 1996). The boundary of *omb* expression seems to overlap the region of 10xStat-eGFP expression within the engrailed domain that was insensitive to *eya* overexpression (Figure III-6), thus making *omb* an attractive candidate

negative regulator that attenuates Eya's potential to positively regulate Jak/Stat signaling. To test this, future experiments include confirming that the expression of *omb* does not overlap the region of increased 10xStat-eGFP in *en>Eya^{WT};10xStat-eGFP* wing discs. If this is the case, then the next step would be genetic verification that *omb* is masking Eya's ability to provide positive input to the Jak/Stat pathway in both the wing and the eye. This could either be done by simultaneous knockdown of *omb* and overexpression of *eya* or by overexpressing *eya* in *omb* heterozygous animals. Both experiments predict increased 10xStat-eGFP levels if *omb* prevents Eya from activating Jak/Stat signaling.

My preliminary experiments with *eya* clones in the 3rd instar eye disc suggest that Eya is not necessary for Jak/Stat pathway activity. At this point in development, pathway activity occurs in the absence of *upd* (Bach *et al.* 2007; Tsai *et al.* 2007), suggesting that positive feedback loops ensure continued signaling. Although the wing experiments suggest that Eya can activate the pathway, at least in the eye it does not seem to provide this positive feedback.

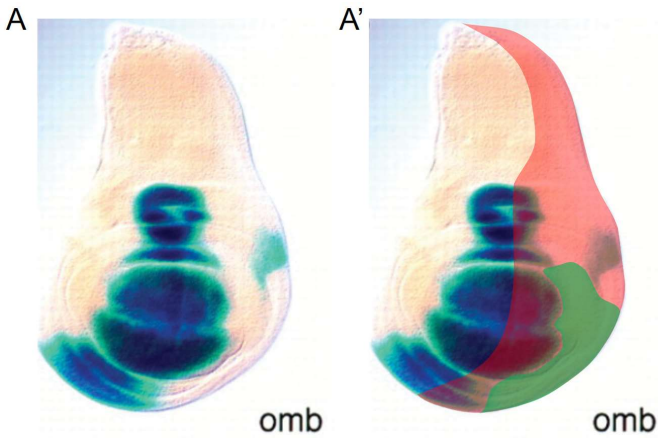


Figure III-6 | Omb expression pattern in 3rd instar wing discs
The posterior border of *omb* expression may abut the anterior boundary of the region of increased 10xStat-eGFP reporter in *en>Eya* wing discs. (A) *omb-LacZ* showing *omb* expression in 3rd instar wing disc. Taken from Figure 2 of (Nellen *et al.* 1996). (A') False colored superimposition onto (A) showing region of *en>Eya* in red and ectopic 10xStat-eGFP reporter in green based on results from Figure III-4C.

III.4.2 – Novel role for Eya in egg chambers

Eya is expressed in follicle cells, and *eya* mutants have reduced fertility (Bonini *et al.* 1998). Prior studies of Eya in the follicle cells show that it is required for repression of polar cell fate such that loss of *eya* results in ectopic polar cells and ectopic BC clusters. My work shows that in addition to repressing polar cell fate, Eya potentially plays an additional role in BC migration. In light of the known role for Jak/Stat signaling in this context, my preliminary data on genetic interactions between *eya*, *hop* and *Stat92E* in BC migration fuel two hypotheses: either Eya works in parallel to the Jak/Stat pathway, or Eya antagonizes Hop but not Stat. Further genetic interaction experiments that assess the ability of pathway components to modify *slbo>Eya*^{RNAi} BC migration defects will be needed to distinguish between these two possibilities.

Based on expression patterns, Eya's function in follicle cells occurs independently from So. Since Eya staining in the follicle cells appears largely nuclear, and since its usual partner, So, is not expressed, Eya must interact with other transcription factors if it acts as a coactivator in this context. Alternatively, perhaps the lack of So shifts the balance more towards Eya's cytoplasmic functions. To determine whether Eya's nuclear or cytoplasmic functions are relevant to BC migration, one could leverage NLS and Myr-tagged Eya which target Eya to the nucleus and cytoplasmic membrane, respectively, and assess those UAS-constructs in their ability to generate BC migration defects upon overexpression and to rescue *eya* loss-of-function phenotypes. If cytoplasmic Eya functions prove important for BC migration, the egg chamber would provide a unique and useful context for studying mechanisms for cytoplasmic Eya activity.

My discovery that Eya may be important for BC migration echoes studies with mammalian Eya that implicate it in cancer cell metastasis (Pandey *et al.* 2010). Mechanistically, metastasizing cancer cells and cells undergoing collective cell migration both undergo epidermal to mesenchymal transitions which allow the cells to become motile. Although Eya's tyrosine phosphatase activity is predicted to be relevant in metastasis, because this activity is dispensable for fly development (Jin *et al.* 2013), the specific molecular mechanisms are likely distinct. The genetic screen results reported in Chapter II identify many cytoplasmic factors with which Eya potentially interacts. Further exploration of these interactions during BC migration may provide new insight into cytoplasmic Eya-related functions in cell motility.

III.4.3 – Synergistic interactions between *eya* and Jak/Stat signaling in the wing are mutual and occur transcriptionally

My experiments in the wing suggest transcriptional synergy between Eya and the Jak/Stat pathway such that Eya can induce activity of the Jak/Stat pathway in certain regions, and conversely, that the Jak/Stat effectors, Hop and Stat, enhance output from Eya-So.

Transcriptional synergy could occur in two, non mutually exclusive ways: either independently such that Eya-So or Stat regulates transcription of Jak/Stat genes or RD genes, respectively, or Eya-So and Stat co-regulate other target genes. ChIP-Seq has been performed for both So from third instar antennal-eye imaginal discs (Jusiak *et al.* 2014) and Stat from embryos (Celniker *et al.* 2009). Putting aside the caveat of tissue-specific differences in transcription factor occupancy patterns, analysis of the two data sets identifies both uniquely occurring peaks as well as regions with overlapping peaks (Figure III-7 A). So peaks were identified at multiple Jak/Stat genes including *hop*, *Stat92E*, *Socs36E* and *Socs44A* (Figure III-7 B, only *Socs36E*

shown other peaks shown in Figure IV-3). Within the core RD network, Stat has peaks at *so* and *dac*, but also peaks at several genes related to the RD network, such as *eyegone*, *Optix* and *teashirt* (Figure III-7C, only shows *dac*, other peaks shown in Figure IV-4). While it is possible that Stat ChIP-Seq in the eye might reveal different binding sites, at face value these data suggest it is reasonable to consider regulation of the RD network by Jak/Stat signaling and Eya-So regulation of Jak/Stat gene transcription. These ideas will be discussed further in Chapter IV. There are also genes that may be co-regulated by Stat and So. Genes with overlapping So and Stat peaks include *Socs36E* (Figure III-7 B) and *dac* (Figure III-7 C). To test for Eya-So-Stat coregulation, one could first perform RT-PCR in both gain- and loss-of-function *eya/so*, *Stat92E* or double mutant backgrounds, and second, for those genes that respond appropriately, generate enhancer-reporter transgenes and examine their direct So- and/or Stat-responsiveness.

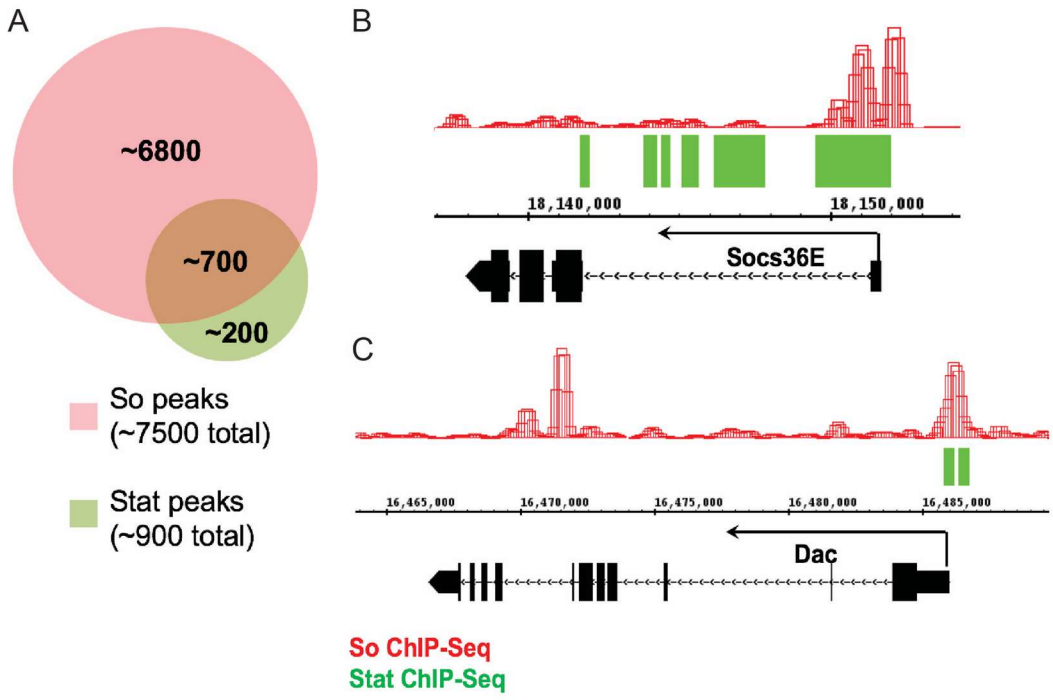


Figure III-7 | So and Stat ChIP-Seq identify genes that are potentially co-regulated

Figure III-7 | So and Stat ChIP-Seq identify genes that are potentially co-regulated (continued)
Analysis of overlapping peaks between So ChIP-Seq from third instar antennal-eye imaginal discs (Jusiak *et al.* 2014) and Stat ChIP-Seq from *white* embryos (Celniker *et al.* 2009). (A) Analysis of these data sets identifies roughly 9,500 So-bound peaks and 900 Stat-bound peaks. Of those peaks, about 700 overlap. (B-C) So read tags and Stat regions with peaks below a 10% false discovery rate show potential co-occupancy at multiple genes. The gene structure is shown below the coordinates, with the arrow starting from the 5' end of the gene and extending over the length of the gene. (B) *Socs36E*, a Jak/Stat target gene that is also important in negative feedback on the pathway, contains So and Stat peaks near its transcription start site. (C) *dac* is an RD gene that contains So and Stat peaks just upstream of the transcription start site.

III.5 – Concluding remarks

The data presented in this chapter showcase Eya's ability to interface with the Jak/Stat pathway in multiple ways. In some contexts, Eya-Jak/Stat synergy may occur at the transcriptional level (Figure III-8), as supported by the wing studies. Candidate genes identified by Stat and So ChIP-Seq provide avenues for exploring which gene targets may be co-regulated by So and Stat, and which RD or Jak/Stat genes may provide points of crosstalk between the two signaling networks. Eya-Jak/Stat interactions may also occur cytoplasmically with Eya-Hop and Eya-SOCS complexes influencing Jak/Stat pathway activity, Eya activity, or both (Figure III-8). Future studies aimed at unraveling the molecular mechanisms by which Eya's transcriptional and cytoplasmic functions contribute to its genetic synergy or antagonism with the Jak/Stat pathway throughout development should reveal novel modes of signal integration.

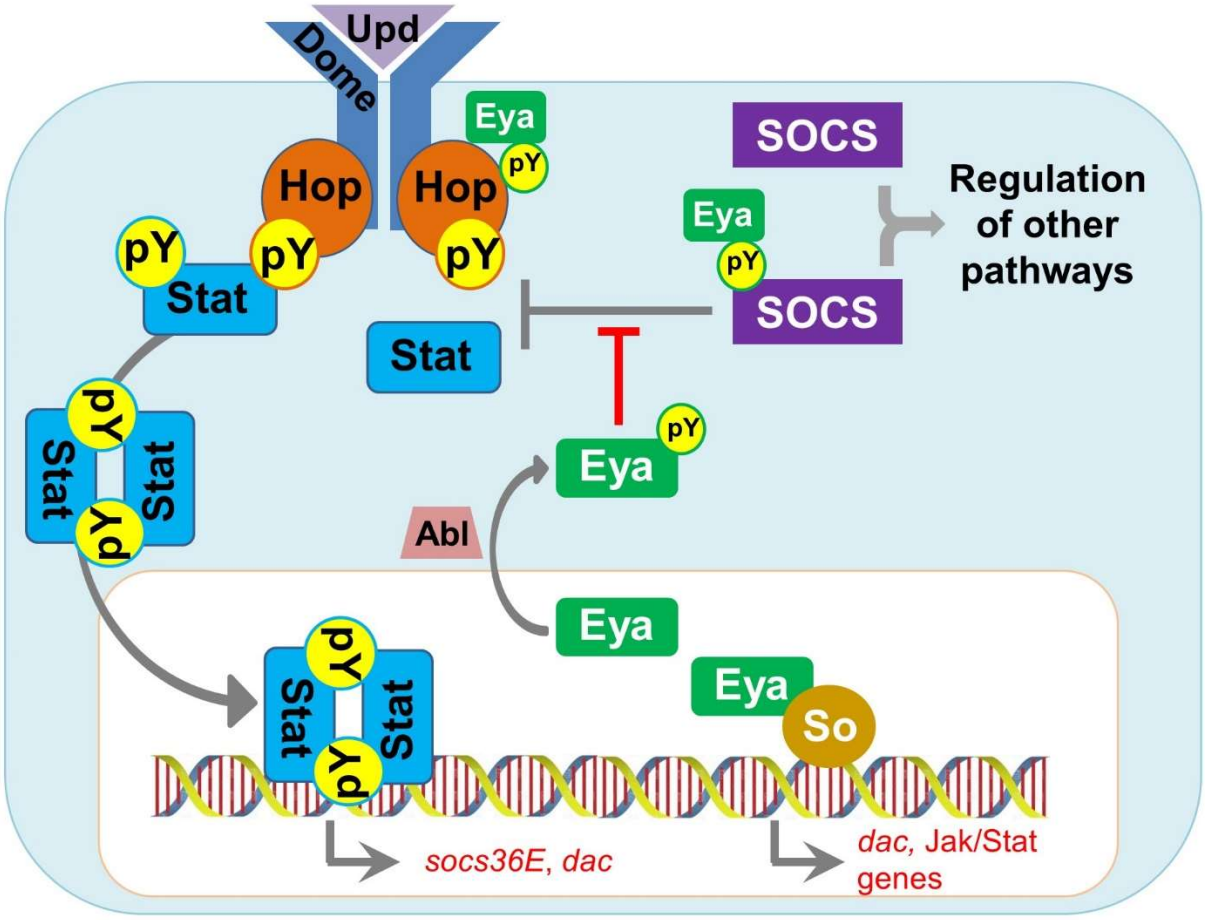


Figure III-8 | Potential interactions between Eya and Jak/Stat signaling components
Schematic of a cell demonstrating potential modes of interaction between Eya and the Jak/Stat signaling pathway. Eya could interface with Jak/Stat signaling in the cytoplasm via physical interactions with Hop and SOCS. Eya could also interface with Jak/Stat signaling by directly regulating transcription of Jak/Stat genes. Jak/Stat signaling could affect Eya/So transcriptional output at target genes such as *dac*. Finally, Eya/So/Stat could co-regulate target genes such as *Socs36E* or *dac*.

III.6 – Materials and methods

III.6.1 – Fly strains

The following strains were used: (1) *10xStat-eGFP*, (2) *act>y>Gal4/CyO; hs-FLP,sb/TM6*, (3) *UAS-Eya;10xStat-eGFP*, (4) *eya^{clift}, FRT40A; ey-FLP*, (5) *FRT40A;10xStat-*

eGFP, (6) *slbo-Gal4*, (7) *c306-Gal4*, (8) Hop^{40037}, (9) Hop^{102830}, (10) $Stat^{43866}$, (11) $Stat^{106980}$, (12) *en-Gal4;10xStat-eGFP*, (13) *en>Eya/Cyo-actGFP; 10xStat-eGFP/TM6b, tub-Gal80*, (14) So^{RNAi} (15) *UAS-Flag-NLS-Eya* (86F), (16) *UAS-Flag-Myr-Eya* (86F), (17) *40C6>EyaIIIa* (18) *57A1>Eya, So*. Superscripted numbers indicate VDRC line.

eya Flp-out clones were generated by allowing flies to lay for 24 hours, then heat-shocking vials 36 hours later for 1 hour at 37°C, then 1 hour off at 25°C, and another hour at 37°C.

III.6.2 – Immunostaining and imaging

Imaginal discs were dissected in S2 cell medium, fixed in 4% paraformaldehyde with .01% Triton, washed 3 times with PBST (PBS+.01% Triton), blocked with PNT (PBS+.01% Triton+5% NGS), incubated in primary antibody overnight at 4°C, washed 3 times with PBST, incubated in secondary antibody for 2 hours at room temperature, and mounted onto slides with N-propyl gallate. Ovaries were dissected in PBS, fixed in 1% paraformaldehyde with 0.025% NP-40 and 75% heptane, dissociated with a cut pipet tip, then stained the same as other tissue. During mounting, stage 14 egg chambers were removed to optimize subsequent microscopy. Primary antibodies used were: gp anti-Eya (1:10,000), anti-FasIII (1:100, DSHB) and anti-Dac (1:10, DSHB). Secondary antibodies from Jackson ImmunoResearch were used 1:2,000. GFP signal in images are all endogenous fluorescence. Images were taken on a Zeiss 510 confocal microscope and processed with ImageJ. Border cell migration phenotypes were quantified by images taken on a Zeiss Axioplan 2 microscope, categorized into stage then binned by BC migration phenotype (<50% or ≥50% BC relative to nurse cell length). Wing immunofluorescence and size quantifications were performed in ImageJ.

III.6.3 – ChIP-Seq data processing

So ChIP-Seq data sets were acquired from the Mardon lab (Jusiak *et al.* 2014) and Stat ChIP-Seq data was downloaded from modENCODE. Peaks were visualized using Integrated Genome Browser. Overlapping peaks were called using intersectBed from the BEDTools Suite (Quinlan and Hall 2010).

III.7 – Acknowledgements

This work would not have been possible without ideas and discussion from Chip Ferguson, slbo-Gal4 and c306-Gal4 flies from Sally Horne-Badovinac, lessons from Jean-Francois in the Rebay lab on ovary dissections, or help from Jemma Webber in the Rebay lab to analyze ChIP-Seq data sets.

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CHAPTER IV

Conclusions, discussion and future directions

Development requires precise interactions between signaling pathways and gene regulatory networks. In this dissertation, I report novel interactions between Eyes absent (Eya), a core member of the retinal determination gene network, and the Jak/Stat signaling pathway. My work reveals genetic synergy during photoreceptor axon targeting but suggests antagonism in border cell migration. Mechanistically, I find in cultured cells that cytoplasmic protein complexes could couple Eya to Jak/Stat signaling. I also identify potential transcriptional synergy from experiments in wing discs between Eya-So and Stat. Taken together, my work lays the foundation for future exploration of the mechanisms by which Eya participates in phosphotyrosine-based signaling networks such as the Jak/Stat pathway. I propose that these interactions may be regulated at multiple levels within the cell, both through post-translational interactions and transcriptional cooperation, and that the specifics of the interaction will be context dependent. In this chapter, I present my ideas for future investigations of these models. I first address the possible function of cytoplasmic Eya complexes with Hop and SOCS, then discuss transcriptional synergy between Eya-So and Stat, and finally expand these models to broader interactions between Eya with other hits from the screen.

IV.1 – Cytoplasmic functions for Eya

There were three main observations from the S2 cell experiments in Chapter II: 1) Eya can CoIP Hop in an Abl-independent manner, 2) Eya can CoIP SOCS in an Abl-dependent

manner, and 3) co-transfection of Eya, Abl and SOCS causes Eya to localize into puncta within the cytoplasm. Since biochemical data were from cultured cells while genetic interactions were performed in eye tissue and egg chambers, the first step is to confirm that the protein complexes described in S2 cells also occur in the tissues where genetic interactions were observed. Assuming yes, then the priority would be in elaborating on the function and mechanism of these complexes by asking whether they are physically and functionally interconnected or if they represent independent mechanisms of biological regulation. Below, I first discuss approaches for studying these complexes *in vivo*, then I consider models for their roles during development.

IV.1.1 – Verifying Eya-Hop and Eya-SOCS interactions *in vivo*

Available reagents for detection of endogenous Drosophila Jak/Stat components include an antibody against Socs36E generated by the Serras group in 2009 (Almudi *et al.* 2009), a Stat92E-GFP line from the modERN project (Celniker *et al.* 2009) and a phospho-Stat antibody generated by the Lin group in 2013 (Zhang *et al.* 2013). To visualize endogenous Hop or Socs44A, one would have to either generate antibodies or protein-trap lines. With these reagents, the first experiment would be assaying whether endogenous Eya associates with components of the Jak/Stat signaling pathway in photoreceptors and egg chambers. Eya-SOCS CoIP in S2 cells required Abl, and despite there being endogenous Abl in photoreceptors and egg chambers (Baum and Perrimon 2001; Xiong and Rebay 2011), Eya is still predominantly nuclear in both these tissues. Thus, additional Abl, removal of endogenous nuclear binding partners such as So, expression of *Myr-Eya*, or all of the above, could be required to fully enrich for cytoplasmic pY-Eya in these experiments. Indeed, previous attempts to relocalize endogenous Eya with ectopic

abl in photoreceptors failed, presumably due to strong Eya-So interactions that retain Eya in the nucleus (W. Xiong and I. Rebay, unpublished).

An alternative to the above experiments, or perhaps an intermediate first experiment, would be generation of tagged, UAS expression constructs which could be used to ask whether overexpressed proteins interact with Eya *in vivo*. One could use *ey-Gal4* to probe interactions early in eye development or *GMR-Gal4* to probe interactions later in eye development. To determine whether Jak/Stat components physically interact with Eya in follicle cells and border cells, one could use *slbo-Gal4*. In addition to experiments in the eye and egg chamber, another potentially useful context is the wing where one can readily observe cytoplasmic relocalization of ectopic Eya by *ptc-Gal4* mediated overexpression of full-length but not kinase-dead Abl (Xiong *et al.* 2009). There are three possible outcomes from these sets of experiments probing into Eya-Jak/Stat complexes *in vivo*: Eya-Hop and Eya-SOCS both occur, discovery that Eya-Stat interactions occur, or no interactions occur between Eya and Jak/Stat components. Below, I will discuss the implications of each possibility. For interactions that are found *in vivo*, further *in vitro* work with recombinant proteins could be performed to assess whether the interactions are direct, and if so, to map the required domains. pY-Eya would be supplied in these experiments by *in vitro* kinase assays previously established in the laboratory (Xiong *et al.* 2009).

IV.1.2 – Eya-Hop and Eya-SOCS complexes in eye development

If Eya-Hop and Eya-SOCS complexes are discovered in photoreceptors, given that Eya synergizes with both *hop* and *Socs*, there are likely two independent mechanisms for how these complexes contribute to axon guidance: Eya-Hop regulates activity of the Jak/Stat pathway,

while Eya-SOCS influences signaling-independent functions such as RTK signaling pathways. Since Eya is involved in both complexes, one idea is that Eya's role may be to balance the two.

IV.1.2.1 – Eya-Hop provides positive input to the Jak/Stat pathway during photoreceptor axon targeting

eya's genetic synergy with effectors of Jak/Stat signaling, *hop* and *Stat92E*, in axon targeting raises the question of whether these interactions reflect Eya's influence on Jak/Stat pathway output. Experiments probing into this question with the 10xStat-eGFP reporter were negative since no changes were observed in either the loss or gain of *eya* clones within the eye (Figure III-2). There is one additional test that could be performed to address this same question. One could ask whether activated, phospho-Stat (p-Stat) changes depending on the levels of Eya. If *eya*'s synergy with *hop* and *Stat92E* reflects Eya acting as a positive regulator, then *eya* null clones show should decrease p-Stat staining while overexpression in clones or by Gal4 should show increased p-Stat levels. This analysis could also be performed biochemically, by comparing p-Stat versus total Stat levels in eye discs in which *eya* had been globally knocked down or overexpressed.

Another question raised by these findings is how *eya* and Jak/Stat signaling synergize in the cytoplasm to regulate axon guidance. As briefly discussed in the introduction, the original thought was that Eya's PTP activity acts in parallel with other known PTPs that activate Cdc42/Rac in growth cones. Although this specific hypothesis centering on Eya's PTP is moot, *eya*'s genetic synergy with Jak/Stat genes raises novel avenues for potential integration of both Eya and Jak/Stat signaling components with the actin cytoskeleton. Endosomal Jak/Stat signaling occurs in Drosophila egg chambers (Devergne *et al.* 2007) and mammalian studies show that IL-

4 endocytosis in HEK293 occur via clathrin-independent mechanisms employing actin regulators Rac1 and Pak (Kurgonaite *et al.* 2015). Rac1 is a member of the Rho family of GTPases that is broadly responsible for driving actin polymerization at the leading edge of migrating cells. Rac1 signals via its effector, p21-activated kinase (Pak) which is a protein kinase that inactivates cofilin, an actin-binding protein that disassembles actin filaments. Thus, one potential model linking Eya, Jak/Stat components and the actin network is that Eya-Hop and/or Eya-SOCS complexes also include Rac1 and/or Pak. To explore this possibility during axonogenesis one could stain for these proteins and assay for co-localization.

IV.1.2.2 – Eya-SOCS antagonizes RTK signaling

One paradox in my data is that Eya genetically synergizes with both antagonists of the pathway, *Socs36E* and *Socs44A*, and effectors, *hop* and *Stat92E*, in axon targeting. Since I confirmed that the *Socs* genes do antagonize *hop* in this context (Figure II-3F) I propose that Eya-SOCS may synergize in Jak/Stat-independent processes. A candidate independent function is SOCS regulation of RTKs. Little is known about the mechanism for Drosophila SOCS proteins to attenuate Jak/Stat and RTK signaling, but in mammalian studies, SOCS proteins bind Jak or RTK receptors, which causes ubiquitination and targeting of the complexes for proteasomal degradation (reviewed in (Trengove and Ward 2013)). Alternatively, some SOCS proteins engage in binding site competition to down-regulate signal transduction (reviewed in (Trengove and Ward 2013)).

Drosophila SOCS proteins show a high degree of sequence conservation with mammalian SOCS proteins (Rawlings *et al.* 2004) and they genetically antagonize Jak/Stat signaling (Callus and Mathey-Prevot 2002; Rawlings *et al.* 2004). Thus the null hypothesis is

that they function similarly to attenuate cytokine and growth factor signaling during development. Indeed, both *Socs* genes antagonize Jak/Stat signaling in the wing as *hop* heterozygosity enhances wing vein phenotypes induced by overexpression of either *Socs36E* or *Socs44A* while overexpression of *hop* suppresses (Callus and Mathey-Prevot 2002; Rawlings *et al.* 2004). Similarly, *Egfr* heterozygosity enhances *Socs36E* overexpression phenotypes while loss of an EGFR negative regulator, *cbl*, suppresses (Callus and Mathey-Prevot 2002). In contrast to this, *Egfr* heterozygosity and overexpression of an EGFR negative regulator, *argos*, suppress *Socs44A* overexpression wing vein phenotypes (Rawlings *et al.* 2004). Thus, in the wing, while both SOCS factors antagonize Jak/Stat activity, Socs36E antagonizes EGFR while Socs44A enhances it. Socs44A synergy with EGFR in the wing is the only example of any SOCS having positive genetic interactions with EGFR signaling. In eye development, Socs36E competes with Drk to limit RTK activity of Sev in R1/R6 and R3/R4 photoreceptor cell differentiation (Almudi *et al.* 2010). Socs44A has not been tested in this context, and therefore it is not known if the two SOCS proteins also show inconsistent behaviors towards RTK signaling in the eye. However, since both *Socs* synergize with *eya* in axon guidance, and since Socs44A-EGFR interactions in the wing appear to be unique, the simplest assumption is that both SOCS proteins antagonize RTK in the eye.

Given that *eya* synergizes with both *Socs36E* and *Socs44A* and assuming that both SOCS antagonize RTK signaling, the prediction is that Eya-SOCS interactions promote the antagonism. Socs36E is already known to antagonize Sev activity during photoreceptor differentiation (Almudi *et al.* 2009), prompting two questions: first, does Socs44A similarly antagonizes Sev?, and second, does Eya synergize with SOCS in this process? To address the second question, one

could ask if *eya* knockdown or heterozygosity dampens suppression of *sev* gain-of-function normally achieved by *Socs36E* overexpression (Almudi *et al.* 2009). If the answer is yes, there are two, non-mutually exclusive mechanisms by which *eya* could synergize with *Socs36E.* One is via cytoplasmic Eya-Socs36E complexes that antagonize Drk, and second is by Eya-So transcriptional regulation of *Socs36E*, which will be discussed later in this chapter. If *eya* does not synergize with *Socs36E* in antagonism of Sev, then perhaps Eya-SOCS regulate another RTK pathway involved in eye development: EGFR signaling.

EGFR is also required for differentiation of photoreceptors (Freeman 1996) and for the development of lamina cartridges which photoreceptors innervate (Lee and Sun 2015). As photoreceptor growth cones enter the developing lamina, they produce Hedgehog, which induces expression of EGFR (Huang and Kunes 1996; Huang *et al.* 1998). Huang *et al*. show that cell-autonomous expression of the EGFR ligand, *spitz* (*spi*), in R2 and R5 photoreceptors is required for lamina differentiation in which expression of an active Spi fragment induces ectopic laminal cells whereas null clones lack lamina cartridges. However, it is not known if EGFR signaling is autonomously required in photoreceptors for axon targeting. To test this idea, one could utilize chaoptin-Gal4 which expresses after photoreceptors have differentiated to ask if knockdown or overexpression of EGFR causes overshooting.

If indeed precise EGFR levels are required for photoreceptor axon targeting, then perhaps Eya-SOCS regulate that balance by targeting EGFR for degradation. To explore this possibility, one could test if *Egfr* heterozygosity enhances *eya* and *Socs* overexpression while suppressing knockdown axon guidance phenotypes. Additionally, if perturbations to EGFR do cause axon guidance defects, one could also ask the reciprocal question of whether *eya* or *Socs* antagonize

Egfr phenotypes. One parsimonious explanation for the function of Eya-Hop and Eya-SOCS complexes during photoreceptor axon targeting is that Eya's interaction with both the Jak/Stat pathway and Jak/Stat-independent functions, such as antagonism of EGFR signaling, are required for axon targeting (Figure IV-1). In support of this idea, a careful balance of Eya is required for proper axon targeting as both overexpression and knockdown of both *eya* and *Socs* produces axon guidance phenotypes (Figure II-1, II-4 and II-S1).

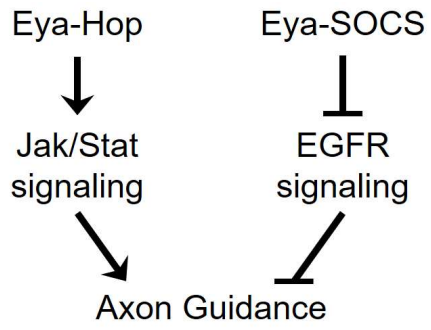

Figure IV-1 | Model for Eya balancing Jak/Stat and EGFR signaling in photoreceptor axon targeting
Model for how precise Eya levels are important for regulating photoreceptor axon targeting. Since Jak/Stat signaling is required for axon targeting, and from my hypothesis that a balance in EGFR signaling may be important for axon targeting, then potential Eya-Hop and Eya-SOCS complexes may serve to regulate these processes.

IV.1.3 – Eya-Hop and Eya-SOCS complexes in border cell migration

If Eya-Hop and Eya-SOCS complexes are found in border cells, given that our preliminary data suggests antagonism between *eya* and *hop*, these complexes likely promote SOCS-mediated repression of signaling. Jak/Stat signaling is required for specification and delamination of border cells (BCs) (Beccari *et al.* 2002; Silver *et al.* 2005) where the balance of

Stat activity is required for determining the optimal number of invasive BCs to migrate with the polar cells. Thus, increased Stat activity will lead to an excess of delaminating cells, which impede BC migration, while a loss of Stat will prevent BC specification and migration (Silver and Montell 2001; Starz-Gaiano *et al.* 2008; Monahan and Starz-Gaiano 2013). Eya's role in BC migration may be to cooperate with SOCS in attenuating Jak/Stat signal transduction, where overexpression and knockdown both perturb the precise levels of Stat required and thus produce the observed BC defects. Given that SOCS proteins can attenuate signaling either by competitive binding or by promoting proteasomal degradation, the hypothesis would be that Eya promotes these mechanisms by a large Eya-Hop-SOCS triple complex. I observed that Eya can CoIP Hop and SOCS, and that Hop and SOCS can both CoIP each other (Figure II-S2). Since these observations were from S2 cells which contain endogenous Jak/Stat signaling, it is not clear whether Eya-Hop-SOCS occurs through mutual intermolecular interactions or if there is one protein bridging an interaction between the other two (Figure IV-2). In order to differentiate between these possibilities, one would have to perform pull-down experiments using purified proteins to determine whether Eya can interact with both Hop and SOCS or if Eya can only interact with Hop or SOCS.

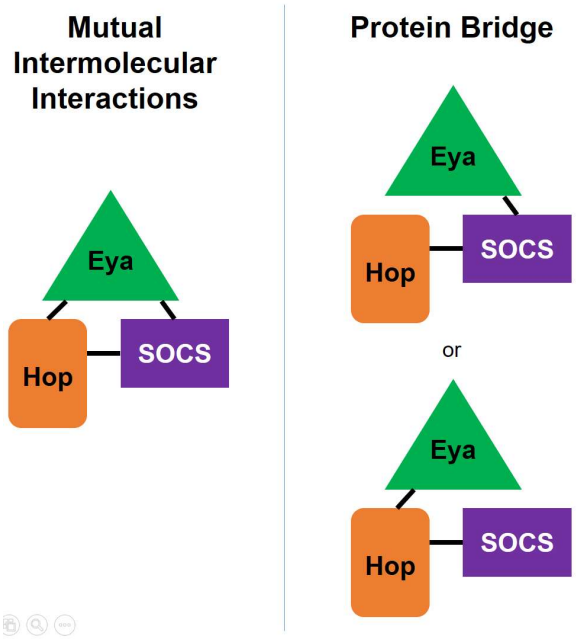

Figure IV-2 | Models for Eya-Hop and Eya-SOCS complexes
Eya-Hop-SOCS complex can form where all parties show interactions with other components (mutual intermolecular interactions, left) or a complex can form where Eya only associates with one of the Jak/Stat components (protein bridge, right). Based on mammalian data, it is assumed that Hop-SOCS can form direct protein-protein interactions.

In addition to deciphering the architecture of an Eya-Hop-SOCS complex, one could also test this model by asking whether Eya is a negative regulator of the pathway in the egg chamber. This can be determined by asking if Eya overexpression results in decreased 10xStat-eGFP reporter or p-Stat staining in BCs. Conversely, one could also test whether loss of Eya by RNAi or mutant clones results in increased 10xStat-eGFP or p-Stat staining in BCs. One inconsistency is that *Stat92E* knockdown by RNAi did not enhance *slbo>Eya*^{WT} (Figure III-3F), which one would predict if Eya does indeed negatively regulate the pathway. However, the more compelling genetic interaction was not performed, which is suppression of *slbo>Eya*^{RNAi} by loss of *Stat92E*.

If *eya* does not contribute to Jak/Stat signaling in BC migration, then Eya-SOCS interactions with EGFR may again be at play. EGFR expression at the anterior of BCs is required for directional migration of this cluster towards *spitz* and *keren* ligands expressed by the oocyte (McDonald *et al.* 2006). Similar to *Stat92E*, levels are important, where live-imaging shows that loss of EGFR by ectopic expression of a dominant negative will result in clusters that extend protrusions in multiple directions and thus fail to migrate, while overexpression will result in BCs that fail to extend protrusions at all and also fail to migrate (Prasad and Montell 2007). One possibility is therefore that Eya-SOCS interactions are required for balancing EGFR levels. This could be determined by live-imaging egg chambers and asking whether Eya overexpression causes increased protrusions, similar to EGFR loss-of-function mutants, and if Eya knockdown results in no protrusions, similar to overexpression of EGFR.

IV.1.4 – Functions for Eya-SOCS puncta

The preceding sections discuss potential functions for cytoplasmic Eya-Hop and Eya-SOCS complexes in photoreceptor axon targeting and border cell migration, but one additional observation from S2 cell experiments that may be relevant *in vivo* is formation of cytoplasmic Eya puncta upon co-transfection of SOCS and Abl. The model is that Abl phosphorylation of Eya causes cytoplasmic relocalization, but it is not known if extranuclear pY-Eya is degraded or if it has functions in the cytoplasm. Supporting the idea that these Eya puncta are functional is the observation that their frequency dramatically increases upon co-transfection of both Abl and full-length Socs44A, but not Socs44A$^{\Delta SH2}$. Further, Myr-Eya only efficiently forms puncta with Socs44A in the presence of Abl, indicating a requirement for tyrosine phosphorylation to redistribute cytoplasmically localized Eya. Thus, it appears these structures are not mere side

effects from overexpressed protein or accumulation of Eya in the cytoplasm, but rather, are the result of specific pY-SH2 initiated events. Arguing against the possibility that cytoplasmic Eya is functional are the caveats that the puncta were observed in overexpression assays in cultured cells and that cytoplasmic Eya is not observed endogenously in fly tissues. In order to gain a better understanding of the potential role of cytoplasmic Eya *in vivo*, this section will speculate on potential functions for Eya puncta and discuss experiments that explore these ideas.

The most common physiological functions for puncta include protein aggregates destined for degradation, vesicle trafficking and focal adhesions during cell migration. The latter would not be expected to occur in S2 cells, which are suspension cells, and thus will not be discussed, but should be kept in mind in the event that Eya puncta are observed *in vivo.* The first model is supported by the fact that SOCS factors are ubiquitin ligases and that cytoplasmic Eya is rarely appreciated *in vivo.* In order to test whether Eya-SOCS complexes are degraded, one could ask whether CoIP levels and frequency of Eya puncta increases upon addition of proteasome inhibitors such as MG132. Additionally, one could live-image cells and ask whether Eya puncta are short-lived, which one would expect if they are aggregates being turned over.

Tentative support for the second model that Eya puncta may be involved in vesicular trafficking comes from studies demonstrating that clathrin-mediated endocytosis of Dome is important for Jak/Stat signaling in both cultured S2 cells and follicle cells *in vivo* (Devergne *et al.* 2007). This hypothesis is highly speculative and requires affirmation that Eya-SOCS complexes include Dome (and Hop), but could highlight a novel mechanism for SOCS regulation of Jak/Stat signaling and provide an explanation to the presently contentious synergistic genetic interactions between *eya* with *hop*, *Stat92E*, *Socs36E* and *Socs44A*. If indeed

Eya puncta are Dome-containing vesicles, then one can stain for endosomal markers and look for co-localization with Eya puncta. Devergne *et al.* show that *clathrin heavy chain* (*Chc*), *rab5* and *Hrs* mutant clones all prevent Jak/Stat signaling as determined by Stat antibody staining showing no nuclear localization; in contrast, *rab11* clones did not alter Stat localization. Rab5 and Hrs are markers of early endosomes while Rab11 is a GTPase that regulates recycling of clathrin-coated vesicles (Ullrich *et al.* 1996). Thus, one can check whether Chc, Rab5 and Hrs co-localize with Eya puncta, but not Rab11. In addition to these markers, one can also check Rab7 which marks late endosomes (Vanlandingham and Ceresa 2009). If Eya puncta represent signaling complexes whose formation are facilitated by interactions with SOCS, then live imaging of cells would be expected to show that Eya puncta are stable structures within the cell. An extension of this model is the hypothesis that pY-Eya is stabilized in the cytoplasm by interacting with Socs44A. This can be tested by knocking down endogenous *Socs44A* in S2 cells in an *abl* overexpression background and asking if this results in less cytoplasmic pY-Eya. Successful experiments can then be extended *in vivo* by performing similar experiments in the wing where Abl-mediated relocalization of Eya is possible.

IV.2 - Transcriptional synergy between Eya-So and Stat

An additional possibility for Eya-Jak/Stat complexes *in vivo* relates to transcriptional cooperativity. This idea is supported by my finding of mutual transcriptional synergy between Eya and Jak/Stat members in the wing where ectopic Eya induces Jak/Stat pathway activity in a So-dependent manner and *hop* and *Stat92E* synergize with *eya* in ectopic Dac induction (Figure III-5A-C). Transcriptional synergy could occur by two possible models: 1) combinatorial

regulation of common target genes by Eya-So and Stat and 2) direct transcriptional feedback between Eya-So and Stat. Below, I will discuss both models and how they may provide additional insight into the interconnectedness between Eya, the RD network, the Jak/Stat signaling pathway, and other networks.

IV.2.1 – Cooperative So-Stat synergy in cell cycle regulation, specification and differentiation

If Eya-So and Stat co-regulate target gene transcription, then So and Stat binding sites should be present at the same regulatory elements of the same genes during development. To test this idea for proliferation and eye specification and differentiation, I have compiled genes important for cell cycle regulation (reviewed in (Noatynska *et al.* 2013)), common signaling pathways, and photoreceptor specification and differentiation (reviewed in (Quan *et al.* 2012)) and analyzed So and Stat ChIP-Seq profiles within 10kb of these genes (Table IV-I and (Celniker *et al.* 2009; Jusiak *et al.* 2014)). Consistent with the idea that So and Stat may co-regulate common target genes, I observe 23/57 (40%) genes showing both So and Stat peaks. Impressively, 22 of these 23 genes have overlapping So- and Stat-bound regions, suggestive of possible So-Stat co-binding to elements. Grouping these genes by process, there were 5/15 (33%) cell cycle regulators, 6/17 (35%) genes in specification and differentiation and 11/25 (44%) genes related to signaling pathways with overlapping So- and Stat-bound elements. Most striking is the EGFR signature, in which the receptor (*Egfr*), ligand (*spitz*), effectors (*pnt* and *yan*) and negative regulator *(argos*) all contain So and Stat co-bound regions. One hypothesis is that So-Stat cooperate to activate EGFR signaling during photoreceptor differentiation. After verifying binding by ChIP-qPCR, a starting point to test this model would be verifying that these

genes are actually regulated by Eya-So and Stat by performing RT-PCR in gain- and loss-of-function *eya*, *so* and *Stat92E* mutant backgrounds. Next would be asking whether Eya-So and Stat binding to these regions results in transcriptional activation and if Eya-So-Stat cooperate in this process. This could be tested by using the bound-regions to create enhancer-reporter constructs and then assessing cooperative regulation by Eya-So and Stat. Mutation of predicted So or Stat binding sites could be included to confirm the requirement for direct binding.

Cell Cycle Regulators		
Gene	**So peaks <10 kb?**	**Stat peaks <10 kb?**
CyclinA	Yes	No
CyclinB	Yes	No
CyclinB3	Yes	No
CyclinD	Yes	No
CyclinE	Yes	Yes
Cdk1 (Cdc2)	Yes	No
Cdk2 (cdc2c)	Yes	No
Cdk4	Yes	No
Myt1	Yes	Yes
Wee1	Yes	Yes
Polo	Yes	No
Aurora A	Yes	No
string (cdc25)	Yes	Yes
twine (cdc25)	Yes	Yes
dacapo	Yes	No

Signaling Pathways		
Gene	**So peaks <10 kb?**	**Stat peaks <10 kb?**
Egfr	Yes	Yes
spitz	Yes	Yes
argos	Yes	Yes
yan	Yes	Yes
pnt	Yes	Yes
Notch	Yes	Yes
Delta	Yes	No
Serrate	Yes	No
Suppressor of Hairless	Yes	No
mastermind	Yes	Yes
hippo	No	No
warts	Yes	Yes
yorkie	Yes	No
bantam	Yes	Yes

Photoreceptor Specification & Differentiation		
Gene	**So peaks <10 kb?**	**Stat peaks <10 kb?**
extradendicle	Yes	No
homothorax	Yes	Yes
twin of eyeless	Yes	No
eyegone	Yes	Yes
teashirt	Yes	Yes
optix	Yes	Yes
extra macrochaetae	Yes	No
Atonal	Yes	No
senseless	Yes	No
rough	Yes	No
seven up	Yes	Yes
sevenless	Yes	No
spalt major	Yes	Yes
phyllopod	Yes	No
Bar	Yes	No
prospero	Yes	No
seven in absentia	Yes	No

Signaling Pathways (cont.)		
Gene	**So peaks < 10kb?**	**Stat peaks <10 kb?**
wingless	Yes	Yes
frizzled	Yes	No
LRP1	Yes	Yes
armadillo	Yes	No
pangolin	Yes	No
hedgehog	Yes	No
*patched**	Yes	Yes
smoothened	Yes	No
fused	No	No
cubitus Interruptus	Yes	No
decapentaplegic	Yes	No

Table IV-1 | Patterns of So and Stat at genes relevant to cell cycle regulation, signaling pathways, and photoreceptor specification and differentiation

Table IV-1 | Patterns of So and Stat at genes relevant to cell cycle regulation, signaling pathways, and photoreceptor specification and differentiation (continued)
So ChIP-Seq (Jusiak *et al.* 2014) and Stat ChIP-Seq (Celniker *et al.* 2009) were cross referenced for presence of peaks within 10 kb up- or downstream of genes involved in cell cycle regulation, signaling pathways, or photoreceptor specification and differentiation. Grayed boxes highlight those genes containing regions with overlapping So- and Stat-bound regions. Note that *patched* (marked with the asterisk) is the only gene with So and Stat peaks without any of these elements overlapping. Signaling pathways are grouped into darker outlined boxes.

IV.2.2 – Eya-So and Stat transcriptional feedback

An alternative mechanism for genetic synergy between Eya-So and Stat that does not involve direct co-regulation of target genes is transcriptional feedback between Eya-So and Jak/Stat signaling on Jak/Stat signaling or RD genes, respectively. This idea is supported by the observation that there are So peaks at multiple Jak/Stat genes including *hop, Dome, Stat92E, Socs36E,* and *Socs44A* (Figure III-7B and Figure IV-3) and Stat peaks at two RD genes, *so* and *dac,* and several RD-related genes, *eyegone, Optix* and *teashirt* (Figure III-7C and Figure IV-4). These signatures suggest that there may be significant contribution of Eya-So regulation to transcription of Jak/Stat genes and vice versa. To verify this hypothesis, one would perform similar RT-PCR and enhancer-reporter experiments of bound regions as described above.

One idea is that there are spatial and temporal dynamics for the regulation of these genes. To speculate, Eya-So may regulate pathway effectors (*Dome*, *hop* and *Stat92E*) anterior to the furrow to ensure that those cells are primed for responding to Notch-mediated *upd* (Chao *et al.* 2004) and *cyclinD* transcription (Tsai and Sun 2004), but will regulate *Socs* transcription during photoreceptor cell differentiation for antagonism of RTK signaling (Almudi *et al.* 2009). Analogously, Stat may regulate *eyegone* anterior to the furrow when it is required for early

proliferation in the eye (Chao *et al.* 2004), but later regulates *dac* which expresses around and within the MF (Mardon *et al.* 1994). These hypotheses can be tested by characterizing the expression pattern of enhancer-reporters by cell-type and developmental time. The prediction would be that *Dome*, *hop*, *Stat92E*, and *eyegone* enhancer-traps show expression anterior to the furrow while *dac* expresses within the furrow and *Socs* enhancer-traps express posterior to the furrow.

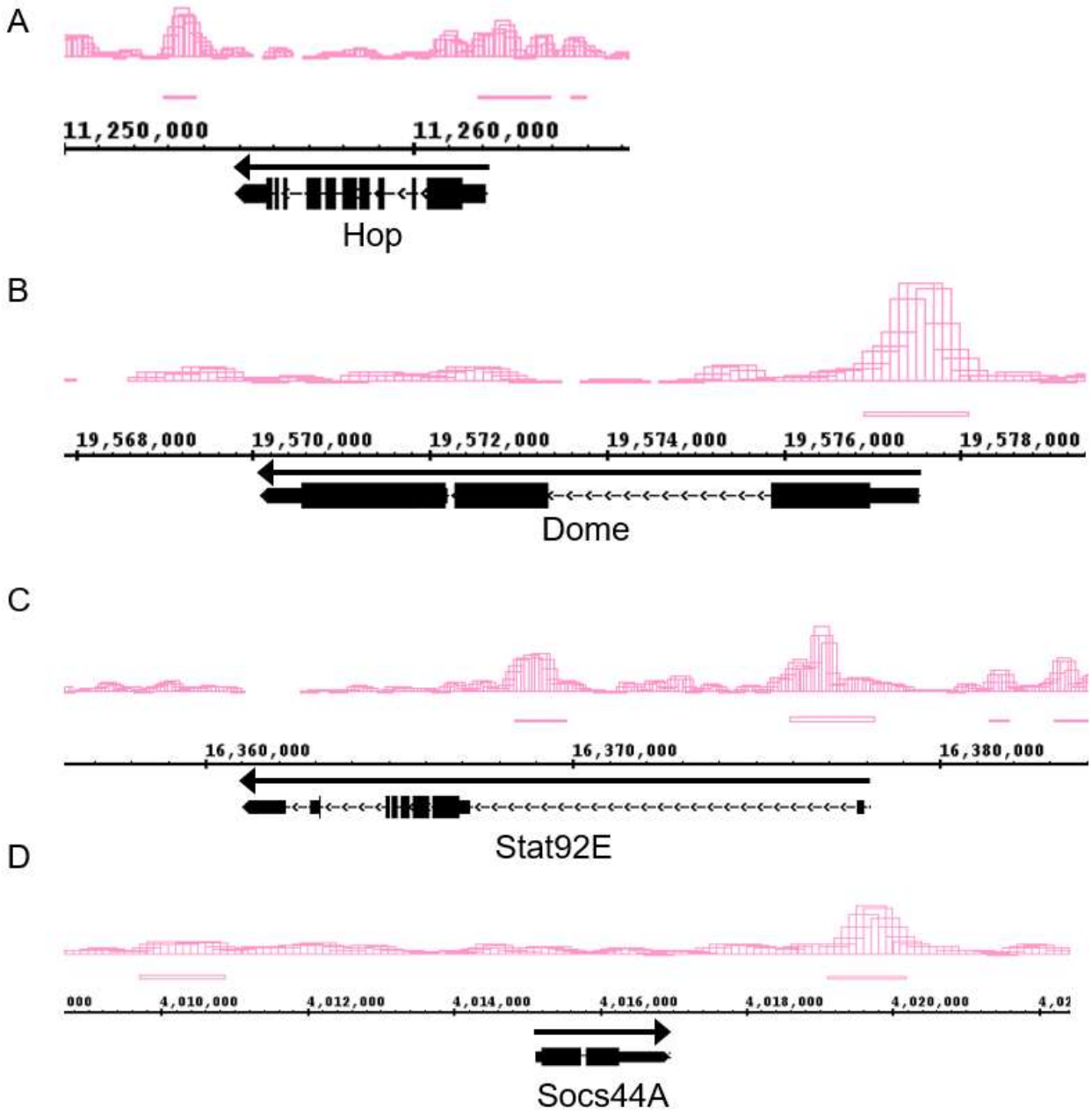

Figure IV-3 | Sine oculis binding sites at multiple Jak/Stat genes
(A-D) So ChIP-Seq data (Jusiak *et al.* 2014) for Jak/Stat components, Hop (A), Stat (B), Socs44A (C) and Socs16D (D). The peaks are constructed from read tags and likely So-bound sites are shown by red rectangles below the peaks, assessed by a false discovery rate cutoff of 10%. The gene structure is shown below the coordinates, with the arrow starting from the 5' end of the gene and extending over the length of the gene.

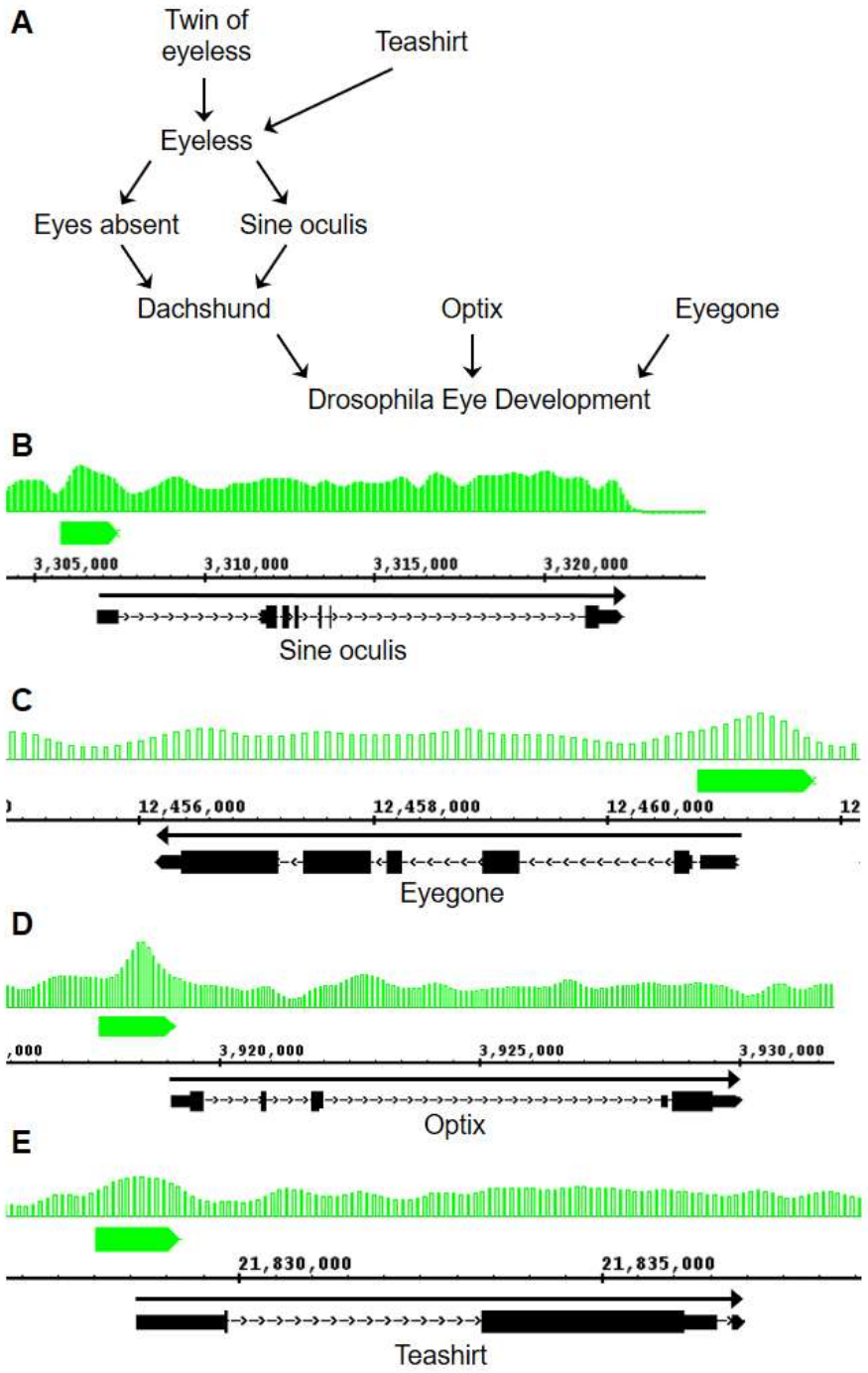

Figure IV-4 | Stat binding sites at multiple genes involved with the RD network
(A) Genetic interaction between core RD network with other genes important for eye development. (B-E) Stat ChIP-Seq data (Celniker *et al.* 2009) at *so* (B), *eyegone* (C), *Optix* (D) and *teashirt* (E). The peaks are constructed from read tags and called peaks are marked by blocks underneath (Celniker *et al.* 2009). The gene structure is shown below the coordinates, with the arrow starting from the 5' end of the gene and extending over the length of the gene.

IV.3 – Interactions between Eya with tyrosine kinases

The screen for suppressors of *eya*'s axon guidance overshooting phenotype (Chapter II) was designed to identify proteins that may bind pY-Eya and mediate its cytoplasmic functions. The hit rate from this screen was quite high, with 12/36 screened genes identified as candidate Eya interactors. We interpret the high hit rate as indication of the importance of tyrosine kinase networks to Eya's cytoplasmic function during photoreceptor axon targeting. Previous work has highlighted that phosphotyrosine-based signaling is important for axonogenesis, as a number of tyrosine phosphatases are critical for this process (Garrity *et al.* 1999; Sun *et al.* 2001; Jeon *et al.* 2008). Our screen identifies a number of non-receptor tyrosine kinases (Csk, Src42a, Src64b, Shark and Hop) that may provide the phosphorylation inputs. Taken together, it appears that Eya is highly integrated with phosphotyrosine signaling networks to regulate photoreceptor axon morphogenesis, and future work may choose to focus on exploring these interactions. One tantalizing avenue is exploration of interactions between Eya with Src signaling, which I discuss below.

IV.3.1 – Eya-Src interactions potentially balance Eya-Abl interactions

Src family kinases are a family of non-receptor tyrosine kinases conserved across metazoans (reviewed in (Parsons and Parsons 2004)). Drosophila have two Src homologs, *Src42A* and *Src64B*, both of which have a high degree of homology to their vertebrate counterparts (Hoffman-Falk *et al.* 1983; Hoffmann *et al.* 1983). They have been implicated in a variety of developmental processes such as cell proliferation, apoptosis, differentiation, regulation of cell-cell contacts, embryonic axon guidance and ovarian ring canal morphogenesis (Pedraza *et al.* 2004; O'Donnell and Bashaw 2013). In addition to these *in vivo* roles,

experiments in cell culture have also shown that Src family kinases regulate actin cytoskeletal remodeling via activation of Rac1 and Cdc42 in growth cones (Antoine-Bertrand *et al.* 2011). A screen performed to identify phosphotyrosine signaling pathways in which Eya participates as a phosphatase that identified *abl* also identified *Src64B* (I. Rebay, unpublished). While mammalian studies have shown that Src family kinases can phosphorylate and activate c-Abl (Chen *et al.* 2008), their relationship in Drosophila have not been tested.

It is unknown what regulates the balance between nuclear and cytoplasmic Eya, either in flies or in mammals. Unpublished data from a former graduate student, Santiago Morillo, showed that Src64B overexpression cause nuclear aggregates of Eya in S2 cells, that Src64B tyrosine phosphorylates Eya in its PST-rich transactivation region, but that Src64 fails to affect Eya's function as a transcriptional co-activator in both transcription assays and in ectopic Dac induction assays in the wing (S. Morillo and I. Rebay, unpublished). These observations leave us with a model in which Src-mediated interactions with Eya limit the cytoplasmic pool of Eya by restricting it to the nucleus (Figure IV-5). This activity potentially opposes Abl-mediated cytoplasmic localization of Eya, such that Abl and Src together regulate the distribution of Eya in subcellular compartments. Since Morillo identified candidate tyrosines phosphorylated by Src, one could test this hypothesis by asking whether Eya with mutations in these tyrosines no longer stays in the nucleus. Intriguingly, work by Xiong et al. show that Abl potentially phosophorylates Eya at the same sites as those regulated by Src. Thus, another interesting idea is that it is not necessarily the identity of the kinase or the sites that become phosphorylated that regulates Eya localization, but instead that any Eya PST phosphorylation prevents it from shuttling between compartments. To test this idea, one could express an NLS-tagged Abl and ask

whether pY-Eya^{Abl} forms aggregates similar to those observed with Src overexpression. Conversely, one could express Myr-Src and ask whether pY-Eya^{Src} accumulates in the cytoplasm rather than in the nucleus.

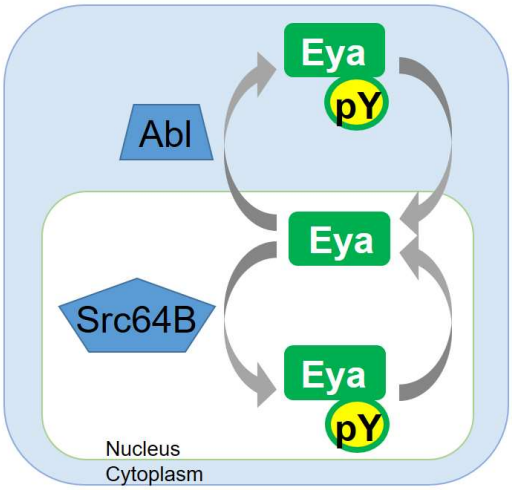

Figure IV-5 | Model for Src and Abl balancing pY-Eya distribution
Abl, which is cytoplasmic, while Src64B, which can be nuclear or cytoplasmic but shows effects on nuclear aggregation of Eya, may potentially balance the distribution of Eya.

IV.3.2 – Eya-Src genetic interactions may be context dependent

Eya and Src's genetic relationship differs by context and thus, like Eya-Jak/Stat interactions, may reflect complex, context-dependent modes of integration. Morillo showed that *Src42A* and *Src64B* antagonize *eya* in ectopic eye formation, but we show that *eya* synergizes with both in axon guidance. One potential mechanism explaining Eya-Src synergy in axon guidance comes from experiments in mouse fibroblasts showing that EGFR activation stimulates Src to regulate rearrangement of the actin cytoskeleton (Chang *et al.* 1995). To test whether this is the case in photoreceptors, one could test whether Src and EGFR synergize in axon guidance.

Such a scenario would be yet another process requiring balanced regulation, as I previously suggested that Eya-SOCS reduces EGFR, but here, Eya-EGFR interactions would promote Src.

IV.4 – Eya links the RD network with cytoplasmic signaling pathways

My work shows that Eya interacts with members of the Jak/Stat signaling pathway in multiple developmental contexts and potentially in distinct compartments within a cell. One tantalizing idea that arises from this conclusion section is that Eya, perhaps through interactions with Jak/Stat genes, may also integrate with RTK signaling. This pathway interface could occur cytoplasmically, possibly involving Eya-SOCS, or nuclearly, by collaboration between So and Stat. Much remains to be studied to parse out how Eya's dynamic roles in the nucleus and cytoplasm are regulated and how these affect its function as a transactivator and its role in tyrosine signaling cascades.

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APPENDIX I

Screen for tyrosine phosphatases redundant with Eya in Drosophila eye development

Charlene Hoi, Nora Peterson and Ilaria Rebay

IR recombineered and double balanced all flies used for the crossing scheme shown in Figure AI-1. NP performed a pilot screen which included 4/9 lines that were ultimately screened. CH designed the project and performed all other experiments.

AI.1 – Introduction

Phosphorylation of proteins on serine, threonine and/or tyrosine residues is a common mechanism employed by cells to regulate protein activity. Appropriately balancing these marks by the opposing activity of kinases and phosphatases is critical for maintaining proper protein functions throughout the cell and over development. Phosphatases are generally highly substrate-specific and non-redundant with other phosphatases while kinases are more promiscuous and will often share substrates; despite this, there are examples of redundant phosphatases in flies (Kirchner *et al.* 2007; Jeon *et al.* 2008).

AI.2 – Results and discussion

The axon guidance-based screen performed in Chapter II was motivated in part by the hypothesis that Eya's protein tyrosine phosphatase (PTP) activity would be relevant in the cytoplasm. Since SH2/PTB domains recognize phosphotyrosines, we hoped that interactors might pinpoint potential substrates for Eya's PTP. Work in the Mardon lab in 2013 revealed that

Eya's PTP is dispensible for normal development (Jin *et al.* 2013), thus nullifying Eya's absolute requirement in the cytoplasm as a PTP while raising the possibility that Eya may act redundantly with other PTPs. To test if redundancy is the reason that Eya's PTP is not required for normal development, we screened for secondary phosphatases whose loss, by deficiency or by allele, might reveal an inability for BAC constructs with phosphatase-dead Eya (BAC-Eya^{E728Q}) to fully rescue *eya* null flies compared to wild-type Eya (BAC-Eya^{WT}) (Figure AI-1). Unpublished data demonstrated that BAC-Eya^{E728Q} is in fact a sensitized background in which heterozygosity of Eya's partner genes, *so* and *dac*, are sufficient to reveal a requirement for Eya's PTP (C. Hoi, T. Davis and I. Rebay), thus providing evidence that such a rescue assay would be suitable for identifying Eya-interactors.

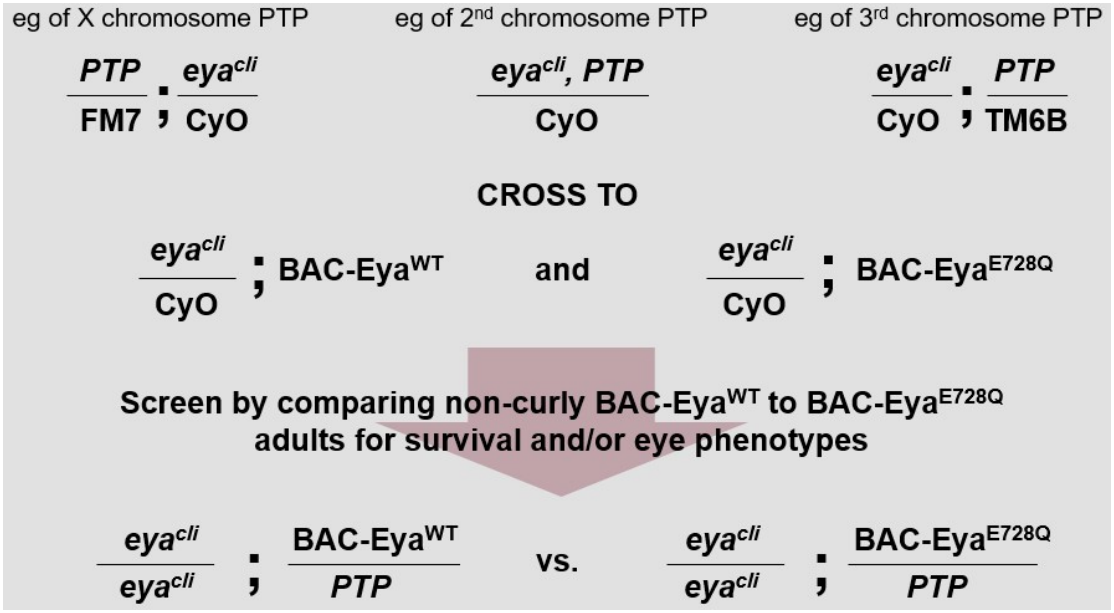

Figure AI-1 | Screen Schematic
The screen will be performed by asking whether alleles or deficiencies of other PTPs in *Drosophila* (denoted by *PTP*) modify genomic rescue by WT (BAC-Eya^{WT}) versus phosphatase-dead *eya* (BAC-Eya^{E728Q}) in an *eya^{clift}* background.

There are 37 tyrosine phosphatases in the fly, including *eya* (Figure AI-2 and Table AI-1). This list is based on annotated function and sequence conservation from Flybase. Many of these are localized to the cytoplasm and expressed in the eye, with only MKP-4 being known to be exclusively nuclear and CG14297 to not show RNA transcript in the eye. Furthermore, many PTPs in this list have characterized functions in cells, thus providing additional contexts to study interactions with Eya should they come out as hits from this screen. All lines listed in Table AI-1 have been acquired from the Bloomington stock center, and many have been recombined with eya^{clift} in order to perform the screen. To identify potential PTPs redundant with Eya, we asked whether heterozygosity of secondary phosphatases might reveal an inability for BAC-Eya^{E728Q} to rescue *eya* null fly eye development and survival (Figure AI-1). 9 lines which include 11 total genes were screened in this way for eclosure rate and eye phenotypes (Figure AI-3). At least 3 lines showed defects in eclosure rate – Df 6003, Df 7309 and Ptpmeg – and 3 lines show eye phenotypes – Df 6003, Df 473 and Ptp69D.

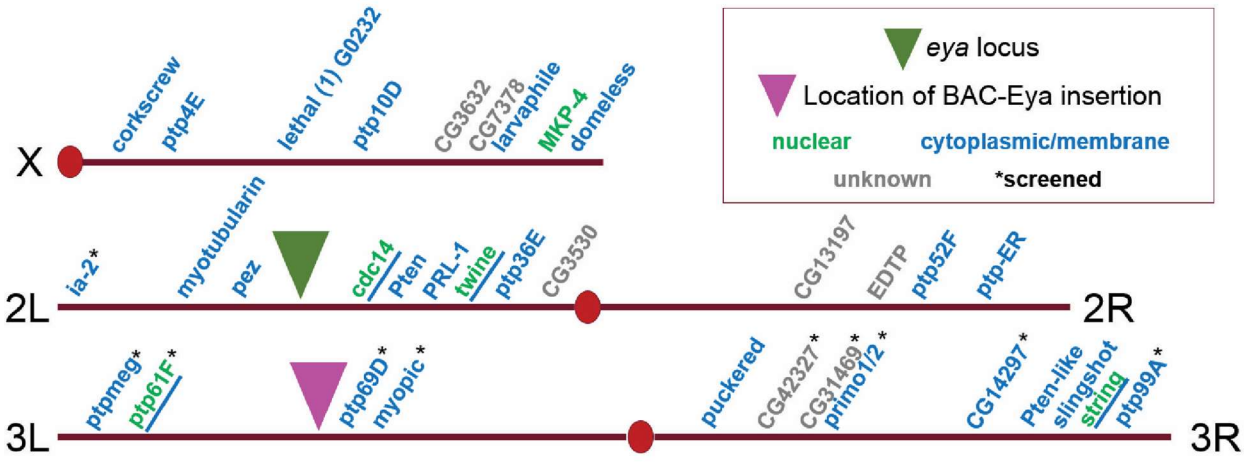

Figure AI-2 | Location of 37 PTPs in Drosophila genome

Figure AI-2 | Location of 37 PTPs in Drosophila genome (continued)
Schematic showing approximate location of 37 PTPs across the Drosophila genome, organized by chromosome arm. Inverted green triangle shows genomic location of *eya* while inverted purple triangle shows location of the BAC construct used for genomic rescue of *eya* null animals by (Jin *et al.* 2013). PTPs are color coded to show localization (green for nuclear, blue for cytoplasmic and gray for unknown). If a PTP localized to both the nucleus and cytoplasm it shows in green text underlined by blue. Those genes screened are annotated with an asterisk.

Name	Stock	In Eye?	Localization	Function	Screen
CG14297	7660	N	cytoplasm?	N/A	x
CG31469	7649	Y	N/A	N/A	x
CG42327	7960	Y	N/A	N/A	x
corkscrew	23874	Y	cytoplasm	regulates RTK signaling by Torso pY918, complexes with drk/dos	
eya		Y	nucleus, cytoplasm	eye/axon/gonad development	
IA-2	7490	Y	secretory vessicles	digestive tract development, insulin secretion	x
larvaphile	970	?	transmembrane RPTP	motor neurogenesis, defasciculation of R1-6, axon guidance (not PTP)	
lethal (1) G0232	11956	Y	cytoplasm, trafficking	neurogenesis, phagocytosis, CRAL/TRIO lipophilic binding domain	
myopic	15817	Y	endosome	regulates EGFR/Toll/Hippo signaling pathways, spindle assembly, complexes with Rab4/5/Drk/Yki	x
pez	7800	Y	cytoplasm	Ferm domain (not PTP) in Hippo signaling, scaffolding	
primo-1	7649	Y	LMW-PTP, cytoplasm	retina development, neurogenesis	x
primo-2	7649	Y	LMW-PTP, cytoplasm	retina development, neurogenesis	x
PTP10D	5810	Y	transmembrane RPTP	motor neuron axon guidance, CNS, tracheal development, complexes with ena/sas, redundant with Ptp4E, genetic interaction with lar/Ptp99A/Ptp52F/Ptp69D	
	40961				
PTP36E	7840	?	cytoplasm?	N/A – identified as putative PTP by sequence alignment	
PTP4E	6003	Y	transmembrane RPTP	motor neuron axon guidance, CNS, tracheal development, redundant with Ptp10D, genetic interaction with Ptp52F/Ptp69D	
	42019				
	40961				

Table AI-1 | List of protein tyrosine phosphatases in the fly

PTP52F	6590	Y	transmembrane RPTP	organ subdivision, axon guidance, motor neurogenesis, genetic interaction with lar/Ptp10D/Ptp4E	
PTP61F	23674	Y	nucleus, cytoplasm, perinuclear	axon guidance, JAK/STAT regulation, complexes with dock	x
PTP69D	5088	Y	transmembrane RPTP	axon guidance, neurogenesis, genetic interaction with Ptp99A/Ptp10D/Ptp4E	x
	7596				
PTP99A	5089	Y	transmembrane RPTP	motor neurogenesis, genetic interaction with lar/PTP69D/PTP10D	x
	24977				
PTP-ER	5764	Y	cytoplasm	Ras signal transduction	
Ptpmeg	9756	Y	cytoplasm	axon guidance, mushroom body development, autophagic cell death	x
cdc14	7806	Y	N/A	M/G1 transition, rho signaling during cytokinesis	
CG13197	21336	Y	N/A	N/A	
CG3530	14361	Y	endosome?	N/A	
CG3632	18249	Y	endosome?	regulates BMP (dpp) signaling - myotubularin in mammals?	
CG7378	44989	Y	N/A	MKP-like	
domeless	10263	Y	transmembrane RPTP	Jak/Stat signaling receptor	
	11953				
EDTP	17050	Y	N/A	oogenesis	
MKP-4	13783	Y	nucleus	regulates JUN activity	
myotubularin	9185	Y	membrane-associated	regulates PI3P activity, cell cycle, endocytosis	
PRL-1	43456	Y	membrane-associated	antagonizes Src	
Pten	24134	Y	membrane-associated	actin binding, cell organization, cell size	
	16275				
Pten-like phosphatase	7991	Y	membrane-associated	regulates PI5P	
puckered	773	Y	cytoplasm	regulates JUN/MAPK activity, G2/M transition, actin organization	
	9699				
slingshot	9110	Y	cytoplasm	cofilin phosphatase, cell organization, cell size	
	9111				
string	7690	Y	nucleus, cytoplasm	(aka cdc25) G2/M transition	
twine	4274	Y	nucleus, cytoplasm	(aka cdc25) G2/M transition	

Table AI-1 | List of protein tyrosine phosphatases in the fly (continued)

Table AI-1 | List of protein tyrosine phosphatases in the fly (continued)
Expression in the eye (N = no, Y= yes, ?=unknown), localization data and function are from Flybase (RPTP = receptor PTP, LMW-PTP = low molecular weight PTP, question marks indicate assumed localization based on listed protein domains/motifs). Note that almost all PTPs are expressed in the eye and that many localize to the cytoplasm.

AI.2.1 – Eya^{E728Q} compromises survival

Jin et al. show that BAC-Eya^{E728Q} is sufficient to rescue survival of *eya* null flies (Jin *et al.* 2013), however we failed to recapitulate this result and observed compromised eclosure rates for those flies (Figure AI-3B, Chi^2 value). The BAC-Eya^{E728Q} continued to behave oddly in our screen in which some genotypes that are now heterozygous for other PTPs no longer show survival defects. This could be interpreted as suppression of the BAC-Eya^{E728Q} phenotype, however this occurred too frequently for that to be the case (6/9 lines show "suppressed" reduced rate of survival). Thus, for the remaining discussion, we will focus on eye phenotypes.

AI.2.2 – Df 6003, Ptp69D and Df 473 show compromised eye development in Eya^{E728Q} backgrounds

Jin et al. show that BAC-Eya^{E728Q} is sufficient to rescue eye development, an observation which we recapitulated in our experiments (Figure AI-3 A-B). Our preliminary screen identified 3 lines showing modification of eye development in the phosphatase-dead background. Those lines in order of severity were: Df 6003, a deficiency covering the *IA-2* locus, Ptp69D and DF 473, a deficiency covering the *Ptp61F* locus.

Df 6003 in the BAC-Eya^{WT} background show rough eyes with disorganized ommatidia (Figure AI-3S), immediately suggesting without further experiments that this deficiency contains at least one gene important for eye development. This rough eye phenotype was enhanced with phosphatase-dead BAC-Eya^{E728Q}, in which eyes were much smaller and showed more dramatic

disorganization of ommatidia, often with fusion and necrosis (Figure AI-3T). Future experiments would include first verifying that *IA-2* is the relevant gene within the deficiency that interacts with *eya*. To date, *IA-2* in Drosophila function has only been described in insulin signaling for gut development and morphogenesis (Kim *et al.* 2008), however work from our laboratory has shown that *IA-2* may be regulated by the Yan/Pnt network as there is a significant single Yan ChIP peak at the *IA-2* locus and transcription assays demonstrate that *IA-2* can be activated by Pnt and repressed by Yan (Webber *et al.* 2013).

Ptp69D heterozygotes in the BAC-Eya^{WT} background were normal (Figure AI-3E) while those in the BAC-Eya^{E728Q} background showed defects in the dorsal posterior eye with many necrotic and disorganized ommatidia (Figure AI-3F). Ptp69D functions in axon targeting (Garrity *et al.* 1999; Newsome *et al.* 2000), thus we wondered whether it synergizes with *eya* in that context. To test that, I asked whether *Ptp69D* heterozygotes could dominantly modify Eya axon guidance phenotypes. I failed to observe any enhancement or suppression with either *GMR>Eya^{WT}* or *GMR>Eya^{RNAi}*, suggesting Ptp69D does not dominantly interact with Eya in this context (Figure AI-4 A-C). It is possible that *Ptp69D* heterozygosity was not sufficient to reveal an interaction; future studies could repeat these experiments utilizing a strong RNAi against *Ptp69D*.

Df 473 in the BAC-Eya^{WT} background were normal (Figure AI-3O) while those in the BAC-Eya^{E728Q} background showed defects in the dorsal posterior eye with necrotic and disorganized ommatidia (Figure AI-3P). Df 473 includes the *Ptp61F* locus. No follow-up experiments were performed, however Ptp61F has been characterized as a negative regulator of the Jak/Stat signaling pathway (Müller *et al.* 2005), an intriguing hit given our findings that Eya

interfaces with Jak/Stat signaling in multiple contexts (Chapter II and III). The direction of the interaction is curious in light of the fact that our screen here suggests synergy between *eya* and *Ptp61F* while our prior studies of Eya and members of the Jak/Stat pathway suggest that Eya synergizes with effectors of the pathway. However, like SOCS proteins, Ptp61F has other roles in regulating EGFR/MAPK signaling in addition to regulating the Jak/Stat pathway (Tchankouo-Nguetcheu *et al.* 2014). Ptp61F also opposes Abl activity in regulation of actin polymerization, thus an Eya-Abl-Ptp61F axis may be another potential context for interactions.

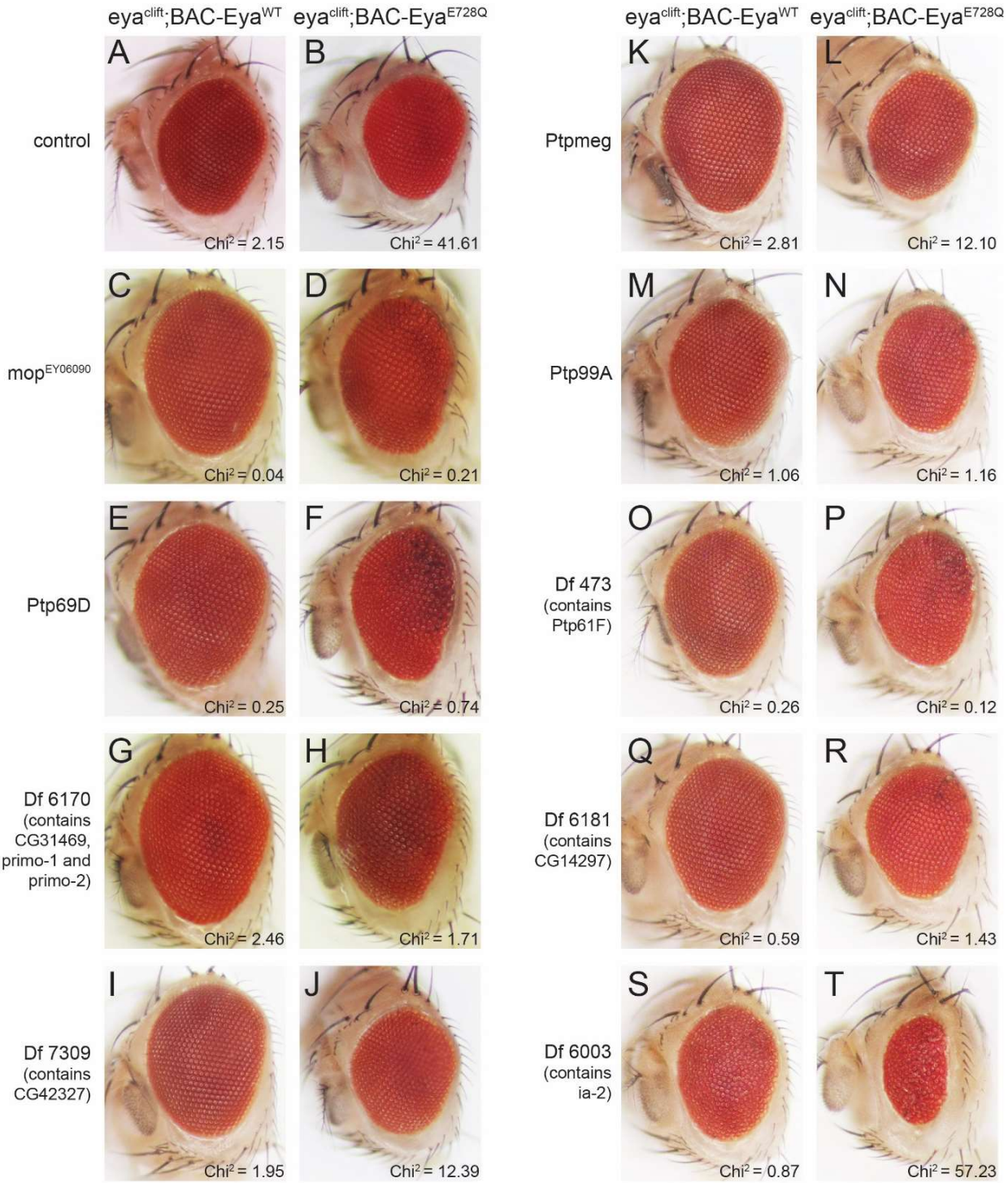

Figure AI-3 | Screen of 9 lines for sensitivity to phosphatase-dead Eya genetic backgrounds

Figure AI-3 | Screen of 9 lines for sensitivity to phosphatase-dead Eya genetic backgrounds (continued)
Alleles and deficiencies shown were recombined with *eya*^{clift} then crossed to the genotypes shown in the column headers: either *eya*^{clift} heterozygotes that possess a BAC construct with wild-type Eya (Eya^{WT}) or phosphatase-dead Eya (Eya^{E728Q}). Adult eye formation was used to assess eye development. Shown are representative eyes from 3-5 day old decapitated females. Eclosure rate of non-curly:curly flies was used to assess survival. Shown in the bottom right corner of each image is the chi-squared value associated with that genotype (p-value 0.05 corresponds with a chi-squared value of 5.99).

AI.2.3 – *Ptp69D* potentially synergizes with *eya* in earlier steps of eye development

Since *eya* and *Ptp69D* do not seem to interact in photoreceptor axon targeting, I shifted my focus to earlier steps in eye development. We noticed subtle effects on eye size in our screen, in which BAC-Eya^{E728Q} appeared to be compromised in being able to generate fully sized eyes (Figure AI-4D). *Ptp69D* heterozygosity enhances this effect (Figure AI-4E), thus suggesting that Ptp69D synergizes with Eya in proliferation of the eye. Previous analysis of *Ptp69D* mutant eye tissue showed that X-ray induced clones generated wild-type photoreceptor (Garrity *et al.* 1999), indicating that Ptp69D is not required for differentiation of photoreceptors, however the authors do not mention whether the frequency or relative size of clones was smaller than controls, which one would expect if Ptp69D has a role in proliferation. Eya is known to have a role in proliferation (Chapter I), in which knockdown of *eya* early in eye development with *eyeless-Gal4* driver will cause reduced eyes. If Ptp69D is interacting with Eya in proliferation, then it should dominantly enhance the *ey>Eya*^{RNAi} small-eye phenotype. Indeed, heterozygosity for *Ptp69D* synergized with Eya-mediated eye reduction (Figure AI-4F).

Ptp69D is a transmembrane receptor protein tyrosine phosphatase (RPTP), and although its specific ligand(s) or substrate(s) are not known, there is precedence for RPTPs having roles in

proliferation (reviewed in (Xu and Fisher 2012)). Eya already has its link to proliferation by regulating the cdc25 homologue, *string* (Jemc and Rebay 2007). Redundancy between Eya and Ptp69D may either hint that Ptp69D regulates *stg* as well, or that Eya shares functions with Ptp69D in proliferation that have not yet been discovered. To explore these hypotheses, one would first want to characterize Ptp69D's expression profile to check whether it overlaps with that of Eya. If they are indeed acting redundantly, then there should be at least some co-expression of the two. Next, to test if Ptp69D contributes to Stg regulation, one could analyze *stg* transcript levels and Stg phosphorylation levels. Stg regulation is primarily through serine phosphorylation (Donzelli and Draetta 2003), thus if Ptp69D regulates Stg activity, it must either be transcriptionally or indirectly by secondary proteins.

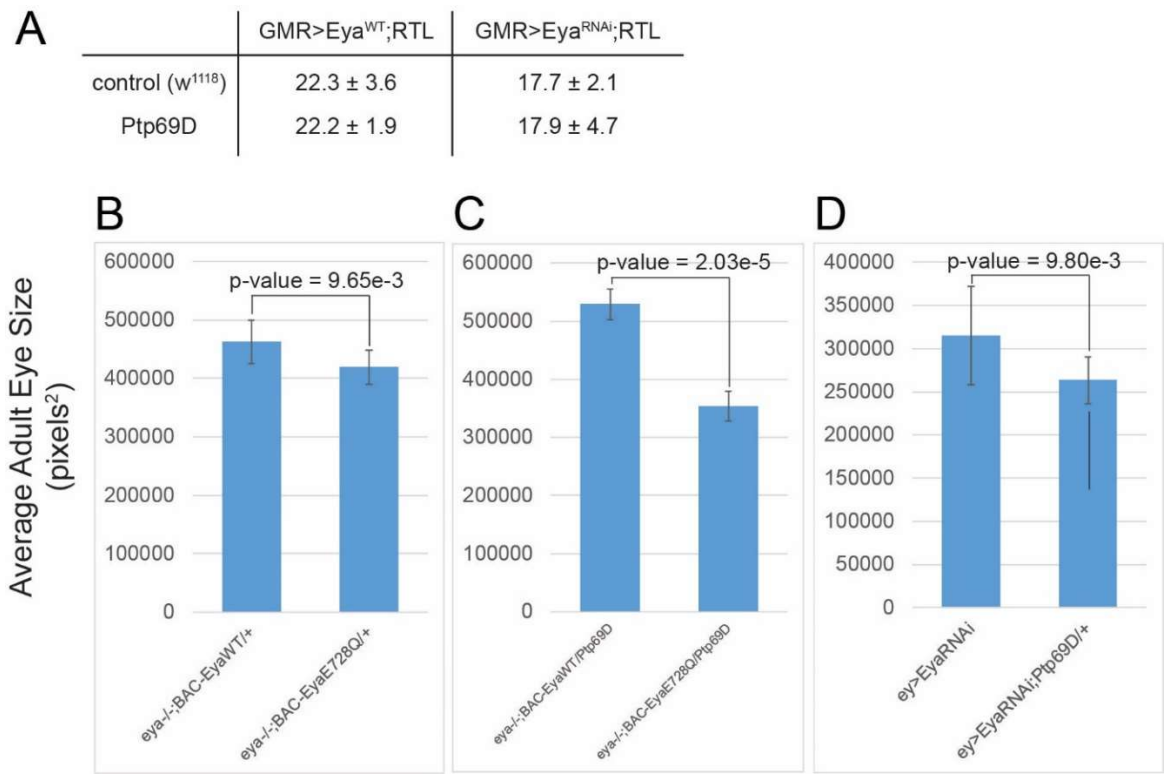

Figure AI-4 | *Ptp69D* synergizes with *eya* early in development

Figure AI-4 | *Ptp69D* synergizes with *eya* early in development (continued)
(A) *Ptp69D* heterozygosity does not modify Eya-mediated axon overshooting phenotypes. *GMR*-driven *eya* overexpression and knockdown, as has previously been demonstrated (Chapter II and Xiong *et al.* 2009), causes R2-R5 photoreceptor axons marked by Ro-lacZ^{tau} (RTL) to overshoot the lamina. *Ptp69D* heterozygosity does not dominantly modify these phenotypes. (B-D) *Ptp69D* dominantly enhances Eya-mediated small eye phenotypes. (B) Quantification of adult male eyes shows that phosphatase-dead Eya (BAC-Eya^{E728Q}) is less efficient at rescuing eye size than wild-type Eya (BAC-Eya^{WT}). (C) Quantification of adult female eyes shows that *Ptp69D* heterozygosity dominantly enhances the small eye observed from BAC-Eya^{E728Q}. (D) Quantification of adult female eyes showing that *Ptp69D* heterozygosity dominantly enhances the *ey>Eya^{RNAi}* small eye phenotype.

AI.3 – Concluding remarks

Many genes remain to be screened. Among the list, we expect *string* and *dome* to show interactions given previous findings that Eya/So regulate *string* (Jemc and Rebay 2007) and that Eya synergizes with components of the Jak/Stat pathway (Chapter II). *twine* and *csw* might also be expected to be pulled out in the screen. *twine* is a cdc25 homolog that is only required in meiosis (Courtot *et al.* 1992), however *eya* has roles in gonadogenesis (Bai 2002; Fabrizio *et al.* 2003) and thus in this screen may interact with *twine* in survival but not eye development. *csw* was identified in the axon guidance screen in Chapter II and thus may be expected to also interact with BAC-Eya^{E728Q} in this screen. Csw is a PTP with roles in RTK pathways that also interacts with Src kinases (Herbst *et al.* 1996, 1999; Cooper *et al.* 1996). Src-Csw interactions have been reported in phagocytosis of necrotic axons and dendrites (Ziegenfuss *et al.* 2008), thus one potential hypothesis is that Eya interactions with Src and Csw identified in the axon guidance screen are reaffirmed by this screen in which Eya and Csw act redundantly with respect

to Src. Taken together, our preliminary results indicate that this screen will be useful for identifying novel Eya interactors and pinpointing potential contexts for Eya's PTP activity.

AI.4 – Materials and methods

AI.4.1 – Fly strains

BAC-Eya^{WT} and BAC-Eya^{E728Q} stocks were generously shared by G. Mardon. All other stocks were acquired from the Bloomington stock center.

AI.4.2 – Immunostaining and imaging

Adult eye pictures were taken by anesthetizing 3-5 day-old flies, decapitating them with a 27 gauge syringe needle, orienting the heads on double-sided tape then imaging with a mounted Canon EOS Rebel T2i camera. Image processing was performed using iSolution Lite software with the "Stereo focus enhancement with perfect focus" macro. Adult eye size was quantified using ImageJ. Statistical p-values were calculated by performing student's T-tests between the indicated genotypes.

Eye imaginal disc-brain complexes were dissected from pre-pupal third instar larvae and stained as previously described (Xiong *et al.* 2009) with rabbit anti-β-gal (1:20,000, Promega) and secondary antibodies (1:2000, Jackson ImmunoResearch). Samples were mounted with N-propyl-gallate solution in 90% glycerol. Images of axons were taken on a Zeiss LSM 510 confocal microscope with 0.5-1.5µm steps for the entire depth of the brain and then projected maximally in ImageJ.

AI.5 – Acknowledgements

Thank you to Graeme Mardon for sharing his genomic rescue flies, Nora Peterson for performing a pilot screen and identifying Df 6003 and Trevor Davis and Ilaria Rebay for collaborative work on establishing that G.M.'s genomic rescue stocks are sensitized backgrounds suitable for screening.

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APPENDIX II

Restricting Eya to the nucleus by a Nuclear Localization Sequence (NLS)

AII.1 – Introduction

The evolutionally conserved dual-function transcription factor and phosphatase *eyes absent* (Eya) localizes to both the nucleus and cytoplasm, however only limited examples for requirement of cytoplasmic Eya have been found in mammalian cells and none for Drosophila cells or tissue. Eya was initially discovered to be a transcription co-factor necessary and sufficient for retinal development in flies, and later four mammalian homologs were also described as being important for eye development in addition to kidney, muscle, and gonad development (Xu *et al.* 1997; Abdelhak *et al.* 1997; Zimmerman *et al.* 1997; Bonini *et al.* 1997; Pignoni *et al.* 1997; Söker *et al.* 2008). Although Eya function seems to be conserved from invertebrates to vertebrates, regulation of subcellular localization is not. While Drosophila Eya is mainly nuclear, which aligns with its function as a transcription co-factor, mammalian Eya is cytoplasmic until it's transcription co-factor Six is expressed (Ohto *et al.* 1999). In order to ask whether *Drosophila* require Eya in the cytoplasm, I developed nuclearly-localized Eya expression constructs by addition of SV40 NLS sequences.

AII.2 – Results and discussion

Eya has three isoforms, all of which contain one intrinsic NLS between the Eya Domains while A and B contain an extra N-terminal NLS. The N-terminal NLS appears to be functional, as previous mutagenesis showed that mutation of conserved basic residues into alanines to the N-

terminal NLS but not the internal NLS caused cytoplasmic accumulation of Eya (W. Xiong, I. Rebay, unpublished). This raises the interesting possibility that isoform C, which intrinsically lacks the N-terminal NLS, may be functioning in the cytoplasm. Early studies of *eya* missed this third isoform (Bonini *et al.* 1993), and thus it may be that this transcript is very specifically regulated or transcribed at low levels. To perturb this potential function, I added strong SV-40 NLS sequences by quikchange to EyaB cDNA expression constructs that would be common to all isoforms (Figure AII-1). Our ultimate goal is to put a similar construct in the fly, where these experiments will be informative on the appropriate NLS insertion site.

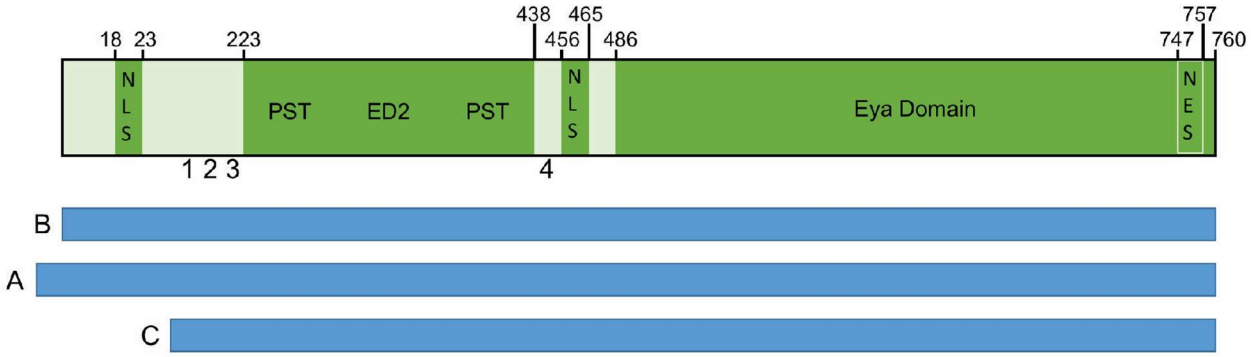

Figure AII-1 | Schematic of NLS insertions in *eya*
Eya's three isoforms are depicted below. Isoform B is the most highly transcribed form, and thus positions are shown relative to its sequence. Conserved regions of Eya include one N-terminal NLS (18-23), the PST-rich domain housing the Eya domain 2 (ED2) (223-438), a secondary internal NLS (456-465), and the C-terminal Eya domain (ED) which contains a NES (747-757). 4 potential insertion sites were chosen, numbered 1-4, for the addition of the SV-40 NLS sequence: PKKKRKV.

In order to confirm whether these NLS tags are functional, I transfected S2 cells and assessed Eya's subcellular localization. All four PAFW-Eya constructs with added internal NLSs, termed Eya intNLS1-4, produced cells with very strong nuclear localization. Normally,

Eya transfected into S2 cells will express strongly in the nucleus with some faint cytoplasmic localization (Figure AII-2A). Previous work has shown that co-transfection of Abl will cause cytoplasmic sequestration of Eya in the cytoplasm (Figure AII-2B and (Xiong *et al.* 2009)). My Eya intNLS constructs all express nuclearly (not shown), and this is robust even with co-transfection of Abl (Figure AII-2 C-F). This behavior is consistent across multiple cells and transfection experiments, as demonstrated by the quantification of subcellular localization from cells across two transfections (Figure AII-2G).

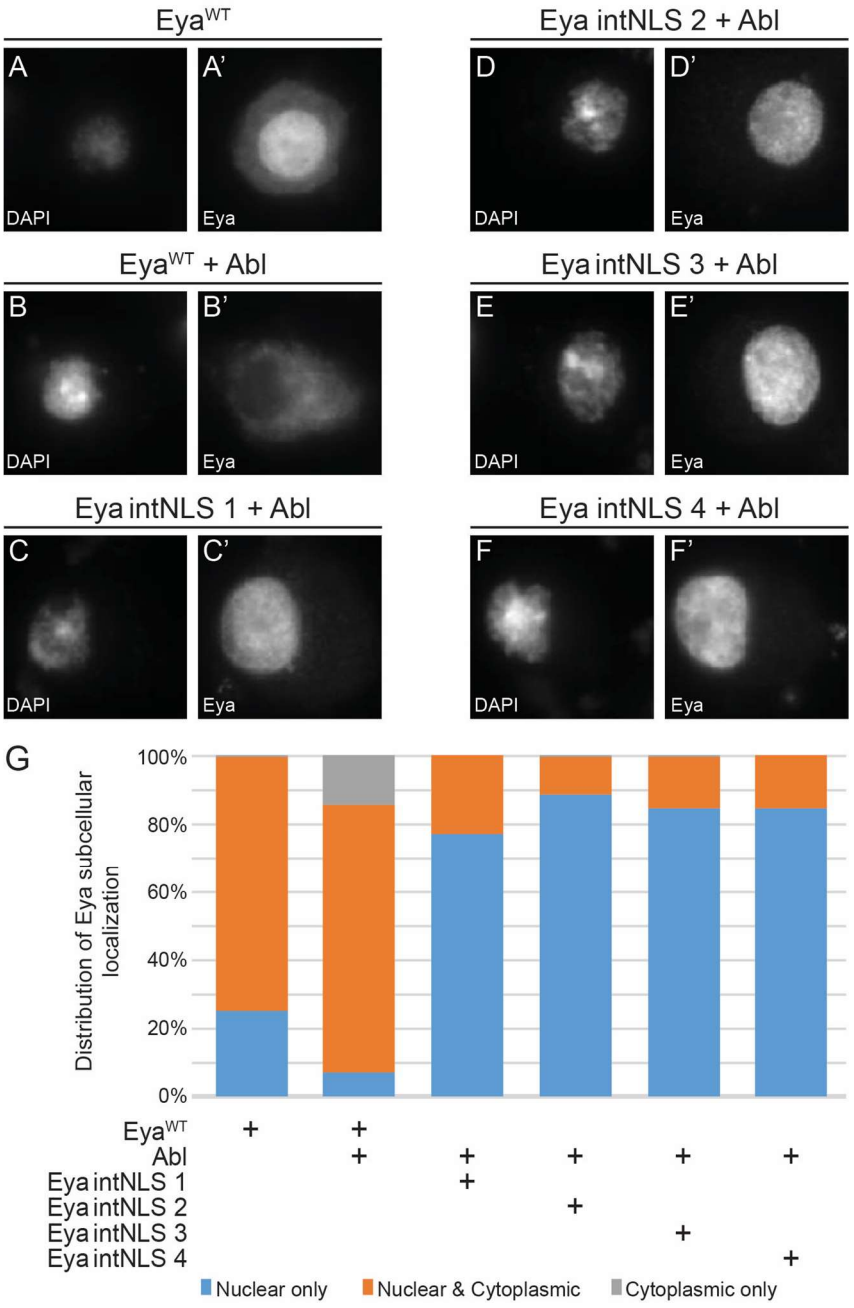

Figure AII-2 | Eya intNLS 1-4 are strongly nuclear and resist Abl relocalization
S2 cells transfected with Eya, Eya intNLS variants, and Abl. (A) WT Eya is mostly nuclear with some cytoplasmic staining. (B) Eya co-transfected with Abl causes an increase in the proportion of cells with cytoplasmic Eya staining. (C-F) Eya intNLS 1-4 all show very strong nuclear Eya staining even with co-transfection of Abl. (G) Quantification of subcellular distribution of Eya across two transfection experiments.

I next wanted to test whether Eya is still a functional transcriptional coactivator with these internal NLS sequences. I assessed this by performing transcription assays on the LMEE-luciferase reporter construct which is a verified Eya-So transcriptional target (Yan *et al.* 2003). Wild-type Eya cooperates with So to activate this reporter, where Eya alone will only minimally activate the enhancer but co-transfection of So increases activity of the element by 6-fold (Figure AII-3). I found that all Eya intNLS constructs still retain some So-responsiveness, with Eya intNLS 4 having the highest fold induction compared to the others (Figure AII-3). One caveat to Eya intNLS 4 is that it also had the highest basal activation of LMEE in the absence of So. Phi-integrase constructs have been cloned for both intNLS 2 and intNLS 4, where intNLS 2 will represent an NLS-Eya with appropriate basal transcriptional activity while intNLS 4 will present an NLS-Eya with proper So-responsiveness.

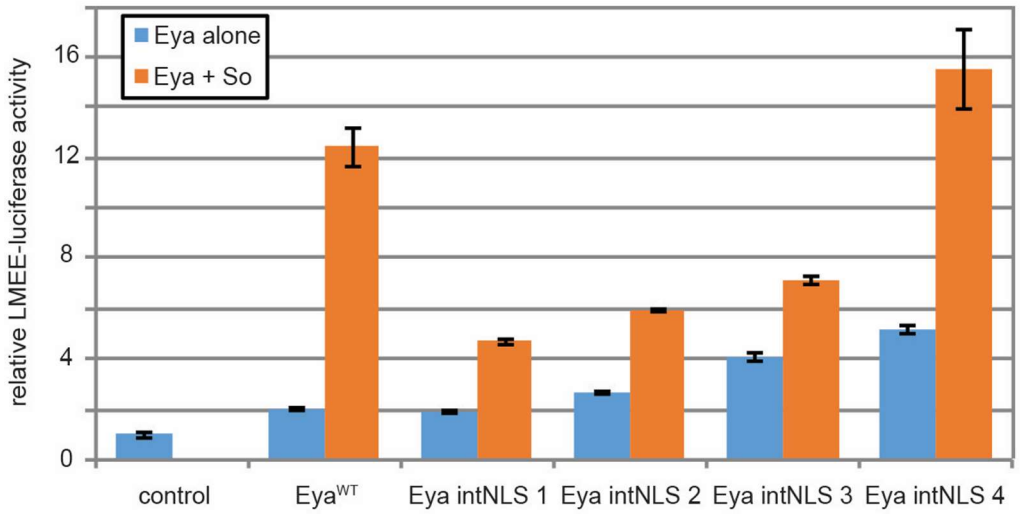

Figure AII-3 | Eya intNLS are still responsive to So in transcription assays
LMEE-luciferase transcription assays were performed to test the transactivation capability of Eya intNLS constructs. Shown are average intensities of LMEE-luciferase to actin-renilla luciferin across four transfections normalized to control transfections containing just actin-renilla and LMEE-luciferase. Wild-type Eya is only able to very minimally activate the LMEE-luciferase reporter, however there is a 6-fold increase when co-transfected with So. All Eya intNLS constructs show some fold increase upon co-transfection of So, however they are not as great as that of wild-type Eya.

AII.3 – Materials and methods

AII.3.1 – Cloning

The SV-40 sequence (PKKKRKV) was added to Eya by quikchange addition of the sequence CCG CCA AAG AAG AAG CGC AAG GTG into PAFW-Eya for S2 cell experiments. Quikchange primers used were: 1) intNLS1 F: TAGCGCCGGTGCCGGCATGCC GCCAAAGAAGAAG 2) intNLS1 R: CCCACACCGACACCCACCTTGCGCTTCTTCTTTGG 3) intNLS2 F: CCAGCCGCTCCAGCCGACAATACCGCCAAAGAAGAAG 4) intNLS2 R: GCGAGGCCAGCGATCCCACCTTGCGCTTCTTCTTTGG 5) intNLS3 F: GCGCAGTCCGC CGGCCCGCCAAAGAAGAAG 6) intNLS3 R: GTGGAGAAACTGCTCACCTTGCGCTTCT TCTTTGGC 7) intNLS4 F: CCGCCGGCTCTGGGCCGCCAAAGAAGAAGC 8) intNLS 4 R: CTCGTTGCCACGCCCACCTTGCGCTTCTTCTTTGGC. Flag-Eya intNLS2 and Flag-Eya intNLS4 were subsequently subcloned into the pUAS-attB vector using EcoRI and XbaI restriction sites: F: GTGGAATTCGCCACCATGGACTAC and R: CACTCTAGATCATAAGA AGCCCATGTCGAG.

AII.3.2 - S2 cell transfection, transcription assays and imaging

Drosophila S2 cells were cultured at 25°C in Schneider's medium (Sigma-Aldrich) supplemented with 10% Insect Medium Supplement, penicillin and streptomycin (Invitrogen). Cells were transiently transfected with 1.0ug of each plasmid for cell staining or 20ng of actin-renilla and 250ng of each plasmid for transcription assays using dimethyldioctadecyl-ammonium bromide (DDAB, Sigma) transfection and induced with 0.1M $CuSO_4$. Expression constructs included pMT-Myc-Abl (Xiong *et al.* 2009), pMT-Myc-So (Silver *et al.* 2003), pBS-LMEE-luciferase (Mutsuddi *et al.* 2005), PAFW-Eya^{WT}, PAFW-Eya intNLS1, PAFW-Eya intNLS 2,

PAFW-Eya intNLS 3, PAFW-Eya intNLS 4, and pMT-HA as an empty vector to normalize amount of transfected DNA. Transcription assays were performed as previously described in (Silver *et al.* 2003) except that luciferase and luciferin activity was measured on a Berthold Technologies AutoLumat Plus LB 953 luminometer with Berthold TubeMaster software.

For imaging and quantification, cells were settled onto poly-L-lysine treated slides, fixed with 4% paraformaldehyde for 10 minutes and stained with mouse anti-Flag (1:1000, Sigma) and secondary antibody (1:2000, Jackson ImmunoResearch). Samples were mounted with N-propyl-gallate solution in 90% glycerol. Imaging and quantification were performed on a Axioplan 2 imaging fluorescent microscope with a 63x objective.

AII.4 – References

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APPENDIX III

Eya RNAi axon overshooting phenotypes persist into adult brains

AIII.1 – Introduction

Eya is required for photoreceptor cells (R) 1-6 targeting in third instar larvae (Chapter II and Xiong *et al.* 2009), however it is not known whether these phenotypes resolve themselves in later pupal stages or if the phenotypes remain. Work on *runt*, another gene whose misexpression causes R1-R6 to overshoot the lamina, have shown that these overshooting photoreceptor axons still synapse in the lamina, and that they can sometimes also switch fate from R1-R6 into R7 and R8 (Edwards and Meinertzhagen 2009). Edwards and Meinertzhagen assessed this fate change by morphology and rhodopsin expression. R1-R6 have larger rhabdomeres which express *rhodopsin 1 (rh1)* while R7 and R8 have smaller rhabdomeres that express *rh3/rh4* and *rh5/rh6*, respectively. Analysis of adult brains from flies overexpressing *runt* show some *Rh1-LacZ* improperly innervating medullar cartridges. Upon analysis of morphology of thin sections, they find that random R1-R6 fates have smaller rhabdomeres and express Rh3 and Rh5, thus indicating that they have switched to R7 or R8 cell types.

AIII.2 – Results and discussion

To investigate whether something similar may happen with Eya overshooting phenotypes, I analyzed adult brains from *GMR>Eya^{RNAi}, Rh1-LacZ* flies. In wild-type brains, β-gal is only visible in the laminar region (Figure AIII-1A') and absent from the medulla, which can be visualized with chaoptin (Figure AIII-1A''). Furthermore, the columns within the medulla

are highly organized in distinct columns. In *GMR>Eya*^{RNAi} brains, I observed β-gal within the medulla (Figure AIII-1B') and that the medullar columns are very disorganized (Figure AIII-1B''). Thus, Eya knockdown causes axon mistargeting phenotypes in third instar larvae that persist into the adult. Future experiments will include characterizing whether there is evidence of fate switching in this genotype and whether *eya* overexpression also produces phenotypes into adulthood.

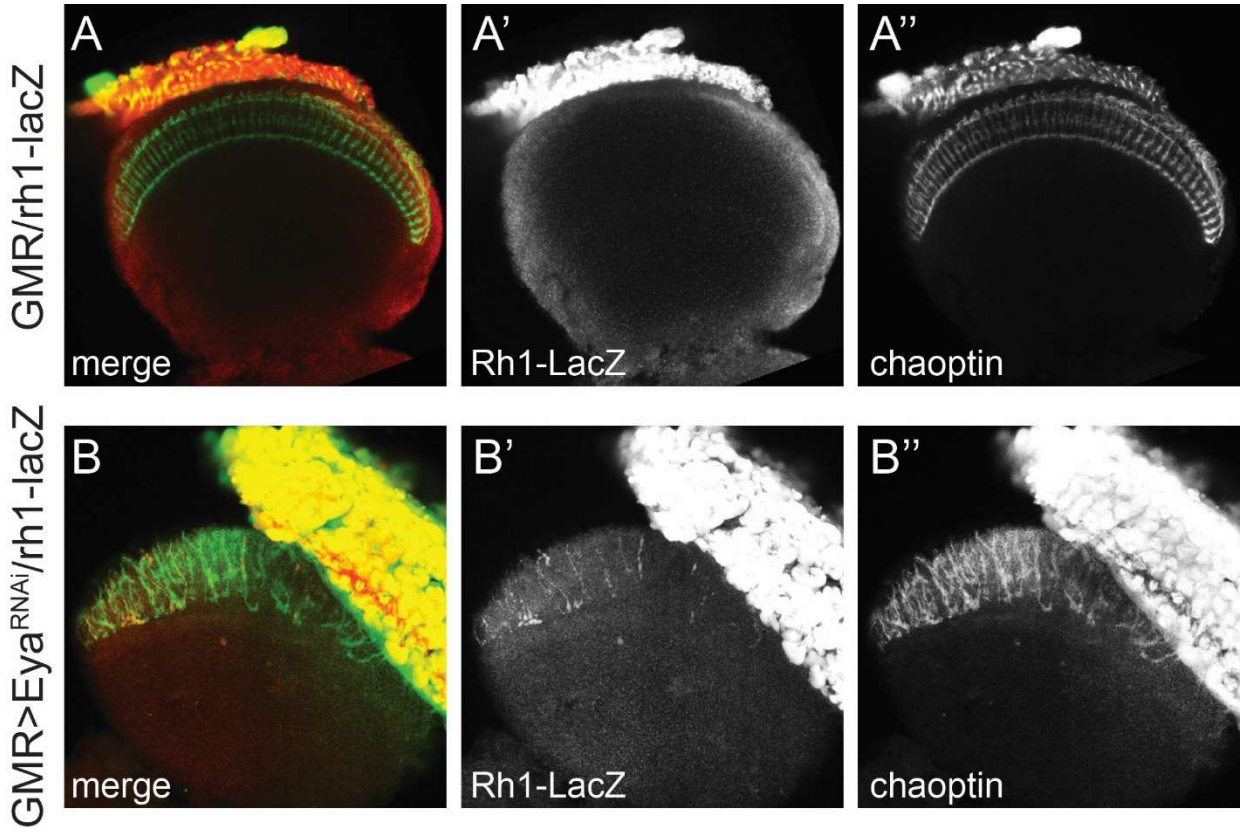

Figure AIII-1 | Eya^{RNAi} axon mistargeting phenotypes persist into adulthood
Adult brains were dissected and stained with β-gal and chaoptin. Wild-type brains (A) show *Rh1-LacZ* within the lamina (bright staining immediately adjacent to the brain, tissue above is left over photoreceptor bodies) (A') which does not cross into the medulla (A''). Note that the medulla is very well organized into discrete columns. Brains in which developing photoreceptors were knocked-down for *eya* (B) show inappropriate β-gal staining within the medulla (B'). The medullar cartridges themselves are also highly disorganized and do not form discrete columns (B''). Much of the very bright tissue in these B-panels are photoreceptor bodies such that the lamina is not visible.

AIII.3 – Materials and methods

AIII.3.1 – Fly strains

Rh1-LacZ flies were ordered from the Bloomington stock center (Stock #5766). These were crossed into recombinant *GMR>Eya*^{RNAi} lines for the analyses shown.

AIII.3.2 – Immunofluorescence

3-5 day old adult flies were dissected as described by (Williamson and Hiesinger 2010), fixed in 4% paraformaldehyde for 20 minutes, then stained as previously described (Xiong *et al.* 2009) with mouse anti-chaoptin (mAB 24B10, 1:10, DSHB) and rabbit anti-B-gal (1:20,000, Promega) and secondary antibodies (1:2000, Jackson ImmunoResearch). Samples were mounted with N-propyl-gallate solution in 90% glycerol. Images were taken on a 510 Zeiss confocal microscope with 0.5-1.5µm steps for the entire depth of the brain and then projected over 5 Z-slices in ImageJ.

AIII.4 – References

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APPENDIX IV

Drosophila H702 does not contribute to PTP activity

AIV.1 – Introduction

Although Eya's C-terminal Eya Domain (ED) which encodes the tyrosine phosphatase is highly conserved in sequence from plants to invertebrates to vertebrates, activity is not. In fact, Drosophila Eya has the weakest in vitro phosphatase activity of all Eya family proteins (Rayapureddi *et al.* 2003, 2005; Tootle *et al.* 2003). One hypothesis is that Drosophila Eya tyrosine phosphatase is non-functional, which would corroborate Jin et al.'s finding that it is dispensable for normal development (Jin *et al.* 2013). Closer examination of the sequence alignment between Eya sequences across metazoans shows that a key lysine (Lys, K) that defines the beginning of motif 3 is instead a histidine (His, H) in *D. melanogaster* and glutamine (Gln, Q) in other fruit fly species and the house fly (Figure AIV-1). Lys, His and Gln are all hydrophilic amino acids, where Lys and His are charged while Gln is uncharged. The function of the conserved Lys is to stabilize the negatively charged intermediate during catalysis (Seifried *et al.* 2013). Since the side chain of His is an imidazole ring whereas Lys has a chain of carbons, it is reasonable that the His reside in *D. melanogaster* might fall short in stabilizing intermediates and therefore be a less efficient enzyme.

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                                                Motif 3
                                        KxxxxxxxxxxxxxxxxxϕϕϕϕGDxxxE
HhEya1  human       PIENIYSATKIGKESCFERIIQRF-GRKVVYVVIGDGVEEEQGAKK
HhEya2  human       PIENIYSATKTGKESCFERIMQRF-GRKAVYVVIGDGVEEEQGAKK
HhEya3  human       PIENIYSATKIGKESCFERIVTSL-GKKLTYVVIGDGRDEEIAAKQ
HhEya4  human       PIENIYSATKIGKESCFERIMQRF-GRKVVYVVIGDGVEEEQAAKK
XlEya   xenopus     PIENIYSATKIGKESCFERIIQRF-GRKVVYVVVGDGAEEEQGAKK
DrEya   zebra fish  PIENIYSATKIGKESCFERVIQRF-GRKVVYVVVGDGVEEEQGSKK
TnEya   pufferfish  PIENIYSATKIGKESCFERIMQRF-GRKVVYVVVGDGVEEEQAAKK
BfEya   amphioxus   PVENIYSATKVGKESCFERIVSRF-GRKVTYVVVGDGRDEEQAAKQ
SpEya   sea urchin  NIENVYSATRVGKESCFERIVARF-GRKVTYVAIGDAKDEETAAKQ
EsEya   squid       SIENIYSATKIGKESCFERIVSRF-GRKCTYVVVGDGRDEEAAAKQ
DjEya   planaria    QIENIYSANKIGKESCFERISSRF-GRKSTYVVVGDGKDEETAARQ
CeEya   nematode    PVENIYSISKQGKESVFEKIQSRF-GKKCSFICITSGDTANS-AKR
CbEya   nematode    PIENIYSTLKQGKESIFEKIQTRY-GKKCSFIYVTSRDTSRDVAKR
DmEya   fruit fly   NIENIYSAHKIGHETCYERIVTRF-GRKSTYVVIGDGNEEETAAKA
DvEya   fruit fly   NIENIYSAHKIGQETCYERIVTRF-GRKSTYVVIGDGAEEESAAKA
DwEya   fruit fly   NIENIYSAHKIGQETCYERIVTRF-GRKSTYVVIGDGAEEESAAKA
MdEya   house fly   NIENIYSAHKIGQETCYERIVTRF-GRKSTYVVVGDGAEEESAAKA
AgEya   mosquito    PVENVYSANKIGKEQCFERIVTRF-GRKSTYVVVGDGQDEENAAKN
DpEya   butterfly   PIENIYSATKTGKETCFEKIKQRF-GERCTYVVVGDGQDEEAAA-K
BgEya   cockroach   PIENIYSATKIGKESCFERIVSRF-GRKCTYVVVGDGQDEEAAA-K
TcEya   tribolium   PIDNIYSATKIGKESCFERIVARF-GRKCTYVVIGDGQDEEAAA-K
NvEya   wasp        PIENIYSANKTGKESCFGRIMARF-GRKCTYVVIGDGSDEEAAA-R
AmEya   bee         PIENIYSASKIGKESCFGRVVARF-GRRCTYVVIGDDTDEETAA-R
AtEya   Arabidopsis RHENVYSSIDVGKLQCFKWIKERFNHPKFRFCAIGDGWEECAAAQA
ZmEya   maize       SSENVYSSWEVGKLQCFKWIKERFDGPNVRFCAIGDGHEECSAAQV
OsEya   rice        AFENVYSSWEVGKLQCFKWIKERYDGPNVRFCAIGDGHEECTAAQI
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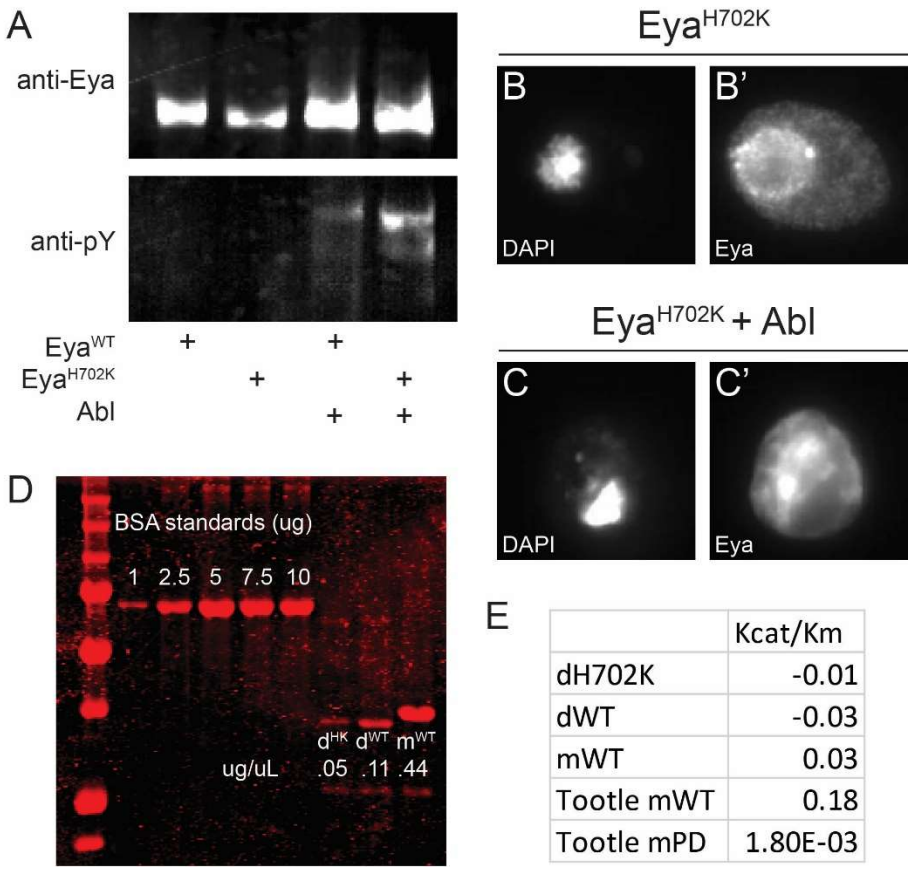
Figure AIV-1 | Sequence alignment of Motif 3 from multiple Eya proteins
Figure adapted from (Rebay 2015). Motif 3 is defined by K--ϕ ϕ ϕ ϕGDxxxE where ϕ represents any hydrophobic amino acid. Drosophila and *Musca domestica*, the common house fly, show divergence of a key lysine (K) within this motif by having either a histidine (H) or glutamine (Q), highlighted in green.

AIV.2 – Results and discussion

In order to test whether Drosophila Eya (dEya) is less effective as a phosphatase due to this His residue, I generated and assayed expression constructs of dEya^{H702K} in which I mutated the His back to a Lys. I tested phosphatase activity by leveraging dEya's reported ability to autodephosphorylate (Tootle *et al.* 2003). I co-transfected either dEya^{WT} or dEya^{H702K} with Abl and asked whether phosphor-tyrosine (pY) levels were decreased in Eya^{H702K} mutants. I instead observed the opposite, in which pY levels appeared increased, suggesting that Eya^{H702K} may actually be less active than wild-type dEya (Figure AIV-2A). This amino acid substitution does not compromise Eya's responsiveness to Abl, since Eya^{H702K} still relocalizes to the cytoplasm upon co-transfection of Abl (Figure AIV-2 C vs B).

Future experiments should focus on testing dEya^{H702K} by *in vitro* phosphatase assays. I attempted this but was unsuccessful, most likely due to inability to purify high-quality protein. I expressed and purified dEya^{H702K}, dEya^{WT} and mEya^{WT} to compare their activities in *in vitro* pNPP assays. dEya did not purify as robustly as mEya, with dEya^{H702K} yielding less than dEya^{WT} (Figure AIV-2D), thus compromising the subsequent pNPP assay. The ratio of Kcat to the Michaelis constant (Km) is commonly used to represent the "efficiency" of an enzyme in which lower numbers equate with lower efficiency. My preliminary experiment yielded negative Km's for both dEya proteins, and my mEya readings showed that it was less efficient than previously reported. While these results are largely inconclusive, they at least suggest that the single amino acid change in dEya^{H702K} does not increase dEya's enzymatic ability to that of mEya.



	Kcat/Km
dH702K	-0.01
dWT	-0.03
mWT	0.03
Tootle mWT	0.18
Tootle mPD	1.80E-03

Figure AIV-2 | H702K does not increase Drosophila Eya's efficiency as a tyrosine phosphatase

(A-C) S2 cell experiments with PAFW-Eya where Eya is either WT (Eya^{WT}) or contains the K-H substitution (Eya^{H702K}). (A) Western blot of IP-ed Eya in the presence or absence of Abl. Staining for pY shows Eya's efficiency in dephosphorylating itself. (B-C) Staining of S2 cells from lanes 2 and 4 of (A) showing that Eya^{H702K} behaves like wild-type Eya in which it is largely nuclear (B) unless co-transfected with Abl (C). (D-E) GST-Eya Domain expression constructs were used to perform a preliminary pNPP assay. (D) Mouse ED expressed the most robustly (m^{WT}) whereas Drosophila Eya^{H702K} (d^{HK}) and Drosophila Eya^{WT} (d^{WT}) expressed at lower levels. These proteins were used in a pNPP assay which shows that d^{HK} is not as active as m^{WT}, which we hypothesized. As comparison, prior values from similar pNPP assays are shown for wild-type mEya-ED (mWT) versus phosphatase-dead mEya-ED (mPD) (Tootle *et al.* 2003).

AIV.3 – Materials and methods

AIV.3.1 – S2 cell transfection and immunofluorescence

Drosophila S2 cells were cultured at 25°C in Schneider's medium (Sigma-Aldrich) supplemented with 10% Insect Medium Supplement, penicillin and streptomycin (Invitrogen). Cells were transiently transfected with 1.0ug of each plasmid using dimethyldioctadecyl-ammonium bromide (DDAB, Sigma) transfection and induced with 0.1M $CuSO_4$. Expression constructs include pMT-Myc-Abl, PAFW-Eya^{WT} and PAFW-Eya^{H702K}. PAFW-Eya^{H702K} was generated by quikchange mutagenesis of PAFW-Eya^{WT} using the following primers: 1) CAGTG CGCACAAAATCGGCAAGGAAACCTGCTATGAG and 2) CTCATAGCAGGTTTCCTTGC CGATTTTGTGCGCACTG. Prior to fixation or lysis, cells were incubated with pervanadate 33μM pervanadate and 66μM H_2O_2. Co-immunoprecipitation assays were performed by lysing cells in lysis buffer (50mM Hepes, 150mM NaCl, 1mM EDTA, 0.5mM EGTA, 0.9M glycerol) supplemented with protease inhibitors (Roche), 0.5mM DTT and 0.1% Triton, incubating the cell lysate with anti-Flag agarose beads (Sigma) overnight at 4°C, washing three times with lysis buffer, and resolving the proteins on 8% SDS-PAGE gels. Proteins were visualized by immunoblotting using rabbit anti-pY (1:400, Upstate Antibodies), mouse anti-Flag (1:1000, Sigma) and secondary antibodies (1:2000, Li-COR Biosciences). Cells were settled onto poly-L-lysine treated slides, fixed with 4% paraformaldehyde for 10 minutes and stained with mouse anti-Flag (1:1000, Sigma) followed by secondary antibodies (1:2000, Jackson ImmunoResearch). Images were taken using an Axioplan 2 imaging fluorescent microscope.

AIV.3.2 – Phosphatase assay

pGEX-Tev-Flag-dED-H702K, pGEX-Tev-Flag-dED-WT and pGEX-Tev-Flag-mED-WT fusion proteins were purified from BL-21 E. coli as previously described (Tootle *et al.* 2003) with the addition of incubating sample with TEV protease overnight at 4°C. Subsequent assays utilized *p*-nitrophenyl phosphate (pNPP, Sigma) were performed as previously described (Tootle *et al.* 2003). PNP anion was detected using the Infinite M200 Pro Tecan plate reader.

AIV.4 – Acknowledgements

Thank you to Matt Hope for help with sequence alignment and discovery of the H-K substitution in *D. melanogaster* and the Kovar lab for use of their Tecan plate reader.

AIV.5 – References

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APPENDIX V

Socs36E can become tyrosine phosphorylated in S2 cells

AV.1 – Introduction

The non-receptor tyrosine kinase, Abelson (Abl), is foundational to the work in this thesis based on its ability to phosphorylate Eya and thereby direct Eya's interactions with phosphotyrosine signaling networks explore in Chapter II. This appendix explores potential interfacing between Abl and one of Eya interactors, Socs36E. In mammals, cAbl has been shown to phosphorylate Socs1 and Socs3, whereby tyrosine phosphorylated Socs1 and Socs3 become less efficient at inhibiting Jak/Stat5 signaling (Qiu *et al.* 2012). Although Socs1 and Socs3 seem to be homologs that are absent in the fly, and there is no data on Abl phosphorylating Socs4 or Socs5 (homologs of Drosophila Socs36E) or Socs6 or Socs7 (homologs of Drosophila Socs44A) (Rawlings *et al.* 2004), our experiments in S2 cell have shown reproducible shifts of Socs36E when co-expressed with Abl.

AV.2 – Results and discussion

In order to determine whether Socs36E becomes tyrosine phosphorylated in the presence of Abl, I performed western blot analyses of Flag-tagged Socs36E and probed these blots with anti-phosphotyrosine (anti-pY) antibody. As before, the shift in Socs36E was noticeable in the presence of Abl, and this shifted band showed significant pY staining (Figure AV-1, lanes 1 and 3). Socs44A was also queried in these experiments but, as before, showed no shift, and does not

show any pY staining (Figure AV-1, lanes 2 and 4). Thus, Socs36E but not Socs44A accumulates pY in the presence of Abl.

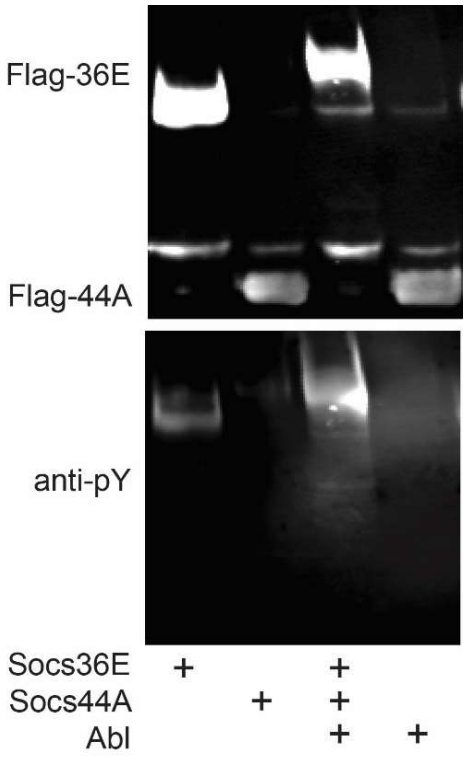

Figure AV-1 | Socs36E accumulates tyrosine phosphorylation when co-transfected with Abl Flag-SOCS proteins were either transfected alone or with Abl. Blotting of immunoprecipitated Socs36E but not Socs44A shows significant phosphorylation in the presence of Abl.

Future steps include exploring whether Abl directly phosphorylates Socs36E, and if so, at which residues. As a start, one could test Y552 and Y606, which are the analogous tyrosines to those phosphorylated by cAbl in human Socs1 (Figure AV-2). One would also want to determine whether Abl-mediated phosphorylation of Socs36E affects its ability to inhibit Jak/Stat activity as has been shown in mammalian cells, perhaps by using the Stat92E-LacZ reporter (Gilbert *et*

al. 2005). If Abl-Socs interactions are conserved, then one would expect reduced Stat92E-LacZ reporter assay in the presence of Abl. If this is the case, it would be interesting to see if and how Eya affects this relationship. Genetically, *eya* synergizes with both *abl* and *Socs36E* in photoreceptor axon targeting, as well as *hop* and *Stat92E*. One intriguing possibility is that Eya synergizes with pY-Socs36E which results in increased Jak/Stat signaling, thus explaining the positive genetic interactions observed between *eya*, *hop* and *Stat92E.*

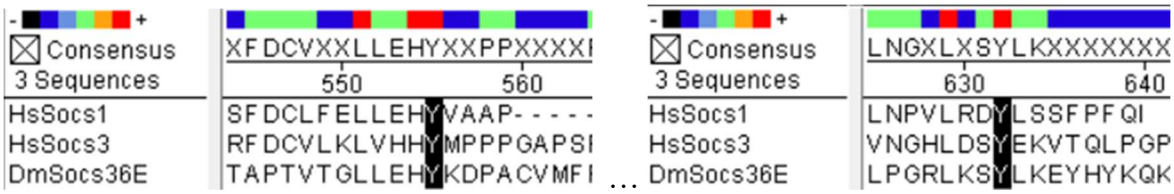

Figure AV-2 | Sequence alignment of human Socs1 and human Socs3 with Drosophila Socs36E
Qiu et al. show that Abl phosphorylates Socs1 at Y155 and Y204. These tyrosines are conserved in hSocs3 as well as Drosophila Socs36E and thus dSocs36E may be phosphorylated by dAbl.

AV.3 – Materials and methods

AV.3.1 – S2 cell transfection, co-immunoprecipitation and immunostaining

Drosophila S2 cells were cultured at 25°C in Schneider's medium (Sigma-Aldrich) supplemented with 10% Insect Medium Supplement, penicillin and streptomycin (Invitrogen). Cells were transiently transfected with 1.0ug of each plasmid using dimethyldioctadecyl-ammonium bromide (DDAB, Sigma) transfection and induced with 0.1M $CuSO_4$. Expression constructs include pMT-Myc-Abl, PAFW-Socs36E and PAFW-Socs44A. Prior to fixation or lysis, cells were incubated with pervanadate 100µM pervanadate and 200µM H_2O_2. Immunoprecipitation was performed by lysing cells in lysis buffer (50mM Hepes, 150mM NaCl,

1mM EDTA, 0.5mM EGTA, 0.9M glycerol) supplemented with protease inhibitors (Roche), 0.5mM DTT and 0.1% Triton, incubating the cell lysate with anti-Flag agarose beads (Sigma) overnight at 4°C, washing three times with lysis buffer, and resolving the proteins on 8% SDS-PAGE gels. Proteins and phosphotyrosine were visualized by immunoblotting using rabbit anti-pY (1:400, Upstate Antibodies), mouse anti-Flag (1:1000, Sigma) and secondary antibodies (1:2000, Li-COR Biosciences).

AV.4 – References

Gilbert M. M., Weaver B. K., Gergen J. P., Reich N. C., 2005 A novel functional activator of the Drosophila JAK/STAT pathway, unpaired2, is revealed by an in vivo reporter of pathway activation. Mech. Dev. **122**: 939–48.

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Rawlings J. S., Rennebeck G., Harrison S. M. W., Xi R., Harrison D. a, 2004 Two Drosophila suppressors of cytokine signaling (SOCS) differentially regulate JAK and EGFR pathway activities. BMC Cell Biol. **5**: 38.